Uropathogenic *Escherichia coli* of Dogs and Cats:

Pathotypic Traits and Susceptibility to Bacteriophages

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Für meine Eltern

Doris und Dr. rer. nat. Karl-Heinz Freitag

Die ich liebe und respektiere

&

For Derek

Whom I love and respect

The History of Medicine

2006 BC

Here, eat this root.

1000 AD

That root is heathen. Here, say this prayer.

1850 AD

That prayer is superstition. Here, drink this potion.

1920 AD

That potion is snake oil. Here, swallow this pill.

1945 AD

That pill is ineffective. Here, take this penicillin.

1955 AD

Oops . . . bugs mutated. Here, take this tetracycline.

1960–1999 AD

39 more "oops" . . . Here, take this more powerful antibiotic.

2000 AD

The bugs have won! Here, eat this root.

2006 AD

Or maybe try that pill again?

Modified from Anonymous

Abstract

The purpose of this study was to investigate the feasibility of using bacteriophages - viruses that can lyse bacteria - to control infections caused by uropathogenic *Escherichia coli* (UPEC) in dogs and cats. Prior to phage experiments, UPEC were subjected to virulence factor genotyping by multiplex polymerase chain reaction assay and phylogenetic 'fingerprinting' by Pulsed-Field Gel Electrophoresis (PFGE). Twenty-five of 30 assessed virulence factor gene (VFG) markers were detected at least once in 31 UPEC isolated from 20 UK cats and 89 UPEC isolated from dogs (56), cats (22) and people (11) living in New Zealand (NZ). The PFGE banding patterns of UPEC isolates from different individuals were markedly dissimilar unless isolates had been collected at the same hospital within one month of each other. In contrast, ≥2 UPEC strains isolated from each of 3 UK cats diagnosed with multiple UTIs were indistinguishable by PFGE. Antibiograms inaccurately predicted UPEC clonality and, of clinical importance, underestimated the number of relapsing or persistent infections in these cats. A comparison of VFG profiles and PFGE banding patterns of UPEC isolated from NZ and UK cats demonstrated a geographically uneven distribution of pathotypic and phylogenetic traits and indicated that, among other factors, the source of UPEC must be considered when comparing UPEC from different host species. When comparing UPEC isolates from NZ dogs, cats and people, strains with similar VFG profiles were found among the different host species. Other strains, with VFG profiles that differed according to the host species of origin were also detected. The latter finding, which is in contrast to the results of previous studies, may be of interest to researchers aiming to predict the potential zoonotic risk posed by particular UPEC strains sourced from dogs and cats.

Forty bacteriophages (phages for short) were isolated from sewage waters and propagated on UPEC strains. The ability of these phages to cause bacterial lysis was tested on 31 canine UPEC, 22 feline UPEC and 7 faecal *E. coli*. In contrast to faecal *E. coli*, UPEC strains were highly susceptible to phages. Ten phages with a particularly broad host range each lysed ≥27/53 (≥51%) UPEC strains. Used in combination, these 10 phages were predicted to be able to lyse 49/53 (92%) of the UPEC strains in the collection. Morphological and genotypic studies on 5 of these 10 phages demonstrated that 4 of them belonged to the lytic T4-like genus, while one phage showed similarity to the temperate phage P2. Overall, results of this project indicate that the majority of canine and feline UPEC - with very diverse PFGE banding patterns and VFG profiles - are susceptible to lysis by naturally occurring phages. Hence, phages show promise as therapeutic agents for treatment of canine and feline UTI and, perhaps, for other infections caused by UPEC.

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Preface

It has been almost a century since Felix d'Hérelle first applied bacteriophages, viruses that can infect and kill bacteria, to combat bacterial infections in animals and people (reviewed in Summers, 1999). In the first half of last century, bacteriophages (phages for short) were used enthusiastically to treat various bacterial infections (Carlton, 1999; Sulakvelidze et al., 2001; Summers, 2001). However, with the introduction of antibiotics in the early 1940s, interest in phage therapy waned dramatically (Carlton, 1999; Sulakvelidze et al., 2001; Summers, 2001). In recent decades, antimicrobial resistance has become increasingly apparent, generating fear of an impending 'post-antibiotic era' (Alanis, 2005). With the emergence of bacteria that are resistant to multiple antimicrobials, interest in phage therapy has been renewed (Barrow and Soothill, 1997; Merril et al., 2003; Sulakvelidze et al., 2001).

The principal intention of this PhD project was to carry out a preliminary investigation on the feasibility of using phages for treatment or prevention of bacterial infections in dogs and cats. Urinary tract infections (UTIs) caused by uropathogenic *E. coli* (UPEC) were chosen for study. This was because *E. coli* UTI in dogs and cats constitute one of the main infectious disease processes experienced in daily clinical practice (Ling, 2000). *E. coli* UTI can be readily diagnosed by cystocentesis and culture. Thus, it was expected that an adequate number of canine and feline UPEC could be collected in a short time frame. A further reason for focusing on *E. coli* UTI was that these pathogenic *E. coli* have been shown to spread from the urinary tract to other organs (Ling, 2000). There, they may cause serious, intractable infections, such as prostatitis and discospondylitis. These infections and *E. coli* UTI may become increasingly difficult to treat with conventional antimicrobials if the resistance of UPEC to current antimicrobials continues to increase as it has over the last decades (Cohn et al., 2003; Cooke et al., 2002; Mammeri et al., 2005; Sanchez et al., 2002; Warren et al., 2001).

UPEC isolated from dogs and cats were also considered an interesting study, because their role in the pathogenesis of canine and feline UTI is incompletely understood. In particular, few studies have focused on investigating pathotypic traits of feline UPEC in detail (Feria et al., 2000a; Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Wilson et al., 1988; Yuri et al., 1998).

In chapter 1, the reader will find a detailed review of the history of phage therapy and current therapeutic applications of phage. Related research areas, such as morphological studies of phage, have been reviewed in brief. The reader will also find a review of the current knowledge concerning canine and feline *E. coli* UTI. Special attention has been given to reporting knowledge of the pathotypic traits studied in detail in this project. This was done because published reviews of pathotypic traits of UPEC do not focus on canine or feline UPEC or do not include a detailed description of all pathotypic traits assessed here (Beutin, 1999; Emody et al., 2003; Hacker and Heesemann, 2000a; Johnson, 1991, 2003; Johnson and Russo, 2005; Mühldorfer et al., 2001).

The materials and methods used to establish the results presented in this thesis are described in detail in chapter 2. The reader will find additional information, such as supplemental information about *E. coli* strains, recipes, primer sequences and suppliers of materials, in the appendix 8.4.

In chapter 3, the first results chapter, pathotypic traits of UPEC that were to become targets of phage were investigated. The work presented in that chapter was seen as an important preparation for the intended *in vitro* phage trials of this project, because a review of the phage therapy literature had shown that (i) lack of knowledge about the targeted bacteria had been associated with phage therapy failure (Carlton, 1999; Summers, 2001); and (ii) a characterisation of pathotypic traits of bacteria may contribute to an understanding of why lysis occurred (Smith and Huggins, 1982). In addition, results presented in chapter 3 complement previous studies that compared canine, feline and human UPEC.

In the early stages of this PhD project, it became apparent that UPEC isolated from New Zealand cats would accumulate more rapidly than anticipated. In addition, the opportunity arose to obtain UPEC from London cats that were concurrently affected by chronic renal failure (CRF). Thus, it was possible to investigate, for the first time, the presence of virulence factor genes (VFGs) in a reasonably large number of feline UPEC isolates. Moreover, by acquisition of feline UPEC from 2 different countries it was possible to assess the possible geographic variation of VFG profiles in feline UPEC. The results of these evaluations are reported in chapter 4.

Among the 31 feline UPEC obtained from the Royal Veterinary College in London were 17 *E. coli* isolates that had been collected from 5 CRF-affected cats that had suffered from multiple UTI during a 2-year period. Much could be learned about recurring UTI in cats from a study of these 17 *E. coli* isolates. Thus, at the time I received these isolates, I was highly enthusiastic about the opportunity to explore the clonal relatedness and antimicrobial resistance patterns of these 17 UPEC isolates. The results of this investigation are reported in chapter 5.

Having characterised the pathotypic traits of "future targets" of phage, it was timely to investigate whether phages able to infect and kill canine and feline UPEC exist in the environment. Chapter 6 describes the results of this investigation and further *in vitro* trials that aimed to determine whether phage therapy could potentially become a useful substitute or supplement to conventional antimicrobial therapy of *E. coli* UTI in dogs and cats.

In chapter 7, the reader will find the general discussion of this thesis. There, important findings of this project have been emphasised and are discussed in detail. Furthermore, strengths and weaknesses of this project have been evaluated. A recommendation for future research that may result out of this project has also been given.

Publications arising from this research are listed in the appendix. Furthermore, the appendices contain useful supplemental information concerning current knowledge of the subject of this thesis, including references to historical publications on phage therapy of UTI. These references are not cited in current search engines and have been very cumbersome to obtain. In addition, the appendices contain raw data concerning experiments described in this thesis and supplemental information to chapter 2 (Materials and Methods).

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List of Abbreviations

ABU	Asymptomatic bacteriuria
CRF	Chronic renal failure
EM	Electron Microscopy
ExPEC	Extraintestinal pathogenic *Escherichia coli*
Fur	Ferric-uptake regulator
HPI	High-pathogenicity island
IVABS	Institute of Veterinary, Animal and Biomedical Sciences
LB	Luria Bertani
MIC	Minimal inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
NCCLS	National Committee for Clinical Laboratory Standards
NZ	New Zealand
OMP	Outer membrane protein
ORF	Open reading frame
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PFU	Plaque forming unit
THP	Tamm-Horsfall protein, synonym uromucoid
UK	United Kingdom
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VF	Virulence factor
VFG	Virulence factor gene

Chapter 1

Introduction
and
Review of the Current Literature

1.1. Introduction

Uropathogenic *Escherichia coli* (UPEC) are the most common infectious agents isolated from dogs, cats and people with urinary tract infection (UTI; Foxman, 2002; Lees, 1996; Ling et al., 2001; Russo and Johnson, 2003). Irrespective of the species-origin, UPEC (also termed extraintestinal pathogenic *E. coli*; ExPEC) have been shown to comprise a subset of the general *E. coli* population that is characterised by association with phylogenetic groups B2 and D, expression of limited O-serogroups and possession of specific virulence factors (VFs) that enhance the extraintestinal virulence of these *E. coli* (Beutin, 1999; Feria et al., 2001a; Johnson, 1991; Johnson et al., 2001a; Johnson et al., 2001d; Russo and Johnson, 2000; Whittam et al., 1989; Yuri et al., 1998).

Canine UPEC have been demonstrated to possess virulence factor genes (VFGs) that are similar, or identical, to VFGs of human ExPEC (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d; Low et al., 1988; Senior et al., 1992; Westerlund et al., 1987; Whittam et al., 1989; Wilson et al., 1988; Yuri et al., 2000).

The VFGs of feline UPEC have been studied to a much lesser extent (Feria et al., 2001a; Feria et al., 2001b; Yuri et al., 1998) or in much smaller sample populations (Johnson et al., 2001a; Whittam et al., 1989; Wilson et al., 1988). No such studies have been conducted in New Zealand. Nonetheless, an extensive pathotypic characterisation of UPEC, particularly those isolated from cats, may reveal useful insights concerning the aetiopathogenesis of *E. coli* UTI. In contrast to dogs and people, *E. coli* UTI is considered rare in young cats (Kruger et al., 1991; Lees, 1996). However, the likelihood of UTI rises significantly in older cats (Bartges, 2004; Bartges and Barsanti, 2000; Lees, 1996), particularly those that are concurrently affected by chronic renal failure (CRF; Barber et al., 1999; Mayer-Ronne et al., 2004). A preliminary prospective study has shown that many of the CRF-affected cats suffer from multiple, often clinically silent, *E. coli* UTI during the course of their disease (Barber et al., 1999). Infections that recur several weeks after successful treatment of the initial UTI are thought, in most cases, to be caused by different UPEC clones (i.e. *reinfecting* UPEC; Ling, 2000; Lulich and Osborne, 2004). However, studies that assess the clonal relatedness and antimicrobial resistance patterns of UPEC from cats with multiple diagnoses of *E. coli* UTI have, as far as I am aware, not been conducted anywhere. The

epizootological information gathered in such a study may be crucial for optimising the management plans of CRF-affected cats that develop UTI and may help to reduce or terminate the potentially detrimental loss of nephrons associated with microbially induced inflammation (Horcajada et al., 2004; Slotki and Asscher, 1982).

Failure to eradicate UPEC from the urinary tract of cats or dogs can be a frustrating clinical problem. Clinical management of canine and feline UTI may be particularly difficult if the UPEC strain is resistant to one or many 'front-line' antimicrobials (Drazenovich et al., 2004; Hagman and Greko, 2005; Seguin et al., 2003). In light of a rising resistance of UPEC to "second-line" antimicrobials, such as fluoroquinolones (Cooke et al., 2002), emerging reports of multi-drug resistant UPEC (Sanchez et al., 2002; Warren et al., 2001) and a possible zoonotic transmission of antimicrobial-resistant UPEC to humans (Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2001d; Yuri et al., 1998), a more stringent control of companion animal veterinarians' use of antimicrobials has been suggested (Morley et al., 2005). Moreover, the fear of a 'post-antibiotic era' - an era in which bacterial infections can no longer be eradicated by any of the available antimicrobials (Alanis, 2005) - has led to a surge of effort to find alternative treatment methods for UTI, such as UPEC-specific vaccines (Russo and Johnson, 2006) or bacteriophage therapy.

Bacteriophages (phages for short) are viruses that can infect and kill bacteria (Kutter and Sulakvelidze, 2005). Before the advent of antibiotics, phages were thought by some investigators to offer promise as antibacterial therapeutic agents (reviewed by Summers, 1999). However, the lack of knowledge about these viruses at the time, together with the lack of rigorously controlled treatment trials, led to ambiguous treatment results (reviewed by Summers, 2001 and Sulakvelidze, 2001). Thus, phages were easily eclipsed in the affluent world when the startling efficacy of antibiotics became apparent. In the former Soviet Union and Poland, where effective antibiotics have not always been readily available to physicians, phage medicine continued to be used and developed throughout the 20th century (Alisky et al., 1998; Chanishvilli et al., 2001; Slopek et al., 1983; Slopek et al., 1987; Sulakvelidze and Kutter, 2005; Weber-Dabrowska et al., 2000a). Now, at a time when the development of new antimicrobials has slowed (Powers, 2004), and the emergence of resistance to these antimicrobials goes hand-in-hand with their development (Walsh, 2003a), phage medicine has re-emerged and has been globalised (reviewed by Thiel, 2004). In recent years, well-devised and

executed British studies have demonstrated the efficacy of phages in treating experimental *E. coli* meningitis and septicaemia in mice, chicken and calves (Barrow et al., 1998; Smith and Huggins, 1982). Furthermore, phages have been successfully applied to resolve human UTI caused by antimicrobial-resistant UPEC (Perepanova et al., 1995; Slopek et al., 1987; Weber-Dabrowska et al., 2000a). Despite these encouraging reports, no studies of which I am aware have investigated the use of phages for controlling UTI in companion animals.

Hence, it was the purpose of this study to assess the feasibility of phage therapy for *E. coli* UTI in dogs and cats. Phages are ubiquitous; it was proposed that different phage types would be readily found in pooled canine and feline faeces and human sewage. Upon isolation, these naturally occurring phages were to be incorporated into *in vitro* trials to assess their potential to lyse a variety of UPEC isolated from NZ dogs and cats with UTI. Phages considered for future use in therapy - those lysing numerous UPEC isolated from different dogs and cats - were to be assessed further to elucidate whether these phages were likely to possess deleterious traits, such as the carriage of toxin genes or an excessively broad host range.

A review of the history of phage therapy has shown that lack of knowledge about the targeted microorganism may be associated with failure of phage therapy (Carlton, 1999; Summers, 2001). Conversely, knowledge about pathotypic characteristics of UPEC may contribute to an understanding of why lysis occurs and, ultimately, why phage therapy is successful (Smith and Huggins, 1982). To enable a more science-based approach to selection of candidate phages for use in therapy, it was intended to investigate the pathotypic traits of feline and canine UPEC that were to become the targets of phage.

Such an investigation was considered beneficial - not only for the selection of appropriate phages for therapy of *E. coli* UTI - but also for the understanding of the role of UPEC in the aetiopathogenesis of intractable *E. coli* UTI in dogs and cats from NZ and other parts of the world.

1.2. Bacteriophage Therapy

A Review of the History of Bacteriophage Therapy

and

Current Applications of Bacteriophage

1.2.1. Discovery of Phage

The first reported observation of phages may date as far back as 1896, when Ernest Hankin, a British bacteriologist, isolated a substance that was able to destroy *Vibrio cholerae* from the rivers Ganges and Jumna in India (reviewed by Sulakvelidze et al., 2001 and Summers, 2005). He assumed that the filterable, heat-labile substance was a volatile chemical with bactericidal effect. In the following years, several similar observations of bacterial lysis were made (cited by Raettig, 1958). However, it was not until 1915, that Frederick W. Twort associated bacterial lysis with a possible acute viral infection of the bacterial cells (as discussed in detail by Summers, 1999). Twort noted that micrococcal colonies had different gross appearances. Next to colonies that appeared 'normal', there was a "glassy" morphotype. Microscopically, this morphotype was consistent with degenerated cells. Degeneration of micrococcal colonies could be induced by transferring an invisible, heat-labile, filterable agent isolated from a degenerated colony to a normal colony. Next to the possibility of a viral infection, Twort suggested that an enzyme or a non-particulate infectious protoplasm may possibly be the cause of the bacterial degeneration (cited by Summers, 1999). At approximately the same time, Felix d'Hérelle discovered the antibacterial phenomenon when investigating an outbreak of haemorrhagic dysentery among French Soldiers in Paris (as discussed by Summers, 1999). From samples of the soldiers, d'Hérelle isolated the dysentery-causing bacteria (for the production of a vaccine) and prepared a bacteria-free filtrate, which he thought may contain viruses or other substances that may enhance the pathogenicity of the dysenteric bacteria. However, *in vitro* trials showed that the filtrate contained a bactericidal agent that caused lysis of bacterial cultures (cited by Summers, 1999). D'Hérelle had no doubt that the antibacterial substance was a virus, a parasitic, ultra-microscopic particle that was able to multiply in, and cause death of, living bacterial cells. In 1917, he officially announced the discovery of a suspected antibacterial virus, which he had named "bacteriophage"*, at an Academy of Sciences meeting in Paris (reviewed by Summers, 1999). D'Hérelle suggested that phages may confer a natural, exogenous resistance to bacterial infections and that they

* Bacteriophage – from bacteria and phagein, the Greek word for 'to devour'

may be used to treat bacterial infections (as discussed in detail in chapter 1.2.4; cited by Summers, 1999).

In 1921, years after d'Hérelle had officially claimed the discovery of phages, Twort's notes on the phage phenomenon were recovered by André Gratia, a student of Jules Bordet (Gratia and Jaumain, 1921; cited in Raettig, 1958). Bordet, a Nobel Prize winner of previous years who strongly opposed d'Hérelle's theory of a bactericidal virus (as discussed in more detail below), disputed that d'Hérelle was the first to reveal the phage phenomenon (reviewed by Summers, 1999). After several years of controversy, well-known independent researchers repeated both d'Hérelle's and Twort's initial experiments and confirmed that the phenomena observed by d'Hérelle and Twort were indeed the same (reviewed by Summers, 2005).

1.2.2. The Nature of the Phage Phenomenon

D'Hérelle's opinion that the invisible agent was an infecting virus was not shared by all members of the scientific community at the time. Many believed that lysis occurred as a result of an induction of bacterial products, such as enzymes (i.e. as bacterial autolysis; cited by Ackermann and DuBow, 1987a; Sulakvelidze et al., 2001; van Helvoort, 1994 and Summers, 1999). The 'enzyme theory' was based on the observation that strains that had been subjected to phage did not lyse immediately. Instead, bacteria seemed to lyse suddenly - after subculturing or when there was a change in growth conditions. Jules Bordet was one of the researchers who had observed the induced, 'enzymatic' reaction (reviewed by Summers, 2005). Therefore, he strongly believed that the ability to autolyse was not associated with an infectious particle and was a property of what he had termed *lysogenic* bacteria*. Of note, Lwoff and colleagues revealed in the 1950s that the phenomenon of delayed lysis is associated with a certain kind of phage, termed temperate phage (Lwoff, 1953). Upon infection, temperate phages have the ability to enter the lysogenic lifecycle, that is, replicate in synchrony with the bacterial host. During the lysogenic lifecycle, which is discussed in

* Lysogenic bacteria: Bacteria that have the power to transfer and exhibit the agent of lysis. Lwoff, A. (1953)

detail in chapter 1.2.4.2, no lysis of bacteria is observed. However, under certain circumstances, temperate phages are able to lyse the bacterial host - like lytic phages - they can undergo the so-called vegetative cycle. The vegetative cycle may be induced by changes in growth conditions (as discussed by Ackermann and DuBow, 1987a). This knowledge about the lysogenic lifecycle of temperate phages was not available at the time Bordet defined lysogeny. D'Hérelle could not persuade his opponents that the invisible agent was a virus, despite presenting several sophisticated studies, including those that showed the host specificity of phages and the influence of dilution of filtrate on the plaque count* (indicative of the presence of particles; reviewed by Summers, 1999). Furthermore, d'Hérelle observed that lysis did not occur continuously, which indicated that lysis was the result of an infectious cycle. D'Hérelle's findings were later supported by Ellis and Delbrück (1939), who showed in the one step growth experiment that phages (i) adsorb to bacterial cells (shown by a decrease in number of free phage in the filtrate); (ii) enter a latent period (no change in number of phage in the filtrate is seen); and (iii) burst cells, which results in a rise in the number of phage particles in the filtrate to a plateau level. The one-step growth experiment also allowed the determination of the time span from infection to lysis and of burst sizes†. However, the controversy about the nature of phage continued despite efforts by other researchers who showed that phage differed in size and consisted of protein and DNA (reviewed by Ackermann and DuBow, 1987a). In 1940, the German medical doctor Helmut Ruska visualised the first phages with the newly invented electron microscope (reviewed by Krüger et al., 2000 and Anderson, 1992). In 1941, American scientists Krueger and Scribner insisted that phage forms from a precursor protein in bacterial cells (Krueger and Scribner, 1941a, b). However, by 1942 phages infecting bacteria had been visualised with electron microscopy (Anderson, 1992). Thus, phages were established as replicating, infectious organisms.

* Plaque count: count of bacteria-free spots (plaques) on agar slants or plates

† Burst size: Number of phages released after lysis of one cell (individual burst size). The average of individual burst sizes of many bacterial cells (average burst size) is often chosen, because individual burst sizes may vary greatly for a given phage and bacterial strain.

1.2.3. Non-therapeutic Phage Research

1.2.3.1. Phage as a Model Organism

Parallel to the immediate interest in applying phages as antibacterial agents in the 1920s, researchers investigated the chemical, physical and physiological characteristics of phage. At first, this interest was stimulated by the controversy about the nature of phage. However, soon phages, which could be propagated easily at little expense, became models to study the reproduction of higher organisms (reviewed in Cairns et al., 1992). From the late 1930s onwards, this fundamental phage research became intimately linked to a group of researchers that soon formed the "phage community". In 1944, the "phage community", under the leadership of Max Delbrück, decided to focus on 7 lytic phages (T1-T7) to increase the inter-laboratory comparability of results and the exchange of ideas (reviewed in Cairns et al., 1992). Similarly, Andre Lwoff and his colleagues concentrated on the model phage λ, a temperate phage that confers lysogeny to infected bacterial cells (reviewed in Cairns et al., 1992 and Lwoff, 1953). The fundamental phage studies carried out by the phage community and Lwoff's research group contributed significantly to our current knowledge of (i) DNA as the carrier of genetic information; (ii) the organisation of genetic information in operons; (iii) the recombination, mutation and regulation of genes; and (iv) gene transcription and its translation to proteins (reviewed in detail in Cairns et al., 1992). Furthermore, identification of phage morphology, phage constitution and the life cycle of phages laid the cornerstone for modern phage therapy (as discussed by Ackermann and DuBow, 1987a).

1.2.3.2. The Study of the Phage Morphology

Soon after the first electron microscopic evaluation of phage it became apparent that many different phage types exist. The electron microscopic study of phage morphology became an important descriptive method to categorise the many different phages, to study similarities between phages and to explain possible phylogenetic relations (Ackermann, 2005). The morphological study of phage may also be used to gain an indication of the therapeutic behaviour of phage by comparing them with known phages that are associated with traits that may be desirable or undesirable for therapy.

Today's International Committee on Taxonomy of Viruses classification scheme is primarily based on phage morphology and the nucleic acid type of phages (Ackermann, 2005). Physicochemical properties and replication characteristics play a secondary role in phage classification. Thus, phages have been categorised into one order, 14 families and 31 genera (Fauquet et al., 2005). More than 95% of all phages observed to date belong to the order *Caudovirales* or tailed phages (Ackermann, 2001). Many of these can infect *Enterobacteriaceae* (Ackermann, 2005). All tailed phages consist of a non-enveloped icosahedral head and a helical tail. The length-to-width ratio of the head has been used to define different head morphotypes (type 1-3; Ackermann and Eisenstark, 1974). The head may be isometric (type 1) or prolate (types 2 and 3; Ackermann and Eisenstark, 1974). Three families of tailed phages have been defined based on the morphology of the tail (morphotype A-C; Ackermann, 1998). The *Myoviridae*[*] (morphotype A) are phages with a long contractile tail that consists of a central tail tube, which penetrates the bacterial cell membrane at infection, and a tail sheath. Approximately 25% of tailed phages belong to this family (Ackermann, 2005). Well-characterised genera of this family are the lytic T4-like phages and the temperate P1-like, P2-like and Mu-like phages. *Siphoviridae*[†], including phage λ, constitute approximately 61% of tailed phages (Ackermann, 2005). These phages have a long, flexible non-contractile tail (morphotype B). *Podoviridae*[‡], which constitute approximately 14% of all tailed phages, exhibit a short, non-contractile tail (morphotype C; Ackermann, 2005). Well-characterised genera of the *Podoviridae* are T7-like phages (lytic) and P22-like phages (temperate). A baseplate, spikes and tail fibres may be

[*] From Greek *mys, myos* = muscle.
[†] From Greek *siphon* = hollow tube.
[‡] From Greek *pous, podos* = foot

present at the terminal end of the tail in some phages (e.g. T4; (reviewed by Ackermann, 1998). These structures commonly facilitate the tail-first adsorption to bacterial receptors (as discussed in Ackermann and DuBow, 1987a). Other facultative structures, such as collars, fibres, knobs or discs protruding from the head or tail and spiral filaments may also be present (reviewed by Ackermann, 1998). All tailed phages described to date encode double-stranded DNA (cited by Ackermann, 2005). Phages without a prominent tail constitute less than 4% of the currently recognised phages (cited by Ackermann, 2005). Ten different families of tailless phages have been defined (reviewed by Ackermann, 2005). These phages appear phylogenetically diverse, as they come in many different shapes, are enveloped or not, and have different nucleic acid genomes (e.g. single-stranded RNA, double-stranded DNA). Few of the tailless phages have been shown to infect *Enterobacteriaceae*. Many of these phages are lysogenic or infect bacteria chronically, without causing lysis. Therefore, they are rarely utilised in conventional phage therapy (as discussed by Ackermann, 2005). However, filamentous phages (e.g. coliphage M13) are commonly applied in phage display procedures (reviewed in Calendar, 2006) and are currently being evaluated as transporters for antimicrobial enzyme systems or for phage-mediated vaccination (as discussed by Westwater et al., 2003 and Clark and March, 2004).

1.2.3.3. Phages as Diagnostic Tools in Bacteriology

The potential use of phages for diagnostic purposes was first discussed by Sonnenschein in 1925 (cited by Raettig, 1958). Since then, phages have been utilised (i) to identify bacterial genera or species; (ii) to investigate disease outbreaks associated with bacteria; (iii) to classify bacteria; and (iv) to detect bacteria in living tissue and organic matter (reviewed by Ackermann and DuBow, 1987a and Rees, 2006). Target strains are often human or animal pathogens, such as *Salmonella* Typhi, *Vibrio cholerae*, *Mycobacterium tuberculosis* and *Listeria monocytogenes* (reviewed by Ackermann and DuBow, 1987a). Phage assays have also been applied to discriminate between pathogenic and non-pathogenic bacteria (e.g. *Bacillus cereus* and *Bacillus anthracis*; as discussed by Ackermann and DuBow, 1987a). Results may be obtained rapidly and at little expense. The sensitivity and specificity of phage assays is commonly based on phage-mediated lysis of the target strains. This is particularly the case in traditional phage assays, namely diagnostic assays and phage typing methods, where a positive result is equivalent to the observation of lysis of the tested strain (Ackermann and DuBow, 1987a). The recently developed phage amplification assay is also based on the lysis of the target bacterial strain (reviewed by Rees, 2006). The difference from traditional phage assays is that phages not only infect the bacterial strain of interest, but also a fast-growing standard laboratory strain. After initial multiplication in target strains, progeny phage is subjected to subsequent replication cycles in the laboratory strain. By using this method, the presence of slow growing bacteria, such as *M. tuberculosis*, may be detected more rapidly, fewer target bacteria can be detected and the contact with bacterial pathogens can be kept to a minimum. Other recent phage-based tests are the bioluminescence adenylate kinase assay, the bioluminescence ATP assay and the reporter phage assay (reviewed by Rees, 2006). These tests are applied to screen food and beverage samples for microbial contaminants (e.g. *Salmonella* spp. and *L. monocytogenes*; Stanley, 1989) or to rapidly identify slow-growing microorganisms, such as *M. tuberculosis* (reviewed by Rees, 2006). Phages have also been utilised to determine bacterial characteristics, such as antimicrobial susceptibility or to detect specific molecules, such as antibodies (reviewed by Rees, 2006). Overall, these phage assays utilise phage-specific receptor binding, phage-mediated lysis or the transducing abilities of phages to achieve rapid and specific results.

1.2.4. Phage Therapy

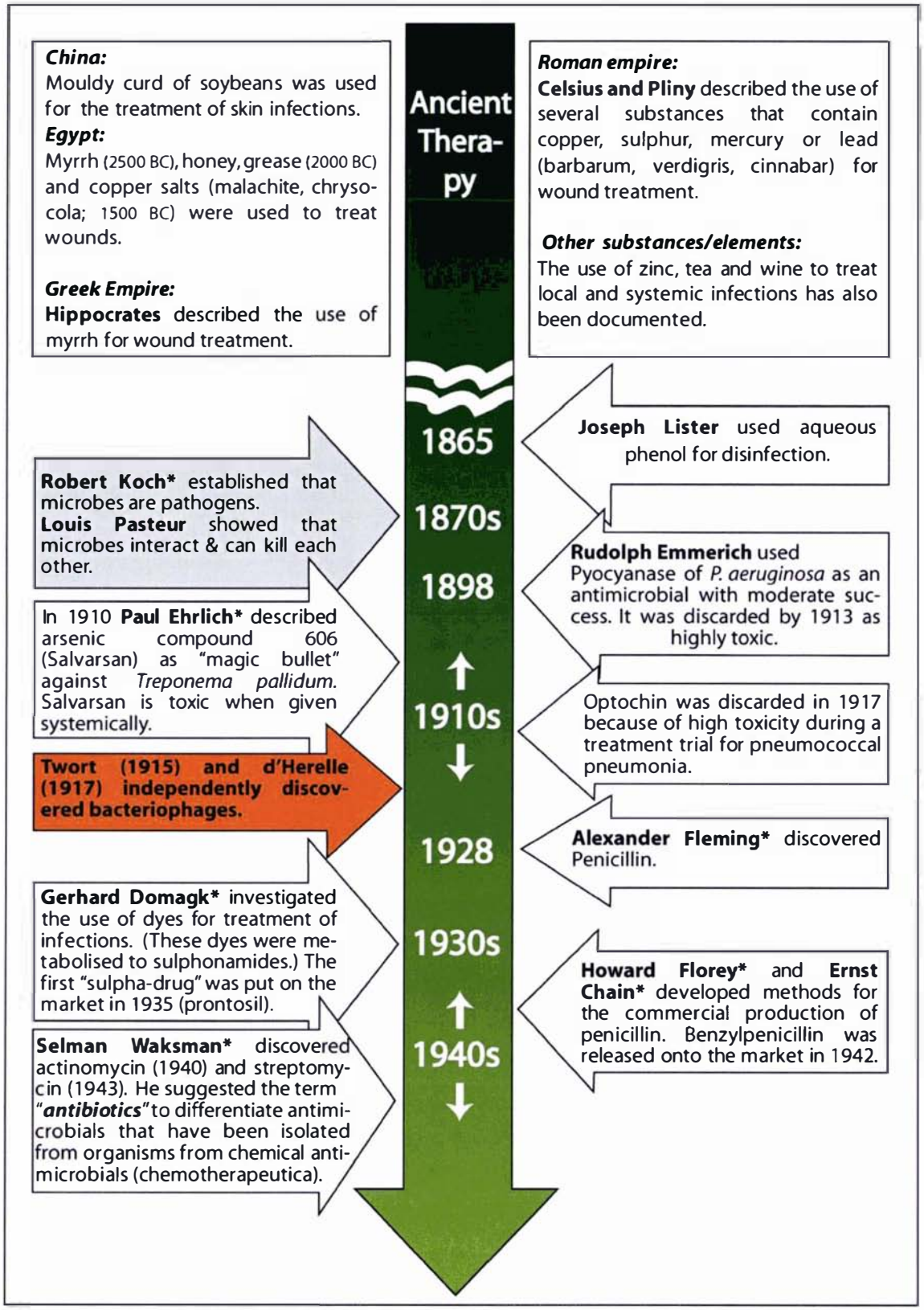
1.2.4.1. History

The Enthusiastic Pre-Antibiotic Era of Phage Therapy

Almost immediately after the discovery of phages, d'Hérelle investigated the potential use of phage to cure infectious diseases (reviewed by Summers, 1999). In 1919, d'Hérelle published the results of his first therapeutic experiments using phages for the treatment of fowl typhoid, the infection of chickens with *Salmonella gallinarum* (cited by Summers, 1999 and Raettig, 1958). According to Summers (2001), these trials were designed carefully, containing both flocks of chickens treated with an oral phage suspension and a control group (flocks without phage treatment). When both groups were subsequently exposed to infected chickens, the disease established primarily in birds that had not received phage. Overall, a lower mortality rate, reduced duration of infection and a reduced risk of reinfection were observed in phage treated flocks when compared to non-treated flocks. When phage was given orally to treat rabbit dysentery caused by enteropathogenic *E. coli*, and parenterally to treat Indonesian buffalos suffering from haemorrhagic septicaemia caused by *Pasteurella multocida*, similarly encouraging results were obtained (cited by Summers, 1999). Having established the therapeutic potential of phages in animals, d'Hérelle focused on the treatment of human epidemics. The first therapeutic use of phages on humans dates back to 2 August 1919, when d'Hérelle treated a 12-year-old boy with culture-confirmed haemorrhagic dysentery caused by *Shigella dysenteriae* at the Hôspital de Enfants-Malades in Paris (reviewed by Summers, 1999). Prior to this treatment, d'Hérelle, his family and staff of the hospital ingested increasing volumes of phage preparation and received a subcutaneous injection containing phage (reviewed by Summers, 1999). No adverse reactions were observed during these 'safety trials'. The boy, and later 3 other moribund children, recovered from the dysentery within a few days.

D'Hérelle was not the only one to recognise the therapeutic potential of phages in an age where antimicrobial chemotherapeutics were often highly toxic and therefore restricted to local treatment (Figure 1.1).

Figure 1.1 Early antimicrobial therapy[a]



[a]: Illustration created from material presented by Moellering, 1995 and Thurston, 2000.
An asterisk (*) indicates Nobel laureates.

Raettig (1958) lists many hundred phage therapy studies conducted in the years after d'Hérelle's discovery of phage. Phages were applied to treat (i) patients with infected traumatic or surgical wounds or osteomyelitis; (ii) dermatological disorders such as pyodermatitis, carbuncles and furuncles; and (iii) medical disorders, such as dysentery, respiratory infections, UTIs and septicaemia. D'Hérelle's application of phage to treat human plague is likely to be one of the most remarkable studies of the 1920s. According to Summers (1999), d'Hérelle treated four patients with serologically confirmed bubonic plague by injecting 0.5 ml of an anti-plague phage preparation into each bubo. Phage treatment quickly resolved plague in all four patients. The successful outcome of the treatment, published in October 1925, initiated a series of phage studies called the "Bacteriophage Inquiry" (1927 – 1936). This project, reviewed in detail by Summers (1999) and Sulakvelidze and Kutter (2005), was set up to investigate the efficacy of phage in treatment and prevention of cholera in Indian provinces. Anti-cholera phage was distributed either directly (in form of concentrated oral preparations) or indirectly (by pouring phage into drinking wells) among tens of thousands of people during the 9 year period. Treatment trials were conducted in hospitals or as field trials in several Indian provinces. Phage treated groups were compared with control groups that received no treatment or the standard cholera treatment at the time. Overall, phage seemed to reduce morbidity and mortality associated with cholera effectively. Fewer cholera outbreaks and fewer cholera-associated deaths were noted in provinces that had received phage as a preventive measure, compared with provinces that had not received phage. Encouraged by the first promising results of these studies, numerous other projects investigated the potential of phage as a treatment for infectious diseases caused by pathogenic microbes such as *Salmonella* Typhi, *Salmonella enterica* and *Clostridia diphtheriae* (cited by Raettig, 1958).

Initial phage therapy trials were soon followed by the commercial distribution of phage preparations. According to Summers (1999), d'Hérelle started his own commercial lab (Le Laboratoire du Bactériophage) that distributed 5 phage preparations, named Bacté-coli-phage, Bacté-rhino-phage, Bacté-staphy-phage, Bacté-pyo-phage and Bacté-intesti-phage. Major pharmaceutical companies such as the American Eli Lilly, Parke-Davis, E.R. Squibb and Sons and Swan-Myers also produced and distributed phage products in the 1930s (reviewed by Sulakvelidze and Kutter, 2005 and Sulakvelidze et al., 2001). These liquid or jelly-based preparations were used to treat a variety of infections,

including abscesses and wound infections, respiratory infections and infections of the genital tract (reviewed by Sulakvelidze et al., 2001 and Sulakvelidze and Kutter, 2005). Commercial companies focused on producing few preparations that supposedly targeted many different bacterial hosts. At the time, the host specificity of phage had been observed. In fact, it had been discovered by d'Hérelle in the early 1920s (cited by Summers, 1999). However, the mechanism of infection was poorly understood. Even d'Hérelle believed that host specificity was only a temporary feature and that a given phage could infect any bacterial strain if it was allowed to adapt its virulence to the targeted bacterial strain (reviewed by Summers, 1999). Thus, it was d'Hérelle's opinion that treatment of a clinical infection required an *in vitro* test prior to phage application, to establish whether phage had previously 'adapted' to the clinical isolate and was virulent against it. These cumbersome susceptibility tests were often not applied when commercial preparations were used. Instead, it was claimed that preparations consisted of phages that had been 'adapted' to various bacterial hosts (called 'polyvalent' phages) or contained a mixture of phages that had been 'adapted' to different hosts. However, these phage preparations often lacked the potential to cure infections (reviewed by Summers, 2001). Ambiguous results were also observed in several research trials conducted in the 1930s (reviewed by Sulakvelidze and Kutter, 2005). Several recent reviews have analysed the problems that led to the ambiguity observed during early phage therapy and concluded that the (i) lack of knowledge about the nature and constitution of phage; (ii) poor understanding of the interaction between phage and its host; and (iii) lack of universal standards when producing phage preparations and assessing their efficacy are to blame for the failure of early phage therapy (Table 1.1; Carlton, 1999; Sulakvelidze et al., 2001; Summers, 2001). During the 1930s, doubts about the efficacy of phage therapy were raised. To address the controversy surrounding the nature of phage and its therapeutic use, the Council on Pharmacy and Chemistry of the American Medical Association appointed Stanehope Bayne-Jones, a renowned bacteriologist, and Monroe Eaton, a specialist in infectious diseases, to provide a detailed review of previously published phage therapy research. In their report, published in 1934, Bayne-Jones and Morton concluded that phages were bacterial enzymes and that the therapeutic potential of phages was limited (Eaton and Bayne-Jones, 1934a, b, c). As stated earlier, a similar report by Krueger and Scribner, published in 1941, stated that phage was a proteinaceous bacterial substance that added

little therapeutic benefit to conventional treatment methods (Krueger and Scribner, 1941a, b). Both reports had a strong negative impact on phage therapy research in America and Western Europe. The interest in the therapeutic application of phage declined and funding of projects ceased. In the early 1940s, when World War II raged in Europe, antibiotics became the most favoured treatment of infectious diseases in America and Western Europe (Figure 1.1).

Table 1.1 Retrospective assessment of problems encountered during early phage therapy

1. Problems associated with a lack of knowledge about the nature and constitution of phage			
Problem	**Consequence**	**Solution**	**Ref**
Phages were claimed to be effective in diseases caused by viruses or fungi, such as Herpes virus, Food and Mouth Disease, eczema and urticaria	Phages did not clear infections.	Correct erroneous claims.	1, 2, 5
Phage preparations were handled like vaccines and sera – preservatives such as mercurial, phenol, oxidising agents were added, or preparations were sterilised using high temperatures.	Viable phage particles were inactivated or destroyed. Preparations did not clear infections.	Use methods that do not affect the viability of phages, such as sterile filtration. Assess the shelf life and viability of phage preparations after sterilisation.	3-5
2. Problems associated with a lack of knowledge about phage-host interaction			
Problem	**Consequence**	**Solution**	**Ref**
The phage lifecycle was unknown. Lysogeny was interpreted as a bacterial property, not as a characteristic conferred by temperate phage.	Bacterial lysis was unpredictable when temperate phages were used. It appeared to occur spontaneously.	Phage life cycles have been determined. Naturally occurring temperate phages are generally considered inappropriate for phage therapy.	4, 5
The host specificity of phage was poorly understood. The susceptibility of bacterial strains to phage was not always determined.	Infections were treated with inappropriate phages that were unable to infect bacteria. Treatment was not successful.	Test the susceptibility of bacteria to the phage used. If susceptibility test results are not available at the time of treatment, use a combination of phages or a single phage known to lyse a large variety of strains from the determined species.	3, 5
When creating phage preparations, phage growth parameters were assumed to be identical for all phages. Thus, 'polyvalent' phage preparations were created by incubating a number of phages with one bacterial strain.	The 'polyvalent' phage preparation contained only one phage that outgrew the other phages. Only infections caused by bacteria susceptible to that phage were cleared.	Assess host range of phages before creating a phage combination. Assess host range and potency of phage combination upon creation.	4
Resistance of bacteria to phage was poorly understood.	Observation of resistance resulted in a reduction of interest in utilising phages for antimicrobial therapy.	Development of bacterial resistance has been accepted as an evolutionary incident. Resistance may be counteracted by isolating or designing phages that are able to lyse the resistant bacterial strain	4

3. Problems associated with trial design and a lack of universal standards when preparing and assessing phage

Problem	Consequence	Solution	Ref
Double-blinded placebo control studies were not established at the time of early phage research.	Results of phage therapy trials were subjective and ambiguous.	Assess efficacy of phage therapy using double-blinded placebo control studies.	3-5
No or inadequate standardisation for: a) Phage preparation methods b) Assessment of phage potency and concentration c) Assessment of phage purity	Lack of standardisation inhibited the inter-laboratory comparability. Results were not reproducible. Phage preparations contained bacterial fragments that activated the immune system when phage preparations were given systemically. Subsequently, observed adverse effects and immunogenic effects were attributed to phage.	Many standard methods have been established since the era of early phage therapy. Well known methods include the plaque count technique, the one-step-growth experiment, negative staining of phages for Electron Microscopy and phage density centrifugation. Furthermore, molecular biological methods, particularly DNA sequencing, have commonly been applied in recent phage research.	3
Pharmacokinetic and pharmacodynamic studies were not established at the time of early phage research.	It is possible that phages cleared from the patient's body before infection and multiplication occurred or that they were inactivated by the patient's immune system.	Assess the antigenicity of phages, the distribution in body compartments, dosing quantity and intervals. Methods for selection of phages with low antigenicity have been described. Mathematical models that investigate the influence of factors such as bacterial density and phage concentration on the therapeutic outcome of phage have been published	3, 6-9

[1] Raettig, 1958; [2] Barrow and Soothill, 1997; [3] Carlton, 1999; [4] Summers, 2001; [5] Sulakvelidze et al., 2001; [6] Merril et al., 1996; [7] Bull et al., 2002; [8] Levin and Bull, 2004; [9] Payne et al., 2000

Phage Therapy during the Antibiotic Era

Soon after the introduction of antibiotics, the interest in therapeutic phage preparations declined. The major advantage of antibiotics over phage was that antibiotics could eliminate a broader spectrum of bacterial pathogens at one time. Thus, treatment could commence without the time-consuming culture and susceptibility testing necessary for successful phage treatment. The therapeutic application of phages particularly declined in the American and Western European countries where antibiotics became readily available soon after their discovery (Figure 1.1). Consequently, phage research in the West turned away from therapeutic approaches and towards the study of the fundamental nature of phages. From 1950 to 1980, very few Western studies that focused on phage therapy were published and only a small number of phage preparations was commercially available in Switzerland, France and the USA (reviewed by Sulakvelidze and Kutter, 2005, Summers, 2001 and Häusler, 2003). However, in the former Soviet Union phage therapy continued to thrive, driven by the political and economic situation that arose during World War II (reviewed by Summers, 2001). During the 1980s, a Polish phage therapy centre was also established.

Phage Therapy in the Former Soviet Union

Phage therapy in the former Soviet Union commenced shortly after the discovery of phages by d'Hérelle (reviewed by Summers, 1999 and Sulakvelidze and Kutter, 2005). In 1917, Georgi Eliava, a Georgian bacteriologist, had noticed a bactericidal activity in the river Koura in Tbilisi. He decided to explore this observation further and contacted d'Hérelle to discuss "the phage phenomenon". In the following years, a deep friendship and close collaboration developed between d'Hérelle and Eliava. During the 1920s, Eliava travelled several times to Paris to work alongside d'Hérelle at the Pasteur Institute. Similarly, d'Hérelle spent several months in Tbilisi in 1934 and 1935. Driven by the idea of establishing an institution for phage research, Eliava set up a microbiology institute in Tbilisi in 1923. During the 1920s and early 1930s, the complex expanded with the help of d'Hérelle and became the "Institute of Bacteriophage Research". Initially, Eliava and d'Hérelle's work at the institute was strongly supported by the Soviet government. However, in the late 1930s the institute was classified as a threat to the Soviet society. Conflicts escalated in 1937, when Eliava

was arrested by the Soviet secret police, declared an enemy of the Soviet people and executed without trial. D'Hérelle, who had been a follower of Stalin's political system up to that time, left Tbilisi disillusioned and devastated and never returned. However, work at the Bacteriophage Institute, later renamed as the "Eliava Institute of Bacteriophage, Microbiology and Virology", continued. In subsequent years, the institute became one of the key institutions of phage research in the former Soviet Union. Other institutions that contributed to the development of phage pharmaceuticals were the phage production facility in Khabarovsk (Russia), the Phage Research Unit at the Gorky Research Institute of Epidemiology and Microbiology (now ImBio, Nizhniy Novgorod, Russia) and the Biomed Pharmaceutical Company (Perm, Russia; cited by Kutter, 1997 and Sulakvelidze and Kutter, 2005).

According to Alisky et al (1998), many hundred of papers concerning phage therapy have been published in the former Soviet Union since the 1930s. However, few have been made available to the international research community (Alisky et al., 1998). In recent years, several reviews of important Russian literature on phage have been published in English (Alisky et al., 1998; Chanishvilli et al., 2001; Kutter, 1997; Sulakvelidze et al., 2001; Sulakvelidze and Kutter, 2005). Here, points of interest concerning phage research in the former Soviet Union are summarised. Reviewed historical studies are also listed in the appendix (Table 8.2).

During World War II, phage research focused on the treatment and prophylaxis of war-associated infections, such as dysentery epidemics caused by *Shigella* spp. and wound infections caused by *Staphylococcus* spp. and *Streptococcus* spp. Even gas gangrene, caused by *Clostridium* spp., was treated with phage. Overall, phages were reported to decrease the morbidity and mortality associated with these bacterial infections. Furthermore, it was claimed that phage therapy reduced the recovery period and the rate of reinfection (reviewed by Chanishvilli et al., 2001).

From the beginning of the 1950s, an increasing number of clinical trials compared the efficiency of phage treatment with antibiotic therapy or evaluated treatment success when a combination of phages and antibiotics were used. Alisky et al. (1998) and Chanishvilli et al. (2001), who reviewed the numerous clinical research trials from the former Soviet Union, concluded that phage therapy was successful in treating infections with antibiotic-resistant bacteria and that a combination of phages and antibiotics yielded better therapeutic results than antibiotic treatment alone. Furthermore, the

reviewers observed that a combination of phages and antibiotics decreased or increased the therapeutic success of phage-only treatment. It is possible that different results were observed because antibiotics either inhibited phage multiplication by reducing the number of bacteria for phage replication or antibiotics helped to release viable prophage from bacterial cells, thus increasing the phage titre and the therapeutic success. Possible interactions between phage and antibiotics are discussed in more detail in chapter 1.2.4.2.

Another noteworthy approach was the combination of phages with symbiotic organisms. In 1978 and 1981, two independent studies investigated whether a combination of bifidobacteria and phages can be used to treat intestinal dysbacteriosis of infants or immunodeficient patients (Litvinova et al., 1978 and Tolkacheva et al., 1981; cited by Chanishvilli, 2001). Phages were used to lyse pathogenic bacteria in the gastrointestinal tract. Subsequently, bifidobacteria were administered to prevent dysbacteriosis and establish a non-pathogenic gastrointestinal flora. According to Chanishvilli (2001), both studies reported a marked clinical improvement in the patients who had received phage and bifidobacteria.

Over the decades, phages have been applied to treat a large variety of infectious conditions, including respiratory infections, genitourinary infections, meningitides, osteomyelitides and cellulitides, otitides and conjunctivitides, dermatitides and septicaemiae (Appendix, Table 8.2). Phage preparations were created by combining a number of lytic phages with known host range and different receptor specificities (reviewed by Kutter, 1997). Thus, preparations were effective against different strains of a bacterial genus or against strains from different genera that caused similar conditions. According to Kutter (1997), phage preparations were constantly adjusted to avoid cross-resistance and to include phages against strains that became resistant to the preparations. Phages were administered orally (as suspensions or tablets with an acid-resistant coding), topically (as rinses, baths, creams or in drenched bandages and tampons), rectally (as enemas), in aerosols or as injections (e.g. intravenous or intrapleural; reviewed by Sulakvelidze and Kutter, 2005).

Commercial preparations had to pass the drug safety regulations of the Soviet Health Authorities before they were released onto the market. Overall, the work conducted at the Eliava Institute of Bacteriophage, Microbiology and Virology and other bacteriophage institutes of the former Soviet Union has been described as impressive

(Kutter, 1997). During its most productive times, the Eliava Institute of Bacteriophage, Microbiology and Virology had more than 1000 staff and produced several tons of various phage formulations that were distributed to hospitals or pharmacies in the former Soviet Union (Sulakvelidze and Kutter, 2005). With the collapse of the Soviet system in the 1980s, the bacteriophage institutions faced severe economic difficulties that strongly limited the efforts to continue phage therapy research. However, many of the original research centres managed to survive the critical economic situation of the 1980s and 1990s. Additionally, private research and development companies have been formed (Sulakvelidze and Kutter, 2005; Thiel, 2004). These institutions continue to develop new phage preparations and apply phage preparations with established efficacy in clinical settings (reviewed by Sulakvelidze and Kutter, 2005).

Phage Therapy in Poland

Extensive phage therapy research and clinical trials were also conducted in Poland, at the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw. The institute, founded in 1952, initially used phages as diagnostic tools. In the early 1980s, Stefan Slopek and colleagues expanded the phage work conducted at the institute and started to study the potential use of phages as antimicrobial agents (Slopek et al., 1983). Phage studies were conducted as clinical trials rather than scientific experiments. Phage preparations contained a variety of phages or a single phage that had proven *in vitro* efficacy against the target bacterial strain (Slopek et al., 1983). Crude sterile lysates (prepared by chloroform or thymol extraction) were used for therapy (Slopek et al., 1983). Targeted bacteria included gram-positive microorganisms (*Staphylococcus* spp.) and gram-negative microorganisms (*Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp. and *E. coli*). Phages were administered orally (3x daily, after neutralisation of gastric acids) or topically (3x daily; Slopek et al., 1983). Systemic application was avoided to minimise the risk of adverse effects (Slopek et al., 1983). The success of phage treatment was assessed by evaluation of clinical signs associated with the bacterial disease and by assessment of bacterial cultures from clinical samples obtained before, during and after phage therapy (Slopek et al., 1983). No untreated control groups were included in the studies. Treatment results of 550 patients that underwent phage therapy from 1981 to 1986 and 1307 patients treated from 1987-1999 have been published

(Slopek et al., 1987; Weber-Dabrowska et al., 2000a). Overall, the results strongly suggest that phage therapy is safe and effective in treating a variety of infectious diseases, irrespective of the location of infection (Table 1.2), its type (monoinfection or polyinfection) and the age, sex and health status of the patient. A complete recovery was observed in 61-100% of patients. It is noteworthy to mention that many of the infections treated with phage had been treated unsuccessfully with antibiotics (Weber-Dabrowska et al., 2000a). Phage therapy appeared most successful in the treatment of furunculosis (complete recovery of all patients treated) and least successful in treatment of ulcerative varicose vein infections (61% complete recovery, 27% clinical improvement but bacterial sterility not achieved, 12% no improvement). Adverse effects, such as gastrointestinal intolerance or local allergic reaction, were observed in less than 0.5% of all cases (Slopek et al., 1983). Phages were reported to penetrate the intestinal barrier readily when administered orally, and were found in the bloodstream and urine of patients up to 10 days after oral administration (Weber-Dabrowska et al., 1987). In two subsequent studies, the effects of phage on the immune system were evaluated (Weber-Dabrowska et al., 2000b; Weber-Dabrowska et al., 2002). The authors of these studies observed a stimulation of neutrophil production and a normalisation of cytokine levels that had been either increased or decreased prior to phage administration. The authors suggested that the use of phages had stimulated the immune system. However, other factors that could have caused the observed immunomodulatory effects were not excluded. Thus, it is uncertain whether the observed effects were truly caused by an unknown interaction between phage and the immune system or, for example, by bacterial antigens that were released during bacterial cell lysis.

Table 1.2 Examples of infections treated with phages at the Hirszfeld Institute of Immunology and Experimental Therapy

	Categories	Example
Location of infection	Systemic	Septicaemia
	Nervous system and sense organs	Meningitis, conjunctivitis, otitis
	Circulatory system	Infections of varicose veins, pericarditis
	Respiratory system	Rhinitis, pneumonia, pleuritis
	Digestive system	Dysentery, ulcerative stomatitis
	Genitourinary system	Vaginitis, cystitis
	Skin and subcutaneous tissue	Furunculosis, decubitus ulcers
	Musculoskeletal system	Arthritis, myositis, ostitis
	Various postoperative or posttraumatic infections	Infections of burn wounds and laparotomy wounds, ostitis associated with fractures

Critique of Historical Studies from the Former Soviet Union and Poland

Overall, studies from both the former Soviet Union and Poland strongly suggest that phage can be applied successfully to combat bacterial infections. However, as discussed by the reviewers of early work in the former Soviet Union, the results of phage therapy studies remain somewhat subjective (Alisky et al., 1998; Chanishvilli et al., 2001; Sulakvelidze et al., 2001; Sulakvelidze and Kutter, 2005). This is largely due to the failure to include placebo control groups in historic clinical trials. Reviewers of publications from the former Soviet Union also raised the criticism that information that is vital for a rigorous evaluation of research results, such as how phages were selected and prepared, was often lacking. Furthermore, phage preparation methods, the titre of phage and its dosing regimen were rarely described in sufficient detail. It is uncertain, whether, and to what extent, phage characteristics were studied before phage was given to patients. It is also uncertain, whether other factors that could have caused a therapeutic effect were excluded. Few manuscripts published in the former Soviet Union have been indexed on websites such as Pub Med and CAB (Alisky et al., 1998). Thus, it is possible that well-prepared phage therapy trials have been conducted and published results are available through local libraries at the research institutes.

Polish publications also lack information about the phage titres used in the preparations. Furthermore, the source of the phages is not disclosed when the phage preparation method is described (Slopek et al., 1983). It is also uncertain whether genetic or morphological characteristics of therapeutic phages were studied. Reports that summarise the therapeutic outcome of patients treated with phage and antibiotics lack sufficient information about antibiotic treatment protocols (Weber-Dabrowska et al., 2000a; Slopek et al., 1987). For example, in a report published in 1987, phage treatment was stated to be significantly more effective than therapy with a phage-antibiotic combination (Slopek et al., 1987). However, the choice of antibiotic and the treatment protocols are not disclosed. Thus, observed results cannot be reproduced at other institutions and the suggested superiority of phage over antibiotics cannot be confirmed. One weakness of the Polish phage therapy trials is that treatment progress was based on the subjective observations of patient and clinician (Slopek et al., 1983). Another weakness is that, as far as I am aware, no control groups were included in any of the phage therapy trials. Thus, it remains uncertain whether the apparent treatment success was truly associated with lysis of pathogenic bacteria by phage.

Newer phage therapy trials (as discussed in detail below) address many of the issues raised from the review of historical phage therapy trials. Fundamental factors that need to be considered when conducting phage therapy trials are reviewed below.

1.2.4.2. Therapeutic Considerations

The Phage Life Cycle

All phages infect bacterial cells and interact with the bacterial replication apparatus to multiply. The vegetative cycle of phages consists of attachment, penetration, multiplication, maturation and release of progeny. Lytic or "virulent" phages start the active replication cycle immediately after introduction of their genome into the host cell. Release of progeny phage is associated with bacterial cell lysis (as discussed by Ackermann and DuBow, 1987a). Filamentous phages and some pleomorphic phages possess the ability to infect bacteria chronically (reviewed by Abedon, 2006 and Russel and Model, 2006). These phages also enter the vegetative cycle immediately upon introduction of their genome. However, progeny are released by extrusion or budding from the bacterial cell. This is not associated with cell lysis. Temperate phages have the ability to enter a quiescent stage (the so-called lysogenic lifecycle) during which the phage genome replicates in synchrony with the bacterial genome (reviewed by Ackermann and DuBow, 1987a). The vegetative lifecycle may be entered subsequently. As in lytic phages, the vegetative cycle results in release of progeny phage by lysis of the bacterial host. The vegetative lifecycle and lysogeny of tailed phages shall be described in more detail.

The Vegetative Lifecycle

Attachment

The vegetative lifecycle of phages starts with the random contact between phage and bacterium. Upon initial contact, phages attach to specific receptors displayed by the bacterial host. Such receptors may be constituents of cell walls, plasma membranes, capsules, pili and flagellae (reviewed by Ackermann and DuBow, 1987a). Tailed phages use specialised proteins, such as tail spikes and tail fibres, to attach to these host cell receptors (as discussed by Ackermann and DuBow, 1987a). Some tailed phages (e.g. K1-5-specific phage) increase their host cell reservoir by encoding different tail fibres or by encoding several molecules that recognise different receptors (Scholl et al., 2001). Attachment of phages to host cells is often dependent on co-factors, such as

cations (Ca^{++}, Mg^{++}) or sugars (e.g. Maltose). These co-factors induce the proper conformation of tail fibres or allow proper positioning of tail fibres by inducing bacterial cells to increase the receptor density. The proper conformation and position of spikes or fibres is essential for progression of the vegetative cycle (as discussed by Guttman et al., 2005). Most phages complete the attachment process in 10 minutes (Ackermann and DuBow, 1987a).

Insertion of the Phage Genome

Upon completion of attachment, phages introduce their genome into the host cell, while the proteinaceous coat remains bound to the outside of the host (as discussed by Anderson, 1992). In T4-like phages, penetration of the bacterial cell wall is achieved by tail contraction and an enzymatic reaction that lyses the peptidoglycan layer (reviewed by Mosig and Eiserling, 2006). The genome is released from the phage particle and enters the bacterial cell. In the cytoplasm, the naked nucleic acid is susceptible to destruction by host exonucleases and endonucleases. Most phages avoid these host cell defences by circularising their genomes or by protecting the ends of linear DNA or RNA strands (as discussed by Guttman et al., 2005). Other phages have developed special defence mechanisms, such as (i) disguise of the genome by encoding unusual nucleotides (e.g. hydroxymethyl cytosine in T-even phages; Wyatt and Cohen, 1952); (ii) deletion of restriction enzyme recognition sites from the phage genome (e.g. in T7; Rosenberg et al., 1979); or (iii) inhibition of host nucleases by phage-encoded enzymes (e.g. P1, T3 and T7; Kruger and Bickle, 1983; Kruger et al., 1977).

Multiplication and Maturation (Latent Period*)

Completion of host cell penetration marks the start of the eclipse – that is, the time during which no mature phage particles are found in the bacterial cell. In T4-like phages, the start of eclipse is associated with a take-over of the host metabolism, which commences when early phage genes are recognised and transcribed by the bacterial RNA polymerase (Mailhammer et al., 1975). These early genes encode proteins that 'customise' the bacterial cell metabolism to enable phage formation. Thus, the replication of bacterial DNA and bacterial protein synthesis are shut down. Most virulent phages will bring the non-phage associated metabolism of a host to a complete

*Latent period: Time from infection to release of progeny, determined by the one-step-growth experiment (Ellis and Delbrück, 1939)

standstill. Some phages, such as T4, even destroy the DNA of the bacterial host (Bouet et al., 1994). Once phage controlled-protein synthesis is established, transcription and translation of the middle genes (encoding enzymes that control phage DNA and protein synthesis) and late genes (encoding structural proteins and enzymes that package the DNA) commences (Guttman et al., 2005). Heads (procapsids), tails and long tail fibres are produced via separate enzyme pathways. The phage DNA is created as a long strand of repeated DNA sequences that are linked end to end (i.e. a concatemer; reviewed by Birge, 2000). The DNA is packaged into the formed procapsids and, once the capsid is filled with DNA, cut from the concatemer by a terminase. Finally, late proteins assemble DNA-filled capsids, tails and long tail fibres. Completion of the maturation process marks the end of the eclipse. More and more mature phage particles assemble inside the bacterial host cell until lysis of the host cell occurs. Lysis marks the end of the latent period.

Lysis

Rapid lysis of bacterial cells by the lytic systems of phage is one of the main features explored in phage-based antimicrobial therapy. Cell lysis is a phage-induced process that results in the release of progeny phage from the host cell. In tailed phages, the holin-endolysin system is responsible for lysis (reviewed in detail by Wang et al., 2000). The holin, a phage-encoded protein, is programmed to trigger lysis when enough progeny phage has accumulated in the host cell and conditions for survival of the progeny phage after cell lysis are optimal. Thus, holin activation determines the individual burst size and the end of the latent period. At a defined time, the holin that accumulates in the host cell during phage multiplication attacks the host cell wall and forms holes. After hole-formation, the muralytic phage-encoded endolysin rapidly cleaves peptidoglycan bonds of the bacterial cell wall. This process results in destruction of the bacterial membrane potential, disruption of the protective cell wall and, ultimately, cell death. Lysis may not occur for some phages, such as T4, if another T4 phage attempts to infect the bacterium that already hosts a T4 phage. In that case, a complex enzymatic pathway is activated that aborts the scheduled lysis of the bacterial cell. Consequently, progeny continues to accumulate inside the bacterial cell until a change in the energetic state of the cell wall causes bacterial lysis (reviewed by Young and Wang, 2006).

The Lysogenic Lifecycle

As mentioned previously, temperate phages have the ability to enter a latent or lysogenic lifecycle in addition to the vegetative cycle (Lwoff, 1953). During the lysogenic lifecycle, no mature infectious phage particles are produced (Lwoff, 1953). Induction of the lysogenic cycle is a complex process regulated by several early phage genes that are transcribed immediately after infection. In general, a phage will enter the lysogenic cycle if conditions for the production of progeny virions are suboptimal (e.g. when the bacterial host is starving; as discussed in detail by Kutter and Sulakvelidze, 2005). If that is the case, the transcription of phage repressor proteins will be promoted (reviewed in Calendar, 2006). Subsequently, repressor proteins bind to the phage genome and suppress its transcription. The latent phage genome, the so-called prophage, may integrate at specific or random sites into the bacterial genome (reviewed by Birge, 2000). Less commonly, the prophage persists as an episome in the cytoplasm of the bacterial host (cited by Ackermann and DuBow, 1987a). The replication of the phage genome occurs in synchrony with the bacterial genome and during bacterial cell division, each daughter cell inherits a copy of the prophage (Lwoff, 1953). The lysogenic lifecycle is maintained until the prophage escapes the regulation of the repressor (as discussed by Ackermann and DuBow, 1987a). Once activated, integrated prophages are excised from the bacterial genome and enter the vegetative cycle. Spontaneous prophage excision and replication occurs approximately once every 10,000 cell divisions (Ackermann and DuBow, 1987a). However, prophage excision may be induced by agents that damage DNA (e.g. UV light) or by substances that inhibit DNA synthesis (e.g. nalidixic acid; reviewed in detail by Ackermann and DuBow, 1987a).

Phage Therapy and Temperate Phage

The ability of temperate phages to undergo a lysogenic lifecycle poses several problems to phage therapy:

A. Phage therapy may fail if phage infection is not associated with lysis of bacterial cells

As mentioned previously, bacterial cell lysis does not occur when temperate phages enter the lysogenic lifecycle (Lwoff, 1953). It may be hard to predict whether a temperate phage will enter the lysogenic lifecycle *in vivo* and phages may react differently in different hosts.

B. Prophages may repress the replication of other phages

A bacterium that carries a prophage (a so-called lysogen) may be protected from lysis by other phages if these phages are susceptible to the repressor protein encoded by the prophage. The repressor protein may then bind to the genome of the superinfecting phage and suppress its replication (as discussed by Guttman et al., 2005).

C. Temperate phages may inhibit the infection and spread of other phages

Temperate phages may encode phage exclusion proteins (reviewed in detail by Snyder, 1995). Phage exclusion proteins exist in the cytoplasm of lysogenic bacteria in inactive form. They are activated by a different phage that superinfects the lysogenic bacterium. In contrast to phage repressor proteins, phage exclusion proteins do not inhibit transcription of phage DNA. Instead, they cause death of the bacterial host. Cell destruction occurs before the superinfecting phage completes replication and assembly. Thus, these phage exclusion systems inhibit the spread of superinfecting phages and protect other bacterial cells carrying the prophage from lysis by superinfecting phages.

D. Temperate phages may confer pathogenic traits on their hosts

The genome of temperate phages may contain genes that encode virulence-associated traits. These virulence traits may be transcribed during the lysogenic lifecycle or prophage induction (as discussed in more detail below).

Due to the associated problems, most clinicians that conduct phage therapy trials avoid the use of temperate phages (Sulakvelidze and Kutter, 2005). If a specific temperate phage exhibits highly desirable host specificity, genetically engineered lytic mutants of temperate phages may be developed using recombinant DNA technology (Merril et al., 1996).

Phage as Virulence-Enhancing Agent

Phage may contribute to the virulence of a bacterial strain by transferring virulence traits, such as toxins, adhesins or antibiotic resistance properties, from one bacterial strain to another (as discussed by Wagner and Waldor, 2002). This process is called transduction (reviewed in detail by Birge, 2000). Transduction has been categorised into specialised transduction or generalised transduction. Specialised transduction is a feature of temperate phages that consistently integrate at a certain place in the bacterial genome (e.g. λ, Φ80). During subsequent induction, excision of the phage genome from its integration site may be imprecise, that is, part of the bacterial genome may be excised along with the phage genome by mistake. During replication of the excised sequence (including the bacterial part), numerous transducing phage particles are produced. Specialised transduction is complete when the transducing phage infects another bacterial host cell and recombines with the 'innocent' bacterial genome.

In generalised transduction, the transduction event occurs during phage assembly. Phage enzymes involved in cutting and packaging of DNA erroneously recognise bacterial DNA sites, which are then packaged into the procapsid. Generalised transduction is mainly observed in temperate phage (e.g. P22, P1; cited by Birge, 2000). However, packaging errors may also be observed in lytic phage particles. These "gene transporters" are rarely able to replicate, because most, if not all, of the phage genome is replaced by bacterial DNA (as discussed by Birge, 2000).

Transduction contributes to the virulence of bacterial strains only if the introduced DNA encodes a virulence trait that is functional upon recombination and if the virulence trait is expressed by the recipient bacterial host. Well-known virulence traits that are conferred by transducing phage include the cholera toxin of *Vibrio cholerae* (Waldor and Mekalanos, 1996), some types of the botulinum toxin of *Clostridium botulinum* (Fujii et al., 1988) and the shiga toxins (stx1 and stx2) that can be found in Shiga toxin-producing *E. coli* (STEC) and *Shigella dysenteriae* (Schmidt, 2001). *E. coli* lysogens may also carry (i) the *bor* gene, a serum resistance conferring gene with high similarity to the *iss* gene of ExPEC (Barondess and Beckwith, 1995); (ii) the *eib* gene, which encodes an immunoglobin-inhibiting and serum resistance-conferring OMP (Sandt and Hill, 2000); (iii) the *lom* gene, which encodes an OMP that may promote adherence to buccal epithelial cells (Vica Pacheco et al., 1997); and (iv) an enterohaemolysin (*Ehly*;

(Beutin et al., 1993). Other phage-encoded VFs of gram-positive and gram-negative bacteria other than *E. coli* have recently been reviewed by Wagner and Waldor (2006) and Boyd and Brussow (2002).

In addition to transduction, phage may contribute to the pathogenesis of bacterial disease by (i) promoting the expression of virulence traits during the induction of prophage; (ii) releasing virulence-associated molecules, such as lipopolysaccharides, during cell lysis; or (iii) promoting the expression of surface proteins that have pathogenic properties (reviewed by Wagner and Waldor, 2002).

Phages that enhance the virulence of bacterial pathogens are inappropriate for phage therapy (as discussed by Sulakvelidze et al., 2001). The exclusion of temperate phage from treatment plans may greatly minimise the introduction of undesirable phage into therapeutic products. The risk of applying undesirable phages may be further minimised by screening the genomes of candidate phages for known virulence-associated traits (as discussed by Skurnik and Strauch, 2006).

The Influence of Chemical and Physical Agents on Phage Viability

Before phages adsorb to susceptible bacteria in the host, they are likely to be exposed to substances or conditions that may influence their infectivity. Susceptibility to chemical or physical agents may differ from phage to phage. Therefore, the resistance of a certain phage to chemical or physical agents that are encountered before its designated use (i.e. during preparation and storage) or *in vivo* should be tested to evaluate the likelihood of therapeutic success.

Much of what we know today about phage resistance originates from historical phage research and preparation methods, laboratory experiments with phage or from preventative measures against phage in the dairy industry (as discussed by Ackermann and DuBow, 1987b and Raettig, 1958). The effect of a few substances or conditions of interest for phage therapy is given below.

Substances that Affect Adsorption

As discussed in chapter 1.2.4.2, some phages need co-factors (mainly cations) to attach properly to the target bacterial strains (Ackermann and DuBow, 1987a). Natural or artificial chelating agents (e.g. chlorophyll, citrate or ethylenediaminetetraacetic acid; EDTA) may bind cations and thus considerably decrease the adsorption rate of phage that require cations. Bile salts and carbohydrates may prevent phage adsorption to bacteria in a similar way (Gabig et al., 2002). Alternatively, these substances may act on bacterial cells and prevent the expression of phage-recognised receptors (Wegrzyn and Thomas, 2002).

Substances that Affect the Viability of Phages

The lack of knowledge about the susceptibility of phage to heat, mercury components or disinfectants (e.g. phenol) may have contributed significantly to the failure of early phage therapy (as discussed by Carlton, 1999; Summers, 2001 and Sulakvelidze et al., 2001).

In general, the viability of phages may be affected by damaging the phage protein coat and structures necessary for adsorption or by damaging the nucleic acid that is contained within the phage capsid. Substances that damage the protein coat include acids and alkalis. The pH resistance of phage to acids needs to be considered - particularly if phages are administered orally and pass the stomach before they reach the site of infection. Phage applied by Smith and Huggins (1983) for the treatment of diarrhoea in calves and lambs, for example, was indefinitely resistant to a pH 3.5. However, at pH 2.5 and 2 the viability of most *E. coli* phages decreased by 100-fold in less than 30 minutes and one minute, respectively (Smith et al., 1987b). *In vivo* titres of phages in the gastrointestinal tract of calves were significantly reduced during passage of the acidic abomasum (pH 1.6-2.3; Smith et al., 1987b). To maintain the viability of phages, some practitioners of phage therapy prefer to administer oral phage in tablets that have a protective coating (reviewed by Sulakvelidze and Kutter, 2005) or administer antacids before phage is given (Slopek et al., 1983). If no protective or neutralising agent is administered, some phages sensitive to acids *in vitro* may pass the stomach and, possibly protected by the gastric chyme, reach the gastrointestinal tract without losing their viability (Weld et al., 2004). The exact mechanisms of this *in vivo* resistance of

phages to acids remain to be elucidated. Overall, pharmacokinetic studies that determine phage titres in different parts of the gastrointestinal tract should be conducted when considering the oral application of phage.

Other substances that may damage the phage coat include proteolytic enzymes or amines, such as urea (reviewed by Guttman et al., 2005). The detrimental effect of these substances is dependent on the concentration of the substance and environmental conditions (e.g. temperature). Vitamin C has been suggested to "reactivate" phage that has been damaged by oxidation (Lominski, 1935 cited in Raettig, 1958).

The destruction of the phage nucleic acid may occur in the presence of mutagens (e.g. UV-light, nitric oxide or carcinogenic dyes; reviewed by Ackermann and DuBow, 1987b). Mutagens are commonly applied to induce temperate phages. In phage therapy, the effect of UV light and temperature on the viability of phage during storage of phage preparations may need to be considered.

The Interaction of Phage and Bacterial Host in the Animal

The *in vivo* efficacy of phage that is able to lyse the target bacterium *in vitro* will largely depend on the distribution and density of phages in different tissues of the host and their elimination from the host (i.e. the pharmacokinetic and antigenic properties of phages) and the interaction between phage and bacteria in living hosts (i.e. the pharmacodynamic properties of phage). These properties are likely to differ for every animal-phage-bacterial system.

Pharmacokinetic Characteristics and Immunogenicity of Phage

Phages must reach the site of infection in order to eliminate bacterial infections in the animal under treatment. Furthermore, phages must adsorb to bacteria at the site of infection before they are eliminated by the immune system of the animal. Previous pharmacokinetic studies of phages in mice, rabbits and humans indicate that phages can enter the bloodstream of the host, irrespective of the route of administration (i.e. oral, intraperitoneal or intramuscular; Biswas et al., 2002; Dabrowska et al., 2005; Dubos et al., 1943; Geier et al., 1973; Smith and Huggins, 1982; Weber-Dabrowska et al., 1987). Phages are readily distributed via blood vessels to other organ systems, such as the liver,

kidney, thymus and spleen (Geier et al., 1973). Phages have been shown to cross the blood-brain barrier (Barrow et al., 1998; Dubos et al., 1943; Smith and Huggins, 1982) and diffuse from the circulation into the urine (Keller and Engley, 1958; Reynaud et al., 1992; Weber-Dabrowska et al., 1987). In healthy mice, phages were readily found in the urine when a plasma titre of approximately 10^5 plaque-forming units (PFU)/ml was exceeded (Schultz and Neva, 1965 cited by Dabrowska et al., 2005). Reynaud et al. (1992) reported that an O103-specific coliphage, administered orally at a dose of $5x10^9$ PFU, persisted in the kidneys of uninfected rabbits for up to 4 days, but was not detected in the urine after 2 days. This may indicate that phages sequester to some extent in the kidneys. However, the exact mechanism of diffusion of phages from the circulation into the kidneys remains to be elucidated.

Several studies indicate that phage is rapidly removed from the circulation and trapped in the liver or spleen, where phage is inactivated by Kupffer cells and macrophages, respectively (Barrow et al., 1998; Geier et al., 1973; Inchley, 1969; Merril et al., 1996; Smith and Huggins, 1982). The elimination of phage by the immune system may have a detrimental effect on the treatment outcome if phages need to enter the circulation to reach the site of infection (Merril et al., 1996). However, phage sequestration by the splenic reticuloendothelial system may be avoided by engineering or isolating phage that lacks antigens recognised by the reticuloendothelial system (so-called long-circulating phage; Merril et al., 1996). Phage therapy may also fail if anti-phage antibody inactivates therapeutic phages (Gachechiladze, 2005; Smith et al., 1987b). Anti-phage antibody formation may decrease the phage adsorption rate and infectivity by clumping phages together or by binding to structures of individual phages that are necessary for adsorption and infection of bacterial host (Gachechiladze, 2005). If the host was previously exposed to (environmental) phage that exhibits identical or similar antigens, anti-phage antibodies may be already present at the time treatment is initiated and titres in these patients may rise further during treatment (Kucharewicz-Krukowska and Slopek, 1987; Smith et al., 1987b). However, the degree of antibody formation has been shown to differ for each phage and phages that provoke a very limited immune response have been identified (Smith et al., 1987b). Furthermore, the risk of formation of long-lasting, high affinity antibodies (IgG) may be minimised by administering phage locally and only once (as discussed by Sulakvelidze and Kutter, 2005). If multiple treatments are necessary, IgG formation may remain low when phage is given

repeatedly within a short time interval (as discussed by Sulakvelidze and Kutter, 2005). In the case where no alternative phage is available, a therapeutic phage titre may be maintained by administering the original (antigenic) phage at a dose or rate that is higher than the amount or rate at which phage is inactivated or eliminated by the host (Carlton, 1999). Ultimately, phage will be eliminated from the host if it cannot replicate in susceptible bacteria. During the active replication process, the phage titre in different tissues depends on (i) the density and reproduction rate of target bacteria; (ii) the initial titre and phage growth parameters*; and (iii) the rate of elimination of phage by the immune system (Levin and Bull, 1996; Payne and Jansen, 2003).

Pharmacodynamic Properties of Phage

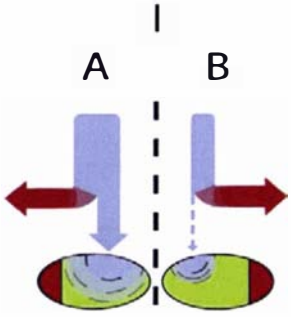
In conventional phage therapy, replicating, evolving phages interact with living, evolving bacteria. To date, our understanding of the dynamic interaction between phages, the bacterial population and animals is almost entirely based on mathematical models (Levin and Bull, 1996; Levin and Bull, 2004; Payne and Jansen, 2001, 2003; Weld et al., 2004). In generic mathematical models, phages and bacteria are likened to predators and their prey (Payne and Jansen, 2001). Phage therapy is considered successful, when the bacterial population (prey) is eliminated by phages (predators) in a particular situation influenced by the host (environment). At present, the accuracy of such generic mathematical models is controversial. Bull et al. (2002), who repeated a Smith and Huggins experiment (Smith and Huggins, 1982), reported that the observations made in that study can be explained with mathematical models. Conversely, Weld et al. (2004) stated that generic models suggested by Payne and Jansen (2001) and Levin and Bull (1996) oversimplified the complex *in vivo* relationship between phage and bacteria in the animal. By comparing theoretical phage growth parameters with observed data derived from experiments with rats, they found that generic models generally overestimated phage growth rates (Weld et al., 2004). Nonetheless, mathematical models have contributed to defining factors that may influence the dynamics of the animal-phage-bacterium system and thus the outcome of

* phage growth parameters: attachment rate, latent period and burst size

phage therapy. Such factors are (i) the growth rates of phages and bacteria; (ii) the phage titre at administration in relation to the population of bacteria; (iii) the timing of phage administration; and (iv) factors that influence the replication rates of phages and bacteria, such as the immune response, antibiotics and the development of bacterial resistance to phage (Levin and Bull, 1996; Payne and Jansen, 2001, 2003).

Four animal-phage-bacterium scenarios that are discussed in the literature are described below (Bull et al., 2002; Levin and Bull, 1996; Levin and Bull, 2004 and Payne and Jansen, 2001, 2003).

Scenario I - Dose in Relation to the Bacterial Population Density



Scenario I A:

Phage therapy will be successful if the initial dose is high enough to establish a phage titre that can eliminate all bacteria during the initial phage replication cycle or subsequent replication cycles. The bacterial titre must be high enough for phage to encounter bacteria (as discussed in scenario II).

Scenario I B:

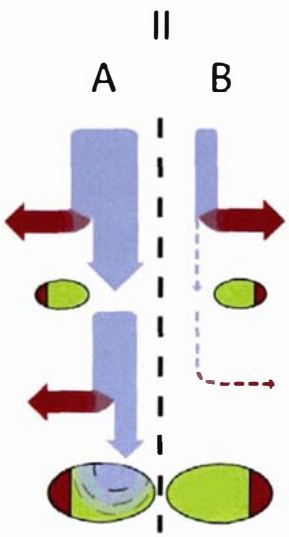
Phage therapy will fail if more bacteria are produced than are lost. This may be seen if the initial phage dose is too low to establish a phage titre that is able to halt the exponential growth of bacteria. Alternatively, the phage dose may be adequate, but the replication rate of phage may be too low.

Removal of phage by the immune system of the animal should always be considered.

Key

Phage Bacterial Population Host immune system

Scenario II – Dose in Relation to the Time of Administration

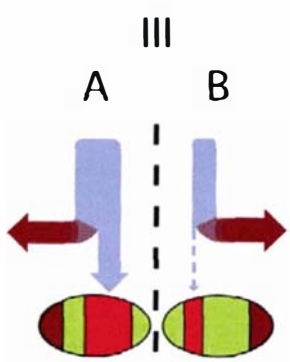


Scenario II A and B.

Phage therapy will fail if the density of the bacterial population at the time of phage administration is too low for phage to encounter bacteria. However, bacteria may grow to sufficient numbers over time. If phage can maintain its presence in the animal until bacteria are present in sufficient numbers (scenario II A), elimination of bacteria by phage is possible (as illustrated in scenario I A). Phage therapy will fail if the phage is lost before bacteria have grown to sufficient numbers (scenario II B).

If phage is given too late, valuable time during which phage can successfully replicate may be lost. Morbidity and mortality rates may rise due to the increasing release of bacterial toxins or bacterial tissue destruction. Moreover, bacteria may become inaccessible to phage (e.g. if bacteria are walled in granulation tissue) or bacteria may become resistant to phage by entering a dormant stage.

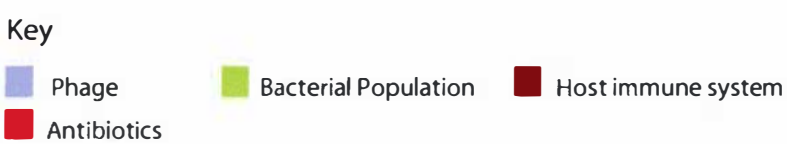
Scenario III - Combination of Phage with Antibiotics



Antibiotics may decrease the efficacy by decreasing the density of the bacterial population (see scenario II).

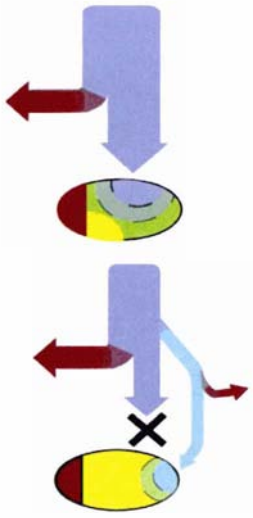
If a low phage titre is present, even a minimal effect of antibiotics may have a detrimental effect on phage therapy (as illustrated in scenario III B).

The specific influence of antibiotics on phage-infected cells and phage replication may depend on the mechanism of action of antibiotics (as discussed in more detail below).



Scenario IV – Bacterial Resistance to Phage

IV



Treatment with phages will select for bacteria that are resistant to that particular phage. Over time, the density of the resistant bacterial population will increase. However, phage types that are able to infect resistant strains may evolve from the phage population. If mutant phage types evolve, phage therapy has the potential to succeed.

Key

Phage
Mutant Phage
Bacterial Population
Resistant Bacterial Population
Host immune system

Phage and Antibiotics

One key difference between phage therapy and antibiotic therapy is that the phage titre can increase *in vivo* due to replication in target bacteria while the *in vivo* concentration of antibiotics declines invariably from the time they are administered (as discussed by Sulakvelidze and Kutter, 2005). Furthermore, phages may co-evolve and counteract the development of bacterial mutants resistant to the original phage. When bacterial resistance to antibiotic emerges, a different antibiotic or a higher concentration of the same antibiotic must be used to counteract antibiotic resistance. Phage therapy and antibiotic therapy also differ in regards to the likelihood that widespread bacterial resistance will develop, host specificity, their mode of action (i.e. bactericidal or bacteriostatic) and the frequency of adverse effects (Table 1.3).

Table 1.3 Phage and antibiotics: Key therapeutic differences[a]

Parameter	**Phage**	**Antibiotic**
Host Specificity	**High**	**Depends on the antibiotic class. Commonly lower than phage.**
Comment	Contrary to most antibiotics, phages may eliminate specific pathogens while leaving the commensal bacterial flora intact. Thus, adverse effects associated with an imbalance of the commensal microbial flora are unlikely to occur. However, more than one phage may be necessary to lyse all pathogens of one bacterial species or subspecies and *in vitro* susceptibility tests before administration of phage are likely to be essential in all cases of phage therapy.	
Development of wide-spread bacterial resistance in non-target species	**Considered unlikely**	**Possible**
Comment	Bacteria are likely to develop resistance to phage. However, in contrast to antibiotics, the use of phage may not select for resistance in non-targeted (environmental) bacteria. Furthermore, phage may co-evolve with bacteria and phage mutants able to infect resistant bacteria may develop.	
Mode of action	**Bactericidal**	**Bacteriostatic or bactericidal**
Comment	Contrary to bacteriostatic antibiotics, phage may eliminate pathogenic bacteria from the host without the help of the immune system. Thus, the use of phages may not be limited to immunocompetent patients.	
Occurrence of adverse effects	**Considered rare**	**Allergies or adverse effects caused by elimination of commensal bacteria or by the release of endotoxins may commonly occur.**
Comment	Most adverse effects reported in phage therapy may be due to the release of endotoxins during bacterial lysis.	

[a] reviewed by Sulakvelidze and Kutter, 2005

Smith and Huggins (1982) showed that carefully selected phage was more effective than conventional antibiotic therapy in rescuing mice from potentially lethal *E. coli* infections. Similar results were also observed in several human studies conducted in the former Soviet Union (cited by Alisky et al., 1998; Chanishvilli et al., 2001 and Sulakvelidze et al., 2001; refer to appendix Table 8.2). When using phages and antibiotics in combination, treatment outcome is likely to depend on the interaction between phage and antibiotic and the timing of their administration. Previous clinical trials show that a combination of phage and antibiotics may be either more or less effective than the use of phage or antibiotics alone. For example, Kochetkova (1989) observed a higher recovery rate from various infections when patients were treated with

a combination of phage and antibiotics, compared to treatment with antibiotics alone (reviewed by Sulakvelidze and Kutter, 2005; listed in appendix Table 8.2). Conversely, Slopek et al. (1987) showed that phage-only treatment was more effective than treatment with a combination of phage and antibiotics. At present, little is known about the influence of antibiotics administration on the efficacy of phages. As discussed previously, it has been hypothesised that administration of antibiotics may be detrimental to phage therapy, because the number of viable bacteria in the host may decrease below a threshold at which phage are likely to encounter bacteria (Payne and Jansen, 2001). Furthermore, antibiotic application is likely to decrease the efficacy of phages if antibiotics interfere with phage replication and maturation by inhibiting protein synthesis or DNA synthesis. This could occur if aminoglycosides and tetracyclines or rifampin, fluoroquinolones, trimethoprim and sulphonamides are used (Walsh, 2003b). However, if antibiotics act synergistically with phage enzymes to shut down specific metabolic pathways of bacteria, treatment efficacy may increase. Similarly, the administration of antibiotics that inhibit bacterial cell wall synthesis (e.g. β-lactams, bacitracin) may be detrimental if the associated cell destruction occurs before sufficient progeny phage has accumulated in the bacterial cell. However, if cell wall destruction by antibiotics occurs concurrently with the action of the holin-lysin system, overall treatment success may increase. The exact influence of specific antibiotics on phage therapy remains to be elucidated.

Bacterial Resistance to Phage

In addition to the intrinsic resistance that exists when bacteria do not exhibit the receptors necessary for phage attachment, resistance to phage may evolve. In 1943, Luria and Delbrück showed with the so-called fluctuation test that the development of bacterial resistance to phage was not induced by phage, but occurred at random through mutation (Luria and Delbrück, 1943). Subsequently, phage-resistant strains may grow to sufficient numbers when phage is present, while susceptible bacterial strains decline in numbers (i.e. the phage selects for resistant bacteria). Bacteria may become resistant to a specific phage if mutation affects the phage receptor gene. Mutation may render the receptor unrecognisable for phage or results in the complete loss of the phage receptor (Levin and Bull, 2004). Phage that adsorbs to bacterial VFs may select for resistant strains that lack these factors and are therefore much less pathogenic than the parent strains (Smith and Huggins, 1982). This selection for avirulent mutants is considered an important advantage of phage over antibiotics, where resistant mutants often exhibit the same pathogenicity as parent strains (Smith and Huggins, 1982).

Partial resistance to phage may develop if bacteria produce extracellular matrix (e.g. slime, capsules) that covers the receptor (Levin and Bull, 2004). This type of resistance is not necessarily associated with mutation and may inhibit attachment of many phages, irrespective of the receptor specificity. Of note, some phages, such as K1 coliphages, are able to infect encapsulated bacteria by secreting enzymes that depolymerise capsules (Mushtaq et al., 2005). Other mechanisms of resistance development are (i) the acquisition of endonuclease genes that encode proteins which destroy phage DNA; (ii) the mutational change of genes that encode proteins essential for phage replication (as discussed by Skurnik and Strauch, 2006); or (iii) the acquisition of a prophages that encode repressor or phage exclusion proteins (as discussed in chapter 1.2.4.2.)

1.2.4.3. Revival of Phage Therapy in Western Europe and America

The emergence of antibiotic resistance became increasingly apparent in the late 1970s and early 1980s, when pathogens emerged that were resistant to (i) reserve antibiotics (e.g. methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant enterococci); or (ii) the majority of antibiotics available (e.g. multi-drug resistant *Mycobacterium tuberculosis*; Alanis, 2005). In fear of a so-called 'post-antibiotic era', international research focused on finding alternative or new antimicrobials – and turned once again towards phage.

Smith and Huggins were the first to re-evaluate phage therapy in the Western World after its abandonment in the 1940s (Smith and Huggins, 1982, 1983; Smith et al., 1987a, b). In a series of well-designed experiments, they showed that phages were able to rescue or protect mice from *E. coli* meningitis caused by an O18:H7:K1 strain (Smith and Huggins, 1982) and protect or cure calves, piglets and lambs from diarrhoea caused by a potentially lethal enteropathogenic *E. coli* (Smith and Huggins, 1983). As mentioned previously, phage treatment of systemic infections in mice proved to be superior to treatment with a number of commonly used antimicrobials (streptomycin, tetracycline, ampicillin, chloramphenicol and trimethoprim and sulphurazole; Smith and Huggins, 1982). These experiments were set up to study the *in vivo* dynamics of phage and *E. coli*, not just the clinical effects of phage (Smith and Huggins, 1982). Bacterial strains had been isolated from natural disease outbreaks. Their serotypes and disease-associated VFs had been determined. Meningitis strain O18:H7:K1 showed *in vitro* susceptibility to all antimicrobials used in the study (as assessed by determining the minimal inhibitory concentrations (MIC; Smith and Huggins, 1982). Lytic phages (a set of K-antigen-specific phages and phages that did not recognise K-antigen) were isolated from sewage and chosen because they were highly virulent against the test pathogens *in vitro*. Clinical trials included negative control preparations (an ultrasonic preparation of bacteria in broth) and untreated control animals. A non-blinded trial design was chosen. All animals were inoculated with a standard dose of bacteria that was given either intramuscularly (causing septicaemia), orally (causing diarrhoea) or intracerebrally (causing meningitis). Phages were given at pre-determined doses intramuscularly (for the treatment of meningitis and septicaemia) or intravenously (to assess the distribution of phage in the mice). Phages were given either prior to,

concurrently with or after bacterial inoculation. For the treatment of diarrhoea, phages were given orally. The possibility of a direct contact between phage and bacteria at the inoculation was minimised by injecting phages and bacteria into different muscles or leaving a time-gap between the oral administration of phages and bacteria. The minimal effective dose of a specific phage type was determined by injecting different mice with an increasing titre of phages. At pre-determined times the efficacy of phage treatment was evaluated (i) by assessing the clinical condition of mice, calves, piglets or lambs; and (ii) by determining the phage titre. In trials assessing the efficacy of phages for the treatment of diarrhoea of calves, piglets or lambs, the concentration of enteropathogenic *E. coli* and its mutants in the faeces was also determined. The distribution of phage, original bacteria and mutants in different body compartments (e.g. blood, spleen, brain, different parts of the gastrointestinal tract) was assessed after sacrificing animals (treated and untreated) at different times during the course of the trial. Treatment or prophylaxis with phages was associated with a significant decline in the number of pathogenic bacteria. After intramuscular injection, phage swiftly entered blood vessels and reached distant sites, including the brain. Phage was present in the circulation for more than 24 hours and the titre at the side of infection and in the spleen remained high at least as long as significant numbers of sensitive bacteria were present. Resistant mutants were observed *in vivo*. However, *in vivo* resistance was observed less frequently than predicted from *in vitro* tests conducted as part of the experiment. A possible explanation for this discrepancy is the action of the host immune system. The application of phage that targeted virulence factors (K-antigen) resulted in phage-resistant mutants that were often significantly less virulent than their parent strains. Thus, they were probably less resistant to removal by the immune system than their parent strain. Phage that had evolved from the original phage types and was able to infect phage-resistant mutants was also readily found. Smith and Huggins concluded that single-dose phage treatment is efficacious, both prophylactically and therapeutically. They suggested that phages might be particularly useful in the control of diseases of animals in intensive farming environments. This hypothesis was confirmed in a subsequent study, which showed that phage protected or cured calves from experimental *E. coli* diarrhoea by administering phage in milk or simply by subjecting calves to phage contaminated housing (Smith et al., 1987a).

The success of the experiments by Smith and his colleagues, together with emerging information concerning successful phage treatment of human infectious diseases in the East, rekindled the interest in phage therapy in the West.

1.2.4.4. Phage Therapy after its Revival

From the early 1990s, research has focused on (i) phage treatment of disease epizootics in farmed animals, such as chicken, calves and fish; (ii) the potential use of phage for treating infections with bacteria that are resistant to antimicrobials; and (iii) the potential use of phage to eliminate human health threats. In recent years, new approaches that utilise phage components or genetically modified phage have been added to conventional phage therapy protocols.

Phage to Control Animal Disease Epidemics

Phage may have the potential to fill the antimicrobial treatment gap that has formed since particularly stringent restrictions on the use of antimicrobials in animal foods were put in place (Phillips et al., 2004). In 1998, Barrow et al. successfully applied phage to cure or prevent potentially lethal *E. coli* meningitis in chickens and septicaemia in chickens and calves. They also studied the distribution of phage and bacteria in different body compartments of chickens or calves. Phages readily reached the site of infection (i.e. brain, circulation) after intramuscular injection. Furthermore, Barrows et al. (1998) demonstrated that phages multiplied at the site of infection, which resulted in a significant decrease in bacterial numbers. The authors concluded that phages are potentially useful in treating or preventing *E. coli* infections of farmed animals. However, for phage treatment to be practical, phage administration would need to be simple and swift (Barrow et al., 1998). Subsequently, Huff and colleagues conducted a series of studies in which phage containing drinks, phage aerosols or intramuscular phage preparations were used to treat experimental *E. coli* respiratory disease in chicken (Huff et al., 2002a, 2003a, b; Huff et al., 2002b). Phages selected for these trials were naturally occurring O2-specific *E. coli* phages that produced clear plaques when incubated with the target *E. coli* strain. These phages did not protect chickens from an experimental air sac infection when given with the drinking water at a dose of 10^6

pfu/ml water (Huff et al., 2002b). However, a significant decrease in mortality was seen when 2 different phage types were given in aerosol form at higher doses ($2x10^8$ to $2x10^9$ pfu/ml aerosol) either one day prior to or on the day of the *E. coli* challenge (Huff et al., 2002a). Administration of the phage aerosol 3 days before *E. coli* challenge was non-protective. A subsequent study, which determined the phage titre in the blood after aerosol administration, showed that phages persisted less than 24 hours in the circulation of unchallenged birds (Huff et al., 2003b). If serum phage titre correlated with the phage titre in the lung, it is likely that treatment was unsuccessful because the phage titre in the lung had declined below a therapeutic level. Compared to aerosol administration, intramuscular injection of phage (0.1 ml of a preparation with 2 phage types at $6x10^8$-$1.34x10^9$ pfu/ml) resulted in a greater titre and longer persistence of phage in the circulation of unchallenged birds (Huff et al., 2003b). Furthermore, it was associated with a significant decrease in mortality if given up to 2 days after the *E. coli* challenge. Overall, treatment delay was associated with a higher mortality compared to treatment immediately after *E. coli* challenge (Huff et al., 2003a). However, once-only intramuscular injection of phage on the day of *E. coli* challenge was less effective than the conventional 7-day treatment with enrofloxacin (given in the drinking water from the day of the challenge; Huff et al., 2004). A combination of phage and enrofloxacin resulted in a complete recovery of all birds challenged. While the mechanism of interaction between phage and antibiotics was not investigated, the authors suggested that phage preparations might be useful supplements to antibiotics when treating avian colibacillosis. However, more practical approaches to phage therapy in chickens remain to be elucidated.

Nakai et al. (1999) and Park et al. (2000) successfully applied phages to decrease the mortality in fish infected with *Lactococcus garvieae* (haemorrhagic septicaemia of yellowtail) or *Pseudomonas plecoglossicida* (haemorrhagic ascites of ayu). Phages were isolated from diseased fish or water. Prior to the treatment trials, morphological and genotypic characteristics of phages were assessed by electron microscopy and restriction enzyme analysis, respectively. Phages were shown to maintain viability *in vitro*, in conditions similar to those observed in water and fish. Phage was applied by intraperitoneal injection (Nakai et al., 1999) or, in a more practical approach, as phage-impregnated feed (Nakai et al., 1999; Park et al., 2000). Phages crossed the intestinal barrier within 3 hours after feeding. They replicated and persisted in the intestine and

the spleen or kidney at least until the bacterial infection was eliminated. When phage resistant bacteria were observed, they were less virulent than the parent bacterial strain (Park et al., 2000). Resistant mutants did not result in death even if administered at doses that were 2 orders of a magnitude higher than the dose of the parental bacterial strain that caused 100% mortality. Treatment was shown to be successful even when the phage was administered 24 hours after bacterial challenge.

Phages to Control of Bacterial Infections in Humans

Antimicrobial resistant bacteria, which are considered a severe health threat for immunocompromised humans, have been the target of several studies that aim to use phage for human therapy (Biswas et al., 2002; Broxmeyer et al., 2002; Matsuzaki et al., 2003; Soothill, 1992). In one such study, Biswas et al. (2002) applied naturally occurring phage to treat experimental infections with vancomycin-resistant *Enterococcus faecium* in mice. This well-designed study showed that (i) lytic phages against vancomycin-resistant *E. faecium* can be readily isolated from sewage; (ii) the therapeutic effect observed is due to a phage-specific elimination of bacteria from the body (as opposed to an immunogenic effect); and (iii) systemic application of phage therapeutics prepared by caesium chloride centrifugation was not associated with anaphylactic reactions. In accordance with other studies, a dose-dependent and time-dependent effect of phage therapy was observed (Biswas et al., 2002; Huff et al., 2003a; Matsuzaki et al., 2003; Nakai et al., 1999; Park et al., 2000; Smith and Huggins, 1982). The use of phage to treat systemic infections in mice, caused by methicillin-resistant *Staphylococcus aureus* was explored by Matsuzaki et al. (2003). Interestingly, they used a temperate phage that was isolated from a *S. aureus* strain after induction. The complete phage genome was sequenced and screened for undesired traits before its use in therapy. Intraperitoneal injection of caesium chloride-purified phage preparations at a titre equivalent to a multiplicity of infection rate* of $\geq$ one significantly reduced the lethality in mice and was not associated with adverse effects. Apparently, the use of a temperate phage did not reduce the efficacy of phage treatment. It is possible that

* Multiplicity of infection rate: The average number of particles that infect a single bacterial cell in a specific experiment

phages entered primarily the vegetative cycle, because *in vivo* conditions were in favour of immediate phage replication. However, in conditions different to those observed, lysis may be inconsistent. It remains to be elucidated whether the temperate phage used by Matsuzaki et al. (2003) is consistently associated with therapeutic success.

Broxmeyer et al. (2002) presented a particularly interesting approach to treating intracellular bacterial infections with phage. They reported that a bacterial strain with attenuated virulence (*Mycobacterium smegmatis*), capable of entering host cells, may be used to deliver lytic phages into host cells infected with pathogenic bacteria *(M. tuberculosis* or *M. avium*). Upon delivery, phages reduced the number of viable *M. tuberculosis* and *M. avium* organisms significantly. This initial *in vitro* study indicates that phage therapy is not necessarily limited to the treatment of extracellular infections. However, the *in vivo* efficacy of such "bacterial delivery systems" remains to be elucidated.

Another interesting piece of recent research is the potential use of phages to eliminate pathogenic bacteria from an environment permanently inhabited by bacteria (e.g. intestinal tract) without affecting the commensal organisms (Bruttin and Brussow, 2005; Chibani-Chennoufi et al., 2004). Chibani-Chennoufi et al. (2004) and Bruttin et al. (2005) reported that lytic *E. coli* phages did not eliminate susceptible intestinal *E. coli* that had established in the intestinal tract (i.e. commensal organisms). However, phages were able to eliminate *E. coli* that had been recently introduced to the intestinal tract. The authors concluded that the observed *in vivo* resistance of established *E. coli* might be due to the inhibition of phage attachment by physical barriers (e.g. other bacteria, organic matter, mucus) or the lack of bacterial reproduction in the intestinal lumen.

The use of lysis-deficient phage has recently been suggested by researchers of the "phage company" Gangagen (cited by Thiel, 2004). This concept is based on the observation that phage T4 exhibits a bactericidal effect before lysis of the bacterial cell occurs (Padmanabhan et al., 2004). Genetically engineered lysis-deficient phage T4 has been shown to infect and replicate in *E. coli* similar to its wild-type relative. However, instead of destroying the bacterial cell, it accumulates and remains inside the shell of the dead bacterium. This type of therapy is currently been tested on *E. coli* O157:H7. *In vivo* therapy with lysis-deficient phage has been suggested to be more useful than therapy with conventional phages, because (i) the shell of the dead bacterium may serve as an inactivated whole cell vaccine (Bharathi et al., 2004); (ii) endotoxin-associated

adverse effects are unlikely to occur since the bacterial cell wall remains intact; (iii) transduction cannot occur; (iv) the number of viable particles in the body may be better controlled and may be adjusted to levels where an immune response against phage is less likely to occur; and (v) bacterial resistance to phage may be minimised by reducing the number of phages in the body and the environment. However, it remains to be elucidated whether lysis-deficient phage can reach and eradicate deep-seated infections with only one replication cycle.

Phages also showed potential in eliminating bacteria from dental roots (Paisano et al., 2004) and in preventing skin graft failure (Soothill, 1994). Many historical trials suggest that phages may be particularly useful for chronically infected skin, including infected burn wounds. Newer *in vivo* trials confirm that lytic phages have the potential to cure infections with important and often antimicrobial-resistant, skin pathogens (Lazareva et al., 2001; Soothill, 1992). A recently developed wound dressing is PhagoBioDerm, a biodegradable non-toxic polymer impregnated with several lytic phages, ciprofloxacin, benzocaine, α-chymotrypsin and sodium bicarbonate (Markoishvili et al., 2002). The combination of ciprofloxacin and phages apparently does not decrease treatment success - clinical treatment trials with human patients suggest that PhagoBioDerm may be successfully applied to wounds that are refractory to conventional treatment, such as diabetic ulcers (Markoishvili et al., 2002) and skin burns caused by radioactive substances (Jikia et al., 2005).

Phage to Eliminate Human Health Threats

Phages may also be useful for eliminating human pathogenic bacteria before they infect people. Compared to phage preparations for use in human medicine, these so-called "phage decontamination systems" may take less time to reach a commercial stage. This is because (i) the complex *in vivo* interactions between host, phage and bacterium can be disregarded if phages are used *ex vivo*; and (ii) timely and costly clinical safety trials necessary in human medicine may not need to be conducted. Pathogens that have been the target of phage decontamination are bacteria that cause food-borne diseases, such as *Salmonellae*, *Listeria monocytogenes*, *Campylobacter jejuni* and *E. coli* O157:H7 (reviewed by Sulakvelidze and Barrow, 2005). Phage decontamination systems are being developed for (i) livestock produced for human consumption; (ii) excrement of

farm animals that is used as fertiliser and may contaminate food or groundwater; (iii) raw food during food processing; (iv) ready-to-eat food during food storage; and (v) surfaces of facilities that produce, process or store food (reviewed by Thiel, 2004 and Sulakvelidze and Barrow, 2005). Furthermore, phage-based washes and creams have recently been suggested for decontamination of the hands of people working in the food or health industry (O'Flaherty et al., 2005).

Alternative Phage Therapy Approaches

Next to the development of therapeutics that utilise the replicating, evolving nature of living phage particles, antimicrobial strategies are being developed that are based on phage enzymes or use phage to deliver antimicrobial compounds into bacterial cells.

Antimicrobial Therapy Based on Phage-encoded Enzymes

The desired therapeutic effect of phage is bacterial death that commonly coincides with lysis of the bacterial cell. Lysis occurs at the end of the vegetative cycle when phage-encoded enzymes (holins and lysins) attack and destroy the bacterial cell wall (see chapter 1.2.4.2). Lysins destroy bacterial cell walls even in the absence of viable phage particles. Thus, purified lysins have been considered as new small molecule antimicrobials (Fischetti, 2005). Contrary to most antimicrobials, lysins are only active in a subset of bacteria that is identical or very similar to the bacteria that host phages encoding the lysins. This is because lysins bind to specific carbohydrate moieties, which are commonly only present in the cell wall of that subset of bacteria (Loeffler et al., 2001). Therefore, they may be used to target certain pathogenic bacteria specifically (Loeffler et al., 2003; Loeffler and Fischetti, 2003; Loeffler et al., 2001). The use of lysins is currently limited to gram-positive organisms, because lysins that bind to moieties in the cell membrane of gram-negative bacteria have yet to be identified. Lysin antimicrobials have been suggested for (i) protection from anthrax (*Bacillus anthracis*; Schuch et al., 2002); (ii) for eliminating pathogenic *Staphylococcus spp.*, *Streptococcus* spp. and *Pneumococcus* spp. from mucosal surfaces before they can gain entry to human or animal hosts (Cheng et al., 2005; Loeffler et al., 2001; Nelson et al., 2001); (iii) for the treatment of individuals that carry and excrete pathogenic bacteria

Fischetti, 2005); and (iv) for treating patients with potentially life-threatening, systemic infections with gram-positive organisms (Loeffler et al., 2003). Compared to the therapy with viable phages, lysin antimicrobials may be easier to introduce to the commercial market, since it may be less time-consuming and more cost-effective to determine the *in vivo* efficacy and safety of the non-evolving lysins. Furthermore, a significant *ex vivo* amplification and distribution of lysins, and thus possible spread of resistance in the external environment, is unlikely to occur upon completion of therapy. At present, resistance to lysins has not been observed. Fischetti (2005) suggested that resistance development might occur rarely, because lysins commonly bind to structures that are essential for bacterial viability. Lysins have been shown to act synergistically with antimicrobials that target the bacterial cell wall (Loeffler and Fischetti, 2003). Furthermore, they have been observed to remain active in the presence of anti-lysin antibodies (Loeffler et al., 2003). The reason for the retained activity in the presence of antibodies is not completely understood. Results of preliminary studies indicate that binding domain of the lysin binds cell wall receptors with a higher affinity than antibodies (Loessner et al., 2002). However, it is not known why lysis is not inhibited by antibodies that target the enzymatic domain of the lysin (as discussed by Fischetti, 2005).

Another recently developed antimicrobial strategy is based on the ability of phage to shut down the bacterial metabolism shortly after the infection of the host cell (Liu et al., 2004). The "phage genomics approach" utilises phage inhibitory enzymes to identify bacterial proteins that are essential for host cell growth (e.g. proteins involved in DNA replication or RNA transcription). Following the identification of these key bacterial proteins, compounds that inhibit these proteins are found by screening commercially available small molecule libraries. Then, available inhibitors are checked for therapeutic suitability during *in vitro* and *in vivo* trials. Importantly, this concept does not aim to use phage-derived inhibitory proteins as final antimicrobials. Thus, knowledge of the exact structure and pharmacological properties of the phage protein is unnecessary. The inhibitory function of the proteins is discovered by cloning inducible ORFs of the phage genome into target bacteria. If bacterial growth after induction of the phage ORF is inhibited, the ORF is likely to contain a phage enzyme with inhibitory function. By applying this concept, ORFs with inhibitory function from many different phages may be identified in a short time.

Phage as a Transporter of Genes that Encode Bactericidal Proteins

The ability of phages to gain entry into bacterial cells has recently been used as a means to deliver bactericidal proteins (Westwater et al., 2003). In this approach, a filamentous coliphage M13, unable to cause lyse cells*, was "equipped" with genes that encode proteins expressed during the programmed cell death of bacteria (so-called bacterial suicide proteins). These genes were expressed in susceptible *E. coli* upon infection with M13, resulting in the death of *E. coli.* A significant reduction in numbers of susceptible bacteria was seen *in vitro* and *in vivo* in mice (Westwater et al., 2003). Similar to lysis-deficient phages, phage delivery systems may be of potential use as *in vivo* antimicrobials or as agents that induce *in vivo* whole cell vaccination.

* M13 is a phage that infects bacterial hosts chronically. Phage progeny are released by extrusion and does not cause cell death (cited by Calendar, 2006).

1.2.4.5. UTI Studies

The first reported use of phages in *E. coli* UTI dates back to the early 1920s (Courcoux et al., 1922; cited by Raettig, 1958). Furthermore, there is evidence that at least another 36 studies that investigated the potential use of phage for treating UTI were published in several languages in the 1920s and 1930s (studies cited by Raettig (1958) are listed in appendix, Table 8.3). Little is known of the content of these early studies. Chanishvilli et al. (2001) reviewed a historic study by Tsulukidze (1938). This gives valuable information on how phages may have been used to treat UTI during the 1930s. Tsulukidze applied phages to 37 patients with acute or chronic cystitis, pyelonephritis or paranephritis*. Prior to treatment, *E. coli* was identified as the cause of the majority (78.4%) of the infections. Other bacteria isolated from the infection site were *Staphylococcus aureus* and *Staphylococcus epidermidis*. Polyvalent phage mixtures or specific phage preparations were injected into the bladder or the renal pelvis. Paranephritic infections were cured by surgical puncture and subsequent injection of phage. In acute cases, clinical signs declined within three to four hours after treatment. A temporary improvement was noted in patients with chronic cystitis. More significantly, phage treatment led to a total recovery in all cases of acute cystitis and paranephritis within three days. A similar successful rate was apparently observed in the pyelonephritis group, where 80% of patients regained complete health. In 1995, Perepanova et al. conducted a study on forty-six patients with urogenital infections. Phages were shown to eradicate Staphylococci, *Proteus* and *E. coli* from 39 of the patients. Three patients showed significant reduction in clinical signs. At the Hirszfeld Institute of Immunology and Experimental Therapy, 78 patients suffering from UTI underwent treatment with phages between 1981 and 2000 (Slopek et al., 1983; Slopek et al., 1987; Weber-Dabrowska et al., 2000a). The phages were multiplied on their target bacterial strains (*S. aureus, E. coli, Klebsiella* spp., *Proteus* spp. and *Pseudomonas* spp.) that had been isolated from the patients. Phages were given orally (after neutralisation of gastric acids) or a lavage of the bladder was applied (Slopek et al., 1983). Orally applied phages were shown to be able to cross from the intestine into blood vessels and

* Paranephritis is defined as the inflammation of the connective tissue around or near the kidney [O'Toole, M. 1997. Miller-Keane Encyclopedia and dictionary of medicine, nursing, and allied health (Philadelphia, Pennsylvania, USA, W.B. Saunders)] In this case, inflammation was caused by bacterial infection.

subsequently into the urine (Weber-Dabrowska et al., 1987). Following treatment, clinical signs and results of urine cultures were assessed for the efficacy of phage treatment. A complete recovery, marked by cessation of clinical signs and negative bacterial culture, was achieved in 59 patients. Nine patients showed marked improvement, defined as a cessation of clinical signs but no bacteriological cure. Neither improvement nor deterioration was reported in 10 patients (Weber-Dabrowska et al., 2000a). The authors stressed that these results were of particular interest, since phage therapy was mostly applied when the infections had not responded to treatment with available antimicrobials (Weber-Dabrowska et al., 2000a). In contrast to these encouraging *in vivo* results, an *in vitro* study from the Hirszfeld Institute of Immunology and Experimental Therapy suggests that only a very small percentage of coliphages may be potentially useful for targeting UPEC (Drulis-Kawa et al., 2002). Only 3 of 44 phages tested from a "phage library" at the Hirszfeld Institute of Immunology and Experimental Therapy were able to lyse more than 50% of 76 *E. coli* isolated from children with UTI (range 51.3-61.8%). A further 11 phages were able to lyse between 15.8 and 31.6% of the *E. coli* strains studied, while the remaining phages lysed less that 10% of the *E. coli* strains studied.

To date, there is no *in vivo* or *in vitro* study of which I am aware that focuses on investigating the potential use of phages to treat *E. coli* UTI in dogs and cats. Thus, it is not known whether the treatment of canine or feline UTI with phages is feasible. This study aimed to investigate whether (i) phages that target a large variety of canine or feline UPEC are available in the environment; and (ii) the susceptibility of canine or feline UPEC to phage varies.

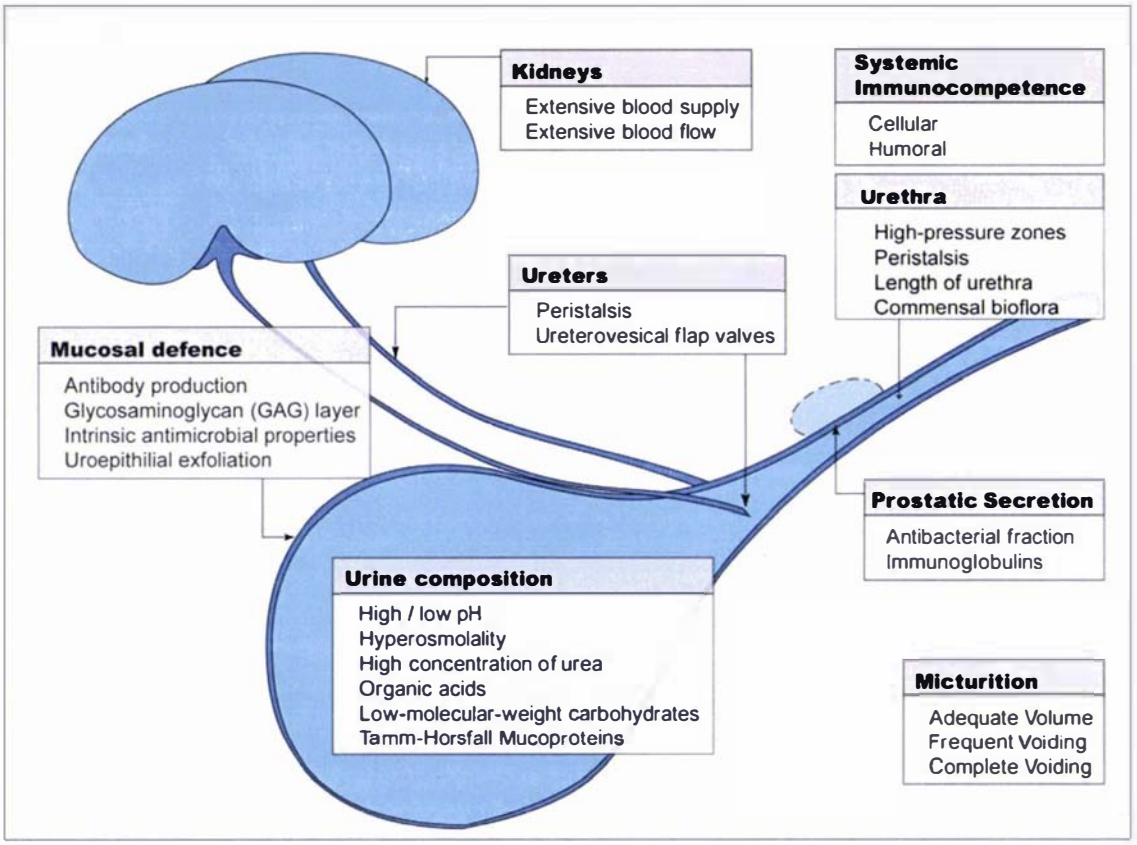
1.3. Urinary Tract Infections in Dogs and Cats Caused by Uropathogenic *Escherichia coli*

A Review of the Current Knowledge

1.3.1. Urinary Tract Infections in Dogs and Cats

UTIs occur when infectious agents overcome the host's defences (Figure 1.2) and multiply within the urinary tract (Bartges, 2004, 2005). A urinary tract infection (UTI) is diagnosed when microorganisms are isolated from parts of the urinary tract that are considered normally to be sterile (Ling, 2000).

Figure 1.2 Preventing UTIs – accepted host defence mechanisms[a]



[a]: Derived from material presented by Bartges (2005)

UTI may be classified according to the causal agent, possible evidence of previous infections, complexity of infection, their localisation and the number of different microorganisms involved (Table 1.4). Parasites, viruses and fungi rarely cause UTI in companion animals (Bartges, 2004, 2005; Osborne et al., 2000). Bacterial infections are by far the most common and shall be discussed in more detail.

Table 1.4 Classification of UTI

Classification	Term	Example/ Explanation	Ref.
Causal agent	Bacterial UTI	*E. coli, Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp., *Enterobacter* spp., *Mycoplasma* spp., *Providencia* spp., other	1-4
	Fungal UTI	*Candida* spp, *Aspergillus* spp, *Cryptococcus* spp, *Rhodotorula* spp, *Prototheca* spp	
	Viral UTI	Herpesvirus (Bovine herpesvirus (BHV-4)), Feline calicivirus, Feline syncytia-forming virus)	
	Parasitic UTI	*Capillaria feliscati*	
Episode of infection	First episode UTI	No previous history of UTI	5-6
	Persistent UTI	Microorganism persists despite treatment, urine at no time sterile.	
	Recurrent UTI	Previous UTI episode, now again UTI. Intermittent urine sterility observed.	
	Reinfection	Form of recurrent UTI. Episode caused by different microorganism to the microorganism that caused previous UTI.	
	Relapse	Form of recurrent UTI.Episode caused by microorganism (clone) that caused previous UTI.	
Localisation	Upper UTI	Pyelonephritis (kidney(s)) Ureteritis (ureter(s))	
	Lower UTI	Cystitis (bladder) Urethritis (urethra) Prostatitis (prostate) Vaginitis (vagina)	
Number of distinguishable organisms causing UTI	Monoclonal infection	One microorganism is causal agent	
	Mixed infection	Several distinguishable microorganism isolated from the urinary tract	
	Superinfection	A different microorganism establishes infection additional to already existing UTI with a different microorganism	6
Complexity	Uncomplicated UTI	No predisposing conditions present. Resolves easily with standard antimicrobial treatment	7-8
	Complicated UTI	Altered host defences (e.g. sub-normal host immune responses, abnormal micturition, anatomical abnormalities), underlying disease processes and/or partial or total resistance of microorganism to antimicrobials require a multifaceted treatment	6

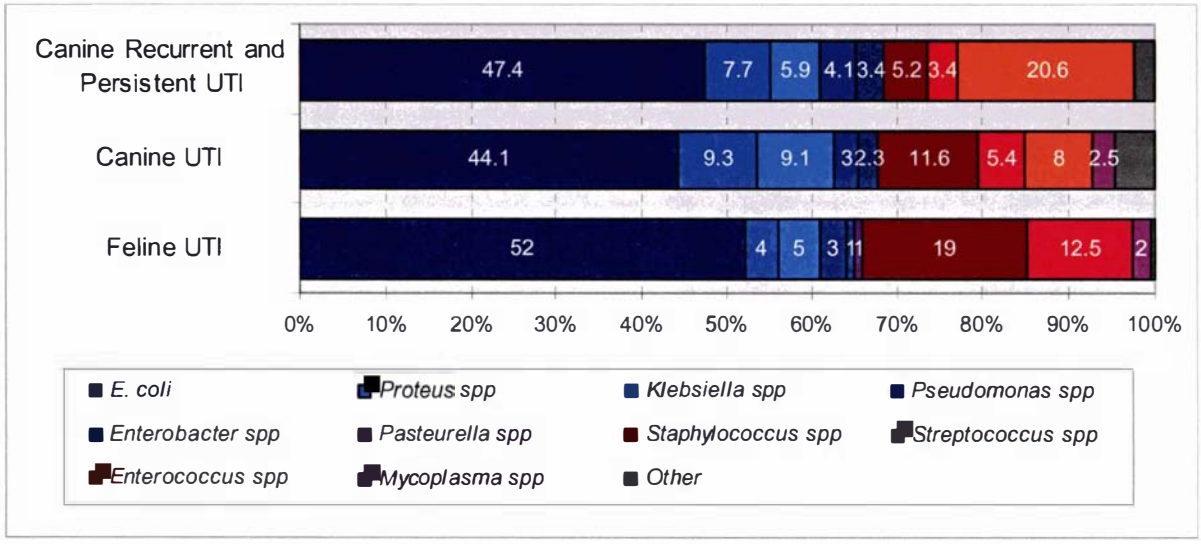
[1] Osborne et al., 2000; [2] Ling et al., 2001; [3] Kruger et al., 1996; [4] Pressler et al., 2005; [5] Norris et al., 2000; [6] Seguin et al., 2003; [7] Bartges, 2005; [8] Elliott, 1996

1.3.1.1. Bacterial UTI in Dogs

Bacterial UTI is considered the most common infectious disease in dogs, affecting an estimated 14% of the dog population during their lifetime (Bartges, 2004; Ling, 2000; Norris et al., 2000; Polzin, 1994). *E. coli* is the predominant organism isolated from dogs with UTI, causing 33% to 65% of all canine UTI (Figure 1.3; Forrester and Troy, 2000; Ling, 2000; Ling et al., 2001; Norris et al., 2000; Seguin et al., 2003). Gram-positive bacteria and gram-negative bacteria, other than *E. coli*, cause further 25% of canine UTI, respectively. Approximately 75% of all UTI are caused by a single organism (Ling, 2000; Ling et al., 2001). In 17% to 20% of all UTI cases, two different organisms are isolated and 3 or more organisms cause 6-8% of all UTI (Ling et al., 2001). Mixed infections occur mainly in female dogs (Ling et al., 2001). This may be because female dogs have a shorter urethra than males (Forrester and Troy, 2000; Seguin et al., 2003). Approximately 75% of all canine UTI are considered uncomplicated, caused by a temporary breach of host defence mechanisms (Figure 1.2) or sheer abundance of uropathogens (Ling, 2000). If host defence is chronically impaired, complicated UTI may develop (Bartges, 2005; Forrester and Troy, 2000; Seguin et al., 2003; Senior, 2000). Conditions predisposing to complicated canine UTI and potential mechanisms of disease development have been outlined in several studies (Forrester and Troy, 2000; Forrester et al., 1999; Norris et al., 2000; Seguin et al., 2003). They include underlying disease processes (e.g. diabetes mellitus, hyperadrenocorticism, CRF, urolithiasis, neoplasia, primary or secondary immune deficiency), anatomical abnormalities (e.g. patent urachus, abnormal uroepithelium) and iatrogenic causes (e.g. catheterisation). Prostatitis, orchitis, discospondylitis and septicaemia are UTI-associated sequelae that may complicate treatment (Ling, 2000). UTI may occur without any clinical signs in 54% to >90% of all patients, particularly when an immune impairment is diagnosed (Forrester and Troy, 2000; Forrester et al., 1999; Ling, 2000; Seguin et al., 2003). A further 10% to 20% of UTI-patients only show mild pyuria or bacteriuria on urine sediment evaluation (Forrester et al., 1999; Seguin et al., 2003). Consequently, the importance of performing regular urine cultures in predisposed animals was emphasized (Forrester et al., 1999; Lulich and Osborne, 2004; Seguin et al., 2003). Recurrent UTI (more than 2 UTI episodes per year) and persistent UTI are considered uncommon in dogs (Ling, 2000; Norris et al., 2000).

They may affect up to 4.5% of dogs with UTI and less than 0.5% of a total patient population in a primary care and referral setting (Drazenovich et al., 2004; Ling, 2000; Norris et al., 2000). Relapses or persistent infections may be identified in approximately 42% of all recurrent UTI (Seguin et al., 2003). Persistent infections are usually caused by gram-negative organisms, particularly *E. coli* (Ling, 2000). Superinfections are rare (Seguin et al., 2003).

Figure 1.3 The Prevalence of Bacterial Uropathogens in Dogs and Cats[a]



[a]: Graph derived from material presented by Ling, 2000; Ling et al., 2001 and Seguin et al., 2003

1.3.1.2. Bacterial UTI in Cats

UTIs are considered uncommon in otherwise healthy cats and may occur in <5% of cats that present with dysuria, haematuria or pollakiuria (Buffington et al., 1997; Kruger et al., 1991; Lees, 1996). UTIs occur more often when host defence impairment is present (Bartges and Barsanti, 2000; Lees, 1996; Lekcharoensuk et al., 2001; Thoresen et al., 2002). Moreover, the likelihood of UTI increases dramatically in cats older than 6 years (Bartges and Barsanti, 2000; Lees, 1996). An estimated 15 to >45% of older cats presenting with dysuria, haematuria or pollakiuria are diagnosed with UTI (Bartges and Barsanti, 2000; Lees, 1996; Lekcharoensuk et al., 2001). Most male cats may be concurrently affected by urethral obstruction or had a perineal urethrostomy. Female cats are often concurrently affected by diseases altering the urine composition, micturition or immune defence, such as CRF, treatment for hyperthyroidism,

diabetes mellitus, FIV, FELV, neoplasia (Barber et al., 1999; Bartges and Barsanti, 2000; Lees, 1996; Mayer-Ronne et al., 2004). Preliminary results of studies by Barber et al. (1999) and Mayer-Ronne et al. (2004) suggest that UTIs occur in 20% to >30% of cats with CRF. UTI is also seen in 12% of cats that are either treated for hyperthyroidism or affected by diabetes mellitus. Treatment with corticosteroids or diuretics may also predispose cats to the development of UTI. Furthermore, renal scar tissue development and intrarenal obstructions have been proposed as a predisposing cause of pyelonephritis (Thoresen et al., 2002). Feline UTI, like canine UTI, may occur without any clinical signs and without changes in urinalysis and complete blood count (Barber et al., 1999; Bartges and Barsanti, 2000; Ling, 2000; Mayer-Ronne et al., 2004; Senior, 2000). Hence, clinically silent UTI may go undetected if urine cultures are not performed (Lulich and Osborne, 2004). The incidence rate of clinically silent UTI has not yet been established (Ling, 2000). However, recent preliminary studies indicate that >50% of detected UTIs are clinically silent (Barber et al., 1999; Mayer-Ronne et al., 2004). Previously, recurrent UTI was considered to occur rarely in cats (Ling, 2000). However, a recent prospective study indicated that recurrence or persistence of UTI may occur more commonly than previously thought when cats are affected by CRF (Barber et al., 1999). *E. coli* caused all 22 infections reported in that study (Barber et al., 1999). This emphasises that *E. coli* plays a particularly important role in causing UTI in old female cats that have CRF. In feline UTI that develops in young cats, cats with concurrent urolithiasis, diabetes mellitus and medically-controlled hyperthyroidism or cats with perineal urethrostomy it may be less important. Overall, *E. coli* has been reported to cause 46% to 52% of feline UTI (Figure 1.3; Lees, 1996; Ling, 2000; Mayer-Ronne et al., 2004).

1.3.2. Commensals and Pathogens – The Diversity of *E. coli*

E. coli may live as nonpathogenic commensals in the intestinal tract of animals and humans. Conversely, pathogenic *E. coli* may infect the host and cause intestinal or extraintestinal disease. Commensal, extraintestinal and intestinal *E. coli* differ from each other in regards to distribution, phylogenetic group and possession of VFs (Table 1.5; Russo and Johnson, 2000). To date, the evolutionary mechanisms and selection factors that led to these differences are poorly understood (Johnson and Russo, 2002a). Overall, it is believed that pathogenic *E. coli* have evolved from commensal *E. coli* by acquisition of mobile genetic entities - namely virulence plasmids, lysogenic phages that carry VFGs and pathogenicity-associated islands (PAIs; Table 1.7; Hacker and Heesemann, 2000a). PAIs have been defined as chromosomal genetic elements that (i) contain at least one VFG; (ii) are associated with elements that promote mobility (e.g. transposases, insertion elements, excionases); (iii) have a G+C* content that differs from the G+C content of *E. coli* core genes; and (iv) are found in vicinity to chromosomal tRNA sites (Hacker et al., 1997). The sudden change in virulence associated with the acquisition of mobile genetic elements has been termed "evolution in quantum leaps" (Groisman and Ochman, 1996).

Table 1.5. Differences of *E. coli* populations

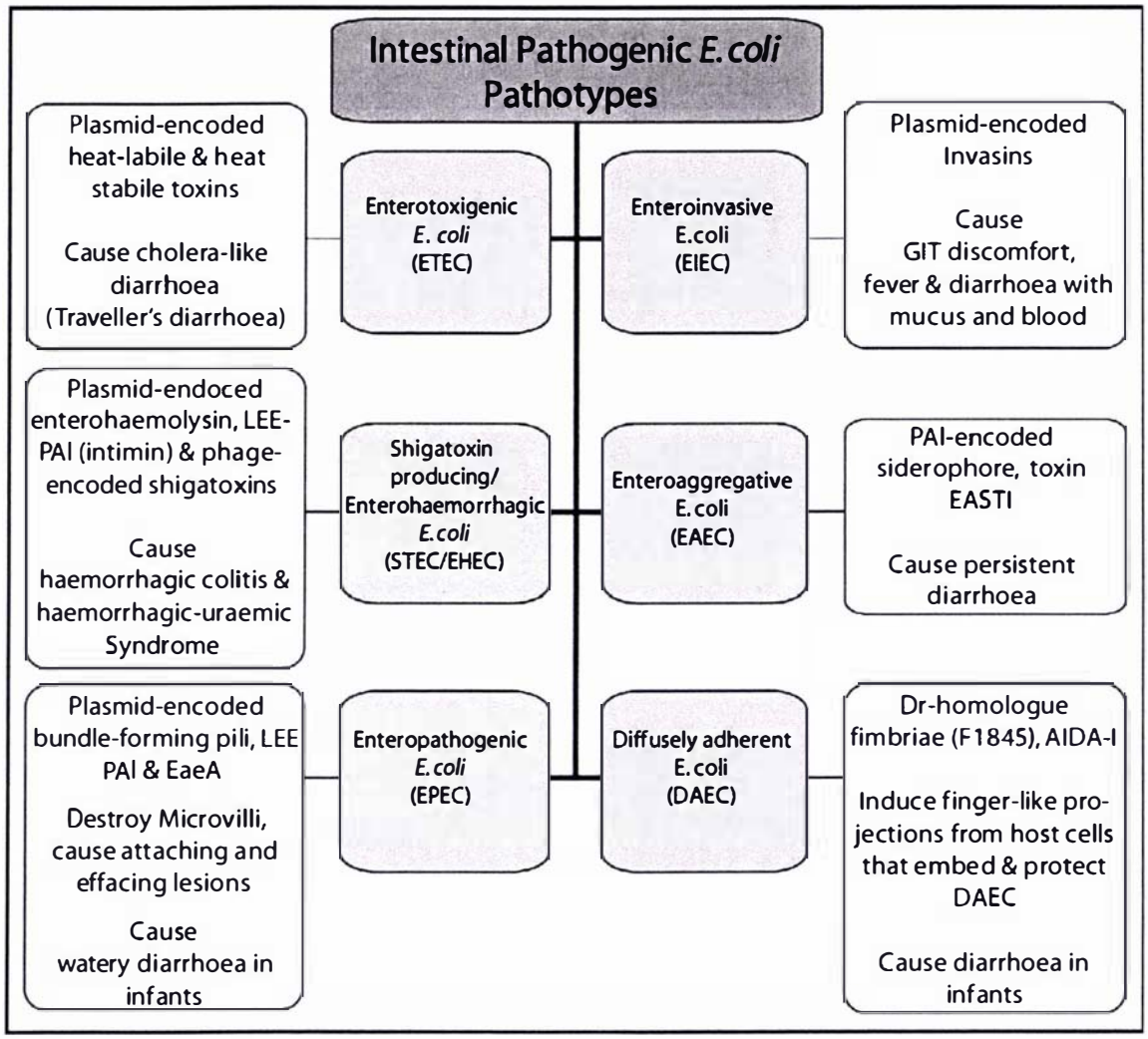
E. coli group / Characteristic	Commensal *E. coli*	Pathogenic *E. coli*	
		Intestinal	Extraintestinal
Present in the gastrointestinal tract of healthy animals/humans?	Yes, definitely	No	Yes, possibly
Associated with intestinal disease?	No	Yes	No
Associated with extraintestinal disease?	Only in compromised patients or when present in abundance[c]	No	Yes
Phylogenetic group[a]	A, B1	Not significantly associated with a certain group	B2, D
VFG presence	Few, if any	Yes, may contribute to a specific intestinal disease[b]	Yes, may contribute to different extraintestinal diseases[c]

[a]: as assessed by Multi-Locus Enzyme Electrophoresis (MLEE; Johnson and Russo, 2002a; Whittam et al., 1989)
[b]: refer to Figure 1.4
[c]: refer to Russo and Johnson (2003)

*G+C: Guanine and Cytosine, bonding nucleobases found in DNA and RNA.

Intestinal pathogenic *E. coli* has been grouped into 6 different pathotypes (Figure 1.4). Each of these pathotypes encodes specific VFG that enable it to cause intestinal infections with specific symptoms, at certain sites or in specific populations (Hacker, 2000; Nataro and Kaper, 1998).

Figure 1.4 Pathotypes of intestinal pathogenic *E. coli*[a]



[a]: Derived from material presented by Hacker and Heesemann (2000a)

Conversely, ExPEC may encode VFs that enable them to cause different clinical syndromes and infect multiple sites in different host populations (Johnson and Russo, 2002a; Russo and Johnson, 2000). Some VFs have been associated with specific extraintestinal diseases (e.g. ExPEC causing pyelonephritis often encode P fimbriae and ExPEC causing meningitis often encode K1 capsules, S fimbriae and Invasion-of-brain-endothelium protein (IbeA; Johnson and Russo, 2002a). However, the association between VF and disease syndrome is rarely absolute (Johnson and Russo, 2002b). That

is, an infection at a specific site may be caused by ExPEC that do not encode VF associated with that particular disease. Furthermore, ExPEC encoding VF associated with a specific disease may also cause extraintestinal infections at other sites. ExPEC have been shown to cause UTI, meningitis and bacteraemia (Johnson and Russo, 2002b; Johnson and Stell, 2000; Korhonen et al., 1985) and less commonly other diseases like pneumonia, intraabdominal infections, osteomyelitis, wound infections and cellulites (Johnson and Russo, 2002b; Russo and Johnson, 2000). In the following chapters, the term UPEC will be used instead of ExPEC to illustrate that this study focuses on *E. coli* that cause UTIs.

1.3.3. *E. coli* –The Major Infectious Uropathogen

UPEC is frequently encountered in canine, feline and human UTI (Johnson, 1991; Ling, 2000). Over the last decades, UPEC have been extensively studied in humans (reviewed by Johnson and Russo, 2005) and in dogs (Beutin, 1999; Feria et al., 2000a; Feria et al., 2000b; Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2003; Senior et al., 1992; Westerlund et al., 1987; Whittam et al., 1989; Yuri et al., 2000; Yuri et al., 1998). Conversely, virulence characteristics of feline UPEC have been studied in less detail and in smaller populations (Beutin, 1999; Feria et al., 2000a; Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Yuri et al., 1998)

Below, an overview of the aetiopathogenesis of *E. coli* UTI in dogs and cats is given. VFs of UPEC that were focused upon in this study will be discussed in detail. Furthermore, a summary of studies that compared UPEC from different hosts and identified potential reservoirs will be given. Current and future therapeutic approaches will be outlined.

1.3.3.1. Aetiopathogenesis of *E. coli* UTI in Dogs and Cats

The majority of feline and canine *E. coli* UTIs are caused by *E. coli* ascending from the distal urogenital tract and possibly the intestine (reviewed by Bartges, 2005). In some instances, *E. coli* may subsequently cause ureteritis and pyelonephritis by ascending the ureters. Vesiculoureteral reflux of urine may aid the migration of UPEC to the kidneys (Finco and Barsanti, 1979). Vesiculoureteral reflux is thought to occur when the intravesical pressure overcomes the intramural ureterovesical tone or an anatomically abnormal ureterovesical junction is present (Figure 1.2; Finco and Barsanti, 1979; Kipnis, 1975). Migrating of gram-negative organisms towards the kidney may also be facilitated by endotoxins that subsequently inhibit ureteral peristalsis (Finco and Barsanti, 1979). Haematogenous spread of *E. coli* rarely causes UTI (as discussed by Bartges, 2005 and Finco and Barsanti, 1979).

In order to cause a UTI, *E. coli* must adhere at the site of infection. As previously mentioned, the adsorption of *E. coli* is counteracted by various host defence mechanisms in healthy hosts (Figure 1.2). UTI occurs when the interaction between host defence mechanism and invading organisms is out of balance (as discussed by Bartges, 2005). This may be the case when (i) the host's defence mechanisms are hypofunctional; (ii) large numbers of *E. coli* that are present in the perineal, genital or intestinal environment invade the urinary tract (Cooke, 1974; Gruneberg, 1969); or (iii) *E. coli* exhibiting a variety of specific VF gain access to the urinary tract (as discussed by Finlay and Falkow, 1997).

1.3.3.2. Virulence Factors of UPEC

VFs enhance the viability and infectivity of UPEC in the urinary tract. They may facilitate attachment to the epithelial layer of the urinary tract (adhesins), interfere with signal transduction of host cells and lyse host cells (toxins) or allow bacterial invasion of host cells (invasins). VFs may also provide protection to the bacterial cell membrane (e.g. O-antigens, capsular antigens) or confer resistance to complement (e.g. TraT, Iss). Some VF, such as iron acquisition molecules (siderophores*), are 'fitness-elements' that may also be found in non-pathogenic *E. coli* (cited by Johnson, 2003 and Hacker and Heesemann, 2000a). They optimise the metabolism and increase the bacterial vitality in a certain environment. VF may be multifunctional, that is, they may act as adhesin and siderophore (e.g. Iha) or as adhesin and invasin (e.g. Type 1 fimbriae).† As previously mentioned, VFs are commonly found on readily transferable genetic elements (Hacker and Carniel, 2001; Hacker and Kaper, 2000). Due to frequent uptake or loss of PAIs, plasmids or phages, UPEC with numerous different genetic combinations of VFs exist (reviewed by Johnson, 2003). Observed statistical associations between VFGs may indicate that these VFGs are co-located on mobile genetic element. However, frequent recombination of PAIs and plasmids contributes to the variety of VF genotypes and prohibits prediction of VFG profiles by identification of few VFG markers (Johnson and Kuskowski, 2000). The virulence of a certain UPEC strain and its pathogenic versatility increases with the number of VFs it possesses. Accordingly, epidemiological studies in people have shown that UPEC with few VF predominantly establish infections in compromised patients, that is, patients that concurrently suffer from an illness other than UTI or a pathological abnormality (Karkkainen et al., 2000; Otto et al., 2001). *E. coli* isolated from human patients with asymptomatic bacteriuria (ABU) have also been shown to lack a number of VF found in 'highly-virulent' strains. In particular, they commonly lack the ability to express P fimbriae, Type 1 fimbriae, F1C fimbriae and haemolysin (Blanco et al., 1996; Roos et al., 2006a).

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* Greek for Iron Carrier

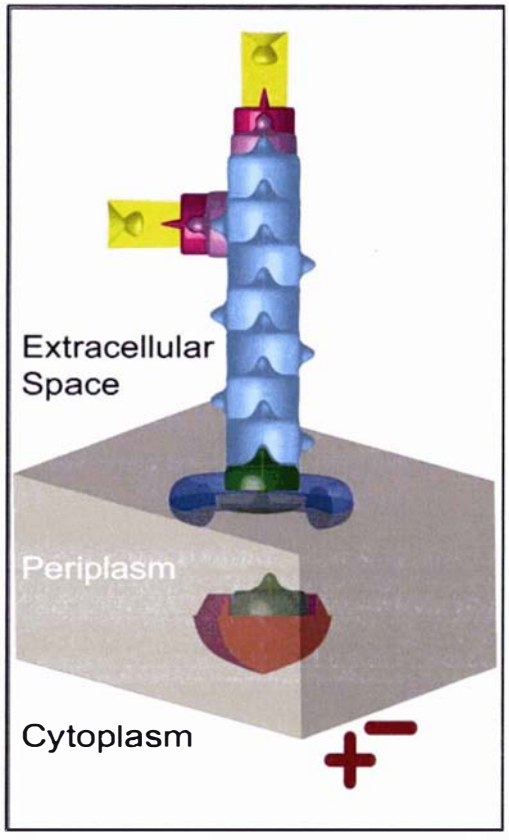
† In the following chapters, VFs are classified according to the function that was initially determined, or is considered the primary function of the VF.

'Highly-virulent' strains, those that encode one or several adhesins, toxins and protectins, are commonly isolated from otherwise healthy human patients or human patients with severe forms of UTI (i.e. pyelonephritis and urosepsis Johnson and Stell, 2000; Johnson et al., 1994; Bingen-Bidois et al., 2002).

Adhesins

Adhesion of UPEC to the uroepithelium enables UPEC to resist the urinary outflow and facilitates UPEC persistence, multiplication and, in some cases, even invasion. Adhesion may be mediated by fimbriae (also called pili) or non-fimbrial adhesins.

Figure 1.5 Schematic illustration of a fimbrium



Please refer to Table 1.6, page 68, for an explanation of subunits.

Fimbriae are hair-like proteinaceous structures protruding from the cell wall. Fimbriae that have been associated with extraintestinal pathogenicity are P fimbriae, S fimbriae, F1C fimbriae, Type 1 fimbriae and Dr fimbriae. They share a similar heteropolymerical composition, formed via the so-called chaperone-usher pathway (reviewed by Klemm and Schembri, 2000 and Sauer et al., 2000). In general, they consist of a major subunit and several minor subunits (Figure 1.5 and Table 1.6). The major subunit resembles a repeated sequence of one protein, encoded by a single gene. The resulting polymer represents the fimbrial rod.

The length of the rod is determined by a minor subunit, which anchors the fimbrium in the outer cell wall. The adhesin, which interacts with host cell receptors in a lock-and-key-like fashion (Johnson, 2003), may be located at the tip, as is the case for P fimbriae, or sides, seen in Type 1 fimbriae (reviewed by Klemm and Schembri, 2000).

It may be linked to the rod by other minor subunits, which may interact with eukaryotic cells themselves (reviewed by Sauer et al., 2000). The position of fimbriae is determined by the so-called usher, which forms a platform in the outer cell wall. Transport proteins (so-called chaperones) transfer fimbrial subunits through the periplasmatic space to the usher, where assembly takes place. During transport, the chaperones also protect the proteinaceous subunits from degradation and inhibit premature aggregation (reviewed by Sauer et al., 2000 and Schilling et al., 2001b). Fimbrial genes are often organised in large operons, which also contain genes for the regulation of expression and genes encoding usher and chaperone subunits.

Table 1.6 Fimbrial assembly[a]

Fimbriae / Subunit & Function	P fimbriae	Type 1 fimbriae	S fimbriae	F1C fimbriae	Dr adhesin [AFA][b]
 Adhesin	PapG	FimH	SfaS	FocG	DraE [AfaE]
 Linker	PapE, PapF	FimF FimG	SfaG, SfaH	FocF? FocG?	
 Core unit	PapA	FimA	SfaA	FocA	
 Anchor	PapK				DraC [AfaC]
 Usher	PapD	FimD	SfaF	FocD?	
 Chaperone	PapC	FimC	SfaE	FocC? FocI?	DraB [AfaB]
 Regulator	PapB	FimB, FimE	SfaB, SfaC	FocB	DraA [AfaA; AfaF]
Reference	1, 2	3	4		5,6

[a]: see also Figure 1.5

[b]: AFA: afimbrial adhesin (discussed in more detail below)

[1] Marklund et al., 1992; [2] Mühldorfer et al., 2001; [3] Klemm and Schembri, 2000; [4] Hacker and Morschhauser, 1994; [5] Servin, 2005; [6] Nowicki et al., 2001

P Fimbriae

P fimbriae were named according to their ability to agglutinate human erythrocytes possessing the P-antigen (i.e. P1, P2 or P1k; cited by Johnson, 1991). P-fimbrial adherence to uroepithelial cells significantly contributes to UPEC virulence (Wullt et al., 2001b). Furthermore, P-fimbrial modulation of the host's transmembrane signalling may cause an increased release of cytokines, such as IL-4, IL-6, IL-8 and p73, into the urine, thus resulting in local inflammation and recruitment of neutrophils (Söderhäll, 2001; Wullt et al., 2001a). It has been estimated that up to 80% of UPEC may bear P fimbriae on their surface (cited by Mühldorfer et al., 2001). The P-fimbrial operon, *papA-K*, is located on the chromosome of UPEC and commonly found in association with other VFGs in PAI (Table 1.7).

Table 1.7 PAIs detected in UPEC

Designated PAI[a]	Putative urovirulence genes[b]	Associated tRNA	Ref.
PAI IV_{J96}	*pap* (*papG I*), *hlyCABD*	*pheU*	[1]
PAI V_{J96}	*prs* (*prsG III*) , *hlyCABD, cnf1, hrad*	*pheV*	[2]
PAI I_{536}	*hly*, ORF-PVF[c]	*selC*	[3]
PAI II_{536}	*prs* (*prsG III*), *hly*, ORF-PVF[c]	*leuX*	
PAI III_{536}	*sfa* , *iroBCDEN*, ORF-PVF[c]	*thrW*	
PAI IV_{536}	*fyuA, irp*	*asnT*	
PAI V_{536}	kps_{K15}, ORF-PVF[c]	*pheV*	[4]
PAI I_{CFT073}	*hlyCABD*, *pap* (*papG II*), *iha*?, *iucABCDiutA*	*pheV*	[5-7]
PAI II_{CFT073}	*pap* (*papG II*),putative iron-regulating genes, ORF-PVF[c]	*pheU*	[7, 8]
PAI $?_{CFT073}$	*iroBCDEN, sfa/foc*	*serX*	[7]
PAI_{CP9}	*prs* (*prsG III*), *foc, iroDEN, hly, cnf1*	?	[9, 10]

[a]: First identified on subscript archetypal UPEC
b: *Pap* or *prs* operons encode *papG* or *prsG* alleles displayed in brackets, respectively
[c]: ORF-PVF: also contains ORF encoding putative VF and several VF that were not focused upon in this project
[d]: *hra* encoding heat-resistant agglutinin
[1] Swenson et al., 1996; [2] Blum et al., 1995; [3] Dobrindt et al., 2002; [4] Schneider et al., 2004; [5] Guyer et al., 1998; [6] Kao et al., 1997; [7] Welch et al., 2002; [8] Rasko et al., 2001; [9] Russo et al., 1999; [10] Johnson et al., 2000b

Adhesion is mediated via the minor subunit PapG (Pilus-associated-with-pyelonephritis subunit G) that recognises the component Gal-(α1-4)-Galβ of the globoseries of glycolipids in eukaryotic cells (Table 1.8). To date, four different *papG* variants have been identified (Table 1.8; Johnson and Stell, 2000). The *papG* III variant, as identified in archetypical strain J96, may also be called *prsG* (pap-related substance adhesin; Johnson et al., 2000a). Accordingly, the J-96-like *papG* III bearing *pap* operon and associated fimbriae may be termed *prs* operon and Prs fimbriae, respectively. The *prs* designation is based on differences in agglutination and adherence pattern between PrsG (PapG III protein variant) and other PapG proteins. PrsG was originally believed to be associated with fimbriae that are similar, but not identical, to P fimbriae (Stromberg et al., 1990). However, this was negated in later DNA sequencing studies (Marklund et al., 1992). In human UTI, different *papG* variants have been associated with different clinical syndromes. For example, *papG* II has been associated with UPEC causing human pyelonephritis or urosepsis (Johnson, 1998; Johnson and Stell, 2000). It is also commonly found in UPEC isolated from human patients with concurrent medical illness (Otto et al., 2001). *PapG III* is more commonly identified in human UPEC causing cystitis (Johnson et al., 1998b). Thus, it is possible that PapG variation contributes to tissue tropism. However, many UPEC possess more than one PapG variant and may consequently have the potential to adhere to various tissues. In UPEC isolated from dogs and cats, *papG III* has been identified as the predominant allele (Feria et al., 2001b; Johnson et al., 2000a). It is encoded by 41% to 95% of canine or feline UPEC (Feria et al., 2001b; Johnson et al., 2003). Approximately 5% to 23% of canine UPEC encode *papG II* (Feria et al., 2001b; Johnson et al., 2003). Conversely, *papG II* has not yet been identified in feline UPEC. *PapG I/I'*-encoding strains are rarely isolated from UTI in dogs, cats and people (Feria et al., 2001b; Johnson et al., 2003; Johnson et al., 2005c). The epidemiologic significance of these alleles has, as far as I am aware, not yet been clarified (Feria et al., 2001b; Johnson et al., 1997).

Table 1.8 Adhesin Binding Specificity

Adhesin	Host receptor (contains)	Host receptor examples	Clinical Importance	Ref.
PapG I PapG I'	Globotriosylceramide [GbO3]		J-96-like strains O4:K5 strains Other clonal groups	1-3
PapG II	Globotetraosylceramide [GbO4]	Globoside	Human pyelonephritis and urosepsis Canine UTI	1, 3-5
Pap G III	Globopentosylceramide [GbO5]	Forssman-antigen Globo-A	Human cystitis and urosepsis in compromised hosts Canine and Feline UTI	3-5
FimH	Manno-oligosaccharides (Collagen, Plasminogen	Uroplakin Ia	Cystitis Invasion of uroepithelium	6-9
SfaS	Sialyl galactosides; e.g. sialic acid α 2-3 lactose	Glycophorin A	NBM[a] UTI	10-11
FocG	Glycosphingolipids; e.g. globotriaosylceramide, galactosylceramide		Putatively ascending UTI and pyelonephritis	12
AfaE, DraA	Different epitopes of Dr antigen on decay-accelerating factor, CEACAM[b], Type IV collagen site	Dr antigen (on DAF), Type IV collagen	Human gestational pyelonephritis, cystitis, diarrhoea, persistent UTI?	13-15
BmaE	M blood group antigen; i.e. epitope that is part of glycophorin A (different to SfaS epitope)	Glycophorin A	?	16
GafD	G-fimbriae? N-acetylglucosamine (GlcNAc)		?	17

[a] NBM: New Born Meningitis

[b]CEACAM: carcinoembryonic antigen-related cellular adhesin molecules

[1] Sung et al., 2001; [2] Johnson et al., 1997; [3]Feria et al., 2001b; [4] Otto et al., 2001; [5] Johnson, 1998; [6] Bouckaert et al., 2005; [7] Pouttu et al., 2000; [8] Duncan et al., 2004; [9] Mulvey, 2002; [10] Hacker and Morschhauser, 1994; [11] Korhonen et al., 1984; [12] Backhed et al., 2002; [13] Servin, 2005; [14] Johnson, 1991; [15] Nowicki et al., 2001; [16] Vaisanen et al., 1982; [17] Vaisanen-Rhen et al., 1983

Type 1 Fimbriae

Type 1 fimbriae, or common fimbriae, have been identified in numerous *Enterobacteriaceae* (Klemm and Krogfelt, 1994). Expressed by up to 70% of UPEC during UTI (Johnson, 1991), Type 1 fimbriae facilitate colonisation of epithelial surfaces and contribute to biofilm formation and invasion (reviewed by Emody et al., 2003). Type 1 fimbriae have been shown to play an essential role in ascending UTI (reviewed by Klemm and Krogfelt, 1994), bladder colonisation (cited by Johnson, 1991) and possible subsequent inflammation (Connell et al., 1996). FimH, the adhesin subunit of the fimbrium, recognises manno-oligosaccharide receptors, which are components of surface-glycoproteins of many host tissues (e.g. epithelium of intestinal tract, ureter, erythrocytes and phagocytic cells; Bouckaert et al., 2005; Klemm and Krogfelt, 1994). Furthermore, FimH has been shown to facilitate invasion of uroepithelial cells (discussed in more detail below; Mulvey, 2002). The associated gene, *fimH*, can mutate spontaneously to alter receptor recognition or to enable fimbriated *E. coli* to bind to additional protein receptors (Hung et al., 2002; Pouttu et al., 2000). This functional variability of the fimbrial adhesin FimH may play an important role in tissue tropism of Type 1-fimbriated *E. coli*. Type 1 fimbriae also adhere to Tamm-Horsfall protein (THP, also called uromucoid), a glycoprotein produced by the kidney and excreted in the urine (Klemm and Krogfelt, 1994). THP-bound UPEC may subsequently be eliminated during micturition. However, THP-coating of UPEC may also prevent opsonin-mediated phagocytosis, thus protecting non-excreted UPEC. Active immunisation may also inhibit colonisation of Type 1-fimbriated *E. coli* (Langermann and Ballou, 2003). However, antigenic variety and phase variation of Type 1-fimbriated *E. coli* may decrease the efficacy of a vaccine drastically (Pouttu et al., 2000).

S Fimbriae and F1C Fimbriae

S fimbriae and F1C fimbriae are genetically highly similar. They differ mainly in binding specificity (Table 1.8; Hacker and Morschhauser, 1994). S fimbriae recognise sialyl-galactosides, which are part of cell membrane glycolipids present in numerous extraintestinal host tissues (Hacker and Morschhauser, 1994). S fimbriae particularly enhance the virulence of ExPEC causing newborn meningitis. They enable ExPEC to cross the blood-brain-barrier and to adhere to brain cells (Korhonen et al., 1984; Korhonen et al., 1985). More than 50% of meningitis-causing ExPEC encode S-fimbrial genes (Johnson et al., 2002). In the urinary tract, S fimbriae enable UPEC to bind to glomeruli, distal tubules and collecting ducts of the kidney, the vascular endothelium of kidney and bladder and the extracellular matrix protein laminin (Marre and Hacker, 1987). UPEC expressing S fimbriae are more commonly isolated from human patients with pyelonephritis than from patients with cystitis (Hacker and Morschhauser, 1994; Marre and Hacker, 1987). This may be because S-fimbrial binding to THP, and subsequent elimination of UPEC during micturition, may prevent bladder colonisation (cited by Hacker and Morschhauser, 1994).

In UPEC isolated from dogs and cats, the S-fimbrial adhesin gene marker *sfaS* has been detected in 12% to 54% and 50% to 100%, respectively (Feria et al., 2001a; Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d; Yuri et al., 1998).

F1C fimbriae, expressed by up to 30% of UPEC (cited by Hacker and Morschhauser, 1994), bind to a range of glycosphingolipids with a ceramide composition (Table 1.8; Backhed et al., 2002; Khan and Hacker, 2000). These molecules are found in the epithelium of the bladder, ureter and kidney and the vascular endothelium of bladder and kidney (Backhed et al., 2002). Thus, F1C-fimbrial adherence may facilitate the migration of UPEC to the kidneys and contribute to the development of pyelonephritis (Backhed et al., 2002). The F1C-fimbrial adhesin gene marker *focG* has previously been identified in up to 25% of canine and feline UPEC (Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d). Adhesion of S and F1C fimbriae to host cells has been shown to initiate an inflammatory response by induction of IL-6, ICAM1 and IL-8 production (Backhed et al., 2002; Hacker and Morschhauser, 1994).

Afimbrial Adhesins (AFA) and Dr Fimbrial Adhesins

Afimbrial adhesins (AFA I-VIII) and Dr fimbriae belong to a group of 13 genetically similar colonisation factors of *E. coli* that cause UTI or diarrhoea (reviewed by Nowicki et al., 2001). AFA are found in close association to the bacterial cell wall (integrated or on the surface), while Dr fimbriae display a structured fimbrial organisation. AFA variants differ predominantly in adhesin (i.e., AfaE) specificity (Nowicki et al., 2001). The receptor of most Dr fimbriae and AFA is the Dr antigen, an epitope on the decay-accelerating factor (Nowicki et al., 1990). The decay-accelerating factor, a complement regulator protein, is present on human Dr (a+) blood group erythrocytes and various other tissues. In UTI, AFA and Dr fimbriae may contribute to virulence of UPEC by binding to decay-accelerating factor that is present in the uroepithelium and interstitium. Additionally, Dr fimbriae bind to the Bowman's capsule and renal interstitium via type IV collagen membrane receptor recognition (as discussed by Schmidt, 1994). Dr fimbriae-specific type IV collagen binding to renal tissue and resistance to polymorphonuclear granulocytes may promote persistence of Dr^+ UPEC (reviewed by Nowicki et al., 2001 and Servin, 2005). Dr fimbriae and AFA have the potential to invade epithelial cells that display decay-accelerating factor (Goluszko et al., 1997; Jouve et al., 1997). The respective invasins are encoded by genes *draE* and *afaD*, respectively (Table 1.6). The operons of AFA and Dr Fimbriae (*afaABCDE* and *draABCDE*) can be located on the chromosome (e.g. AFAI-II) or plasmids (e.g. AFA III; cited by Schmidt, 1994). UPEC encoding AFA and Dr fimbriae are often associated with human gestational pyelonephritis (approximately 1/3 of UPEC isolated) or cystitis in young children (up to 50% of UPEC isolated; as discussed by Nowicki et al., 2001). Canine or feline UPEC encoding *afa* have been identified rarely (Feria et al., 2001a).

M Haemagglutinin

The M haemagglutinin, an afimbrial adhesin, was first found in an *E. coli* isolate causing human pyelonephritis (Rhen et al., 1986a; Vaisanen et al., 1982). The M haemagglutinin operon consists of 5 genes (*bmaABCDE*), of which *bmaE* encodes the receptor (Rhen et al., 1986b). BmaE comprises a 21kDa protein that recognises a terminal serine residue on the glycophorin A^M present in the human M blood group (Rhen et al., 1986a). It shows high similarity to the AfaE VIII adhesin that has been found in bovine diarrhoeagenic and septicaemic *E. coli* (Lalioui et al., 1999). The M agglutinin phenotype or *bmaE* gene marker is rarely observed in UPEC isolated from people. If present, it is commonly associated with UPEC that cause human pyelonephritis (Johnson et al., 2005b; Johnson and Stell, 2000). To my knowledge, *bmaE* has not yet been detected in any feline UPEC and in only one canine UPEC (Johnson et al., 2001a).

G Fimbriae

G fimbriae were first described in a UPEC strain isolated from a human patient with pyelonephritis (Rhen et al., 1986a; Vaisanen-Rhen et al., 1983). This strain also expressed M haemagglutinin. G fimbriae are ca. 1 μm x 5 nm in size (Saarela et al., 1995). They show high similarity to F17 and F17b fimbriae of bovine diarrhoeagenic and septicaemic *E. coli* (Saarela et al., 1996). The operon of these fimbriae consists of 4 genes (*gafABCD)*, of which *gafD* encodes the adhesin (Saarela et al., 1996). GafD adheres to terminal N-acetylglucosamine residues and laminin, an extracellular matrix substance found in basement membranes (Rhen et al., 1986b; Saarela et al., 1996). It has recently been hypothesised that GafD may have evolved from FimH, as both adhesins share a similar structure (Westerlund-Wikstrom and Korhonen, 2005). G fimbriae are rarely found in human and canine UPEC (Johnson, 1991; Johnson et al., 2001a; Johnson et al., 2005b; Johnson and Stell, 2000) and have yet to be identified in feline UPEC. The significance of G fimbriae in *E. coli* urovirulence remains to be elucidated.

Iron-regulated Gene A Homologue Adhesin (Iha)

In 2000, Tarr et al. described the iron-regulated gene A homologue adhesin (Iha), a novel non-haemagglutinating adhesin encoded by *E. coli* O157:H7. Since then, the Iha adhesin has been shown to enhance the pathogenicity of UPEC by enabling adherence to the uroepithelium (Johnson et al., 2005a). The Iha adherence mechanism and putative receptors on uroepithelial cells remain to be identified (Johnson et al., 2005a). However, Johnson et al. (2005a) demonstrated that mutants that lacked the *iha* gene were unable to compete with *iha*$^{+}$ strains and could not colonise murine bladders after 48h. The gene sequence of *iha* shows 53% similarity to the iron-regulated gene A (*irgA*), a siderophore receptor and putative adhesin gene of *Vibrio cholerae* (Tarr et al., 2000). Furthermore, *iha* is highly similar to a putative exogenous ferric siderophore receptor gene encoded by ORF R4 of PAI I from uropathogenic strain CFT073 (Guyer et al., 1998; Tarr et al., 2000). Leveille et al. (2006) recently confirmed that Iha not only confers adherence, but also acts as a catecholate siderophore receptor.

Iha gene markers have been detected in 34% to 74% of human UPEC. They are common in isolates causing pyelonephritis or urosepsis in adults (56-57%) or pyelonephritis and cystitis in children (50% to 70%; Bauer et al., 2002; Johnson et al., 2001a; Johnson et al., 2005a; Johnson et al., 2000b; Kanamaru et al., 2003). *Iha* gene markers have also been detected in 8 canine and one feline UPEC (Johnson et al., 2001a). *Iha* is strongly associated with phylogenetic group B2 and may be linked to various other VFGs (e.g. *hlyA*, *papG II*, *focG*, Bauer et al., 2002; Johnson et al., 2000b). Less than 35% of faecal *E. coli* isolated from humans possess the *iha* gene marker (Bauer et al., 2002; Johnson et al., 2005b; Kanamaru et al., 2003).

Toxins

The toxins described here (haemolysin, cytotoxic necrotising factor and cytolethal distending toxin) are exotoxins (reviewed by Reidl, 2000). Exotoxins are secreted by the bacterial cells and affect the target cell upon binding. The effect of exotoxins is therefore not dependent on the presence of bacterial cells. In general, toxins disturb metabolic or regulatory processes in the host cell. This may lead to impairment of the cell (e.g. impairment of phagocytes), cause activation of signalling pathways via IL-6 and IL-8 secretion and may interfere with normal cell growth and differentiation (e.g. multinucleation, cell distension). In higher concentrations, all toxins mentioned cause cell death.

Haemolysin

Haemolysin was first named after its ability to lyse erythrocytes. However, this toxin also affects numerous other cell types, such as granulocytes, monocytes and epithelial cells (reviewed by Mühldorfer et al., 2001 and Johnson, 1991). The toxin impairs the immune system, increases the vulnerability of the host by injuring host cells and may provide iron for bacteria (as discussed by Johnson, 1991). Haemolysin has been proven to be nephrotoxic and has been shown to support bacterial colonization in ascending UTI (reviewed by Emody et al., 2003 and Johnson, 1991). Human UPEC that cause pyelonephritis and cystitis may express haemolysin in 50% and 40% of all cases, respectively (cited by Johnson, 1991). UPEC that cause asymptomatic bacteriuria in humans, UPEC isolated from compromised human patients and faecal *E. coli* isolated from humans express haemolysin in ≤ 20% of all cases (cited by Johnson, 1991). In UPEC isolated from dogs and cats, haemolysis or haemolysin-encoding gene markers have been detected in 31% to 100% of all strains studied (Feria et al., 2000a; Feria et al., 2001a; Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d; Westerlund et al., 1987; Yuri et al., 1998). Faecal *E. coli* isolated from dogs and cats were positive for *hly* gene markers in up to 48% of all cases (Yuri et al., 1998). Haemolysin comprises 4 subunits (*hlyCABD*; Hess et al., 1986). *HlyA* encodes the actual toxin, a protein of 110kDa (Ludwig et al., 1996). Before secretion, HlyA must be activated by HlyC, which acts as a fatty acid transferase (Issartel et al., 1991). The secretion of HlyA from the bacterial cell is dependent on the presence of HlyB, HlyD

and TolC (as discussed by Gentschev et al., 2002). HlyB and HlyD form a stable complex, located in the cell membrane. They comprise an ATP-binding cassette and a membrane fusion protein, respectively. HlyB facilitates the transfer of HlyA through the cell membrane. TolC, an outer membrane protein that is also involved in other export systems, assists with the transfer through the outer membrane by forming an export pore. HlyD is required to release HlyA (Thanabalu et al., 1998). HlyA subunits that are incorporated into the host cell may accumulate and, if calcium ions are present, form a pore of 1-2 nm (reviewed by Gentschev et al., 2002 and Mühldorfer et al., 2001). Pore formation increases the permeability of the cell wall, destroys the surface charge of the cell and inevitably causes lysis. In low concentrations, HlyA may cause an inflammatory response caused by toxin-induced changes in the intracellular calcium ion concentration of renal cells (Uhlen et al., 2000). These so-called calcium oscillations subsequently activate second-messenger signals and lead to an increased expression and secretion of IL-6 and IL-8 (Uhlen et al., 2000).

The amount of haemolysin secreted may vary between different haemolysin genotypes, as the regulatory genes may differ substantially (Nagy et al., 2000). Haemolysin transcription is suppressed or induced when the concentration of iron is high in the extracellular environment or low-iron conditions are present, respectively (reviewed by Johnson, 1991). The *hlyCABD* operon may be found on the chromosome of UPEC (in $\leq$ 95% of all cases) or on plasmids (as discussed by Mühldorfer et al., 2001). On the chromosome, *hlyCABD* is often associated with other VF and located in PAI (Table 1.7). Furthermore, UPEC strains that express haemolysin often belong to certain O-serogroups (e.g. O4, O6, O18, O75) and display certain K antigens (K2, K5, K12, K13; cited by Johnson, 1991).

Cytotoxic Necrotising Factor (Cnf)

Cytotoxic necrotising factors - Cnf1, 115 kDa and Cnf2, 110 kDa - interfere with cytoskeleton formation and differentiation of host cells (reviewed by De Rycke et al., 1999). Cnf1 is the primary Cnf in canine, feline and human *E. coli* (De Rycke et al., 1999) and shall be discussed in more detail. *Cnf1* is found on the chromosome of 30% to 50% of human UPEC (Johnson et al., 2005b; Landraud et al., 2000). In canine UPEC, *cnf1* gene markers were detected in 34% to 96% of all strains (Feria et al., 2000a; Feria et al., 2001a; Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d; Yuri et al., 1998). Feline UPEC were positive for the *cnf1* gene marker in 40% to 100% of all cases (Feria et al., 2000a; Feria et al., 2001a; Johnson et al., 2001a; Yuri et al., 1998). *Cnf1* is rarely detected in faecal *E. coli* isolated from healthy people (cited by De Rycke et al., 1999 and Johnson, 1991). However, up to 47% of faecal *E. coli* from healthy dogs and cats tested positive for Cnf1 or its gene marker (Blanco et al., 1993; Yuri et al., 1998). Blanco et al. (1993) reported a particularly high prevalence of *cnf1* (75%) in haemolytic faecal *E. coli* strains isolates from healthy cats. A pre-selection for haemolytic strains may have artificially increased the number of *cnf1* $^{+}$ strains in this population of faecal isolates, since *E. coli* encoding *cnf1* also commonly encode haemolysin (Table 1.7; Boquet, 2001; Johnson, 1991; Landraud et al., 2004). Cnf1 consists of 2 subunits (i.e. it is an A-B toxin; reviewed by Boquet, 2001 and Landraud et al., 2004). The B subunit facilitates binding to the host cell and uptake of Cnf1 by pinocytosis. The A subunit comprises a catalytic domain that permanently activates Rho protein, a host cell GTPase involved in regulation of actin and tubulin formation. Constant activation of Rho protein causes cell shape changes, interferes with cell division, differentiation and migration and promotes multinucleation. Cnf1 toxicosis of epithelial cells and granulocytes may progress to apoptosis and may impede phagocytosis (as discussed by Emody et al., 2003; Johnson, 1991 and Landraud et al., 2004). Long lasting low level Rho protein activation by Cnf1 has also been shown to facilitate uptake of *E. coli* J96 into epithelial cells (Doye et al., 2002).

Cytolethal Distending Toxin

The cytolethal distending toxin (Cdt) was first discovered in 1988 (Johnson and Lior, 1988). The toxin causes an inhibition of cell proliferation, which results in cell distension and, finally, cell death (Cortes-Bratti et al., 2001; Johnson and Lior, 1988). In detail, Cdt has been shown to cause a cell cycle arrest in actively replicating cells. In epithelial cells and fibroblasts, Cdt stops the cell cycle at stages G2 and G1, respectively (reviewed by Frisan et al., 2002). B cells undergo sudden apoptosis, when subjected to Cdt (reviewed by Frisan et al., 2002). Based on differences in gene sequences, 4 different Cdts (CdtI-IV) have been defined for *E. coli* (Mainil et al., 2003; Pickett et al., 2004). CdtII and CdtIII have been shown to be present in *E. coli* associated with diarrhoea (De Rycke et al., 1999; Pickett et al., 2004). CdtI and CdtIV appear to be present in ExPEC (Mainil et al., 2003). Cdt-like effects were particularly often observed in strains that also encode Cnf1 (De Rycke et al., 1999). It was hypothesised that Cnf1 and Cdt work synergistically to facilitate bacterial invasion of deeper tissue layers (De Rycke et al., 1999; Mainil et al., 2003). Cnf1 is thought to activate the DNA synthesis of quiescent cells. Cdt may then arrest the cell cycle and inhibit renewal of affected cells. Weakened by the toxin, cells may not withhold invasion of bacteria. Despite the observation of cell-distending effects in up to 75% of *E. coli* that encode Cnf1, Cdt-related gene sequences were only detected in 12% to 15% of human and canine *E. coli*, respectively (De Rycke et al., 1999; Mainil et al., 2003). Similarly, VF studies of human and canine UPEC showed a *cdt* gene marker prevalence of 12%, 9% and 11% in human pyelonephritis, human cystitis and canine UTI, respectively (Johnson et al., 2003; Johnson et al., 2005b). It may be that UPEC encode other, yet non-detected, types of Cdt (Mainil et al., 2003). All Cdt types described so far comprise of 3 subunits (CdtA-C) that are encoded by different genes (reviewed by Cortes-Bratti et al., 2001). CdtB, the subunit that causes toxicity, has been shown to have strong similarity to mammalian DNAse 1 (Elwell and Dreyfus, 2000). It is thought to act as a phosphodiesterase, damaging the DNA of the targeted cell (Elwell and Dreyfus, 2000). The roles of subunits CdtA and CdtC have not yet been completely defined. CdtA may activate the precursor of CdtC, which may then support the introduction of CdtB into host cells (as discussed by Frisan et al., 2002).

Lipopolysaccharides, Capsular Antigens and Complement Resistance Factors

Capsular polysaccharides, structures that promote complement resistance and factors that facilitate biofilm formation allow UPEC to withstand the host immune response (as discussed by Emody et al., 2003 and Haas and Hensel, 2000). In general, these VF impair the immune response by (i) reducing the binding of opsonins to bacterial cells; (ii) interfering with the classic or alternative complement cascade; (iii) reducing the penetration of antibodies, antimicrobials or phagocytes to bacterial cells; or (iv) disguising bacterial cells as host cells (as discussed by Emody et al., 2003; Haas and Hensel, 2000 and Johnson, 1991). VFs of this kind that were investigated in this project shall be reviewed in detail.

Lipopolysaccharides

Lipopolysaccharides are a major constituent of the cell membrane of gram-negative bacteria (reviewed by Haas and Hensel, 2000). They consist of a Lipid A domain, an oligosaccharide core and the peripheral polysaccharide O-antigen (reviewed by Mühldorfer et al., 2001). Lipopolysaccharides have been shown to enhance the virulence of *E. coli* by conferring toxicosis to host cells. In detail, the lipid A domain may interact with Toll-like or other receptors of host cells and induce a second messenger cascade (reviewed by Haas and Hensel, 2000 and Johnson, 2003). This results in the release of various immune mediators, such as IL-1, IL-6, IL-8, TNFα, which attract neutrophils and macrophages. Neutrophil response may cause a local inflammatory reaction (as discussed by Johnson, 2003). A systemic immune response to lipopolysaccharides may cause fever, malaise, leukocytosis or even septic shock (as discussed by Haas and Hensel, 2000 and Johnson, 2003). In addition to its toxic effect, lipopolysaccharides may confer complement resistance to *E. coli* strains by restricting the access of complement to the cell membrane (reviewed by Johnson, 1991). The O-antigen of *E. coli* generally consists of various oligosaccharides (O-units) that form a heteropolymer (as discussed by Samuel and Reeves, 2003). Genes involved in O-antigen synthesis, reviewed in detail by Schnaitman and Klena (1993), may be grouped into genes encoding biosynthesis enzymes (*rfb* cluster), genes encoding glycosyl transfer proteins (part of *rfa* cluster) and genes involved in translocation (*rfaL*) and polymerisation (*rfc*) of the O-antigen. 186 *E. coli* O-antigens have been identified so far

(Samuel and Reeves, 2003). However, few of these are associated with UTI. O-antigens that are predominant in human UPEC (UTI-associated O-serogroups) are O1, O2, O4, O6, O7, O8, O16, O18, O25, O50, O75 (reviewed by Johnson, 1991). In canine and feline UTI, UPEC with O-antigens O2, O4, O6, O8, O9, O22, O25, O75 and O83 predominate (reviewed by Beutin, 1999). UPEC that possess these UTI-associated O-antigens have been shown to belong predominantly to phylogenetic groups B2 and D (Johnson et al., 2003). They likely also possess various other associated urovirulence factors, such as P fimbriae, S fimbriae, haemolysin, siderophore receptors IroN and FyuA and outer membrane protein OmpT (Johnson, 2003; Johnson et al., 2003; Johnson et al., 1994).

Capsular Antigens

More than 80 capsular antigens (so-called K antigens) have been identified in *E. coli* (as discussed by Johnson, 1991). These antigens have been categorised into 4 different groups, based on various genotypic and phenotypic characteristics (reviewed by Clarke et al., 1999 and Whitfield and Roberts, 1999). Most UPEC are coated by a thin layer of group II capsular antigens, the most common being K1 and K5 (Beutin, 1999; Johnson, 1991). Group II capsular antigens consist of thermolabile polysaccharides that are only expressed at temperatures above 20°C (Cieslewicz and Vimr, 1996). When expressed, capsular antigens may surround the outer cell membrane of *E. coli* as a negatively charged hydrophilic layer that inhibits the binding of (negatively charged) phagocytes and the deposition of complement factors on the cell wall (as discussed by Johnson, 1991). Furthermore, K1 and K5 have been shown to be highly similar to the host surface molecule N-CAM (Bitter-Suermann and Roth, 1987). These antigens may therefore render *E. coli* unrecognisable to the host immune system (as discussed in Hacker and Heesemann, 2000a) and may facilitate invasion of *E. coli* into deeper tissues (reviewed in detail by Kim et al., 2005). Some capsules are considered to confer complement resistance (reviewed by Johnson, 1991) or influence IL-8-mediated neutrophil migration (Russo et al., 2003a). Overall, capsular antigens are thought to contribute to (i) the colonisation of UPEC within the urinary tract; (ii) the ascend of UPEC towards the kidney; and (iii) the haematogenous spread of UPEC from the kidney to other organs (reviewed by Johnson, 1991 and Schneider et al., 2004).

In humans, *E. coli* that cause pyelonephritis and cystitis have been found to express capsular antigens K1, K2, K3, K5, K12, K13 and K20 more often than faecal isolates (cited by Johnson, 1991). Accordingly, 59% to 92% of human UPEC strains and 34% of faecal *E. coli* from healthy humans tested positive for the capsular-group-II gene marker *kpsM II* or *kpsMT II* (Johnson et al., 2005b; Kanamaru et al., 2003). In dogs, the *kpsMT II* gene marker has been identified in up to 29% of faecal strains (Johnson et al., 2003; Johnson et al., 2001c) and 46% to 76% of canine UPEC (Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d). *E. coli* strains that possess Group II capsular antigens often belong to the UTI-associated O-serogroups (reviewed by Beutin, 1999; Johnson, 2003 and Johnson et al., 2001d) and are strongly associated with phylogenetic groups B2 and D (Johnson, 2003; Johnson et al., 2001d). K1 and K5 antigens have also been shown to be associated with P-fimbriae and haemolysin, respectively (as discussed by Johnson, 1991). Capsular gene operons may be located on PAIs (Table 1.7; Schneider et al., 2004).

The group III capsular gene marker (*kpsMT III*) has been detected in 3% of both canine and human UPEC and 2% and 12% of faecal strains isolated from humans and dogs, respectively (Johnson et al., 2003; Johnson et al., 2005b). According to Johnson (2003), Group III capsules (e.g. K10, K54) are putative VF of ExPEC. However, their role in UTI has not been well established.

The polymerisation of Group II and Group III capsular antigens and the translocation of these polysaccharide chains across the cellular membrane are dependent on the existence of a membrane bound polymerisation and export complex (as discussed by Whitfield and Roberts, 1999). In strains that express Group II/III capsules, this polymerisation and export complex is encoded by a complex gene cluster, consisting of 3 different regions (reviewed in detail by Whitfield and Roberts, 1999). Group II and Group III capsular antigens have several homologous genes (*kpsMTCS)* and differ primarily in organisation of gene regions. Hybrids of group II and group III gene clusters also exist (Schneider et al., 2004). The Group II capsule genes and function of the encoding proteins are summarised in Table 1.9.

Table 1.9 Function of proteins encoded by the group II capsular operon[a]

Region	Function	Genes	Protein	Function	Gene sequence variability
1	Maturation	*kpsFEDUCS*	*kpsF, kpsU*	?	low
			kpsE	Subunit anchored in cytoplasm, facilitates kpsD function	
			kpsD	Periplasmatic translocation protein that interacts with cytoplasm and outer membrane by conformation change; facilitates translocation of polysaccharide to the outer membrane	
			kpsCS	Thought to be important for maturation of polysaccharide (attachment of phosphatidyl-Kdo[b]) and entry of polysaccharide into export pathway	
2	Synthesis/ Polymeri-sation	*kfiDCBA*	*kfiDCAB*	Synthesis and polymerisation of group II K antigen	high
3	Trans-location across cell membrane	*kpsMT*	*kpsM*	Transmembrane component of ATP-binding cassette (ABC) transport protein (kpsMT)	low
			kpsT	ATPase component of ABC transport protein (kpsMT)	

[a]: A summary of studies by Whitfield and Roberts, 1999; Arrecubieta et al., 2001 and McNulty et al., 2006.

[b]: phospahtidyl-Kdo: phosphatidyl-2-keto-3-deoxy-manno-octonic acid

Outer Membrane Protein T (OmpT)

Outer membrane proteins (OMP) closely interact with polysaccharides and lipopolysaccharides to fulfil several functions. OmpA, for example, contributes to the invasion of brain microvascular endothelium (Prasadarao et al., 1996) and OMP of the porin family are involved in export and import processes (reviewed by Haas and Hensel, 2000). OmpT, which acts as a serine protease, may degrade defensive proteins and complement that are released from host cells (as discussed by Kukkonen and Korhonen, 2004 and Johnson, 2003). OmpT has also been shown to process plasminogen to plasmin (Lundrigan and Webb, 1992). Therefore, it may contribute to fibrinolysis and subsequent invasion of bacteria from the primary site of infection (Webb and Lundrigan, 1996). Recently, OmpT has also been shown to induce a lipopolysaccharide-independent immune response (TNFα) in monocytes (Brandenburg et al., 2005). Thus, it is likely that OmpT contributes to the virulence of UPEC. More than 70% of UPEC isolated from humans have been reported to possess the *ompT* on their chromosome (Johnson et al., 2005b; Kukkonen and Korhonen, 2004; Webb and Lundrigan, 1996). *OmpT* has been detected particularly often in UPEC that cause pyelonephritis and urosepsis (Johnson et al., 2005b; Webb and Lundrigan, 1996). Faecal *E. coli* isolated from humans have been reported to possess *ompT* in up to 68% of all cases (Kanamaru et al., 2003; Kukkonen and Korhonen, 2004). In dogs, the *ompT* gene marker was found significantly less often in faecal *E. coli* (24%) than in *UPEC* (54%; Johnson et al., 2003).

TraT Outer Membrane Lipoprotein

TraT, a plasmid-encoded outer membrane lipoprotein, has been shown to increase the resistance to serum by inhibiting the correct formation of the membrane attack complex during the alternative pathway of complement activation. In detail, TraT is thought to interfere at the step of C5bC6 complex formation, either by inhibiting the formation or by changing the structure of C5bC6 to produce a non-functioning membrane attack complex (Pramoonjago et al., 1992). Furthermore, TraT may interfere with phagocytosis by restricting or altering opsonisation of bacteria with C3b (Aguero et al., 1984).

The gene *traT* originates from the *tra* operon of F-like plasmids (Sukupolvi and O'Connor, 1990). The *tra* operon contains 23 genes and enables bacteria to exchange genetic information via conjugation (Prasadarao et al., 1993; Sukupolvi and O'Connor, 1990). The *traT* gene may be encoded independently from other *tra* genes on large plasmids, such as R100 and ColV (as discussed by Sukupolvi and O'Connor, 1990). These plasmids may also encode other VF. In detail, *iss* (increased serum survival) and *iut* (aerobactin) are found on ColV and antimicrobial resistance genes *aadA, cat* and *tetA* are found on R100 (as discussed by Waters and Crosa, 1991 and Sampei and Mizobuchi, 1999). TraT may be found in various extraintestinal and intestinal pathogenic *E. coli* (reviewed by Sukupolvi and O'Connor, 1990). Approximately 65% of human *E. coli* that cause UTIs may be positive for the *traT* gene marker (Johnson et al., 2005b). *TraT* has also been suggested as a marker for UPEC causing urosepsis in humans (Johnson and Stell, 2000). In dogs with UTI, the *traT* gene marker was only detected in 17% to 50% of UPEC (Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d). Faecal *E. coli* from dogs and people may harbour *traT* in approximately 40% of all cases (Johnson et al., 2003; Sukupolvi and O'Connor, 1990). The significance of TraT's contribution to UPEC virulence remains to be elucidated (Johnson, 1991; Johnson et al., 2003).

Colicin V

Colicin V is an antimicrobial substance that inhibits other *Enterobacteriaceae* that do not secrete this antimicrobial substance (as discussed in detail by Waters and Crosa, 1991). Thus, colicin V-producing strains may show enhanced survival in dense bacterial populations, like the gastrointestinal tract. Colicin V is commonly encoded on large plasmids (termed ColV plasmids), but has also been found on the chromosome of intestinal *E. coli* (Fernandez-Beros et al., 1990). The colicin V operon consists of 4 genes: *cvi*, the gene that encodes immunity to colicin V, *cvaA* and *cvaB*, encoding export subunits and *cvaC*, the structural gene of colicin V (cited by Waters and Crosa, 1991). Colicin V has been associated with urovirulence (as discussed by Johnson, 1991) and is commonly found in human UPEC strains isolated from patients with pyelonephritis (Johnson et al., 2005b). However, it is uncertain whether the expression of colicin or the association of the colicin operon with other VFGs, such as *iss*, *traT* and

iut on ColV plasmids is the reason for the observed virulence of UPEC strains possessing colicin (as discussed by Johnson, 1991; Waters and Crosa, 1991 and Mühldorfer et al., 2001). Recently, the *cvaC* gene marker was found in 0% to 18% of UPEC causing canine UTI and 9% of UPEC causing human cystitis and prostatitis (Johnson et al., 2003; Johnson et al., 2005b; Johnson et al., 2000a; Johnson et al., 2001d). A significantly larger proportion of human UPEC causing pyelonephritis (41%) harboured the *cvaC* gene marker (Johnson et al., 2005b). This may indicate that *cvaC*-associated VFs are particularly often present in pyelonephritis strains or that strains that harbour *cvaC* show enhanced survival in the kidney (Johnson et al., 2005b).

Increased Serum Survival Factor (Iss)

Iss, a complement resistance factor gene, has been found on plasmids that often also encode colicin V and aerobactin (as discussed by Fernandez-Beros et al., 1990 and Waters and Crosa, 1991). Iss-expressing strains may be 20 x more resistant to complement and 100 x more virulent for one-day old chickens than Iss^- strains (Nolan et al., 2003). *Iss* has high homology to the bacteriophage λ gene *bor* that confers complement resistance to lysogenic *E. coli* (Barondess and Beckwith, 1995). Iss is thought to interfere with the alternative complement cascade by hindering the function of the membrane attack complex (Waters and Crosa, 1991). The *iss* gene marker has been found in up to 35% of human UPEC strains and 25% and 28% of human intestinal and bacteraemic strains, respectively (Fernandez-Beros et al., 1990; Johnson et al., 2005b). However, it appears considerably more important in *E. coli* that cause avian colibacillosis than in UPEC (Nolan et al., 2003). Up to 79% of these *E. coli* may harbour the *iss* gene (Nolan et al., 2003).

Siderophores and Siderophore Receptors

UPEC, like other living organisms, depend on iron for vital metabolic processes, like oxygen transport, DNA synthesis, mitochondrial energy metabolism and electron transport (Andrews et al., 2003; Hacker and Heesemann, 2000b). In a low-iron environment, such as urine or serum, UPEC may express siderophores and siderophore receptors, to increase acquisition of extracellular iron (Hacker and Heesemann, 2000b). Siderophores are small (<1kDa) iron-chelating molecules that may be secreted by UPEC to bind extracellular ferric iron (Fe^{3+}; reviewed in detail by Andrews et al., 2003). Siderophores utilize catecholate, hydroxamate or α-hydroxycarboxylates as Fe^{3+}-chelating ligands. Siderophore-Fe^{3+}-complexes may then attach to siderophore receptors located in the outer membrane of bacterial cells. Subsequently, the siderophore-Fe^{3+}-complex is actively transferred from the outer membrane to the cytosol. There, Fe^{3+} dissociates from the siderophore by reduction to ferrous iron (Fe^{2+}), which may be incorporated into metabolic, transport or storage proteins.

Siderophore receptors are generally barrel-shaped OMP that exhibit a high affinity for siderophore-Fe^{3+}-complexes (reviewed by Andrews et al., 2003). *E. coli* may not just express receptors that bind endogenous siderophores (i.e. siderophores it produced itself), but also siderophores that have been secreted by mammalian cells (e.g. transferrin, lactoferrin) or fungi (e.g. ferrichrome; as discussed by Andrews et al., 2003). *E. coli* commonly encode many functional siderophore systems. This may optimize iron acquisition in different milieu (e.g. urine, serum) or from different sources (e.g. mammalian siderophores; Russo et al., 2002). Furthermore, it may ensure iron acquisition when one siderophore system is targeted by antibodies or becomes dysfunctional after mutation (Russo et al., 2002). Siderophore receptors are a potential target for vaccines (Russo et al., 2003b). Moreover, siderophore uptake systems have been explored as active transport systems for antimicrobials (reviewed by Braun and Braun, 2002). These substances (i) occur naturally (so-called sideromycins, such as albomycin; Hartmann et al., 1979); (ii) are synthetic derivatives of rifamycin (Ferguson et al., 2001); or (iii) are synthetical conjugatives of sulphonamides, β-lactams or other antimicrobials with siderophores (reviewed by Braun, 1999). All molecules contain a receptor recognition epitope similar to siderophore-Fe^{3+}-complexes. Thus, they are

recognised by siderophore receptors and subsequently actively transported into the cytosol (reviewed by Braun and Braun, 2002).

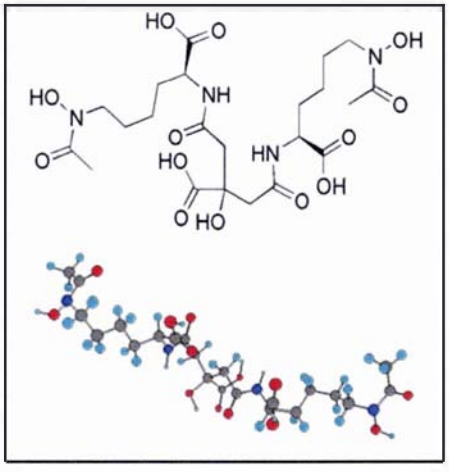
Gene transcription of siderophores and siderophore receptors is suppressed when intracellular and extracellular Fe^{2+} levels are high (reviewed in detail by Andrews et al., 2003). At high Fe^{2+} levels, intracellular Fe^{2+} binds to the ferric-uptake regulator protein (the so-called Fur). Fur acts as global regulator of iron homeostasis. Fur-Fe^{2+}-complexes have a high affinity for specific DNA binding sites. Binding of Fur to DNA suppresses the transcription of Fur-regulated genes.

A detailed description of aerobactin, a hydroxamate siderophore and yersiniabactin, a catecholate siderophore, as well as recently identified putative siderophore receptors IroN and IreA follows. These siderophores and siderophore receptors are considered important in UPEC.

Aerobactin and its Receptor

The Aerobactin siderophore was originally identified on a plasmid of *Aerobacter aerogenes* (now *Klebsiella pneumoniae* 62-1; Gibson and Magrath, 1969). Later, a genetic variant of the original aerobactin was found on plasmids (particularly the ColV plasmid and its replica) and the *E. coli* chromosome (reviewed by Waters and Crosa, 1991). Aerobactin has been shown to increase vitality and promote growth of *E. coli* strains in low-iron conditions, such as urine and serum (as discussed by Johnson, 1991 and Waters and Crosa, 1991). Aerobactin is a small polymer (616 Da), consisting of 2 lysine and one citrate molecules (Figure 1.6; cited by Johnson, 1991). The siderophore utilises a hydroxamate-chelating ligand to bind extracellular Fe^{3+} (Warner et al., 1981). Aerobactin is also able to extract Fe^{3+} from other iron-binding proteins (e.g. transferrin; cited by Johnson, 1991 and Waters and Crosa, 1991). After Fe^{3+} acquisition, the aerobactin-Fe^{3+}-complex is taken up through a 74kDa OMP and actively transported to the iron centres of the bacterial cell (Bindereif et al., 1982; Wooldridge et al., 1992). There, Fe^{3+} is reduced to Fe^{2+}, which

Figure 1.6 Aerobactin Structure



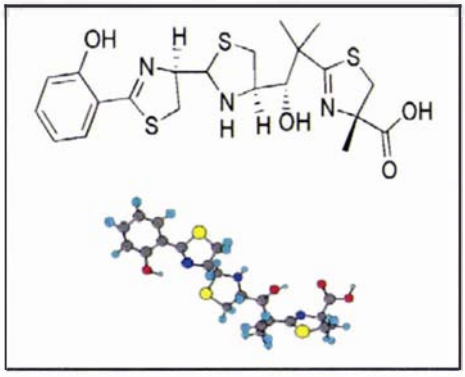
dissociates from aerobactin (as discussed by Andrews et al., 2003). The aerobactin operon comprises 5 genes (*iucDBACiutA*; de Lorenzo et al., 1986). *IucDBAC* (iron uptake chelate) encode proteins important for aerobactin synthesis (de Lorenzo et al., 1986). *IutA* (iron uptake) encodes the OM aerobactin receptor (de Lorenzo et al., 1986). Human UPEC that cause symptomatic UTI have been shown to express aerobactin more commonly than faecal *E. coli* or environmental *E. coli* (>49% *vs.* <41% *vs.* 6%; cited by Johnson, 1991). Aerobactin has been reported in 70% and 58% of strains that cause pyelonephritis and bacteraemia, respectively (Johnson, 1991; Johnson et al., 2005b). Up to 75% of human UPEC strains may encode aerobactin on their chromosome (cited by Johnson, 1991 and Waters and Crosa, 1991). Chromosomally encoded aerobactin may be found in association with haemolysin and P-fimbriae in strains that cause human UTI and urosepsis (Jacobson et al., 1988; Johnson et al., 1988). Plasmids that encode aerobactin may also encode other virulence genes (e.g. *iss*, *iroN*) and antimicrobial resistance genes (as discussed in detail by Johnson, 1991 and Waters and Crosa, 1991). According to Johnson (1991) and Waters and Crosa (1991), *E. coli* that possess plasmid-encoded aerobactin are primarily detected in isolates from habitats that are exposed to antimicrobials, such as human hospitals or intensive farming environments.

The aerobactin gene marker (*aer*) or the aerobactin receptor gene marker (*iutA*) have been found in <34% of canine and feline UPEC (Feria et al., 2000a; Feria et al., 2001a; Johnson et al., 2003; Yuri et al., 1998). In a study by Johnson et al. (2003), *iutA* was even more prevalent in faecal *E. coli* isolated from healthy dogs (44%). *Aer* was detected in <20% of haemolytic canine or feline UPEC (Feria et al., 2001a), which may indicate that it is not located on the chromosome. This theory is supported by a recent study Johnson et al. (2003), which showed that *iutA* of canine UPEC was negatively associated with UTI-associated O-antigens and the phylogenetic group B2. It would have been expected that chromosomal *iutA* is associated with UTI-associated O-antigens and phylogenetic group B2. This is because P-fimbriae and haemolysin have been demonstrated to be strongly associated with strains that belong to UTI-associated O-serogroups and the B2 phylotype (as discussed by Johnson et al., 1991). However, it is possible that aerobactin is encoded independent of P-fimbriae and haemolysin on the chromosome of UPEC.

Yersiniabactin and its Receptor

The Yersiniabactin siderophore system is associated with a high-pathogenicity island that was first discovered in human pathogenic *Yersiniae* (as discussed by Carniel, 2001).

Figure 1.7 Yersiniabactin structure



The high-pathogenicity island has also been found in other *Enterobacteriaceae*, particularly in *E. coli* of the phylogenetic group B2 and D (Schubert et al., 2002). The high-pathogenicity island has been shown to contribute significantly to lethality in mouse models and enhances growth of UPEC in ascending UTI (Schubert et al., 2002; Schubert et al., 2000). Schubert et al. (2002) proposed that pathogenicity conferred by yersiniabactin might not just be due to its siderophore function, but also due to modulation of the immune response or by promotion of the expression of other VFs via yersiniabactin regulator genes. Yersiniabactin has been shown to be well conserved in most (>70%) ExPEC (Schubert et al., 2002). Accordingly, more than 84% of human or canine UPEC tested positive for the genetic marker *fyuA*, which targets the yersiniabactin outer membrane receptor (Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2005b; Johnson et al., 2000a; Johnson et al., 2001d). Faecal canine *E. coli* encode the gene marker *fyuA* to a significantly lesser extent (35% to 44%; Johnson et al., 2003; Johnson et al., 2001c). The yersiniabactin operon approximates 30kb in size and consists of 11 genes that are important for synthesis, transport and regulation of yersiniabactin (Table 1.10; reviewed in detail by Carniel, 2001). Yersiniabactin is a 482 Da catecholate siderophore (Figure 1.7), that binds Fe^{3+} with an affinity higher than aerobactin (Schubert et al., 2002). The yersiniabactin-Fe^{3+}-complex is then recognised by FyuA (Ferric-yersiniabactin uptake), a 71 kDa OMP, which initiates the transport of the yersiniabactin-Fe^{3+}-complex into the cell (cited by Carniel, 2001).

Table 1.10. Gene designation and function of the yersiniabactin gene operon

Functional Region	Synthesis	Transport	Regulation
Gene designation	*irp*1-5, *irp*9	*fyuA*[a], *irp*6-7	*ybtA*
Synomym[b]	*ybtUTES*, *irp*2	*psn*[a], *ybtQP*	

[a]: encoding the yersiniabactin siderophore OM receptor
[b]: synonyms are equivalent to gene designation in *Y. pestis* and *Y. pseudotuberculosis* (Carniel, 2001)

Catecholate Siderophore Receptor IroN

In 1999, Russo et al. identified a putative VF of *E. coli* CP9 with a 77% gene sequence homology to the catecholate siderophore receptor *iroN* of *Salmonella enterica.* The expression of this VF, designated $iroN_{E.\ coli}$, increased 27-fold in iron-depleted urine. IroN was subsequently confirmed as a catecholate siderophore receptor that recognises siderophores enterobactin, enterochelin and similar catecholate siderophores (Russo et al., 2002; Sorsa et al., 2003). A mouse-model of ascending UTI, showing that *iroN* $^{+}$ UPEC outgrew *iroN* $^{-}$ mutants, confirmed that *iroN* possession contributes to *in vivo* urovirulence of UPEC (Russo et al., 2002). Russo et al. (2002) suggested that IroN acts not just as a siderophore receptor, but also interacts with uroepithelial cells in a non-adhesive manner. Despite enhanced urovirulence of *iroN* $^{+}$ strains *in vivo*, IroN did not enhance the growth of UPEC in iron-depleted urine *ex vivo*. Furthermore, it was shown that IroN did not confer adherence to bladder epithelial cells *in vitro* (Russo et al., 2002). *IroN* has consistently been found in significantly more UPEC than faecal *E. coli* (>39% *vs.* <24%; Bauer et al., 2002; Johnson et al., 2003; Johnson et al., 2001c; Kanamaru et al., 2003; Russo et al., 1999). It has been reported in 43% to 76% of human UPEC causing cystitis or pyelonephritis and 67% to 83% of human UPEC causing prostatitis (Bauer et al., 2002; Johnson et al., 2005b; Kanamaru et al., 2003). More than 50% of canine UPEC have *iroN* (Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d). It has been detected in all 4 UPEC isolated from cats that were tested so far (Johnson et al., 2001a). *IroN* has been identified on 3 different PAI in association with *sfa, foc, prsA-G* (*papG III*), *hly* or *cnf1* (Table 1.7; Dobrindt et al., 2002; Johnson et al., 2000b; Welch et al., 2002). Some strains may have IroN-encoding plasmids (Sorsa et al., 2003). The strong association of *iroN* with phylogenetic group B2 and UTI-associated O-serotypes further emphasises its significance in UTI (Johnson et al., 2003; Johnson et al., 2000b). Russo et al. (2003b) suggested IroN as a vaccine candidate. They demonstrated that subcutaneous vaccination with denaturated IroN protects mice from subsequent infection with *iroN* $^{+}$ *E. coli.*

Iron-responsive Element (IreA)

In 2001, Russo et al. described a novel putative siderophore receptor gene isolated from wild-type UPEC strain CP9. The gene, termed iron-responsive element (*ireA*), showed 29% to 38% homology and 48% to 56% similarity to previously identified siderophore receptor genes. *IreA* may be contained within a PAI on CP9 (Russo et al., 2001). Expression of IreA was shown to support growth in urine and bladder in a mouse model of ascending UTI, but not a systemic infection model (Russo et al., 2001). The *ireA* gene marker was found significantly more often in UPEC (13% to 47%, median 25.5%) than in faecal strains (0%; Johnson et al., 2005b; Russo et al., 2001). In particular, *ireA* was detected in 47% of human pyelonephritis strains and 40% of human UPEC isolated from recurrent cystitis (Johnson et al., 2005b; Russo et al., 2001). Epidemiological data about the presence of *ireA* in canine and feline UPEC could not be found in indexes of major search engines (PubMed and CAB abstracts).

1.3.3.3. Regulation of Urovirulence Genes & Phase Variation of Virulence Factors

Regulation of VFG is a complex process that is influenced by numerous environmental and intracellular factors (e.g. temperature, pH, oxygen content and availability of nutrients like iron, precursor amino acids and proteins; reviewed in detail by Emody et al., 2003; Johnson, 2003 and Morschhäuser, 2000b). Expression of many VFs is coordinated by global and local regulatory genes and proteins. Global regulators, such as *rfaH, leuX* and *lrp*, may coordinate the expression of multiple VFs (cited by Emody et al., 2003 and Schmidt, 1994). Local regulators, in general part of a particular VFG operon, promote or inhibit the expression of that particular VF (Gunther et al., 2002). However, regulatory genes of one VF might 'cross-talk' to other unlinked VFG operons (Balsalobre et al., 2000; Holden et al., 2001). For example, the regulator of P-fimbriae, *papB*, may inhibit the expression of Type 1 fimbriae when P-fimbriae are expressed (Holden et al., 2001).

While expression of VFs may be important for the establishment of infection and the vitality of UPEC, the antigenicity of expressed VFs may be disadvantageous for long-term persistence of UPEC in the urinary tract. UPEC may quickly adapt to a new environment by undergoing phase variation, that is, by switching between expression and suppression of VFs or by switching from expressing one gene to expressing another (reviewed by Morschhäuser, 2000a). As a result, the host may be confronted with a pathogen whose modified antigenic determinants are not recognised by the immune system. Thus, phase variation may contribute to UPEC's ability to defeat or escape host defences and may significantly contribute to UPEC virulence (Gunther et al., 2002; Schmidt, 1994).

1.3.3.4. Comparison of UPEC from Different Host Species

Several phenotypic and genotypic studies indicate that UPEC isolated from humans, dogs and to some degree also feline UPEC, share urovirulence determinants and phylogenetic characteristics (listed in the appendix, Table 8.1; Feria et al., 2000a; Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001d; Whittam et al., 1989; Yuri et al., 1998). In detail, UPEC isolated from humans, dogs and cats have been shown to belong predominantly to phylogenetic groups B_2 and D and to possess similar O-antigens (Beutin, 1999; Johnson et al., 2003; Whittam et al., 1989). Within the respective phylogenetic groups and serotypes, strains from different species may be more closely related (as assessed by MLEE and PFGE) than strains from the same species (Johnson et al., 2001a; Johnson et al., 2001d; Whittam et al., 1989). Furthermore, a large number of VFG markers that have been detected in human UPEC have also been found in canine and feline UPEC (Feria et al., 2001a; Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001d; Yuri et al., 1998). A comparison of *papG III* from canine and human *E. coli* revealed highly homogenous peptide sequences (Johnson et al., 2000a). Furthermore, it has recently been shown that UPEC clones can co-exist in human and animal members of one household (Murray et al., 2004). Studies that demonstrate apparent similarities between UPEC from different species and the possible cross-species transfer of UPEC emphasise the possibility that UPEC are not host-specific. However, it is yet uncertain whether some UPEC have adapted to specific hosts. Adaptation of UPEC to a specific host deserves consideration, because host factors that influence the survival of UPEC in the host, such as urine osmolarity or surface receptor repertoire, are likely to differ between species (Lees et al., 1979; Lindstedt et al., 1991). The assessment of differences or similarities in UPEC derived from dogs, cats or people is influenced by a possible variation of VFG within one species. For example, it may need to be considered that VFG profiles within one species differ in different geographic areas. Early work by Grüneberg and Bettelheim (1969) and Peddie et al. (1981) showed that human UPEC from different areas possess different O-antigen and H-antigen serotypes. A possible geographic variation of VFG profiles of UPEC from dogs, cats or people has not yet been the focus of any study. Thus, it is not known whether the geographic origin needs to be considered when comparing UPEC from different species.

1.3.3.5. Reservoirs

According to Mosby's Dictionary of Medicine, Nursing and Health Professions (Myers, 2006), a reservoir of infection is defined as a human, animal or plant that acts as a continuous source of infectious disease. In that sense, humans, dogs or cats may act as a reservoir for UPEC. An interesting notion is that certain tissues or organs may have "*reservoir function*" (Schilling and Hultgren, 2002). Suggested tissues with reservoir function for UPEC are the epithelial cells of the bladder, the distal genitourinary tract or the gastrointestinal tract of humans, dogs or cats. These reservoirs need to be considered when attempting the successful treatment of UTI. Putative or established reservoirs for UPEC are discussed in more detail below.

Uroepithelial Cells as a UPEC Reservoir

During the acute phase of a UTI, UPEC have been shown to establish a 'bladder reservoir' by invading the superficial umbrella-like cells of the bladder epithelium (Mulvey et al., 2001). The bacterial invasion mechanism and the associated host response have been the subject of several recent studies - *in vitro* as well as in the murine model - and numerous detailed reviews (Anderson et al., 2004a; Anderson et al., 2004b; Duncan et al., 2004; Justice et al., 2004; Kau et al., 2005; Mulvey, 2002; Mulvey et al., 2001; Mulvey et al., 2000; Schilling and Hultgren, 2002; Schilling et al., 2002; Schilling et al., 2001a, b). Importantly, the establishment of an intracellular reservoir, which so far has only been shown in murine models, may be one plausible explanation for the occurrence of relapses or long-term persistent infections (Schilling and Hultgren, 2002). Invasion of UPEC is mediated by FimH, the adhesin of Type 1 fimbriae (Mulvey, 2002). FimH has been shown to bind to Uroplakin Ia, a constituent of the lipid rafts that form the superficial layer of the bladder epithelium. Upon binding to Uroplakin Ia, FimH induces a complex signalling cascade that results in the internalisation of the FimH $^{+}$ UPEC (Mulvey, 2002). Intracellularly, UPEC are contained within vacuoles that become the basis for intracellular bacterial communities (Justice et al., 2004; Mulvey, 2002). Intracellular bacterial communities have been shown to undergo 4 growth stages that differ in regards to motility and shape of UPEC, organisation of the intracellular bacterial community and the replication rate (Table

1.11; Justice et al., 2004). After maturation (also called pod formation) UPEC re-emerge from the host cell. This stage is also called efflux. The re-emerging UPEC may subsequently cause a UTI relapse. Alternatively, they may invade deeper epithelial layers and survive several months in a dormant state (Schilling et al., 2001a). Triggered by an unknown signal, they may subsequently re-emerge and cause a UTI (Schilling and Hultgren, 2002). Invading UPEC induce exfoliation and migration of immune cells to the invaded epithelium (Schilling et al., 2001a). However, UPEC may react to these defence mechanisms in a complex manner and may often maintain colonisation (Justice et al., 2004; Schilling et al., 2001a). Moreover, Schilling et al. (2002) recently demonstrated that FimH $^{+}$ UPEC can effectively persist within mice bladders despite 3-day and 10-day treatment with trimethoprim-sulphamethoxazole that rapidly established urine sterility. It is likely that intracellular UPEC also evade other antimicrobials. Thus, alternative treatment approaches, such as vaccines or phage vectors*, may need to be considered.

Table 1.11 Growth characteristics of intracellular bacterial communities[a]

Stage		Duration (h)	Organisation	Shape	Motility	Growth rate
I	Establishment of IBC[b] "Bacterial factory"	< 8	Loosely within a vacuole in the cytosol	Rod-shaped	Non-motile	Rapid growth (double in 35 minutes)
II	Maturation "Pod formation"	10-14	Highly organised biofilm	Coccoid	Non-motile	Slow growth (double in > 1h)
III	Detachment	-	Detachment from IBC, Efflux	Rod-shaped	Motile	
IV	Filamentation	*E. coli* cells grow, but do not divide and become up to 70 µm in length. Observed from late stage III onwards. Failure to divide may be due to *in vitro* artefact or an unknown reaction to host signals.				

[a]: As observed by time-lapse fluorescence video-microscopy (Justice et al., 2004)
[b]: IBC: intracellular bacterial community

* Phage vector: Phages may be delivered to intracellular infections inside attenuated bacteria that have retained the ability to invade host cells (Broxmeyer et al., 2002).

The Intestine and Distal Genitourinary Tract as a Reservoir

The observation of similarities between UTI-causing strains and *E. coli* strains isolated from the intestine at the time of UTI led to the theory that UTI is an opportunistic infection, caused by migration of abundant intestinal *E. coli* strains to the urinary tract (Cooke, 1974; Gruneberg, 1969). Subsequent studies revealed that intestinal *E. coli* strains differ from UPEC (as discussed in chapter 1.3.2). However, UPEC often predominate in the intestinal tract and distal urogenital tract (vagina, urethra) at the time of UTI (Caugant et al., 1983; Johnson et al., 1998a; Yamamoto et al., 1997). Furthermore, many healthy individuals may harbour UPEC strains in their intestine or distal urogenital tract (Manges et al., 2004; Moreno et al., 2006a; Yuri et al., 1998). The frequency at which faecal colonisation with UPEC leads to an establishment of UTI has not yet been established. Manges et al. (2004) suggested that intestinal colonisation with UPEC may rarely progress to UTI in otherwise healthy hosts. Several factors may predispose to the development of an ascending UTI. In humans, established factors that predispose to UTI are the sex of the host, sexual contact, immune status of the host (compromised *vs.* non-compromised), colonisation of the distal genitourinary tract with other microorganisms and virulence of the particular UPEC strain (Foxman et al., 2002; Johnson et al., 1998a; Johnson et al., 1994; Johnson and Russo, 2005; Johnson and Stell, 2000; Roos et al., 2006b; Tseng et al., 2002). In dogs and cats, host compromise may also predispose to UTI (as discussed by Bartges, 2005). However, this has not yet been studied in detail.

External Reservoirs

Members of a close community, e.g. a household, have been shown to share intestinal bacterial strains, including UPEC, commonly (Foxman et al., 2002; Manges et al., 2004; Murray et al., 2004). Furthermore, it has been shown that faecal *E. coli* and UPEC from dogs and cats share virulence characteristics and phylogenetic attributes with human UPEC strains (Feria et al., 2001a; Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001c; Johnson et al., 2001d; Yuri et al., 1998). This evidence has raised the concern that dogs and other pets may act as a reservoir for UPEC that may cause life-threatening extraintestinal diseases in humans (Feria et al., 2001a; Johnson et al., 2001c;

Yuri et al., 1998). This theory is further supported by a recent study by Murray et al. (2004) that identified a strain that co-colonised all members of one household (a human heterosexual couple and a cat) and caused a UTI episode in the woman. However, when closely examining the colonisation pattern of the household members, it appears likely that the women developed a UTI after transmission of UPEC from the man, not the cat. This is because (i) no *E. coli* strain was isolated from the feline faeces during the week the UTI episode occurred in the woman; (ii) a week before, an *E. coli* strain different to the UTI-causing strain predominated in the faeces of the cat; (iii) the UTI-causing UPEC clone was first identified in the urine of the man (who did not have symptoms); and (iv) the UPEC clone co-colonised the urinary tract of the human couple until the UTI episode of the woman was treated. Furthermore, the human couple had regular sexual contact, during which the UPEC clone may have been transferred (Foxman et al., 2002). Studies that estimate how often transfer of UPEC from pets to people occurs could not be found in indexes of major search engines (PubMed and CAB abstracts). A recent study by (Sannes et al., 2004) shows that UPEC isolated from humans are often more antimicrobial resistant than *E. coli* strains found in the faeces of dogs. Thus, Sannes et al. (2004) suggest that transmission from pets to humans occurs infrequently. In fact, the authors state that pets may acquire pathogenic *E. coli* from humans.

1.3.3.6. Current Therapy of *E. coli* UTI in Dogs and Cats

Antimicrobial therapy has been the cornerstone of the treatment of *E. coli* UTI since antimicrobials became available. The selection of appropriate antimicrobials is commonly based on susceptibility of the isolated UPEC in that geographic region (Bartges, 2005). If susceptibility tests are not available at the time antimicrobial therapy is started, selection is based on knowledge about the common susceptibility pattern of UPEC (Bartges, 2005). Previous occurrences of UTI episodes, underlying anatomical or neurological conditions and concurrent diseases other than UTI also need to be considered when treating *E. coli* UTI. Furthermore, the cost of the antimicrobial, dosing (quantity, interval) and documented adverse drug reactions may influence which antimicrobial is chosen (Bartges, 2005; Kelly et al., 1979a; Kelly et al., 1979b, Ling, 2000). In dogs and cats, a 10 to 14 day course of Trimethoprim-Sulphadiazine or first or second generation cephalosporins are currently recommended as the treatment of choice in uncomplicated *E. coli* UTI when susceptibility patterns of the isolate are not known (Table 1.12; Bartges, 2005). Treatment is considered successful when no growth is observed upon culturing cystocentesis-derived urine collected 5 to 7 days after completion of the antimicrobial course (Bartges, 2005). Complicated *E. coli* UTI may require longer treatment periods (>4 weeks) and the use of different antimicrobials, such as fluoroquinolones and gentamicin (Bartges, 2005). In some cases "prophylactic" long-term (>6 months) low-dose antimicrobial treatment may need to be considered (Bartges, 2005). To evaluate efficacy of 'middle' and long-term antimicrobial treatment, urine cultures and susceptibility testing before, during and 5-7 days after antimicrobial therapy have been strongly recommended (Lulich and Osborne, 2004).

To prevent possible sequelae, antimicrobial treatment of any UTI, even those that are only diagnosed by positive urine culture from cystocentesis-derived urine, is currently recommended in dogs and cats (Bartges, 2005). Conversely, 'clinically silent' UTI or asymptomatic bacteriuria (ABU) may not be treated in humans (Nicolle, 2000b). Despite the fact that ABU may develop into symptomatic UTI, it has been shown that treatment of ABU with antimicrobials does not decrease rate of morbidity (Nicolle, 2000a, b). On the contrary, it may be harmful due to the occurrence of antimicrobial-associated adverse effects and the emergence of antimicrobial resistance. Moreover, ABU strains have been used to prevent symptomatic UTIs caused by more virulent

strains in humans with neurogenic bladders after spinal cord injury (Darouiche et al., 2005). This approach utilised the ability of ABU strains to inhabit the urinary tract. By doing so, the ABU strain hindered the virulent UPEC strain from colonisation, much like commensal enteric bacteria prevent intestinal pathogens from colonising the intestinal tract.

Antimicrobial Resistance – Current Situation and Future Prospects

The use of a specific antimicrobials to treat *E. coli* UTI is at some stage followed by the development of resistance of *E. coli* to that antimicrobial (Walsh, 2003a). The speed with which resistance to the antimicrobial develops depends on the quantity of antimicrobial dispensed within a short time, the number of conditions other than UTI for which the antimicrobial is used and the frequency with which subtherapeutic levels occur (as discussed in detail by Walsh, 2003a). Furthermore, resistance to an antimicrobial may develop faster, when resistance to antimicrobials with similar structure or mechanism is already present. The occurrence and timeline of spread of resistance also depends on the resistance mechanism (Table 1.12). For example, chromosomally acquired resistance, like resistance to fluoroquinolones, often develops because of point mutations and may predominantly be transferred vertically. The frequency with which point mutations result in resistance is considered low. Thus, chromosomally conferred resistance may take several years to develop (Walsh, 2003a). However, once present, resistance may persist for years, even if the respective antimicrobial is discontinued (Cohn et al., 2003). Conversely, horizontally acquired resistance, like the plasmid-encoded resistance to β-lactams or sulphonamides, may spread quickly when antimicrobials confer a selection pressure. Resistance plasmids may be lost by the pathogens once the antimicrobials selecting for resistance are discontinued. The frequency with which UPEC resistance to widely-used antimicrobials is nowadays observed and the ongoing spread of resistance to 'front-line' antimicrobials, have raised concerns about the future of antimicrobial treatment (Drazenovich et al., 2004; Feria et al., 2000b; Gordon and Cowling, 2003; Kahlmeter, 2003; Seguin et al., 2003). Even more concerning is the development and spread of resistance to 'second-line' antimicrobials that are used to treat complicated infections. Resistance of canine UPEC to fluoroquinolones, for example, has significantly increased in veterinary hospitals over the last 2 decades (Cohn et al., 2003; Cooke et al.,

2002). This is probably due to a more widespread use of fluoroquinolones. In addition, an increasing number of fluoroquinolone-resistant UPEC are resistant to several other antimicrobials, including extended spectrum β-lactams (Cooke et al., 2002; Sanchez et al., 2002; Warren et al., 2001). Alarmingly, fluoroquinolone resistance-conferring plasmids have been detected in several *E. coli* and *Klebsiellae* that were isolated from humans in Europe, China and the USA (Mammeri et al., 2005; Wang et al., 2004; Wang et al., 2003). These plasmids may also confer antimicrobial resistance to most β-lactams, aminoglycosides, sulphonamides, rifampin, trimethoprim and chloramphenicol (Mammeri et al., 2005).

Table 1.12 Antimicrobials commonly used to treat *E. coli* UTI in dogs and cats – Mechanism of action and mechanism of resistance developed by bacteria[a]

Antimicrobial	Mechanism of action	Resistance	
		Mechanism	Gene location
Trimethoprim	Inhibition of DNA and RNA synthesis by inhibiting the folate metabolism enzyme Dihydrofolate reductase	Modification of the affinity of Dihydrofolate reductase to trimethoprim results in a decreased binding and efficacy of trimethoprim	Transposons, Plasmids, Integrons
Sulphonamides[b]	Inhibition of DNA and RNA synthesis by inhibiting the folate metabolism enzyme Dihydropteroate synthase	Equivalent to trimethoprim	Transposons, Plasmids, Integrons
β-lactams[c]	Inhibit peptide bond formation during cell wall synthesis	Production of β-lactamase, an enzyme that cleaves the β-lactam molecule	Plasmids, Chromosome, Integrons
		Modification of the affinity for β-lactams results in decreased binding and efficacy of β-lactams	Chromosome
Fluoroquinolones[d]	Inhibition of DNA replication by inhibiting DNA-Gyrase function (i.e. supercoil introduction in DNA)	Modification of affinity of DNA-Gyrase or topoisomerase enzymes for fluoroquinolones by point mutation in gyrase or topoisomerase gene	Chromosome Plasmid[A]
Aminoglycosides[e]	Irreversible attachment to the 30S ribosomal subunit and subsequent block of protein synthesis initiation	Production of enzymes (N-acetyltransferase, Phosphotransferase or Adenyltransferase) that render aminoglycosides dysfunctional	Plasmids, Transposons, Integrons

[a]: Material taken from Hacker and Heesemann, 2000a, Walsh, 2003b and [A] Mammeri et al., 2005
[b]: Sulphonamides: e.g. Sulphadiazine, Sulphamethoxazole
[c]: β-lactams: Penicillins, Cephalosporins, Carbapenemes, Monobactames
[d]: Fluoroquinolones: e.g. Enrofloxacin, Marbofloxacin, Ciprofloxacin
[e]: Aminoglycosides: e.g. Gentamicin, Streptomycin, Amikacin, Kanamycin

Interestingly, UPEC that are resistant to multiple antimicrobials have been shown to be less virulent. They may belong to different phylogenetic groups than 'typical' UPEC (i.e. A instead of B_2 and D) and often possess less VFs than antimicrobial susceptible strains Horcajada et al., 2004; Johnson et al., 2004; Moreno et al., 2006b). Thus, it is likely that resistant UPEC derived from non-pathogenic strains that were subjected to a high selection pressure and that pathogenicity of these 'opportunistic UPEC' is predominantly based on their sheer abundance and colonisation of predominantly compromised hosts (Johnson et al., 2004). Nonetheless, such 'emerging' multidrug-resistant UPEC clones may cause persistent, sometimes life-threatening infections (Drazenovich et al., 2004; Sanchez et al., 2002; Warren et al., 2001) and may have the potential to cause 'UTI epidemics' (Johnson and Russo, 2002b; Manges et al., 2001).

Several measures, such as antimicrobial-use surveillance programmes, detection of potential reservoirs and subsequent prevention of spread of resistant pathogens from these reservoirs, have been recommended to slow the development of antimicrobial resistance (Gordon and Jones, 2003; Kahlmeter, 2003; Morley et al., 2005; Sannes et al., 2004). At the same time, new antimicrobial interventions are being developed. Some of these antimicrobials may be specifically tailored to target UPEC. For example, some antimicrobials use siderophores as carriers (as discussed by Braun and Braun, 2002). Others target subunits important for the assembly of fimbriae (Lee et al., 2003). Furthermore, vaccines that target VFs are currently being developed (reviewed by Russo and Johnson, 2006). This study, however, investigates whether can be used to combat UTIs caused by UPEC.

Chapter 2

Materials and Methods

2.1. *Escherichia coli* Strain Collection

2.1.1. Test Strains

A total of 57 canine and 29 feline *E. coli* isolates, collected from NZ patients with suspected UTIs, was assessed in this project. NZ *E. coli* strains were kindly provided by Gribbles Veterinary Pathology Laboratories in Palmerston North, Auckland, Hamilton and Linton or by the Microbiology Laboratory of the Institute for Animal, Veterinary and Biomedical Sciences (IVABS), Massey University (please refer to the appendix 8.4.4 for contact details). Sample collection method and clinical and laboratory information (listed in the appendix 8.4.1) were used to group isolates according to their likelihood of being urinary pathogens or contaminants (Table 2.1).

Table 2.1 *E. coli* from the strain collection - Differentiation between UPEC and non-UPEC

Category No.	Likelihood that *E. coli* was not a contaminant	Features
1	Very likely	Collection by cystocentesis[a] **OR** Collection by cystocentesis not confirmed, but moderate to severe pyuria (± haematuria, crystalluria, bacteriuria) or SG <1.015 **AND** Pure, heavy growth of *E. coli* [b]
2	Likely	Criteria of category 1 not fulfilled **AND** Abnormal urinalysis result indicative of UTI (e.g. mild pyuria, mild-severe haematuria, bacteriuria) **OR** **Cats**: pure, heavy growth of *E. coli* [b] **Dogs**: Mixed growth accepted only when urinalysis result indicative of UTI **AND** History indicative of UTI, but other disease (e.g. idiopathic cystitis, urolithiasis) cannot be excluded

[a, b]: please refer to body text below

According to Comer and Ling (1981) and van Duijkeren et al. (2004), sample collection by antepubic cystocentesis decreases the likelihood of contamination with cutaneous or faecal organisms. Therefore, *E. coli* isolates from urine samples collected by cystocentesis were considered more likely to have derived from parts of the urinary tract that are normally considered to be sterile, that is, the bladder, ureters or kidneys (Ling, 2000).

According to van Duijkeren et al. (2004), growth of mixed organisms is rarely observed in cystocentesis-derived urine samples from cats. Conversely, mixed growth is commonly observed in urine samples from cats collected by free-catch or catheterisation. Thus, mixed growth may indicate a contamination with faecal or cutaneous organisms. Therefore, *E. coli* isolated from mixed-growth cultures of urine from cats were excluded. In dogs, up to 21% of urine samples may yield a mixed growth of organisms irrespective of the sampling method (Ling et al., 2001). Thus, *E. coli* isolated from mixed-growth urine samples of dogs were included into category 2 if additional criteria were fulfilled.

Eleven human urinary *E. coli* were obtained from Medlab Central Ltd (please refer to the appendix 8.4.4 for contact details). The *E. coli* had been isolated from mid-stream urine of 11 female patients (2 treated in the hospital, 9 as outpatients).

Seven faecal *E. coli* were obtained from 3 cats and 4 dogs housed at the Veterinary teaching hospital (dogs) or the Feline Unit (cats) at Massey University. *E. coli* strains were isolated from <4-hours-old droppings of dogs and cats. At the time of sample collection, these animals had no clinical signs of UTI or gastrointestinal disease.

All NZ isolates had been confirmed to belong to the *E. coli* genus by the providing labs using standard biochemical techniques (Quinn et al., 1994). Cultures (on nutrient agar slants) were collected personally or shipped from the providing laboratory to the Microbiology Laboratory of IVABS according to the guidelines for shipment of dangerous goods*.

* Land Transport Rule Dangerous Goods 2005, Rule 45001/1.
http://www.ltsa.govt.nz/rules/docs/dangerous-goods-2005.pdf; IATA Guidance document Infectious

A further 31 feline urinary *E. coli* strains were kindly provided by Professor Jonathan Elliott and Dr. Andrew Rycroft from the Royal Veterinary College, London, UK (please refer to appendix 8.4.4 for contact details). These *E. coli* strains were isolated from cystocentesis-derived urine samples of 20 pet cats with suspected UTI and concurrent renal insufficiency or chronic renal failure. Urine samples were collected at 2 primary cat practices in central London where geriatric cat clinics were run by researchers from the Royal Veterinary College. The isolates had been cultured on sheep blood agar using 2 µl of uncentrifuged urine. Organisms had been identified as *E. coli* using cultural and biochemical criteria (Barrow and Feltham, 1993). Strains were stored at the Royal Veterinary College in 20% glycerol in 10% skimmed milk at -70°C. For shipment to NZ, subcultures were prepared on nutrient agar slants. The samples were shipped and imported according to the Dangerous Goods Regulations of the International Air Transport Association (IATA) and the Ministry of Agriculture and Forestry, NZ.

Upon acquisition of NZ and UK samples, subcultures were prepared and Indoltest or Microbact™ test (Microbact™ gram-negative identification system, Oxoid Ltd., Auckland, NZ) were used to confirm the bacterial species. The isolates were stored at -70°C in 15% glycerol broth until further use.

For each set of experiments, a subset of *E. coli* organisms was chosen from the *E. coli* strain collections (Table 2.2). Detailed information about each subset and the patients *E. coli* derived from is given in each chapter.

Substances: http://www.iata.org/NR/rdonlyres/B8B91553-49BE-4DCC-901B-50DA4E57A98E/0/GuidanceDocument18Nov05.pdf

Table 2.2 Subsets of *E. coli* chosen from the strain collection

No. of isolates	Description	Experiment of Chapter			
		3	4	5	6
15/29	Feline *E. coli* from NZ, category 1, selected for VF-PCR study[a]	X	X	-	X
7/29	Feline *E. coli* from NZ, category 1, not selected for VF-PCR study[a]	-	-	-	X
7/29	Feline *E. coli* from NZ, category 2, selected for VF-PCR study[a]	X	-	-	-
22/31	Feline *E. coli* from 1st time UTI from UK, category 1, selected for VF-PCR study	-	X	9/22	-
9/31	Feline *E. coli* from recurrent UTI from UK, category 1, selected for VF-PCR study	-	-	X	-
45/57	Canine *E. coli* from NZ, category 1, selected for VF-PCR study[a]	X	-	-	30/45
1/57	Canine *E. coli* from NZ, category 1, not selected for VF-PCR study[a]	-	-	-	X
11/57	Canine *E. coli* from NZ, category 2, selected for VF-PCR study[a]	X	-	-	-
11/11	Human *E. coli* from NZ, selected for VF-PCR study	X	-	-	-
7/7	Faecal *E. coli* from NZ dogs (4) and cats (3), not selected for VF-PCR study	-	-	-	X

[a] Selection for VF-PCR study occurred at random.

2.1.2. Control Strains

Control isolates for the multiplex PCR (L31, J96, V27, PM9 and 2H25) with known VFG profile (Table 2.3; Johnson et al., 2001a; Johnson et al., 1997; Johnson and Stell, 2000) were kindly provided by Professor James R. Johnson MD, Department of Infectious Diseases, VA Medical Center, Minneapolis, MN, USA. *E. coli* isolates were obtained on nutrient agar slants and subcultured upon arrival. Subcultures were stored at –70°C in 15% glycerol broth until further use.

2.2. Virulence Factor Genotyping by Multiplex PCR

2.2.1. DNA Isolation

DNA was extracted from all isolates that were subjected to virulence factor genotyping according to the method by Ausubel (1988) with minor adjustments. In detail, the isolates were cultured on Luria Bertani (LB) agar (Merck Ltd, Palmerston North, NZ) at 37°C overnight. One colony was collected from each plate and grown for 16-24 hours in LB broth (Merck Ltd, Palmerston North, NZ) at 37°C in shaking motion. Alternatively, bacteria were cultured on LB agar at 37°C for 16-24 hours. The growth from agar plates was suspended in 1 ml sterile isotonic saline (0.9 M NaCl). After centrifugation of the broth or suspension at 14,000 x g for 2 minutes (ALC Microcentrifugette 4214, Thermo EC, Waltham, MA, USA), the bacterial pellet was collected and resuspended in 250 ml 0.5x TE-buffer. 50 µl 10% sodium dodecyl sulphate (SDS) and 5 µl proteinase K (20µg/ml; Roche, Mannheim, Germany) were added. The suspension was incubated at 50°C until the turbidity resolved; which was usually achieved after 3 hours. After adding 100 µl 5 M NaCl and 80 µl CTAB*/NaCl solution (0.7 M NaCl, 10% CTAB), the suspension was incubated at 65°C for at least 30 minutes. Phenol:chloroform:isoamyl-alcohol (25:24:1) was added at an equivalent volume to the sample volume and the tubes were vortexed carefully. The suspension was transferred to a 1.5 ml Phase Lock Gel™ tube (Eppendorf South Pacific Pty. Ltd., North Ryde, NSW, Australia) and spun at 14,000 x g for 5 minutes (ALC Microcentrifugette 4214). The extraction was repeated until a clear aqueous layer remained. The aqueous sample was transferred to a clean microtube and 3M sodium acetate (pH 5.5) at 1/10 of the sample volume was added. Ice-cold isopropanol was added at a volume equal to the sample volume to precipitate the DNA. The mixture was stored at –20°C for 30 minutes. A DNA pellet was collected after centrifugation at 14,000 x g for 5 minutes (ALC Microcentrifugette 4214). The pellet was washed with 200 µl ethanol (70%) that subsequently evaporated and left a dry DNA pellet. The pellet was resuspended in 250 µl 0.5x TE-buffer and kept at room temperature for 2-4 hours to allow DNA to equilibrate. The DNA samples were stored at 4°C until further use.

* Cetyltrimethylammonium bromide

2.2.2. Evaluation of the DNA Concentration

The concentration of the DNA samples was determined using a Gene-Quant Spectrophotometer (Pharmacia Biotech, GE Healthcare, Auckland, NZ). The concentration of DNA was measured at a wavelength of 260 nm. The purity of DNA was assessed using the ratio 260 nm:280 nm. Samples that contained >50 ng/µl DNA at a ratio between 1.8 and 2.0 were subjected to molecular weight analysis.

2.2.3. Assessment of the Quality of Extracted DNA

The molecular weight of extracted DNA was determined with gel electrophoresis. A high DNA mass ladder (Invitrogen™, Auckland, NZ) was used to specify the size of analysed DNA. A 1:20 dilution of extracted DNA was made and 1/5 of the sample volume of DNA-tracking dye (5x SDS dye) was added. Samples were run on a 1% Agarose gel (certified molecular biology agarose, Bio-Rad, Auckland, NZ) in 0.5x TBE-buffer. DNA was allowed to migrate using a voltage of 100 mV for 50 minutes. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 µg/ml) for 10 minutes and destained in micro-filtered water for 5 minutes. The gel was photographed under UV light using the Gel-Doc™ system (Bio-Rad, Hercules, CA, USA). Samples containing a sufficient amount of DNA (>50ng/µl) with a molecular weight greater than 10 kB were used for further analysis.

2.2.4. Multiplex PCR

Thirty VFG markers were studied in a total of 120 UPEC strains (53 isolated from cats, 56 from dogs and 11 from humans; Table 2.2) using a multiplex PCR much as described by Johnson and Stell (2000). DNA from each *E. coli* isolate was subjected to 5 multiplex PCRs. Primer pair combinations for each set are shown in Table 2.3.

Table 2.3 Multiplex PCR: Primer pair combination and VFG profile of control strains

Set	Gene marker	Gene detected	Gene encodes	VFG profiles of control strains[a]				
				L31	J96	V27	PM9	2H25
1	*MalX*	PAI	Pathogenicity-associated island (CFT073)	+	+	+	-	-
	PapA	*papA*	P-fimbrial core subunit	+	+	+	-	+
	FimH	*fimH*	Type 1-fimbrial adhesin unit	+	+	+	+	+
	KpsMT III	*kpsM III; kpsT III*	Group III capsular protein membrane translocation subunits	-	+	-	-	-
	PapEF	*papE; papF*	P-fimbrial minor subunits linking core and adhesion	-	+	+	-	+
	IreA	*ireA*	Iron-regulated putative siderophore receptor	-	-	+	-	-
	Ibe10[b]	*ibeA*	Invasin (Invasion of brain endothelium)	-	-	-	-	-
2	*Univcnf*	*cnf1*	Cytotoxic necrotizing factor 1	+	+	-	-	-
	FyuA	*fyuA*	Yersiniabactin siderophore receptor	+	+	+	+	+
	Ironec	*iroN*	Siderophore receptor, Transport of catecholate siderophores	+	+	+	+	-
	BmaE	*bmaE*	M-agglutinin subunit	-	-	-	+	-
	Sfa/foc	*sfaDE; focC*	S-fimbrial subunits; F1C- fimbrial subunits	+	+	+	-	-
	AerJ	*iutA*	Aerobactin (siderophore) receptor	-	-	+	+	+
	PapG III	*papG III*	P-fimbrial adhesin unit, Variant III	-	+	-	-	-
3	*HlyD*	*hlyD*	Haemolysin (chromosomal)	+	+	-	-	+
	Rfc	*rfc*	O4 antigen polymerase	-	+	-	-	-
	OmpT	*ompT*	Outer membrane protease T subunit	+	+	+	-	-
	PapG I'	*papG I'*	P-fimbrial adhesin unit, Variant I'	+	-	-	-	-
	PapG I	*papG I*	P-fimbrial adhesin unit,Variant I	-	+	-	-	-
	KpsMT II	*kpsM II; kpsT II*	Group II capsular protein membrane translocation subunit	+	-	+	-	+(blot)
	PapC	*papC*	P-fimbrial usher unit	+	+	+	-	+
4	*GafD*	*gafD*	N-acetyl-D-glucosamine specific fimbrial lectin	-	-	-	+	-
	CvaC	*cvaC*	Colicin V structural (toxic) unit	-	-	-	+	-
	H7[b]	H7 *fliC*	H7 flagellin variant	-	-	-	-	-
	Cdts	*cdtB*	Cytolethal distending toxin B subunit	-	-	+	-	-
	FocG	*focG*	Type F1C- minor fimbrial subunit	-	+	+	-	-
	TraT	*traT*	Complement resistance gene	+	+	-	+	+
	PapG II	*papG II*	P-fimbrial adhesin unit, Variant II	-	-	+	-	+
5	*G allele I*[c]	*papG I*	Complete P-fimbrial adhesin unit I	-	+	-	-	-
	G allele II & III[c]	*papG II/III*	Complete P-fimbrial adhesin unit II or III	-	-	-	-	+
	Iha	*iha*	Iron-regulated gene A homologue adhesin	-	-	+	-	+
	Afa/dra	*afaBC; draBC*	Dr antigen-specific adhesion operons (AFA, Dr, F1845)	-	-	-	-	+
	SfaS	*sfaS*	S-fimbrial adhesin unit	+	-	-	-	-
	Iss	*iss*	Increased serum survival gene, Outer membrane protein	-	-	-	+	-
	kpsMT K1[b]	*kpsM K1, kpsT K1*	Group II capsular protein membrane translocation subunit	-	-	-	-	-

[a]: VFG profile: According to previous studies (Johnson et al., 2001a; Johnson et al., 1997; Johnson and Stell, 2000): +: VFG marker present; -: VFG marker absent. Primer sequences used (Appendix 8.4.3.1) have been published previously (Johnson and Stell, 2000). Updates were kindly provided by Professor J.R. Johnson (personal communication).

[b,c]: Please refer to body text below

The multiplex PCR assay included markers *Ibe10*, *H7* and *kpsMT K1*. However, none of the listed control strains encodes these genes. A positive control strain for these genes (RS218) was kindly provided by Professor Johnson, Department of Infectious Diseases, VA Medical Center, Minneapolis, MN, USA. However, gene amplification from this strain was inconsistent despite repeated DNA extraction. A wild-type strain from the collection of this project could have been used to create a control strain by gel extraction of a PCR product of the size of *Ibe10*, *H7* or *kpsMT K1* and confirmation of the gene identity by DNA sequencing. However, I chose to exclude these 3 markers from the analysis because they have previously been reported in less than 30% of human UPEC analysed (Johnson et al., 2005b) and the time that would have needed to be spent to create a positive control was invested in studying a larger number of UPEC strains.

During initial optimisation, it was observed that markers for *G allele I* and *G allele II/III* were amplified inconsistently in control strains that were considered to possess these markers. Amplification of markers for complete genes *papG II/III* did also not correspond with amplification of markers for individual genes *papG II* (set 4) or *papG III* (set 2). The reason for this inconsistency is unknown. A possible explanation is that primers did not anneal consistently. This and other explanations could have been investigated further. However, this was not done because such an investigation would have added little information about VFG profiles of strains. Instead, I chose to exclude these markers from analysis.

The markers *Ibe10*, *H7*, *kpsMT K1*, *G allele I* and *G alleles II/III* were left in the multiplex PCR assay set because initial experiments showed that a change of the original multiplex PCR assay method led to changes in amplification of other PCR products. The time that would have been needed to optimise a PCR method not including these primers was invested in studying a larger number of UPEC strains.

2.2.5. PCR Conditions

Per sample and set, a 12.5 µl reaction-mixture containing 4 mM $MgCl_2$, 200 µM of each dNTP (Roche, Mannheim, Germany), 0.5 unit Taq DNA Polymerase in 1x PCR buffer (Invitrogen™ Life Technologies, Auckland, NZ), 0.6 µM of each primer (custom-

primer, Invitrogen™ Life Technologies, Auckland, NZ) and 100 ng template DNA was prepared. 0.5 g/l bovine serum albumin (New England Biolabs Inc., Ipswich, MA, USA) was also added to each sample to optimise amplification results (Henegariu et al., 1997). Each set of samples was accompanied by a negative control (reaction mixture without sample DNA) and positive controls (vials containing DNA of control *E. coli* strains with known VFG profile; Table 2.3).

PCRs were run in a Gene Amp PCR System 9600 (Perkin Elmer, Boston, MA, USA). Cycling consisted of denaturation (94°C; one minute), amplification (25x 94°C, 30 seconds; 58°C for set 1-4 or 63°C for set 5, 30 seconds; 68°C, 3 minutes) and final extension (68°C, 5 minutes). Upon completion of the PCR run, the samples were kept at ≤15°C until gel electrophoresis.

2.2.6. Gel Electrophoresis of PCR Samples

For gel electrophoresis of PCR samples, DNA-tracking dye (5x SDS dye) was added to the PCR samples at 1/5 of the sample volume. Five to 10 µl of the so prepared sample was pipetted into a well of a 2% agarose gel (certified molecular biology agarose, Bio-Rad, Auckland, NZ) and run in 0.5x TBE at 90V for 1.5 hours. Gel staining and image capture was done as described in paragraph 2.2.3. Positive amplification of VFGs was identified visually by comparison of band sizes to a 100 bp ladder (Invitrogen™ Life Technologies, Auckland, NZ) and to *E. coli* control strains.

After the initial optimisation of the method, during which PCRs were run at least in duplicate and reproducibility of results was checked visually by comparing gel electrophoresis patterns, PCRs were run once. PCR results that were considered inconsistent (no bands per set or poor separation of bands) were rarely observed. When this occurred, results were reassessed by repeating the PCRs. If DNA integrity was questionable, DNA extraction was repeated or integrity was confirmed by amplification of the housekeeping gene *aroE* in a separate PCR, according to a method by Reid et al. (2000).

2.3. Macrorestriction Analysis by Pulsed Field Gel Electrophoresis

16 feline UPEC, 18 canine UPEC and 5 human UPEC from NZ and 31 feline UPEC from UK were subjected to macrorestriction analysis by PFGE done as described by Alley et al. (2002), with minor modifications.

In detail, *E. coli* isolates were grown on sheep blood agar at 37°C overnight. One colony from each plate was picked and incubated in brain-heart-infusion broth (Difco™, BD, Auckland, NZ) at 37°C for approximately 18 hours. The optical density of samples was measured at a wavelength of 610 nm (Helios Alpha spectrophotometer, Thermo EC, Waltham, MA, USA) and adjusted to an optical density of 1.1. Of each sample, a 150-µl volume was centrifuged at 14,000 x g for 5 minutes (ALC Microcentrifugette 4214). Cells were washed with PETT IV buffer*, centrifuged and resuspended in 50 µl PETT IV buffer*. The cell suspension was mixed with 100 µl low-melt preparative agarose (1%; Bio-Rad, Auckland, NZ). The mixture was dispensed into plug molds and left on ice for one hour to solidify. To achieve cell lysis, the agarose plugs were placed into lysis buffer and incubated at 56°C overnight. Upon completion of cell lysis, the plugs were transferred to 10 ml TE buffer. The vials containing plugs and TE buffer were kept on ice and gently agitated for one hour. This process was repeated a further 4 times. The washed plugs were stored in 1 ml TE buffer at 4°C.

Restriction endonuclease digestion was initiated by transferring 1/3 of the plug into 1 ml restriction buffer, in which the plugs were allowed to equilibrate for 45 minutes on ice. The restriction buffer was removed and replaced by 1 ml cutting buffer, containing *Xba*I (Roche, Mannheim, Germany). The samples were kept on ice for further 45 minutes before incubation at 37°C for 24 hours.

The restriction fragments were separated on a Clamped Homogenous Electric Fields-DR II Mapper (Bio-Red, Hercules, California, USA) in a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad, Auckland, NZ) for 20 hours (6 V/cm; pulse time linearly increasing from 0.5 seconds to 18 seconds) in 0.5x TBE buffer kept at 14°C.

*PETT IV buffer: Please refer to appendix 8.4.2.2, page 249, for the recipe.

Lambda ladder PFG marker and low range PFG marker (New England Biolabs Inc., Ipswich, MA, USA) were included as molecular size standards. Upon completion of electrophoresis, the gel was stained and photographed as described in paragraph 2.2.3.

After image capture, the clonality of isolates was determined according to the criteria presented by Tenover et al. (1995). These criteria correlate the occurrence of genetic events, such as loss or gain of restriction sites and gene insertion or deletions with changes in PFGE banding patterns. Tenover et al. (1995) state (i) that 2 strains belong to one clone if they possess indistinguishable PFGE banding patterns; (ii) that 2 strains are likely to be clonally related if 2 to 3 PFGE bands differ between strains; (iii) that 2 strains are possibly clonally related if 4 to 6 bands differ between strains; and (iv) that strains are considered unrelated if $\geq$7 bands differ between strains.

Dice coefficient similarity matrices and dendrograms (using the Average Linkage method) were created from unweighted PFGE banding patterns using the Diversity Database^{TM} software, version 2.0 (Bio-Rad, Hercules, CA, USA).

The significance of clusters (Figure 5.1, chapter 5 and Figure 3.3, chapter 6), observed at a similarity level between 28% and 45%, was tested using a jacknifing procedure by Schmid et al. (1999). In brief, 30 isolate quartets consisting of 2 randomly chosen isolates within the putative cluster and 2 randomly chosen isolates outside the putative cluster were generated for each observed cluster. Following this, the Dice coefficient similarities of all members of the quartets were assessed. A quartet was considered as supportive for the observed cluster when the Dice coefficient similarity of isolates within a putative cluster to each other was higher than the Dice coefficient similarity between any of the isolates within a putative cluster with any of the isolates outside a putative cluster. The number of supportive quartets was recorded. A z-test determined whether the frequency at which supportive quartets were observed was significantly exceeding 0.33, the maximum frequency at which a random association of 2 isolates in a set of 4 would be expected.

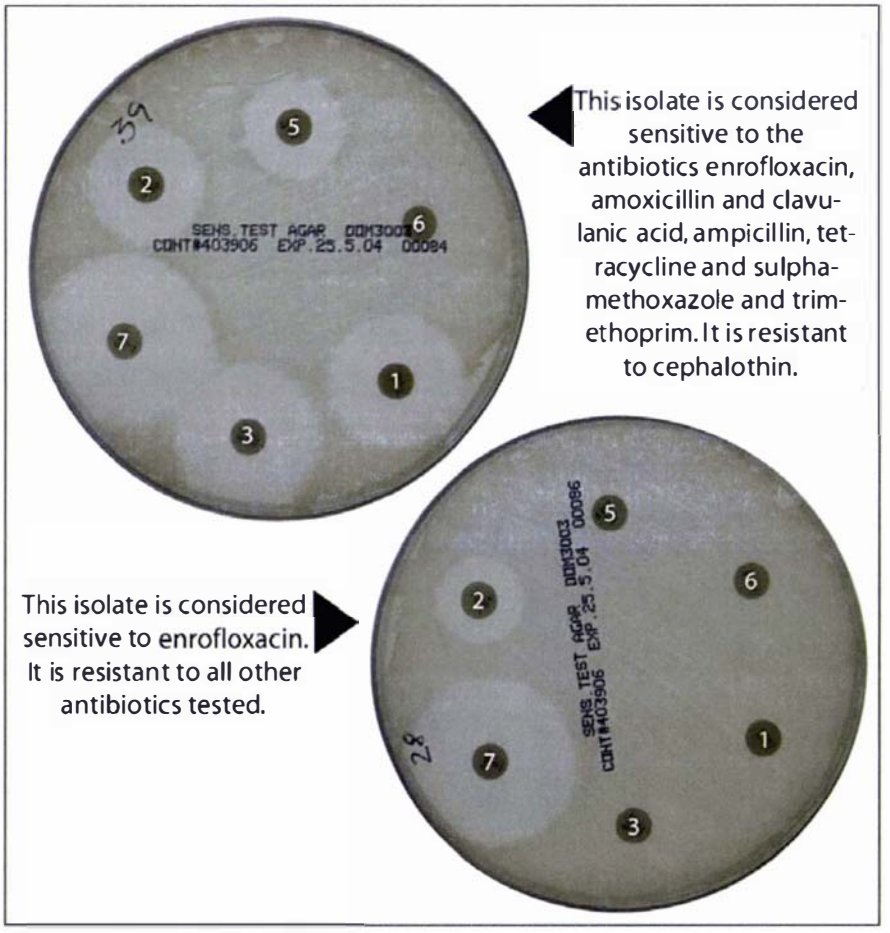
2.4. Antimicrobial Susceptibility Testing

The antimicrobial susceptibilities of 18 feline urinary *E. coli* that were isolated from 6 UK cats with UTI (Figure 5.2; Chapter 5) were tested using antimicrobial discs (Oxoid Ltd, Auckland, NZ) and Etests® (AB Biodisk, Solna, Sweden) for determination of MICs (Figure 2.2). Tests were performed according to the National Committee for Clinical Laboratory Standards (NCCLS) for antimicrobial disc and dilution susceptibility tests for bacteria (NCCLS, 2002). Isolates were classified as susceptible, intermediate or resistant based on NCCLS standards.

The following antimicrobial discs were used:

1. Tetracycline (30 μg),
2. Amoxicillin and clavulanic acid (20/10 μg),
3. Sulphamethoxazole/trimethoprim (23.75/1.25 μg),
4. Cefuroxime (30 μg),
5. Ampicillin (10 μg),
6. Cephalothin (30 μg),
7. Enrofloxacin (5 μg),
8. Ciprofloxacin (5 μg).

Figure 2.1 Examples of Kirby Bauer disc diffusion testing



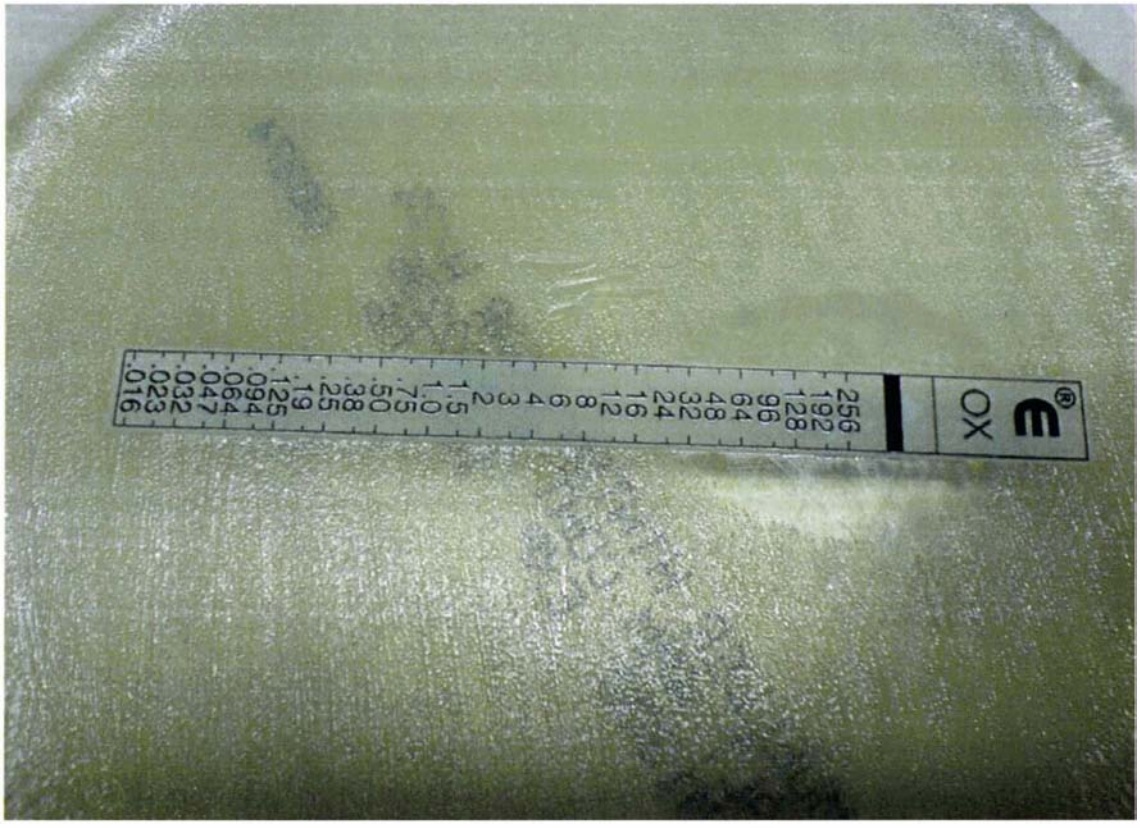
Discs: 1 Tetracycline, 2 Amoxicillin/Clavulanic Acid,
3 Sulphamethoxazole/Trimethoprim, 5 Ampicillin, 6 Cephalothin, 7 Enrofloxacin

The following Etests® test strips with impregnated concentration gradients of antimicrobials (Figure 2.2) were used:

1. Tetracycline (0.016-256 μg/ml),
2. Ampicillin (0.016-256 μg/ml),
3. Amoxicillin and clavulanic acid (2/1; 0.016-256),
4. Cefuroxime (0.016-256 μg/ml),
5. Sulphamethoxazole/trimethoprim (19/1; 0.002-32 μg/ml) and
6. Ciprofloxacin (0.002-32 μg/ml).

An antibiogram was interpreted as having indicated a relapsing or persisting infection when the susceptibility pattern of the current infecting *E. coli* isolate was identical to that of a previous isolate from the same cat (Barsanti, 2006). When the antibiogram of a current infecting *E. coli* differed from that of previous isolates from the same cat, a reinfection was diagnosed.

Figure 2.2 Etest® antimicrobial susceptibility testing

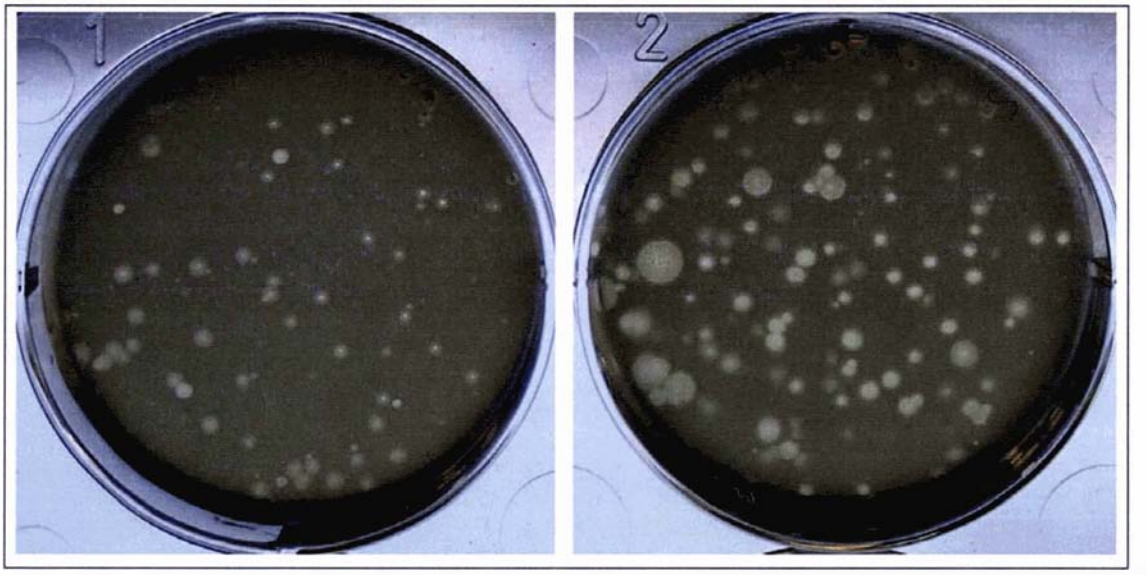


This image was supplied by Lynn Rogers and Roger Athersuch, Medlab Central Ltd. Palmerston North, NZ. The growth of this *E. coli* strain is inhibited at an oxytetracycline concentration of ≤24 μg/ml.

2.5. Preparation of Phage Stock

Phages were isolated from Palmerston North sewage according to a method published by Biswas et al. (2002) using NaCl enrichment, centrifugation at 10,000 x g for 10 minutes (GSA rotor, Sorvall 5C plus, Thermo EC, Waltham, MA, USA), polyethylene glycol 8000 precipitation, chloroform extraction and propagation on selected UPEC strains. Propagation of phages was performed on 21 canine and 21 feline NZ urinary *E. coli*, chosen at random from all *E. coli* strains that were very likely to resemble UPEC (i.e. category 1 strains). Prior to propagation, the chosen 42 *E.* coli strains were grown separately in LB broth overnight at 37°C. Propagation was done using the double-layer technique (as described in Ackermann and DuBow, 1987a). In detail, 100 µl of processed sewage was added to 100 µl of a selected *E. coli* culture. After incubation for 20 minutes at 37°C, the mixture was added to 2 ml molten LB top agar (LB broth containing 0.65% agarose, 50 °C), gently mixed and poured onto 55 mm LB agar plates. After overnight incubation at 37°C, a well-separated phage plaque was picked from each plate displaying plaques (as described in Sambrook and Russell, 2001).

Figure 2.3 Isolation of phages from processed sewage



A variety of different phage plaques was observed after 2 different UPEC strain cultures (1 plate each) were mixed with processed sewage and incubated overnight.

Each of the picked plaques was separately suspended in 1 ml SM buffer. Harvested phages were plaque purified on their target *E. coli* isolates by repeating the double-layer technique 2 more times, using the newly obtained phage suspension instead of processed sewage. Small-scale liquid cultures and large-scale lysates of purified phages were prepared according to standard methods (Sambrook and Russell, 2001). Subsequently, phage stock suspensions were prepared by polyethylene glycol precipitation and chloroform extraction of large-scale lysates (Sambrook and Russell, 2001). Phage stock titres were checked by serial dilution and plaque assay, counting the plaque forming units on the *E. coli* strain on which they had been propagated (Sambrook and Russell, 2001). Stocks containing $\geq 10^7$ phages/ml were considered appropriate for further analysis. Phage stocks were stored in 10 ml SM buffer containing 1% (v/v) chloroform at 4°C until further use.

2.6. Bacteriophage Lysis Experiment

The lysis experiment was designed to test the ability of each phage to lyse strains from a collection of 53 UPEC (31 canine, 22 feline) and 7 faecal *E. coli* (Table 2.2; experiment of chapter 6). Each phage (40 in total) was tested on each of the 60 *E. coli* strains. The 60 bacterial strains were grown separately in LB broth overnight and kept at room temperature until use at the same day. Initially, lysis was tested using a modification of the macroplaque technique described in Sambrook and Russell (2001). In detail, 200 µl of a certain *E. coli* culture was mixed with 1 ml molten LB top agar and poured onto a layer of LB agar (55 mm plate). Phages were transferred from the phage stock suspension to the freshly plated culture using sterile wooden toothpicks. The toothpicks were dipped into phage stock suspension, gently swirled to allow adherence of phage particles and pierced several times into the freshly plated bacterial culture. This procedure was repeated for each of the 40 phages and 60 *E. coli* strains in the collection (i.e. 40 x 60 plates were prepared). After overnight incubation at 37°C, plates were checked for evidence of a clear zone of lysis around the toothpick mark. When lysis was present, a positive result was declared. The modified macroplaque technique was compared with the standard double-layer plating technique (see above) on 30 randomly chosen bacterial cultures, using 7 randomly chosen phages. Positive results of the

modified macroplaque technique were 100% in accord with results of the standard plating technique. When a zone of lysis was not clearly visible using the modified macroplaque technique (i.e. lysis ≤ 1mm around the toothpick mark), the phage's ability to lyse the bacterial strain was tested using a standard double-layer plating procedure. After overnight incubation at 37°C, plates were observed for presence or absence of plaques. When plaques were present, a positive result was declared. Absence of plaques was interpreted as a negative result.

2.7. Electron Microscopy

Electron microscopy (EM) was performed on 5 phages selected from the phage population based on their ability to lyse a broad host range of UPEC. A known T4 phage preparation was used as measurement control. 10 ml of each phage stock suspension was concentrated by ultracentrifugation at 30,000 x g for 2 hours at 4°C (SS 34 rotor, Sorvall RC 5C, Thermo EC, Waltham, MA, USA). Phage pellets were carefully resuspended in 1 ml SM buffer and kept on ice until EM was performed. For each phage EM study, 1 drop of the prepared phage concentrate was placed on Parafilm M® (Brand Scientific Pty Ltd, Silverwater, NSW, Australia). A formvar-carbon-coated copper grid (200 mesh, Agar Brand, Agar Scientific, Stansted, Essex, UK) was floated on top of the drop of phage concentrate for 4.5 to 6 minutes, blotted dry and stained with 2% uranyl acetate (UA; pH 4.2) or phosphotungstate (PT; pH 7.0) for 4.5 to 6 minutes. The copper grid was blotted dry and studied under a Philips 201C transmission electron microscope (Philips, Eindhoven, Holland) using an accelerating voltage of 60 kV. For each of the 5 selected phage strains and the T4 control, phage size was obtained by averaging measurements of > 20 phage particles photographed at a magnification of 72,100 x normal size. Measured sizes were compared with published sizes for T4 (Büchen-Osmond, 2006).

2.8. DNA Sequencing of Bacteriophage DNA Fragments

Four phages that morphologically resembled T4-like phages were subjected to PCR analysis and DNA sequencing of the amplified tail tube glycoprotein gene 18 fragment to compare these phages to phylogenetically well-characterized phages (Tetart et al., 2001). Bacteriophage DNA was extracted and purified from phage stock concentrates using a commercial phage DNA extraction kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia). PCR was done in a 25 µl reaction mix containing 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.4 µM of each primer FT18-N2 and FT18-C3 (Tetart et al., 2001; appendix 8.4.3.2), 1x buffer, 1 u Platinum® Pfx DNA Polymerase (Invitrogen™, Auckland, NZ) and 50 ng template DNA. Cycling consisted of initial denaturation (2 minutes at 94°C) followed by a primary cycle period (10 x 30 s at 94°C; 15 s at 58°C; 50s at 68°C), a secondary cycle period (20 x 30s at 94°C; 15s at 56°C; 45s at 68°C) and a final extension (2 minutes at 68°C). The amplified DNA fragment was separated by gel electrophoresis, excised from the gel and purified using a commercial gel extraction kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia). To increase the yield of phage DNA for sequencing purposes, the PCR was repeated in a second round using each purified DNA fragment as template. Subsequently, DNA fragments were purified with a PCR purification kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia). Sequencing was done with an ABI 3730 Genetic Analyzer, using the BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Inc., Foster City, CA, USA). Obtained nucleotide sequences were compared with previously published nucleotide sequences (Altschul et al., 1997; Tetart et al., 2001). A similarity matrix based on the distance setting uncorrected p and a dendrogram using the Neighbour Joining method were created from Clustal W aligned sequences (Chenna et al., 2003) using the software PAUP* 4.0. (Swofford, 2003).

2.8.1. Nucleotide Sequence Accession Numbers

The nucleotide sequences of the central portion of gene 18 from phages 1-4 have been deposited in the GenBank database under accession no. DQ647771-DQ647774.

2.9. Statistics

Unless otherwise stated, statistical tests were performed using the software SPSS, version 11.5, 13 or 14 (SPSS Inc, Chicago, IL, USA) or Minitab, version 13 or 14 (Minitab Inc., State College, PA, USA).

2.9.1. Studies on VFG profiles of UPEC from Different Species

In chapter 3, the presence and distribution of VFG markers in UPEC isolated from New Zealand dogs (56), cats (22) and humans (11) was investigated. Furthermore, 18 canine, 16 feline and 5 human UPEC were subjected to macrorestriction analysis to determine their clonal relatedness. The statistical methods used as part of the work described in chapter 3 were as follows:

Inter-species differences in the proportions of a particular VFG marker were assessed using the $\chi 2$-test. UPEC from all species (canine, feline and human) were incorporated into the $\chi 2$-test. If significant differences were observed, Fisher's exact test was used to detect significant differences in proportions of VFG markers between UPEC from 2 particular species (i.e. canine and feline UPEC, canine and human UPEC or feline and canine UPEC). For problems of multiplicity, results of $\chi 2$-tests and Fisher's exact tests were adjusted using the Holm's step-down procedure (Ludbrook, 1998). Results with a p-values ≥ 0.05 were considered significant.

Stepwise, cross-validated discriminant analysis, based on the Mahalanobis distance with an F-to-enter value of 3.84 and an F-to-remove value of 1.5 was used to predict species membership (canine, feline or human) from extended VFG profiles. Twenty-five different VFG markers, present at least once in the sample population, were entered into the discriminant analysis.

Inter-species relationships were further assessed by nearest neighbour analysis as described by (Schmid et al., 1992). In detail, a Dice coefficient similarity matrix was constructed using VFGs of all isolates. The observed number of nearest neighbours from the same species (dog, cat or human) was determined and recorded. The number of nearest neighbours from the same species that would be expected by chance (X) was determined with the formula $X = P*n$. P was defined as the probability P that an isolate

from a specific species had a nearest neighbour from the same species. The number of isolates from that species was defined as n. The probability *P* was determined with the formula: $P=(n-1)*(N-1)^{-1}$, with N being defined as the total number of isolates in the test. The expected and observed values were then incorporated into a Fisher's exact test to determine whether the observed number of isolates with nearest neighbours from the same species significantly exceeded the number of isolates that could have had a nearest neighbour from the same species by chance. Results were adjusted for problems of multiplicity using the Holm's step-down procedure (Ludbrook, 1998). Similarly, a nearest neighbour analysis of PFGE Dice coefficient similarity matrices was performed. The purpose of this analysis was to investigate the clonal relatedness of 39 UPEC isolates from different species and different geographic regions.

Principal component analysis of extended VFG profiles was used to illustrate the degree of variance in VFG patterns of UPEC from the same species and to illustrate differences and similarities between UPEC from different species. For principal component analysis, VFG markers that best described the sample population were chosen using the following approach: First, the number of markers that detected genes encoding subunits of one VF was reduced to one, if it was predicted that these VFG markers would be typically detected together in a large majority of UPEC. For example, markers detecting different structural subunit genes of P-fimbriae (*papA, papC* and *papEF*) were considered very likely to be found together. Thus, only one of these markers was included in analysis. Second, one VFG marker was chosen out of a group of VFG markers that were equally or near equally present or absent in UPEC strains. By using this procedure, the number of VFG markers was reduced to 6 (*papEF, papG II, traT, iroN, sfa/foc* and *sfaS*). These markers were chosen to create 3 principal components. For each principal component, the species centroid (mean and median) and the variance around the species centroid (range; first quartile and third quartile) were determined. The association of all UPEC isolates was illustrated by plotting the principal component coefficients for each UPEC isolate in a 3-dimensional graph. In detail, the position of a particular isolate on the x, y and z-axes was determined by the coefficients of principal components 1, 2 and 3 for that isolate, respectively. The squared distances between group centroids of 2 different species in the x-y, x-z and y-z dimensions were calculated from the mean values of PC coefficients.

2.9.2. Investigation of the Genotypic Differences between Feline UPEC from New Zealand and Feline UPEC from Great Britain

In chapter 4, the genotypic differences and similarities between feline UPEC isolated from cats with suspected UTI from the UK (22) and NZ (15) were investigated. The statistical methods used in this chapter were as follows:

Student's t-test was applied to assess age differences between the two different cat populations (UK or NZ). Fisher's exact test was used to compare *E. coli* gene proportions between populations from UK and NZ. The relationship of two individual genes to each other was tested using Cochran's method, setting the geographic origin as the discriminating stratum and individual genes as the dependent factors. A positive or negative relationship between individual genes was identified using the phi coefficient and the common odds ratio. To predict population membership (UK or NZ) the VFGs were subjected to a stepwise, cross-validated discriminant analysis, based on the minimisation of Wilk's Lambda with an F-to-enter value of 1.25 and an F-to-remove value of one. The genetic association between isolates was assessed further using the VFG Dice coefficient similarity matrix in a nearest neighbour analysis method as described previously. The z-test was applied to assess whether observed number of isolates with nearest neighbours from the same country significantly exceeded the number of isolates that could have had a nearest neighbour from the same country by chance. Similarly, a second nearest neighbour analysis was performed on PFGE profiles. A method described by (Al-Samarrai et al., 2000) was used to assess the correlation between PFGE Dice similarity matrix and VFG Dice similarity matrix. For each isolate, corresponding Dice similarity matrix values were determined and plotted in a 2-dimensional graph (displaying the VFG and PFGE Dice similarity matrix values on the y and x-axis, respectively). Because of the large number of data points, isolates were grouped according to their PFGE value in 10% intervals, showing the mean, maximum and minimum VFG value of the group. The correlation between resulting groups was then assessed on mean VFG values using the Student's t-test. Results of statistical tests with a p-value ≤ 0.05 were considered significant.

2.9.3. Assessment of the Reliability of Antibiograms in Distinguishing Relapsing or Persisting Infections from Reinfections in Cats with Multiple Diagnoses of *E. coli* UTI

In chapter 5, antimicrobial susceptibility profiles of *E. coli* from cats with multiple diagnoses of UTI were compared with genomic fingerprints, determined by PFGE, to evaluate whether antimicrobial susceptibility profiles can reliably predict *E. coli* clonality.

The molecular typing method PFGE was chosen because it has been advocated as a typing method with high discriminatory power if a high diversity of restriction patterns is to be expected in the targeted bacteria (Blanc, 2004; Tenover et al., 1997). Previous epidemiologic studies that apply PFGE to UPEC show that a high diversity of restriction patterns, and therefore a high discriminatory power, can be expected in UPEC (Arbeit et al., 1990; Zingler et al., 1992). PFGE consistently distinguished epidemiologically unrelated UPEC with identical serotypes and ribotypes. It has been applied to distinguish between same-strain and different-strain recurrences in humans (Russo et al., 1995) and dogs (Drazenovich et al., 2004).

For the study described in chapter 5, the discriminatory power of PFGE for distinguishing relapsing or persisting infections from reinfections caused by feline UPEC was determined by investigating how often identical patterns were observed in the absence of the possibility of a relapse (i.e. when comparing isolates from different cats). Among 119 possible comparisons, there was only one instance in which 2 isolates had an indistinguishable PFGE pattern*. Thus, the discriminatory power of the PFGE method among the isolates assessed here was at least 0.991 (i.e. the probability that identity between 2 patterns was the result of a relapsing or persisting infection was >99%).

Having established the high discriminatory power of PFGE, previously described criteria presented by Tenover et al. (1995) were used to define relapsing or persisting infections and reinfections. A relapsing or persisting UTI was diagnosed when the *E. coli* isolate causing the current infection had a PFGE banding pattern indistinguishable from that of an isolate that had caused a previous UTI episode in the

* The 2 different cats these isolates were collected from lived in one household.

same cat. A reinfection was diagnosed when the PFGE banding patterns of *E. coli* isolates causing repeated UTI episodes differed by ≥7 bands.

Categorisations made using results obtained from Etests® and disc diffusion tests (as described in paragraph 2.4) were compared with those obtained by PFGE.

2.9.4. Assessment of the Feasibility of Bacteriophage Therapy for Treating Infections Caused by Canine or Feline UPEC

In chapter 6, the potential of phages to lyse UPEC isolated from dogs and cats was investigated. A potential correlation between lysis and the presence of VFG markers that detect surface-encoding molecules was assessed. Furthermore, lysis of faecal *E coli* from dogs and cats by naturally occurring phages was evaluated. The statistical methods applied in chapter 6 were as follows:

The z-test of proportions was used to test whether the proportion of UPEC undergoing lysis was related to the species of origin (i.e., canine versus feline UPEC) and whether the proportion undergoing lysis was related to the category of *E. coli* (i.e. UPEC versus faecal *E. coli*). Fisher's exact test was used to assess whether there were significant differences in presence of VFG markers between UPEC isolated from dogs and cats. Results of z-tests and Fisher's exact test were adjusted for problems of multiplicity using Holm's step-down procedure (Ludbrook, 1998). Stepwise binary logistic regression analyses were performed to assess whether a particular phage's ability to cause lysis was influenced by the presence or absence of 8 VFG markers that encode surface molecules and the origin of the UPEC isolate (i.e. canine or feline). The 9 VFG markers included were *papA*, *papEF*, *papG II*, *papG III*, *sfaS*, *focG*, *ireA*, *iroN* and *kpsMT II*. Initially, all 8 VFG markers and the species origin were included in the regression models. When the initial model indicated a significant influence of at least one of the variables on the phage's ability to cause lysis (i.e. the *P*-value of the model was <0.01*), the number of variables was reduced stepwise in subsequent models to achieve a model that best fits the data.

* To minimize the occurrence of a type II error, only logistic regression models with an overall *P*-value of <0.01 were considered significant.

Chapter 3

Studies on the Virulence Factor Gene Profiles of Uropathogenic *Escherichia coli* isolated from dogs, cats and people living in New Zealand

3.1. Abstract

The presence and distribution of 30 VFG markers, known to be present in UPEC in other parts of the world, was investigated in urinary *E. coli* isolated from dogs (56), cats (22) and people (11) living in New Zealand. Twenty-five VFG markers were amplified at least once in New Zealand UPEC. VFG profiles were very diverse. In total, 74 different VFG profiles were observed in 89 different UPEC strains. A multivariate comparison of VFG profiles indicated that 2 groups of UPEC may exist among dogs, cats and people: UPEC with VFG combinations that are shared among the different host species and UPEC with VFG profiles that differ in relation to the host species from which they were sourced. Up to one-third of UPEC from dogs, cats or people possessed VFG profiles that were highly similar, or identical, to VFG profiles observed in UPEC that originated from one of the other species. These UPEC with a shared VFG profile may be able to infect humans and pets with similar ease. However, discriminant analysis and principal component analysis showed that the majority of feline and human UPEC tested here apparently possessed 'host-specific' VFG profiles. Furthermore, species-specific differences in proportions of single VFG markers were identified in 2/30 (7%) instances, namely for *papG II* and *traT*. These VFG markers were significantly more often detected in human UPEC than in canine UPEC (P=0.003 and P=0.006 for *papG II* and *traT*, respectively) and feline UPEC (P=0.003 for *papG II* and *traT*). Due to limitations associated with the sampling size and the retrospective character of this study, it cannot be ruled out that these apparent 'host-specific' differences in VFG profiles were a result of comparing VFG profiles of subsets of UPEC that did not represent the general UPEC population. Thus, the apparent 'host-species specificity' of UPEC will need to be confirmed in a later study.

3.2. Introduction

UTIs constitute a considerable disease entity in humans, dogs and older cats (Bartges and Barsanti, 2000; Foxman, 2002; Ling, 2000; Polzin, 1994; Russo and Johnson, 2003). The majority of infections in these species are caused by UPEC (Johnson, 1991; Ling, 2000; Ling et al., 2001; Seguin et al., 2003). In other parts of the world, UPEC have been shown to possess numerous VFG that enhance the extraintestinal virulence of these strains (Johnson, 2003). Early phenotypic studies of UPEC had suggested that some VFs are host-specific (Garcia et al., 1988a; Garcia et al., 1988b; Senior et al., 1992; Stromberg et al., 1990). However, more recent American and European studies have shown that UPEC from dogs, cats and people share similar, if not identical, VFG (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d; Low et al., 1988; Yuri et al., 1998). Based on these findings and supporting phylogenetic evidence (Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001d; Whittam et al., 1989), concerns have been raised that UPEC isolated from dogs and cats have the potential to cause potentially life-threatening extraintestinal diseases in humans. A study by Murray et al. (2004) further substantiates these concerns. They reported the occurrence of a UTI in a woman caused by a UPEC clone that had co-colonised the woman, her male partner and her partner's cat. As stated by Feria et al. (2001b), these finding may have "*important implications for disease prevention and antimicrobial resistance surveillance in veterinary practice*". The research described in the previous paragraph was carried out in Europe and North America. At present, it is unknown whether the 'commonality' (Johnson et al., 2000a) or at least substantial overlapping of VFG profiles observed in European and American UPEC isolated from humans, dogs and cats can be extrapolated to other parts of the world and, in particular, to NZ.

The objective of the study reported in this chapter was to determine the presence and distribution of 30 VFG in UPEC isolated from dogs, cats and people living in NZ. This was done for three main reasons. Firstly, it was done to gather background information about the complement of VFG in UPEC isolates that would later become targets in phage susceptibility studies. It was considered of interest to know whether phages able to lyse canine ands feline isolates with substantially different VFG profiles could be found in nature. Some of the 30 VFGs studied here encode bacterial cell surface molecules. Of these, only *kpsMT II*, encoding a transport protein of the group 2 capsules like K1 and K5, has so far been identified as part of a primary receptor for lytic phage (Scholl et al., 2005). Nevertheless, phages from a variety of families have a remarkable ability to alter their adhesins by mutation and by intra- and inter-species recombinations (Kutter et al., 2005; Tetart et al., 1998). Changes of this kind permit phages to evolve to recognise new host receptors. It was considered possible, if extremely unlikely, that in addition to *kpsMT II*, other VFGs encoding bacterial cell surface molecules might be identified to which novel lytic phage can attach.

Secondly, this study sought to identify whether VFG that have been identified in other parts of the world are also present in UPEC isolated from NZ dogs and cats and distributed in comparable profiles.

Finally, the present study sought to complement and extend knowledge concerning the extent of cross-species overlap between UPEC from dogs, cats and people.

A previously validated multiplex PCR assay (Johnson and Stell, 2000) was applied to assess the presence or absence of VFG markers in 89 UPEC isolated from 56 dogs, 22 cats and 11 people living in NZ. The VFG profiles of these 89 UPEC were compared using discriminant analysis, principal component analysis and nearest neighbour analysis. In addition, a subset of 18 canine, 16 feline and 5 human UPEC isolates was subjected to macrorestriction analysis, to determine the degree of clonal relatedness of UPEC that could be expected in the overall sample population.

3.3. *E. coli* Strains and Attributes of Infected Patients

All 89 isolates were acquired between November 2001 and April 2004, originating from individual cases undergoing clinical investigation for suspected urinary tract inflammation or infection.

Fifty-six canine and 22 feline UPEC strains were obtained from a large network of New Zealand veterinary clinical pathology laboratories. Forty-five canine and 15 feline UPEC had been isolated from urine samples of individual patients collected by antepubic cystocentesis. A further 11 canine and 7 feline UPEC had been isolated from urine samples collected by catheterisation (2 canine UPEC), manual expression (3 canine and one feline UPEC) or by a collection method that was not disclosed (6 canine UPEC and 6 feline UPEC). These 18 isolates derived from urine samples of individual patients with marked pyuria and yielded a pure, heavy growth of *E. coli*.
Information about the patient's age and sex was available for 20 cats and 49 dogs*. The mean age of the population of the 20 exclusively female cats was 12.4 years (median 13 years), ranging from 5 years to 19 years. The mean age of the 49 dogs was 8.4 years (median 10 years), ranging from 7 weeks to 19 years. Nineteen of the 49 (38.8%) patients were male and 30/49 (61.2%) were female.

Eleven human UPEC strains were acquired from the clinical laboratory of a local public hospital. These isolates had been collected from mid-stream urine samples of 11 individual female patients aged 6 months to 83 years (mean and median 37 years) in December 2001. Nine of these patients were treated as outpatients for suspected cystitis (7), suspected urosepsis (1) or recurrent pyelonephritis (1). Two paediatric patients (6 months and 8.4 years) were treated in the hospital for diseases other than UTI. In these patients, *E. coli* was isolated from urine that had been taken for screening purposes only.

* Please refer to appendix 8.4.1 for information about individual patients

3.4. Results

3.4.1. The Majority of Assessed VFG Markers are Present in UPEC strains isolated from New Zealand dogs and cats

Twenty-five of the 30 VFG markers known to be present in UPEC of other parts of the world were amplified at least once among all NZ UPEC isolates examined*. All 25 of the markers were detected in the canine UPEC population (Table 3.1). In the feline and human UPEC populations, 24 and 23 VFG markers were amplified, respectively. The feline UPEC population lacked the VFG marker *iss* and none of the human UPEC possessed the VFG markers *cdtB* and *cvaC*. The markers for *papG I* and *papG I'*, *gafD*, *afa/dra* and *bmaE* were detected in none of the UPEC isolates.

The 89 isolates displayed 74 distinct VFG profiles. One VFG profile was present in 4 isolates (3 feline and one canine isolate; Table 3.2) another was present in 3 isolates (2 feline and one canine isolate). 10 VFG profiles were present in 2 isolates each (Table 3.2). When considering the 10 pairs of isolates with indistinguishable VFG profiles, 7 of these pairs originated from the same host species (3 canine, 2 feline and 2 human pairs). In 2 instances, an indistinguishable VFG profile was present in a canine and human isolate. In one instance, a feline and canine isolate shared an indistinguishable VFG profile.

3.4.2. Proportions of Individual VFG Markers in Canine, Feline and Human UPEC

Considering each of the VFG markers individually, 23/25 (92%) were present in a similar proportion of canine, feline and human UPEC. Inter-species differences in single gene proportions were observed for *papG II* and *traT* (Table 3.1). In detail, human UPEC strains were significantly more often positive for the P-fimbrial adhesin

*A multiplex PCR assay as described in detail in chapter 2.2 was applied to detect VFG marker possession. Example images illustrating the visualisation of VFG markers are shown in Figure 3.1.

marker *papG II* than were canine or feline UPEC (P=0.003 for each comparison; Holm's adjusted 2-tailed Fisher's exact test). Furthermore, the VFG marker *traT* was detected in all human UPEC, but was present to a significantly lesser extent in canine UPEC (P=0.01) and feline UPEC (P=0.003).

Canine and feline UPEC differed from each other regarding the presence of VFG marker *traT*, which was overrepresented in canine UPEC strains (P=0.006).

Table 3.1 Prevalence of VFGs in UPEC from different species

Gene Marker	Detection of VFG markers in canine UPEC N=56		feline UPEC N=22		human UPEC N=11		Significance value[x]
	n	%	n	%	n	%	
papA	34	(61)	22	(100)	7	(64)	
papC	33	(59)	21	(95)	9	(82)	
papEF	32	(57)	21	(95)	8	(73)	
papG II	4	(7)	2	(9)	8	(73)	0.003[b,c]; >0.5[a]
papG III	31	(55)	21	(95)	8	(73)	
sfa/foc	39	(70)	21	(95)	10	(91)	
sfaS	7	(13)	7	(32)	2	(18)	
focG	30	(54)	14	(64)	9	(82)	
iha	7	(13)	2	(9)	2	(18)	
fimH	55	(98)	22	(100)	11	(100)	
hlyD	36	(64)	22	(100)	9	(82)	
univcnf	28	(50)	17	(77)	5	(45)	
cdtB	8	(14)	2	(9)	0	(0)	
fyuA	47	(84)	22	(100)	10	(91)	
iutA	14	(25)	3	(14)	5	(45)	
ironec	48	(86)	22	(100)	7	(64)	
ireA	13	(23)	11	(50)	4	(36)	
kpsMT II	24	(43)	12	(55)	8	(73)	
kpsMT III	7	(13)	1	(5)	2	(18)	
rfc	1	(2)	1	(5)	2	(18)	
cvaC	8	(14)	1	(5)	0	(0)	
iss	7	(13)	0	(0)	1	(9)	
traT	30	(54)	3	(14)	11	(100)	0.003[c]; 0.006[a]; 0.01[b]
ompT	42	(75)	18	(82)	10	(91)	
malX	42	(75)	21	(95)	9	(82)	

[x] As determined by 2-tailed Fisher's exact test, adjusted for problems of multiplicity using the Holm's step-down procedure (Ludbrook, 1998). Significant difference between: [a] canine and feline UPEC; [b] canine and human UPEC; [c] feline and human UPEC.

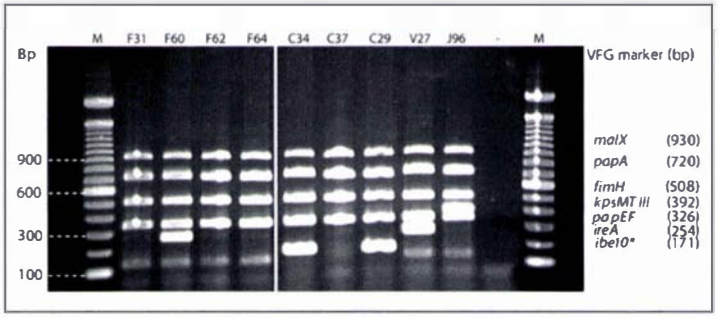
Table 3.2 Twelve VFG profiles found in more than one patient. Source species (dog, cat or human) is shown.

VFG profile	Source and Isolate No. [a,b]	*papA*[c]	*pap G II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
1	C 70; F 26, **F 53, F 142**	+	-	+	+	-	+	-	+	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+
2	C 184; **F 35, F 60**	+	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	+	+
3	C 14; H 2	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	-	-	-	-	+	+	+
4	**C 20; H 1**	+	-	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	-	-	-	+	+	+
5	C 185; **F 121**	+	-	+	+	-	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	+
6	**H 6, H 8**	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	-	-	-	+	+	-
7	**H 7, H 9**	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	-	+	+	-	-	+	+	+
8	**C 79, C 81**	+	-	+	-	-	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	+
9	**F 94, F 140**	+	-	+	+	-	+	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+	+
10	C 15, **C 33**	+	-	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	-	-	-	-	+	+
11	C 171, C 172	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+	+	+	-
12	**F 24**, F 62	+	-	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	-	-	-	-	+	+

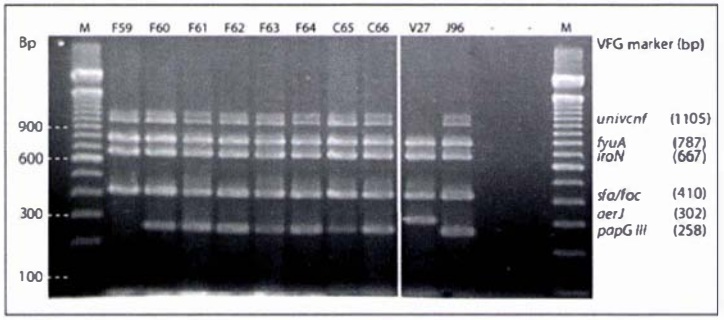
[a]: Source: C: Canine, F: Feline, H: Human. [b]: Isolates in bold letters were included in the PFGE analysis.
[c]: Results for *papC* and *papEF* were identical to those for *papA*.

Figure 3.1 Multiplex PCR of UPEC isolated from NZ dogs and cats

Set1



Set 2



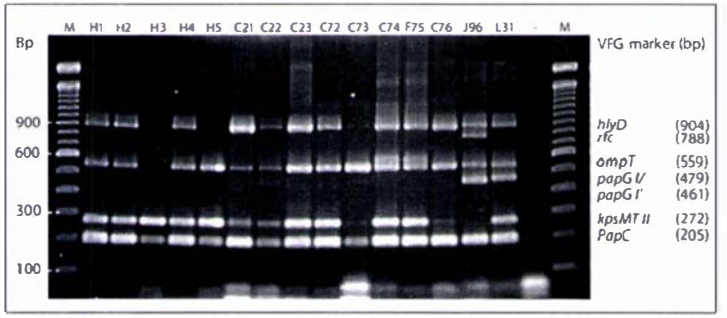
Images illustrating the visualisation of VFG markers for a subset of samples assessed in this chapter. White lanes in Set 1 and Set 2 illustrate the exclusion of strains C57 and C67 from these sets, respectively (Inconsistent results were observed. C57was rerun; C67 was later considered a urine sample contaminant and excluded from the study).

V27, J96, PM9, 2H25 and L31: control strains.

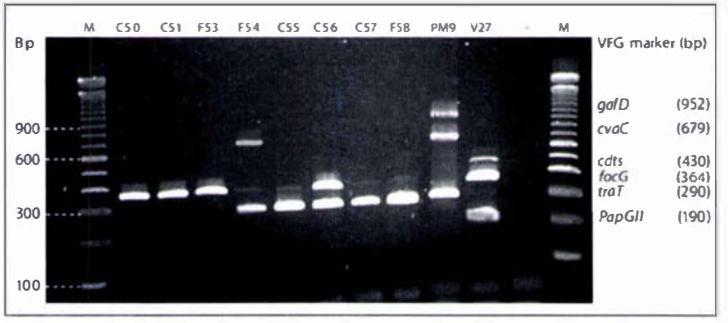
* no control strain was present for markers *ibe10*, *H7* and *kpsMT K1*and ** inconsistent results were observed for *G alleles I/II/III* – as explained in chapter 2.2 these markers were not included in statistical analyses.

Bp: Fragment size in base pairs. **M**: 100bp ladder; per fragment the size is increasing by 100 bp.

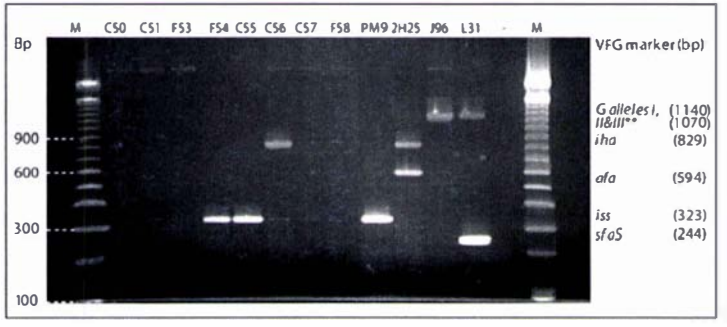
Set 3



Set 4



Set 5



3.4.3. VFG Profiles of New Zealand UPEC from dogs, cats and people overlap partially

Extended VFG profiles were subjected to discriminant analysis as described in chapter 2.9.1 to assess whether the combination of VFG markers could be used to predict the source (canine, feline or human) of UPEC strains. Overall, the source of 62/89 (70%) UPEC was predicted correctly (Table 3.3). Discriminant analysis failed to categorise canine and feline UPEC accurately. Sixteen of 56 (29%) canine UPEC strains were misclassified as having originated from cats, and 2/22 (9 %) feline UPEC were misclassified as having originated from dogs. When comparing VFG profiles from canine and human UPEC, 7/67 (10%) misclassifications occurred. Three human UPEC were falsely predicted to have originated from dogs, and 4 canine UPEC were falsely predicted to have originated from humans. The extended VFG profiles of human and feline UPEC appeared most distinct. Discriminant analysis misclassified only 2/33 (6%) UPEC strains when VFG profiles of human and feline UPEC were compared. In both instances, the misclassified isolates were sourced from cats.

Table 3.3 Prediction of the source of UPEC by discriminant analysis

Predicted host source	Actual host source					
	Canine (N=56)		Feline (N=22)		Human (N=11)	
	n	%	n	%	n	%
Canine	36	(64)	2	(9)	3	(27)
Feline	16	(29)	18	(82)	0	(0)
Human	4	(7)	2	(9)	8	(73)

The relationships between isolates from different species and the variance within VFG profiles of groups of isolates from a particular species were further evaluated by principal component analysis. Evaluation of the principal component coefficients of individual UPEC strains revealed that most feline UPEC displayed similar principal component coefficients, and grouped with each other in 2 major clusters (Table 3.4; Figure 3.2). Human UPEC displayed more variable principal component coefficients than feline UPEC, but clustered distant from feline UPEC (Table 3.4; Figure 3.2). Canine UPEC had highly variable principal component coefficients (Table 3.4). They could not be clearly distinguished from feline and human UPEC in the YZ and XZ dimension, respectively (Figure 3.2).

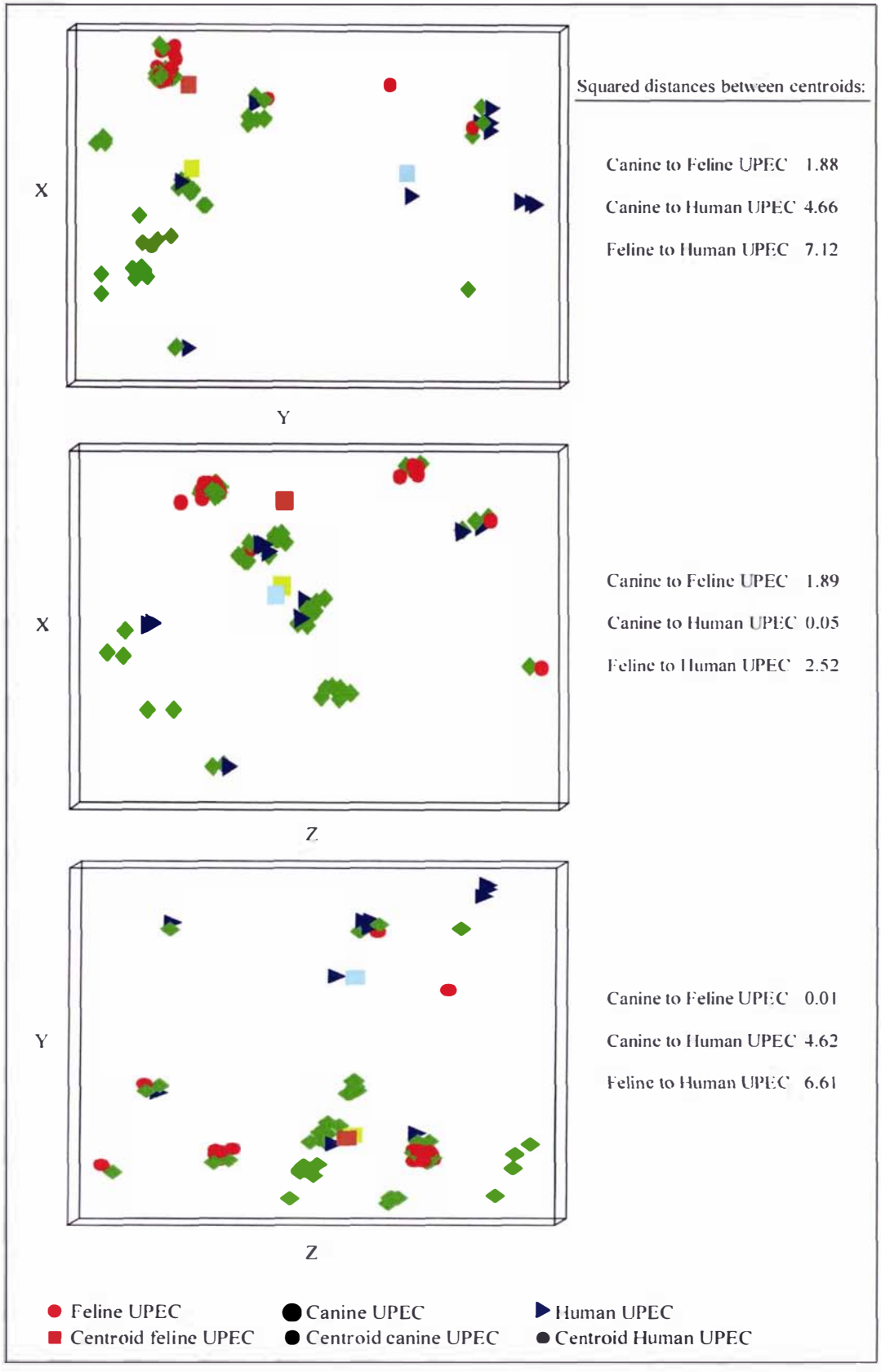
Table 3.4 PC analysis of extended VFG profiles of canine, feline and human UPEC

PC Coefficient	Origin of UPEC	Descriptive Statistics of PC coefficients of individual UPEC					
		Mean	**Min.**	**First Quartile**	**Median**	**Third Quartile**	**Max.**
PC 1; X	Canine	**-0.313**	-3.439	-1.505	**0.071**	1.185	1.536
	Feline	**1.059**	-1.854	1.185	**1.185**	1.273	1.536
	Human	**-0.572**	-3.439	-0.966	**-0.749**	0.268	0.717
PC 2; Y	Canine	**0.266**	-2.548	0.156	**0.533**	0.773	1.223
	Feline	**0.264**	-2.548	0.533	**0.533**	0.534	0.774
	Human	**-1.882**	-2.981	-2.981	**-2.548**	-0.349	0.340
PC 3; Z	Canine	**-0.023**	-1.699	-0.712	**-0.018**	0.516	2.648
	Feline	**0.064**	-0.916	-0.712	**-0.712**	1.376	2.648
	Human	**-0.010**	-1.284	-1.284	**-0.120**	0.384	2.171

Nearest neighbour analysis was performed to evaluate whether or not feline and human UPEC separated into groups with different VFG profiles. A significant separation of UPEC according to their VFG profiles was not observed (P>0.05; Holm's adjusted 2-tailed Fisher's exact test). The nearest neighbour to any given human or feline UPEC originated from a different species in 7/33 (21%) cases. This is 2 cases (6%) more than what would have been expected if human and feline UPEC were separated into different groups with 'species-specific' VFG profiles. Three of 11 (27%) human and 4/22 (18%) feline UPEC strains displayed VFG profiles that were most similar to the VFG profile of a UPEC strain from a different species. In total, 5 individual nearest neighbour pairs with members belonging to different species were present. These pairs did not solely form because of the presence of both *papG II* and *traT* in these strains. In fact, the possession of *papG II* and *traT* in feline strains that paired with human strains varied greatly: One isolate possessed *papG II* and *traT*, 2 isolates possessed only *traT*, and 2 isolates possessed neither *papG II* nor *traT*. Conversely, all 3 human isolates that paired with a feline isolate possessed both *papG* and *traT*.

As expected, nearest neighbour analysis of VFG profiles failed to establish a significant separation of canine and human UPEC, and canine and feline UPEC (P>0.05 for both comparisons).

Figure 3.2 Illustration of the inter-species association of UPEC by principal component analysis



3.4.4. Clonal Relatedness of Canine, Feline and Human UPEC

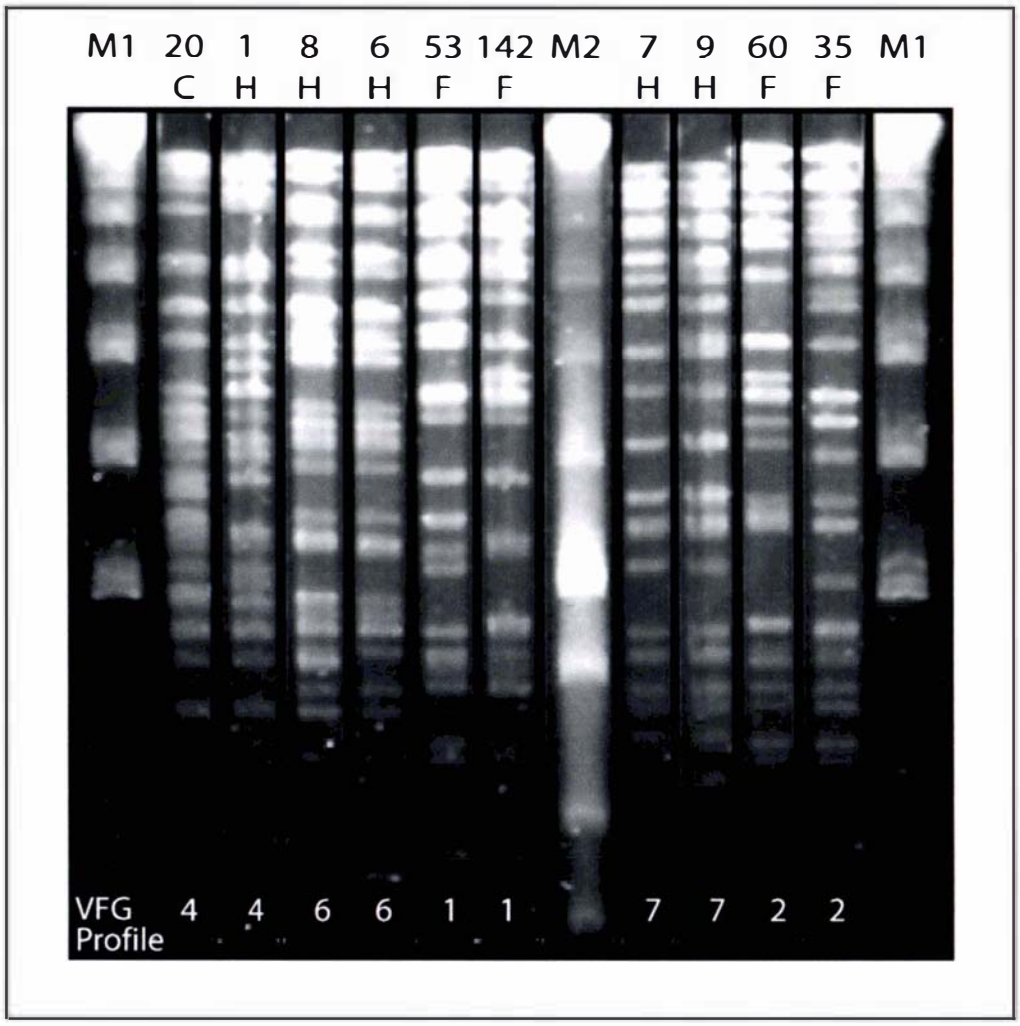
In addition to the comparisons of VFG profiles, PFGE was applied* to 18 canine, 16 feline and 5 human UPEC strains to investigate the clonal relatedness of isolates. Seven pairs of isolates with an indistinguishable VFG profile were included in this analysis.

All strains displayed a distinct PFGE banding pattern. However, the PFGE banding pattern of 2 human strains with an indistinguishable VFG profile (H7 and H9) was highly similar, differing in only one band (Figure 3.3). According to the PFGE interpretation criteria of Tenover et al. (1995), these UPEC strains, which had been isolated from the urine of 2 different Palmerston North women in December 2001, have derived from a common clone in the very recent past. A further 2 human strains with an indistinguishable VFG profile (H6 and H8) were categorised as possibly clonally related (Tenover et al., 1995), as they differed in only 3 PFGE fragments (Figure 3.3). The remaining 35 isolates, including the 5 pairs of isolates with indistinguishable VFG profiles (Table 3.2), were categorised as clonally unrelated (Tenover et al., 1995).

Overall, the PFGE banding pattern varied considerably between the 39 isolates (average similarity level 27.0%, range 0-94.7%; Dice coefficient similarity matrix). The majority of UPEC isolates clustered irrespective of their species-origin and irrespective of the geographic region from which they originated (Figure 3.4; nearest neighbour analysis of PFGE profiles, $P>0.05$ for each analysis).

* Please refer to chapter 2.3 for the detailed method.

Figure 3.3 PFGE banding pattern of a subset of UPEC isolates subjected to PFGE



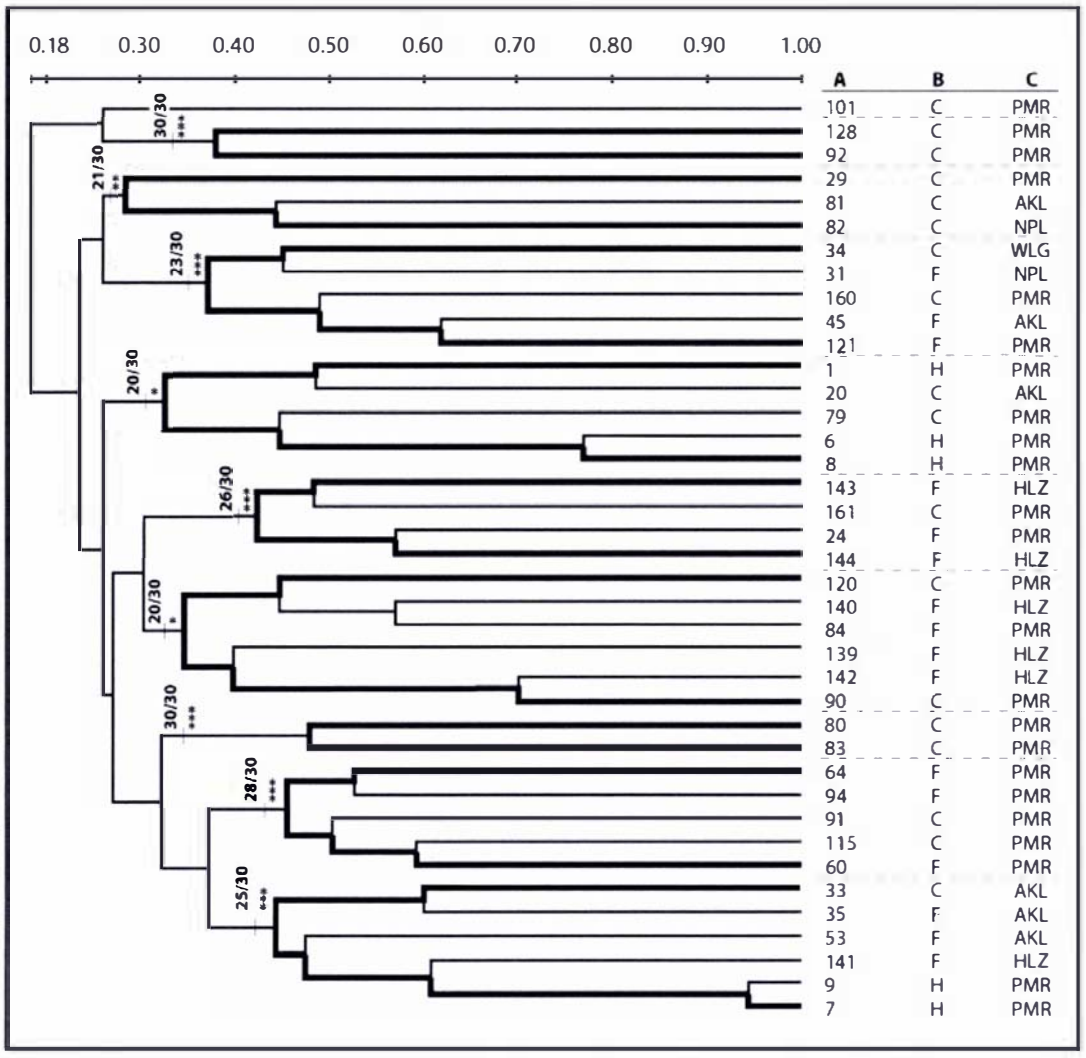
This image illustrates the clonal relatedness of 5 pairs of isolates with an identical VFG profile:

Human isolates 7 and 9 (VFG profile 7) display a highly similar PFGE banding pattern, indicative of a close clonal relationship. The PFGE pattern of human isolates 6 and 8 (VFG profile 6) is also similar. These strains are possibly clonally related. Conversely, all other pairs of UPEC with an indistinguishable VFG profile (i.e. strains C20 and H1, F53 and F142 and F60 and F35) display different PFGE banding patterns. These strains are considered clonally unrelated.

M1: Lambda marker. **M1**: Lambda marker. Size (kb) of visible bands from bottom to top: 48.5, 97.0, 145.5, 242.5, 291.0, 339.5, 436.5+; **M2**: Low molecular weight marker. Size (kb) of visible bands from bottom to top: 6.55, 9.42, 23.1, 48.5, 97.0, 145.5, 194.0, 291.0, 339.5, 436.5+
The black roman numbers indicate isolate numbers.

Origin of UPEC isolates: C: canine; F: feline, H: human. The VFG profile numbers (as listed in Table 3.2) is displayed in white at the bottom.

Figure 3.4 Average Linkage dendrogram illustrating the clonal relatedness of 39 UPEC



A: Isolate number.
B: Species UPEC derived from: C: Canine, F: Feline, H: Human.
C: Geographic origin of isolates: PMR: Palmerston North, AKL: Auckland, HLZ: Hamilton, NPL: New Plymouth, WLG: Wellington.
Clusters were confirmed using a jackknifing procedure as described in detail in chapter 2.3: Proportions of quartets supporting clusters between 28% and 45% Dice similarity coefficient and significance of clusters (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$, z-test) are displayed at the base of the clusters.

3.5. Discussion

The study reported in this chapter revealed that the majority of VFG markers, known to be present in UPEC collected in some other parts of the world, can also be detected in canine, feline and human UPEC from NZ.

When comparing the VFG profiles of canine, feline and human UPEC, it became apparent that up to 29% of UPEC from one species possess VFG profiles that are highly similar to, if not indistinguishable from, VFG profiles of another species. These results are in accord with previous studies (Feria et al., 2000b; Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001d; Low et al., 1988; Yuri et al., 1998). Overall, results from previous studies and this study indicate that a variety of UPEC clones may be transmissible between species, and may infect dogs, cats or people with similar ease. Further studies will be needed to investigate how likely it is that cross-species transmission of these UPEC will be followed by UTI in the recipient host.

VFG profiles of many UPEC isolated from dogs extensively overlapped with, or were identical to, VFG profiles of UPEC from the other two species. Conversely, the VFG profiles of the majority of feline and human UPEC differed considerably. Thus, the source species of individual feline and human UPEC isolates could be predicted with reasonably accuracy using discriminant analysis and principal component analysis. The reason for the apparent differences in VFG profiles of many feline and human UPEC from New Zealand is unknown.

One possible explanation is that some feline and human UPEC have adapted to a particular host by accumulating VFG that render them particularly suitable for colonisation of and persistence in the urinary tract in a particular, preferred host. For example, the apparent lack of VFG markers *papG II* in many feline UPEC (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a) may indicate that these VFG are less important in feline UTI then in human UTI. It is possible that receptors for *papG II* are not, or are to a significantly lesser extent, expressed by the uroepithelial mucosa of cats (Sung et al., 2001). Alternatively, cats may have a particularly active immune response that clears *papG II*-expressing UPEC from the feline urinary tract before they can establish an infection. It is noteworthy to mention that *PapG II* is thought to enhance the pathogenicity of human pyelonephritis and urosepsis-causing UPEC considerably

(Johnson, 1998; Johnson et al., 2005b; Johnson et al., 2005c; Johnson and Stell, 2000; Otto et al., 2001). Thus, the lack of *papG II* in many feline and canine UPEC (Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2003; Johnson et al., 2000a; Johnson et al., 2001d) may indicate that UPEC transmitted zoonotically from cats and dogs may be less likely to cause pyelonephritis and urosepsis in humans (Johnson et al., 2001c). Further studies are needed (i) to explain the apparent lack of *papG II* in many UPEC isolates sourced from dogs and cats; and (ii) to determine whether UPEC transmitted from dogs or cats can cause pyelonephritis or urosepsis in people.

A second possible explanation for the apparent differences in VFG profiles of many feline and human UPEC is that differences are the result of sampling subsets of feline or human UPEC that did not represent the general UPEC population. This explanation deserves serious consideration because patients from whom UPEC derived were not matched for clinical signs of disease or degree of host immune compromise. Indeed, to the best of my knowledge, no comparative study that attempts to match human and feline UTI cases by extent or nature of disease has so far been carried out. In UPEC isolated from humans, VFG profiles have been shown to differ significantly between patients with and without host compromise, such as immune dysfunction or anatomical defects in the urinary tract (Johnson, 1998; Johnson et al., 1994; Johnson and Stell, 2000; Otto et al., 2001). Similarly, VFG profiles have been shown to differ significantly between patients that suffer from urosepsis or pyelonephritis and patients with simple cystitis. As mentioned above, *papG II* has been shown to be overrepresented in UPEC causing human pyelonephritis and urosepsis (Johnson, 1998; Johnson et al., 2005b; Johnson et al., 2005c; Johnson and Stell, 2000; Otto et al., 2001). Presence of *traT* has also been suggested as a predictive marker for likelihood of development of urosepsis in humans (Johnson and Stell, 2000). The high prevalence of these particular VFG in the human UPEC isolates studied here may indicate predominance of urosepsis and pyelonephritis cases in this small sample population. However, the limited clinical information available in this study suggests that this is unlikely: At least 7/11 (64%) UPEC derived from patients suspected to have cystitis. Further 2 patients that were treated as inpatients at the time UPEC was isolated from their urine were not suspected to have UTI. Instead, UPEC was isolated from urine samples taken as part of a routine diagnostic work-up in these patients.

An estimation of the proportion of pyelonephritis isolates and cystitis isolates in feline UPEC was also not possible in this retrospective study. In any retrospective trial, it would be very difficult, if not impossible, to determine which isolates derived from simple cystitis, and which from pyelonephritis *sensu stricto*. Accurate diagnosis would need to be achieved by invasive diagnostic tests, such as tissue and fluid sampling from the affected renal pelvis or kidney. These tests are not part of the routine diagnostic work-up of UTI in dogs and cats. Even if the proportion of feline UPEC causing pyelonephritis had been known, an interpretation of the possible influence of the kind of UTI on differences of VFG profiles would have been ambiguous. This is because it has not yet been determined whether VFG profiles of feline (and canine) UPEC differ with the kind of UTI the UPEC derived from.

The focus of this project was on canine and feline UPEC. Thus, only a small number of human isolates was included. This was an important limitation of the comparative assessments presented in this chapter. Besides the small sample size, PFGE analysis confirmed that at least 2 pairs among the 11 human UPEC isolates were clonally related. Furthermore, all human isolates were obtained within a short time-span (less than one month) from the clinical laboratory of one public hospital. Due to these sampling limitations, the probability that tested human UPEC isolates belong to a subset of UPEC that do not represent the general UPEC population is high. The multivariate comparative analysis bears a low statistical power. Thus, it cannot be excluded that apparent 'host-specific' differences in VFG profiles represent a type II statistical error. Nonetheless, the fact that species-related differences were apparent in this preliminary study may be of interest for researchers aiming, in future, to determine the zoonotic risk of canine and feline UPEC. Further studies are needed that specifically focus on investigating whether some subsets of UPEC pose a much greater zoonotic risk than others.

3.6. Conclusion

Results of this study show that VFG markers that had previously been identified in European and American UPEC can also be detected in UPEC isolated from dogs, cats and people in NZ. The multivariate comparative analyses of extended VFG profiles from canine, feline and human UPEC allowed estimation of the extent of cross-species overlap of UPEC. It indicated that up to 29% of UPEC from dogs, cats and humans display similar VFG profiles, and are, in principle, likely to be able to infect dogs, cats and humans with similar ease. It remains to be elucidated whether (i) any particular VFG facilitate or are essential for cross-species transmission; and (ii) UPEC encoding these VFG can be targeted with prophylactic or therapeutic measures relevant to humans, dogs and cats. In contrast to the extensive cross-species overlap of VFG profiles observed in up to 29% of UPEC, species-related differences in VFG profiles were evident in many UPEC isolated from cats and humans. This may indicate that these UPEC have adapted to different physiological conditions in cats or humans. However, due to sampling limitations in this study, further studies will be needed to confirm or disprove the existence of host-species specific differences in UPEC.

Chapter 4

Feline Uropathogenic *Escherichia coli* from Great Britain and New Zealand have Dissimilar Virulence Factor Genotypes

4.1. Abstract

In this chapter, the presence of 30 known VFG markers in UPEC from 2 geographically distinct feline populations was investigated. UPEC isolates were also subjected to macrorestriction analysis to assess their clonal relationships. VFG profiles differed considerably according to the geographic origin of the isolates, enabling discriminant analysis to predict population membership for 15/15 NZ isolates and 18/22 UK isolates correctly. The prevalence of VFG markers for P-fimbriae (*papA*, *papC*, *papEF* and *papG III*), colicin V (*cvaC*), increased serum survival factor (*iss*), complement resistance factor (*traT*), PAI_{CFT073} (*malX*), iron-regulated siderophore receptor (*ireA*) and haemolysin (*hlyD*) differed significantly between UK and NZ isolates. Significant clonal differences between the 2 UPEC populations were also identified, but VFG profiles could not be predicted based on their clonal relationships. Consequently, a geographically uneven distribution of certain virulence genes, independent of clonal relatedness, is the likely cause of VFG marker differences between populations. Subtle differences in patient disease status or a disproportion in the number of *E. coli* causing clinically silent UTI may have contributed to the dissimilarity of VFG profiles.

4.2. Introduction

UPEC isolates from domestic cats have repeatedly been reported to share similarities with *E. coli* strains that cause serious extraintestinal infections in humans (Beutin, 1999; Feria et al., 2001a; Johnson et al., 2001a; Whittam et al., 1989; Wilson et al., 1988; Yuri et al., 1998). In particular, the discovery of a marked species overlap of VFGs has raised concern that humans may acquire UPEC infections from their pet cats (Feria et al., 2001a). Based on phylogenetic data and extended VFG profiles from UPEC of very few cats, it has been argued that UPEC from cats and humans may not only share pathogenic traits, but essentially be indistinguishable organisms (Feria et al., 2001a; Johnson et al., 2001a; Whittam et al., 1989). However, the evaluation of this potential zoonotic risk is not straightforward. One of several factors complicating this field of study is that it is unknown whether different UPEC genotypes are present in different geographic regions. In humans, urinary *E. coli* from different geographic regions have been shown to vary serotypically (Grüneberg and Bettelheim, 1969; Peddie et al., 1981). It is therefore possible that the VF genotypes of UPEC infecting any host species also differ with geographic origin. It has been suggested that differences in UPEC isolates related to geographic origin need to be investigated (Johnson et al., 2001a), but no study focusing on geographic variation of VFGs within UPEC from one host species has yet been carried out. In this study, a previously validated multiplex PCR assay was applied to determine the presence of 30 VFGs of UPEC from 2 geographically distinct feline populations. Furthermore, UPEC were subjected to PFGE to investigate their clonal relatedness. Finally, a possible correlation between PCR-based VF genotype of individual UPEC isolates and their PFGE profile was examined.

4.3. *E. coli* Strains and Attributes of Infected Patients

4.3.1. UK Strains

Between June 1999 and March 2002, twenty-two UK strains were isolated from urine samples collected by antepubic cystocentesis from 20 cats with suspected UTI and concurrent CRF. In 4/22 (18%) instances, clinical signs associated with UTI (e.g. stranguria, pollakiuria, haematuria) were reported at the time the urine sample was collected*. The mean age of UK cats was 15 years, ranging from 9 to 20.8. All but one of these patients were female. All UK cats belonged to the domestic shorthair breed.

4.3.2. NZ Strains

NZ *E. coli* isolates, cultured from urine of exclusively female cats with suspected UTI, were obtained between January 2002 and September 2003. In at least 7/15 (46%) instances†, clinical signs associated with UTI were present at the time urine samples were collected. The mean age of the NZ cat population was 14 years, ranging from 6 to 18. Five cats were purebred (one Oriental, one Siamese, 3 Burmese), all other cats were domestic shorthaired.

Similar to UK isolates, all NZ *E. coli* isolates were classified as category 1 strains based on the criteria described in chapter 2.1.1. UPEC were derived from urine samples collected by antepubic cystocentesis (12 of 15) or from urine samples for which the collection method was not known, but which showed marked pyuria on sediment evaluation and yielded a pure, heavy growth of *E. coli* (3 of 15).

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*Please refer to appendix 8.4.1 for specific information

† In the remaining 8/15 (54%) the information available did not allow me to specify with certainty whether clinical signs of UTI were observed at the time UPEC were isolated (appendix 8.4.1).

4.4. Results

4.4.1. Presence of VFG Markers in Isolates from UK and NZ

The presence of 30 VFG markers in the 37 UPEC isolates was assessed using a multiplex PCR assay as described in detail in chapter 2.2.4.

Twenty-five of the 30 VFG markers were amplified at least once among the feline UPEC subjected to PCR*. *PapG I, papG I', gafD, afa/dra* and *bmaE* markers were not present in any of the isolates. The marker for *fimH*, encoding the adhesin of Type 1 fimbriae, was found in all feline isolates (Table 4.2). *PapG II* was amplified only twice, both times from NZ isolates.

Four pairs of isolates had indistinguishable VFG profiles. One UK isolate (UK2) had a VF genotype indistinguishable from that of a NZ isolate (NZ 121). Two indistinguishable pairs consisted of members from NZ (NZ35/NZ60 and NZ140/NZ94) and one indistinguishable pair originated from UK (UK5 and UK11).

Cochran's method and the phi coefficient (as described in chapter 2.9.2) were applied to assess whether pairs of VFG markers were significantly more likely to be co-detected or whether individual VFG marker were more likely to be detected in the absence of the other member of the pair. Such a correlation was observed for several VFG markers (Table 4.1). In particular, isolates from which *pap* gene markers were amplified had the markers *sfa/foc*, *focG*, *hlyD*, *univcnf* and *malX* to a significantly greater extent and *iss* to a significantly lesser extent, than did isolates lacking *pap* gene markers.

* Figure 4.1 shows images of some gels generated to assess VFG profiles of UPEC assessed in this chapter.

Table 4.1 Correlation between virulence factors gene markers[a,b]

	PapC[c]	*Sfafoc*	*SfaS*	*FocG*	*Iha*	*HlyD*	*Unicnf*	*FyuA*	*AerJ*	*Ironec*	*CvaC*	*Iss*	*TraT*	*OmpT*	*MalX*
PapC[c]		*		*		**	**					*			*
Sfafoc	+		*	*		**	**	**	0.07	*		**	*	0.10	***
SfaS		+			**	*	0.06								*
FocG	+	+				*					0.10				
Iha			+						***						
HlyD	+	+	+	+			***		**	*		**			**
Unicnf	+	+	+			+		*	**	0.08		**			**
FyuA		+					+					0.06		**	*
AerJ		-			+	-	-			*	*	0.08			*
Ironec		+				+	+		-						*
CvaC				+					+			0.09	**		
Iss	-	-				-	-	-	+		+		*		***
TraT		-									+	+		0.09	*
OmpT		+						+					-		
MalX	+	+	+			+	+	+	-	+		-	-		

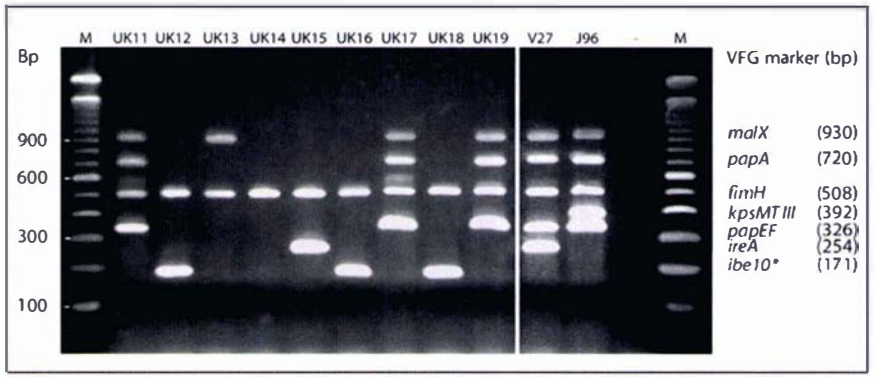
[a] Significance values were determined with Cochran's method. *P* values 0.10 – 0.051 are displayed numerically. *: $P \leq 0.05$. **: $P \leq 0.01$. ***: $P \leq 0.001$.

[b] Correlation was assessed by phi coefficient and common odds ratio. Correlations are displayed when significance value $P \leq 0.10$. +: positive correlation. -: negative correlation.

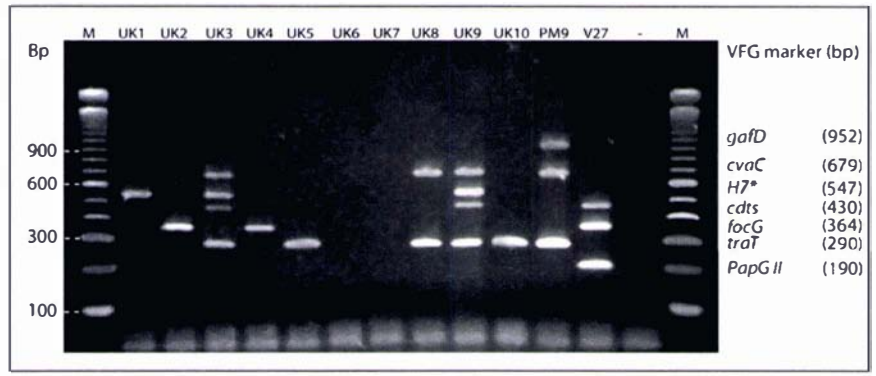
[c] *papC* was chosen as representative for *papA*, *papC*, *papEF* and *papG III*. *Pap* genes (excluding *papG II*) were significantly correlated with each other ($P \leq 0.001$).

Figure 4.1 Multiplex PCR assay of feline UPEC

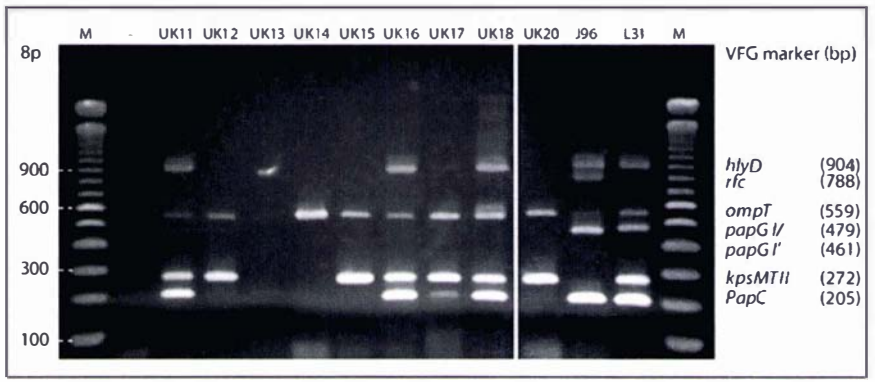
Set 1



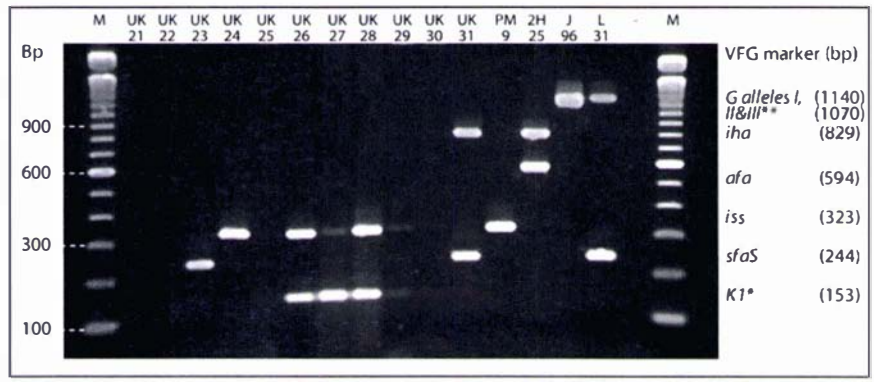
Set 4



Set 3



Set 5



Images illustrating the visualisation of VFG markers for a subset of samples assessed in this chapter (UK 1-31). White lanes in Set 1 and Set 3 illustrate the exclusion of UK 20 and 19 from these sets, respectively (inconsistent results were observed and the sample was rerun). In the original picture of set 3, a faint, distinct positive *ompT* band was present in control strain J96. In the printed version, some reduction of opacity of this band is observed.

V27, J96, PM9, 2H25 and L31: control strains.

* no control strain was present for markers *ibe10*, *H7* and *kpsMT K1*and ** inconsistent results were observed for *G alleles I/II/III* – as explained in chapter 2.2 these markers were not included in statistical analyses. **Bp**: Fragment size in base pairs. **M**: 100bp ladder; per fragment the size is increasing by 100 bp.

4.4.2. Differences in VFG profiles between UPEC from UK and NZ

The proportions of several individual VFG differed between UPEC from UK and NZ (as assessed by 2-tailed Fisher's exact test). In detail, *papA, papC, papEF* and *papG III* gene markers were amplified from all NZ isolates, but to a significantly lesser extent, ≤41%, from UK *E. coli* isolates (Table 4.2). Similarly, all NZ isolates had markers for *hlyD* and *malX*, whereas less than two-thirds (64% in each case) of UK isolates did so. Conversely, the protectin markers *cvaC, iss* and *traT* were not observed in any NZ isolate, but were present in 46%, 50% and 68% of UK isolates, respectively.

Having determined dissimilarities in proportions of single VFG markers between UK and NZ feline UPEC, the complete VFG profile (25 VFG markers) of all UPEC was entered into discriminant analysis (as described in chapter 2.9.2), to assess whether or not VFG profiles differed to an extent that would allow accurate prediction of the geographic origin of UPEC isolates. Discriminant analysis correctly predicted the geographic origin of 15/15 NZ strains and 18/21 UK strains.

In addition, all UPEC isolates were subjected to nearest neighbour analysis of VFG profiles, as described in detail in chapters 2.9.1 and 2.9.2. This was done to assess whether the VFG profiles of UPEC from a particular country (either UK or NZ) had a greater similarity to VFG profiles of UPEC from the same country or to VFG profiles of UPEC from the other country. VFG profiles of UPEC from the same country showed a higher similarity to each other than to VFG profiles of UPEC from the other country (z-test; $P \leq 0.001$). The nearest neighbour to any given VFG profile was observed in UPEC from the same country in 28/37 (75.7%) cases; that is 24.3% (9 cases) more often than would have been expected if nearest neighbours were assigned by chance – irrespective of the geographic origin.

Table 4.2 Prevalence of VFGs in relation to geographic origin

	Gene marker	UK (n=22)		NZ (n=15)		Significance value[a]	
		n	(%)	n	(%)		
Adhesin	*PapA*[b]	8	(36)	15	(100)	***	<0.0005
	PapG III[c]	9	(41)	15	(100)	***	<0.0005
	Pap G II	0	(0)	2	(13)		0.158
	Sfa/foc	16	(73)	15	(100)		0.063
	SfaS	9	(41)	4	(27)		0.491
	FocG	9	(41)	11	(73)		0.092
	Iha	1	(5)	2	(2)		0.554
	FimH	21	(100)	15	(100)	-	
Toxin	*HlyD*	14	(64)	15	(100)	*	0.012
	Univcnf	13	(59)	12	(80)		0.286
	Cdts	1	(5)	1	(1)		1
Siderophore	*FyuA*	19	(86)	15	(100)		0.257
	AerJ	8	(37)	2	(13)		0.153
	Ironec	20	(91)	15	(100)		0.505
	IreA	3	(14)	8	(53)	*	0.025
Protectin	*KpsMT II*	17	(77)	8	(53)		0.164
	KpsMT III	0	(0)	1	(7)		0.405
	Rfc	0	(0)	1	(7)		0.405
	CvaC	10	(45)	0	(0)	**	0.002
	Iss	11	(50)	0	(0)	***	0.001
	TraT	15	(68)	1	(7)	***	<0.0005
	OmpT	21	(96)	11	(73)		0.136
PAI	*MalX*	14	(64)	15	(100)	*	0.012

[a]: Proportions between populations, Fisher's exact test, 2-tailed. *: $P \leq 0.05$. **: $P \leq 0.01$. ***: $P \leq 0.001$.
[b]: Values for *papA* were identical to *papEF*
[c]: Values for *papG III* were identical to *papC*

4.4.3. UK and NZ isolates have dissimilar PFGE banding patterns

All UPEC isolates were subjected to macrorestriction analysis by PFGE as described in chapter 2.3.

According to the criteria of Tenover et al. (1995) for interpretation of PFGE*, all isolates belonged to distinct clones. PFGE banding patterns varied considerably between isolates (average Dice coefficient similarity level: 29.5 %). The maximum level of similarity was observed between two UK isolates (UK7 and UK31; 69% similarity). At a PFGE Dice coefficient similarity level between 30 and 45%, 2 UK-only clusters (5 isolates each), one NZ-only cluster (7 isolates) and 4 mixed clusters were apparent (Figure 4.4). Observed clusters were verified by a jackknifing procedure described in detail in chapter 2.3. All clusters had similar mean sums of individual gene operons† and individual VFG were not confined to single apparent clusters. All pairs of isolates with indistinguishable VFG profiles (see paragraph 4.4.1) were found to be clonally unrelated. The pairs shared PFGE Dice coefficient similarities ranging from 16.2% to 58.5%. Only one NZ pair that had identical VFG profiles also shared the highest level of similarity by PFGE.

Nearest neighbour analysis of Dice coefficients derived from PFGE banding patterns showed a significant clonal separation between UK and NZ isolates (z-test, $P \leq 0.001$). In 27/37 (73%) cases, the nearest neighbour originated from the same country.

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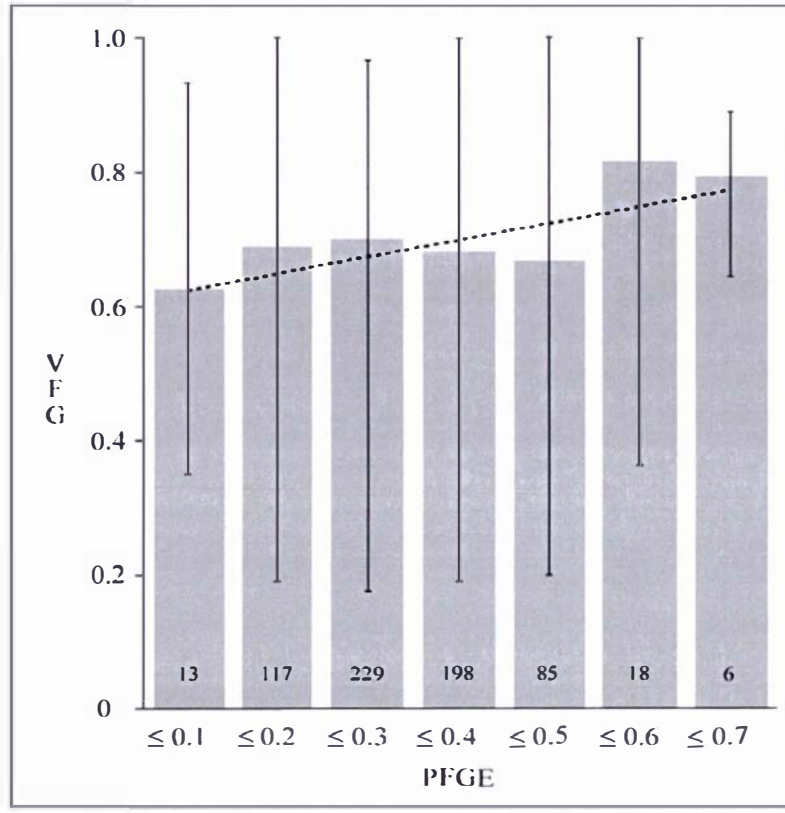
* As stated in chapter 2.3

† Of the total of 30 VFG markers, VFG markers *papA*, *papC* and *papEF* were considered dependent, as they are located within one operon (*papA-K*).

A method by Al-Samarrai et al. (2000; described in detail in chapter 2.9.2) was used to assess whether or not the clonal separation between UPEC isolates from UK and NZ was the predominant cause for observed origin-associated differences in VFG profiles. A significant correlation between PFGE Dice coefficients and VFG was not observed (Figure 4.2). Furthermore, the VF genotype did not predict the PFGE cluster membership of isolates (discriminant analysis, data not shown).

Figure 4.2 Correlation between VFG markers and PFGE types[a]



[a]: Assessed on Dice coefficient similarity matrices derived from VFG profiles and PFGE banding patterns

Isolates were grouped according to their Dice coefficient derived from PFGE banding patterns.

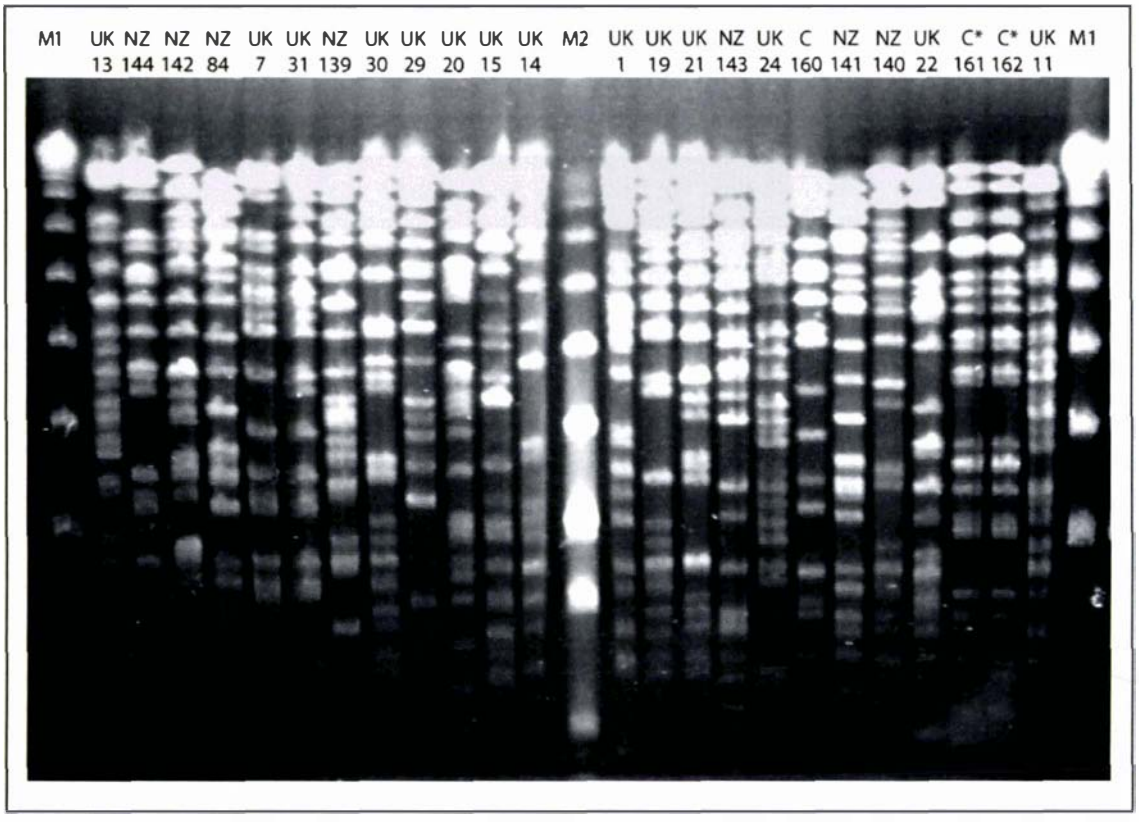
The corresponding mean (grey bar), and range (black vertical line) of corresponding VFG profile Dice coefficients are shown.

Numbers within grey bars show the numbers of VFG-PFGE pairs falling into the PFGE group.

The dashed line illustrates the linear trend of the mean.

The VFG Dice coefficients mean values did not differ significantly between groups (Student's t-test; $P>0.5$)

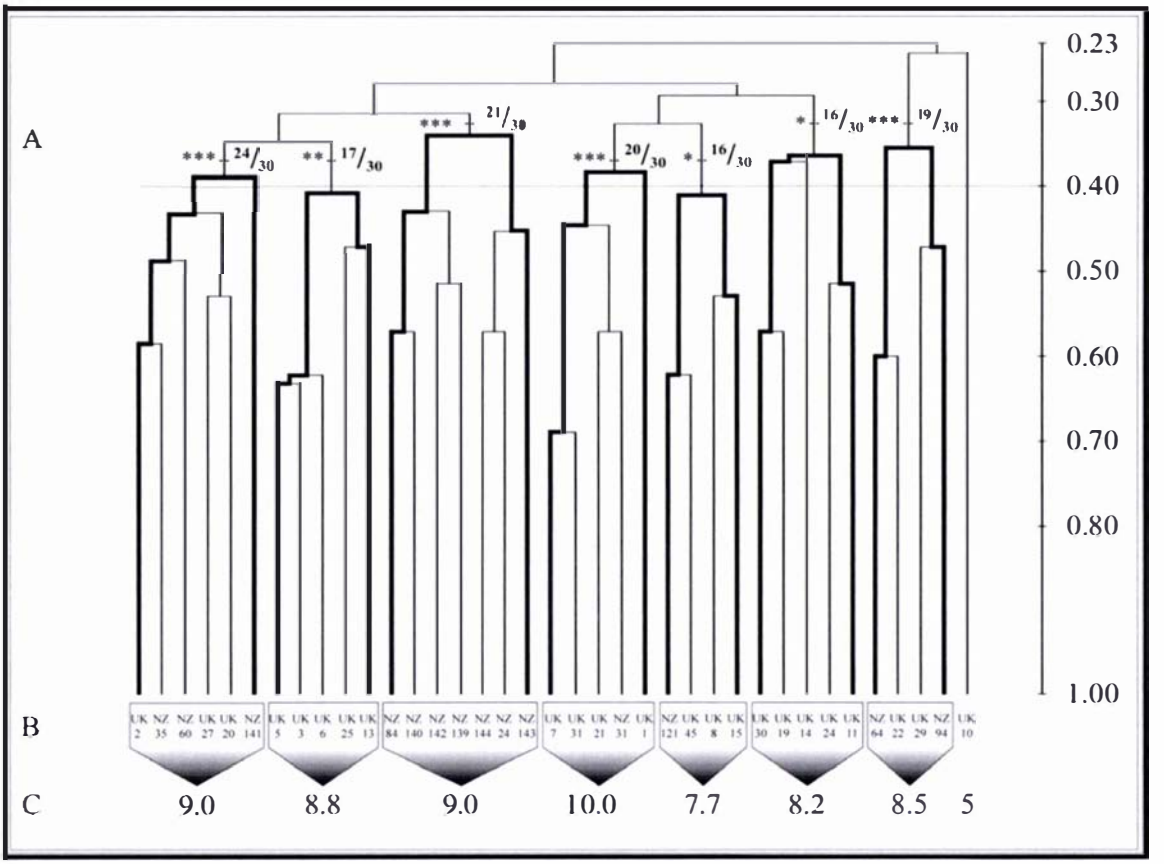
Figure 4.3 PFGE banding patterns of UK and NZ isolates



This image shows PFGE banding patterns of 15 UK isolates and 6 feline NZ isolates subjected to PFGE as part of this study. The image also includes 3 canine NZ isolates (C160-C162) that were not part of this study.
M1: Lambda marker. Size (kb) of visible bands from bottom to top: 48.5, 97.0, 145.5, 242.5, 291.0, 339.5, 436.5+; **M2**: Low molecular weight marker. Size (kb) of visible bands from bottom to top: 9.42, 23.1, 48.5, 97.0, 145.5, 194.0, 291.0, 339.5, 436.5+

All feline UK and NZ isolates show ≥7 PFGE bands difference to any other isolate. According to the criteria by Tenover et al. (1995), they are clonally unrelated. The similarity of these UPEC was further assessed using the Dice similarity coefficients created from banding patterns – please refer to Figure 4.4. Canine isolates C161 and C162 show an indistinguishable PFGE banding pattern. These isolates belong to the same clone (Tenover et al., 1995). C161 and C162 were isolated from the same dog and indicate that the dog suffered from a relapsing or persisting UTI. (Isolate C162 was excluded from the study described in chapter 3.)

Figure 4.4 Clonal relationship of UPEC from UK and NZ



Dendrogram created from Dice coefficient similarity matrix of PFGE patterns using the Average Linkage method

A: Jackknifing procedure: Proportions of quartets supporting between 30% and 45% Dice coefficient similarity and significance of clusters (z-test): $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.005$

B: Isolate Number

C: Mean sum of independent VFG markers. Of the total of 30 VFG markers assessed, VFG markers *papA, papC* and *papEF* were considered dependent as they are located within one operon (*papA-K*; Marklund et al., 1992).

4.4.4. Minimal Differences in Attributes of Patients from UK and NZ

UK and NZ cats did not differ significantly in age (Student's t-test, $P=0.212$).

The uneven distribution of breeds between UK and NZ populations did not influence the results. Observed VFG profile and PFGE differences between both populations were also apparent when only domestic shorthaired cats were included in the statistical analysis (data not shown).

4.5. Discussion

This study has revealed genetic differences between UPEC clones infecting 2 geographically separated populations of cats, both in terms of VFG markers and PFGE patterns. The most likely explanation of this phenomenon is that different genotypes prevail in different geographic locations, explicable as a result of genetic drift, a founder effect or local conditions favouring particular genotypes. This finding indicates that the extent of UPEC cross-species transmission from cats to people cannot readily be predicted from VFG profiles, unless feline and human isolates are from the same geographic region and ideally from the same household.

It cannot completely be ruled out that observed differences in VFG profiles were influenced by differences in the patient populations or differences in the proportions of UPEC derived from the same kind of UTI. In particular, UPEC from UK had been isolated from clinically silent UTI considerably more often than NZ UPEC. Studies on *E. coli* causing ABU in humans show that ABU strains possess P-fimbriae and haemolysin to a significantly lesser extent than *E. coli* causing symptomatic UTI (Blanco et al., 1996; Roos et al., 2006a). Here, P-fimbrial gene markers (*papA*, *papEF*, *papC* and *papG III*) and the haemolysin gene marker *hlyD* were significantly less often detected in UPEC from UK. This may indicate that (i) UPEC causing clinically silent UTI in cats are comparable to *E. coli* strains causing ABU in humans; and (ii) observed differences in VFG profiles were exaggerated because different proportions of UPEC isolated from clinically silent UTI were present in the UK and NZ populations. Furthermore, subtle differences in patient health status may have increased the differences between the *E. coli* populations through a selection of disease-specific genotypes (Karkkainen et al., 2000). All UK cats were known to have suffered from concurrent renal insufficiency or CRF and were therefore predisposed to developing UTI (Barber et al., 1999). However, the proportion of NZ cats suffering from concurrent renal insufficiency or CRF is unknown and may have differed from the proportion of UK cats suffering from renal insufficiency or CRF.

When assessing the correlation between VFGs, a significant linkage between several VFG markers was observed, irrespective of the geographic origin of UPEC isolates.

However, nearest neighbour analysis of PFGE banding patterns of UPEC from NZ and UK clearly demonstrated a significant clonal separation of UPEC strains. To assess whether origin-associated differences in VFG profiles were predominantly observed because different VFG combinations were present in the different PFGE types of UPEC, the correlation of VFG profiles and PFGE types was assessed. A significant correlation between PFGE banding pattern and VF genotype could, however, not be established. In fact, it became apparent that even isolates with indistinguishable VFG profiles may share little more than 15% similarity when subjected to PFGE. This result indicates that the phylogenetic relationship of strains possessing certain VFGs cannot readily be assessed with PFGE. This is because (i) PFGE does not discriminate broad phylogenetic groups (Johnson and Russo, 2005); and (ii) urovirulence genes have the ability to move independently from the *E. coli* genomic background (Hacker et al., 1997; Oelschlaeger et al., 2002; Welch et al., 2002) and are possibly linked on frequently transmitted genomic units, such as PAI or plasmids, that cannot readily be detected with PFGE (Dobrindt et al., 2002; Feria et al., 2001a).

While the VFG profiling and VFG linkage results of this study suggest that some UPEC of this collection may possess PAI such as PAI_{CFT073} or PAI V_{J96} (Blum et al., 1995; Welch et al., 2002), it must be noted that some VFG correlations reported as significant in this study may actually be insignificant. This is because VFG linkage result were based on multiple statistical tests. Compared to a single statistical test, the application of multiple statistical tests increases the chance of false positive results (Bender and Lange, 2001). However, adjustment measures to minimize the chance of false positive results, such as the Bonferroni method, increase the chance of false negative results (Perneger, 1998) and were therefore not applied. Instead, the non-adjusted correlation results may be used as a primary guide and will need to be confirmed or rejected in future studies focusing exclusively on the identification of VFG associations in feline UPEC.

In addition to the geographic examination of VFG markers in feline populations, this study extends previous knowledge (Feria et al., 2000a; Feria et al., 2001a; Johnson et al., 2001a; Yuri et al., 1998) about the prevalence of VFG markers of feline urinary *E. coli* substantially. The majority of VFG markers, known from canine and human ExPEC, was amplified from feline UPEC in this study.

The marker for the fimbrial adhesin allele *papG II* was detected for the first time in feline UPEC. This allele was amplified from 2/37 (5%) feline NZ isolates. Both isolates also possessed the marker for *papG allele III*[*]. In humans, *papG II* has been suggested as a marker for pyelonephritis or urosepsis (Johnson, 1998; Johnson and Stell, 2000). Clinical information indicated that the *papG II*-possessing UPEC isolates in this study were likely to have derived from cats with cystitis. This may explain the apparent rarity of *papG II* in feline UPEC studied here. However, it is noteworthy to mention that (i) a positive correlation between *papG II* and pyelonephritis or urosepsis has not yet been established for UPEC isolated from cats; and (ii) it cannot be excluded that patients suffered from pyelonephritis and cystitis at the same time. Other possible explanations for the apparent rarity of *papG II* in cats are that (i) a particularly effective immune response eliminates *papG II*-positive bacteria from the feline urinary tract (Söderhäll et al., 1997; Wullt et al., 2001a); or (ii) globotetraosylceramide (Gb4), the receptor for PapG II (Sung et al., 2001) is lacking in the feline urinary tract.

In agreement with previous studies, the *papG* allele *III* was identified as the predominant *papG* allele in feline UPEC (Feria et al., 2001b; Johnson et al., 2001a).

FimH, the adhesin for Type 1 fimbriae, was amplified in all feline strains studied. Type 1 fimbriae have been shown to be abundant not only in canine, feline and human ExPEC, but also in commensal *E. coli* (as discussed by Sauer et al., 2000). FimH came to attention recently, when it was shown that it triggers the uptake of *E. coli* into murine bladder cells (Anderson et al., 2003; Mulvey et al., 2000). Following intracellular multiplication in an environment sequestered from immune cells and some antimicrobials, *E. coli* may cause therapy-resistant, relapsing infections.

Five VFG markers (namely *gafD*, *afa/dra*, *bmaE*, *papG I* and *papG I'*) were not amplified from any of the isolates in this collection. These gene markers have been detected in similarly low numbers in previous studies of companion animal and human UPEC (Feria et al., 2000a; Feria et al., 2000b; Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2005b; Johnson et al., 2005c; Johnson and Stell, 2000; Johnson et al., 2001d; Yuri et al., 1998). They may be of low prevalence in UTI.

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[*] Please refer to appendix 8.3.2 for complete VFG profiles of UPEC isolates studied here.

4.6. Conclusion

VF genotypes of feline UPEC from UK and NZ differ considerably according to geographic region. PFGE banding patterns of UPEC isolates from UK and NZ also differed significantly. The reason for the observed differences may be the local predominance of certain *E. coli* lineages and VFGs. Patient-related health factors and the presence of different proportions of *E. coli* derived from clinically silent UTI may have contributed to observed differences in VFG profiles. Nonetheless, this work emphasises the need for a careful consideration of the source of isolates when comparing UPEC from different host species.

Chapter 5

Antimicrobial susceptibility Profiles Do Not Reliably Distinguish Relapsing or Persisting Infections from Reinfections in Cats with Chronic Renal Failure and Multiple Diagnoses of *Escherichia coli* Urinary Tract Infection

5.1. Abstract

Older cats with CRF commonly develop *E. coli* UTI. UPEC infections are often complicated and may persist or recur in affected cats. In cats with multiple diagnoses of *E. coli* UTI, antimicrobial susceptibility profiles have been used to determine whether same-strain recurrences (i.e. relapses or persistent infections) or different-strain recurrences (i.e. reinfections) occurred. However, the accuracy with which antibiograms discriminate different feline urinary *E. coli* clones is uncertain. Here, 17 cystocentesis-derived UPEC isolates collected from 5 cats with stable CRF and multiple diagnoses of UTI were studied. UTI were classified as relapsing or persistent infections *versus* reinfections using antibiograms determined by Kirby-Bauer discs and Etests®. Subsequently, the 17 UPEC isolates were subjected to PFGE to determine their clonal relatedness. A comparison of PFGE results with antibiograms indicated that antimicrobial resistance patterns varied considerably within several individual *E. coli* clones. Both antimicrobial susceptibility tests differentiated between relapsing or persistent infections and reinfections with only 58% overall efficiency. Thus, antimicrobial susceptibility profiles cannot be relied upon to distinguish between persisting or relapsing infections as compared to reinfections in cats with CRF and multiple diagnoses of *E. coli* UTI.

5.2. Introduction

Escherichia coli UTI has been reported to occur frequently in older cats that show signs of urinary tract disease (Bartges, 2004; Bartges and Barsanti, 2000; Lees, 1996). One factor predisposing older cats to development of UTI is declining renal function. Approximately two-thirds of older cats with UTI may be concurrently affected by CRF (Barber et al., 1999; Bartges and Barsanti, 2000; Mayer-Ronne et al., 2004). A prospective study conducted at the Royal Veterinary College showed that 11/36 (30.5%) cats with stable CRF developed at least one *E. coli* UTI (Barber et al., 1999). During a study period of 2 years, repeat diagnoses of infection were made in 6/11 (54.5%) patients after an initial UTI episode. Repeated diagnoses of UTI in an individual cat may be made because of incomplete eradication of the initial infection (i.e. relapsing or persistent infection; Seguin et al., 2003) or later episodes of UTI may be caused by different bacterial strains (i.e. reinfections). At present, antibiotic treatment is advocated in cats with confirmed UTI (Bartges, 2005). Prompt antimicrobial treatment may reduce the severity of microbial-induced inflammatory damage to the kidneys (Horcajada et al., 2004; Slotki and Asscher, 1982). To design a treatment plan for a cat with multiple diagnoses of *E. coli* UTI, it may be useful to determine whether each diagnosed infection is a relapse or reinfection (Drazenovich et al., 2004; Seguin et al., 2003). Comparison of antimicrobial susceptibility patterns of UPEC from different UTI episodes has been used commonly to predict UPEC clonality in a given cat, because other phenotypic or genotypic information about infecting UPEC strains is often limited in a clinical setting. However, the precision with which antimicrobial susceptibility patterns distinguish feline UPEC clones has not been thoroughly investigated. In this chapter, 17 cystocentesis-derived UPEC isolates from 5 cats with multiple diagnoses of UTI and CRF were studied. One further cystocentesis-derived UPEC isolate from a cat that suffered from a single UTI that lived in the same household as another cat included in this study, was also examined. Antibiograms were compared with genomic fingerprints, done by PFGE, to evaluate whether antimicrobial susceptibility profiles can reliably predict *E. coli* clonality.

5.3. *E. coli* Strains and Attributes of Infected Patients

Six female domestic shorthaired cats with *Escherichia coli* UTI and concurrent stable CRF, diagnosed and treated for UTI between January 1999 and February 2002, were included in this study. A total of 18 *E. coli* was isolated from these 6 cats.

In all cases, UTI was diagnosed on culture of *E. coli* from cystocentesis-derived urine. Urine was collected routinely as part of an evaluation of geriatric cats with suspected or confirmed chronic kidney disease. Signs of UTI (e.g. stranguria, pollakiuria, hacmaturia) were present on only 2/18 occasions (i.e. Cat A, infection 1* and cat D, infection 1†). All other UTI occurred in the absence of noticeable clinical signs. Cat A-C, E and F were concurrently treated for hyperthyroidism. In cat A, abdominal ultrasonography showed diffuse thickening of the bladder wall. Permission to biopsy the bladder wall was not granted.

Five of the cats studied had multiple diagnoses of UTI (Figure 5.2, cats A-C, E and F). One cat (D) with a single diagnosis of UTI lived in the same household as a cat that had 2 diagnoses of UTI (cat E). Of the 5 cats with multiple UTI diagnoses, 3 cats were found to be infected on 2 occasions, one cat on 3 occasions and one cat on 8 occasions over a period of 23 months. The interval between successive diagnoses in individual cats ranged from 6 weeks to 2.5 years. An association between the severity of CRF (as assessed by urine SG and creatinine concentration) and the number of UTI diagnoses was not apparent. None of the cats suffered from rapid progression of CRF during the 2-year study period. Upon diagnosis of UTI, cats received antimicrobial therapy based on the antimicrobial susceptibility of the isolated *E. coli*. In the majority of cases, intermittent urine sterility was demonstrated by bacteriological urine cultures obtained during antimicrobial therapy. No information was available whether urine was cultured at least 3 days after completion of each antimicrobial course. Therefore, relapsing infections (bacteriological cure observed) were grouped with persisting infections (no bacteriological cure observed).

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* Strain UK2 and

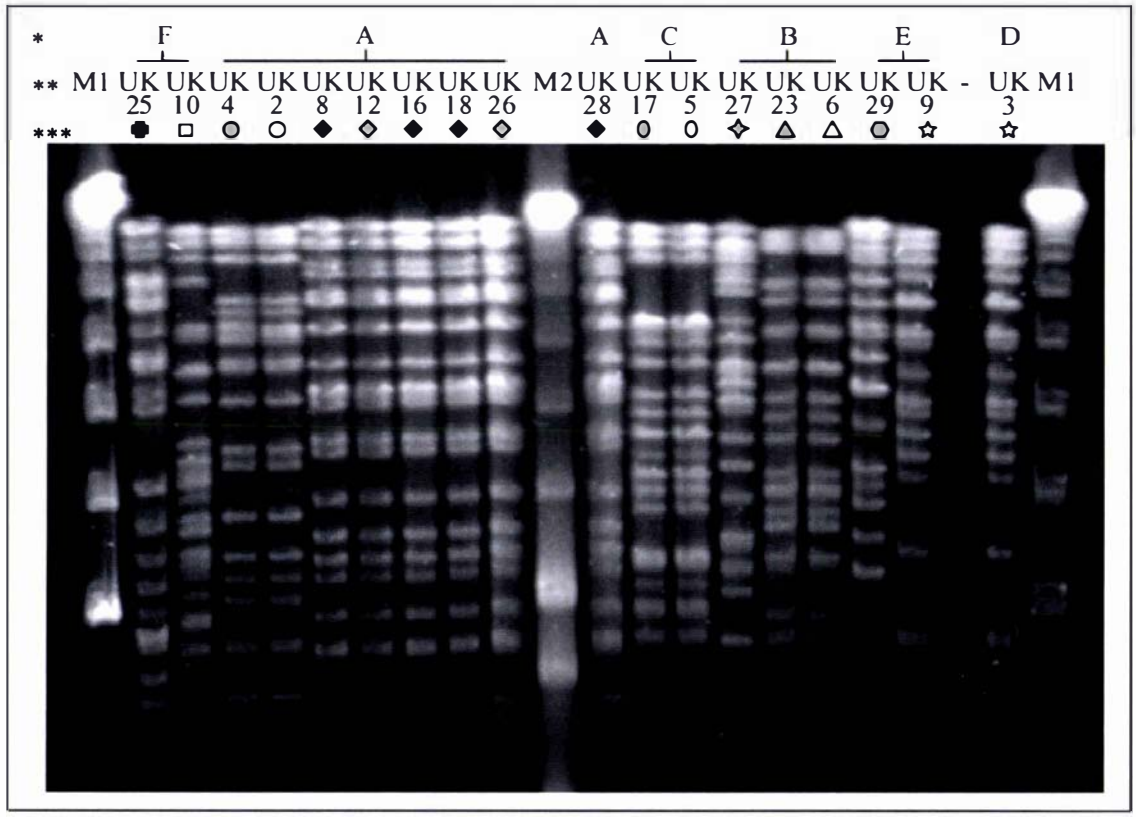
† Strain UK3 – please refer to appendix 8.4.1 for information on respective clinical signs.

5.4. Results

5.4.1. Observation of Identical PFGE Banding Patterns in UPEC isolates

PFGE, carried out as described in detail in chapter 2.3, identified 9 unique *E. coli* clones among the 18 isolates from the 6 cats (Figure 5.1).

Figure 5.1 Pulsed Field Gel Electrophoresis banding patterns of UPEC isolates



* Cat; ** Isolate Number (from left to right: UK25-3);

*** Nine different *E. coli* clones were detected in the six cats. Identical shapes indicate indistinguishable PFGE types. Fill colours indicate whether a relapsing/persistent infection or a reinfection was predicted using Etest®:
Grey, relapsing/persistent infection; Black, reinfection. Initial infections are shown in white.
The timeline of infections is shown in Figure 5.2.

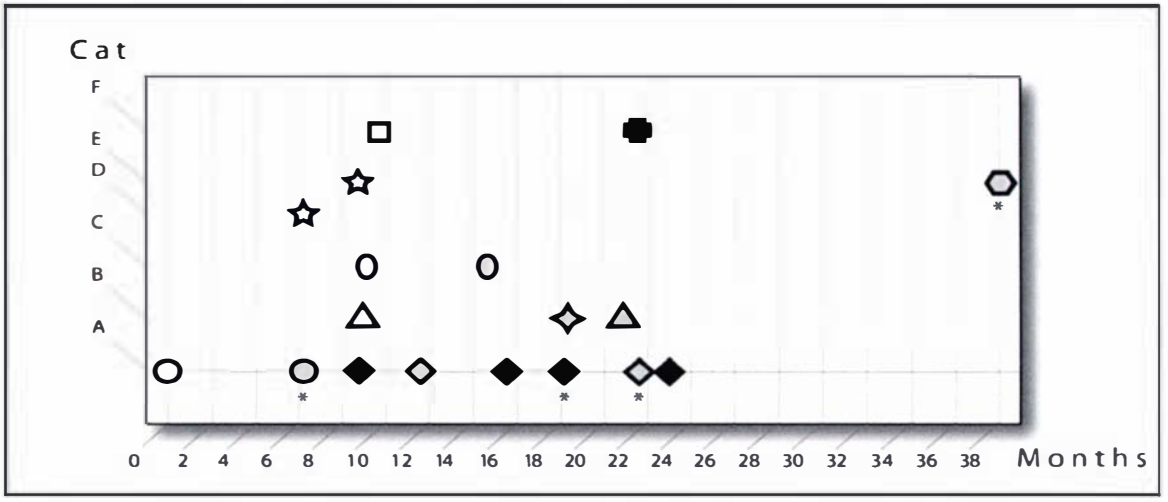
M1: Lambda PFGE marker. Size (kb) of visible bands from bottom to top: 48.5, 97.0, 145.5, 242.5, 291.0, 339.5, 436.5+; **M2**: Low molecular weight marker. Size (kb) of visible bands from bottom to top: 23.1, 48.5, 97.0, 145.5, 194.0, 291.0, 339.5, 436.5+

\- empty lane.

Two or more UPEC strains with an indistinguishable PFGE banding patterns were isolated from each of the cats A, B and C. UPEC strains that possessed distinguishable PFGE banding patterns were isolated from cats A, B, E and F. PFGE banding patterns of distinguishable strains were markedly dissimilar (Mean Dice coefficient similarity level: 35.9%).

Based on the establishment of the high discriminatory power of PFGE in distinguishing UPEC clones in this test setting* and criteria defined by Tenover et al. (1995), it was defined that isolation of UPEC strains with indistinguishable PFGE banding patterns corresponded with relapsing or persistent infections. Isolation of UPEC strains with distinguishable PFGE banding patterns corresponded with a reinfection. Thus, 4/5 (80%) cats had experienced reinfections and 3/5 cats (60%) had experienced at least one relapsing or persistent UTI (Figure 5.2). One of the 9 unique *E. coli* clones also was found in a sixth cat, which lived in the same household as one of the 5 cats with multiple diagnoses of infection.

Figure 5.2 Timeline of detected *E. coli* clones in 6 cats.



Isolate symbols are identical to those in **Figure 5.1**.

Identical shapes indicate identical clones as determined by PFGE. Fill colours indicate whether a relapsing/persistent infection or a reinfection was predicted using Etest®:

Grey, relapsing/persistent infection; Black, reinfection. Initial infections are shown in white.

An asterisk is shown below each isolate for which disc diffusion tests disagreed with Etests®.

Interestingly, PFGE determined that 6 *E. coli* isolates obtained sequentially from cat A over a period of 385 days were of the same clone. Lengthy antimicrobial therapy with a fluoroquinolone (marbofloxacin) resulted in intermittent urine sterility while the animal was receiving antimicrobial. However, post-antimicrobial urine sterility was not achieved. Severe bladder wall thickening was diagnosed sonographically, but not investigated further due to severe concurrent pulmonary illness and subsequent euthanasia of the animal.

* described in detail in chapter 2.9.3

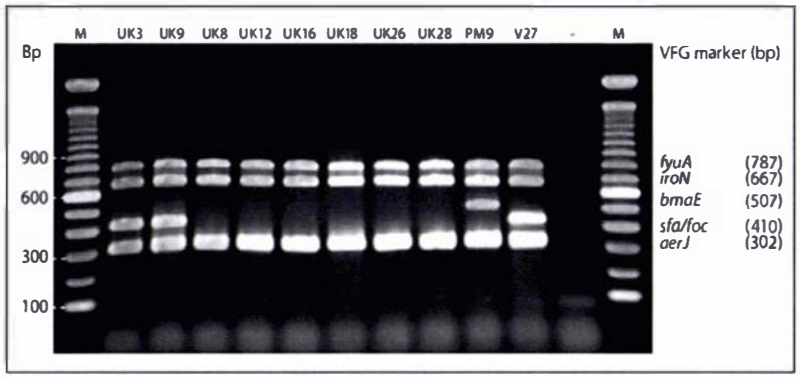
Cat B was diagnosed with a relapsing or persisting infection after an intervening UTI episode caused by a different *E. coli* clone. The interval between the first and third UTI diagnoses in cat B, associated with the same *E. coli* clone, was 354 days. This was the longest infection-to-relapse interval recorded in this study, although cat A was infected by the same *E. coli* clone over a longer total period.

Two cats that were members of the same household (cats D and E) were found to be infected by the same *E. coli* clone. Cat D was diagnosed with UTI first, was treated and was not subsequently observed to be infected (as assessed by clinical evaluation and urinalysis). Three months after the UTI in cat D was detected, cat E was found to be infected by the same *E. coli* clone. Cat E was treated and not subsequently found to be infected until 2.5 years later, when a reinfection with a different clone was diagnosed.

5.4.2. UPEC of the same PFGE type have identical VFG profiles

VF genotyping results* were 100% in accord with results obtained by PFGE typing. Each of the clones identified by PFGE displayed a unique VFG profile (Table 5.1). All isolates that belonged to one clone displayed the same VFG profile. 20/30 (33%) VFG markers were detected at least once in the 9 different clones. The VFG markers *papG I*, *papG I'*, *papG II*, *gafD*, *afa/dra*, *bmaE*, *iha*, *ireA*, *kpsMT III* and *rfc* were detected in none of the 9 different clones. The mean sum of independent gene markers observed in these strains (9.6) was comparable with that observed in other UK and NZ feline *E. coli* (chapter 4, Figure 4.4)

Figure 5.3 Visualisation of representative VFG markers (Set 2) in isolates from cats A, D and E



Legend:
UK3 was isolated from cat D
UK 9 was isolated from cat E
UK8-28 (from left to right) were isolated from cat A
M: 100 bp marker; per fragment the size is increasing by 100 bp.
PM9; V27: control strains
-: negative control
Bp: Fragment size in bp.

* As assessed by multiplex PCR assay described in detail in chapter 2.2

Table 5.1 VFG profiles of the 9 UPEC clones determined by PFGE

Cat	Clone	No. of Isolates[a]	*PapA*[b]	*Pap G III*	*sfa/foc*	*SfaS*	*FocG*	*FimH*	*HlyD*	*Univcnf*	*CdtB*	*FyuA*	*IutA*	*IroN*	*KpsMT II*	*CvaC*	*Iss*	*TraT*	*OmpT*	*MalX (PAI)*	Sum of Independent VFG markers[c] (N=28) n (%)
A	○	2	+	+	+	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	11 (39)
A	◇	6	-	-	-	-	-	+	-	-	-	+	+	+	-	+	+	+	+	-	8 (28)
B	△	2	-	-	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	+	10 (36)
B	✧	1	-	-	+	-	-	+	+	+	-	+	-	+	+	-	-	-	+	+	7 (25)
C	⬯	2	+	+	+	+	-	+	+	+	-	+	-	+	+	-	-	+	+	+	13 (46)
D; E	☆	1	-	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	13/ (46)
E	⬡	1	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	-	11 (39)
F	□	1	-	-	-	-	-	+	+	-	-	-	-	+	+	-	+	+	+	-	7 (25)
F	✿	1	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	+	-	7 (25)
Overall Prevalence of VFG (%)			22	22	67	33	22	100	56	44	11	89	44	89	67	44	56	33	100	56	**Mean 9.6/23 (34)**

[a]: Number of isolates belonging to this clone and isolated from an individual cat.
[b]: Results for *papC* and *papEF* were identical to those for *papA*.
[c]: Of the 30 VFG markers assessed, the VFG markers *papA*, *papC* and *papEF* were considered dependent, as they are located within one operon (*papA-K*; Marklund et al., 1992).

5.4.3. Antimicrobial susceptibility Test Results do not Concur with PFGE Results

Antimicrobial susceptibility results obtained by applying Etests® and Disc Diffusion tests as described in detail in chapter 2.4 are shown in Table 5.2.

When comparing antimicrobial susceptibility test results with PFGE results, it was observed that Etest® antibiograms incorrectly categorised 3/8 relapsing or persisting infections and 2/4 reinfections in 3/5 cats (Figure 5.2). Sensitivity and specificity of Etest® antibiograms for detection of relapsing or persisting infections were 63% and 50%, respectively. Etests® discriminated between relapsing or persisting infections and reinfections with 58% efficiency. Disc diffusion test-based antibiograms, which included 2 additional antimicrobials, incorrectly categorised 4/8 relapsing or persisting infections and 1/4 reinfections in 2/5 cats. Thus, disc diffusion tests detected relapsing or persisting infections with 50% sensitivity and 75% specificity. The overall efficiency of disc diffusion test-based antibiograms to distinguish accurately between relapsing or persisting infections and reinfections was 58%.

There was 93% categorical agreement between Etests® and Disc Diffusion tests. Differences were mostly related to cefuroxime (5/8 (62.5%) disagreements).

MICs differed between UPEC isolates that were grouped within one NCCLS category. Differences in one or more MICs that were not associated with a change of the NCCLS category were observed between ≥2 UPEC strains isolated from each of the cats A, B, C and E.

The last UPEC isolate (UK28) from cat A, an *E. coli* strain that had been demonstrated to have an indistinguishable PFGE banding pattern to 5 other UPEC strains isolated from that cat, had acquired resistance to fluoroquinolones and β-lactam antibiotics.

Table 5.2 Results of antimicrobial susceptibility testing

Antimicrobial			Tetracycline			Sulphamethoxazole/ Trimethoprim			Ciprofloxacin			ENR[a]	Amoxicillin/ Clavulanic acid			Ampicillin			Cefuroxime			KF[b]
Cat	Clone[c]	Isolate No.	MIC[d]	Category		MIC[d]	Category		MIC[d]	Category		Category	MIC[d]	Category		MIC[d]	Category		MIC[d]	Category		Category
				Etest	Disc		Etest	Disc		Etest	Disc	Disc		Etest	Disc		Etest	Disc		Etest	Disc	Disc
A	○	UK2	3	S	S	0.125	S	S	0.008	S	S	S	4	S	S	3	S	S	3	S	S	I
		UK4	1.5	S	S	0.094	S	S	0.008	S	S	S	6	S	S	3	S	S	3	S	S	R
	◇	UK8	1.5	S	S	0.094	S	S	0.25	S	S	I	6	S	S	4	S	S	6	I	S	R
		UK12	1	S	S	0.094	S	S	0.25	S	S	I	6	S	S	4	S	S	6	I	S	R
		UK16	1.5	S	S	0.19	S	S	0.38	S	S	I	8	S	S	4	S	S	4	S	S	I
		UK18	1.5	S	S	0.125	S	S	0.25	S	S	I	6	S	S	4	S	S	6	I	S	I
		UK26	0.5	S	S	0.19	S	S	0.19	S	S	I	8	S	S	6	S	S	8	I	S	S
		UK28	2	S	S	0.38	S	S	1	I	R	R	8	S	S	12	I	R	48	R	R	R
B	△	UK6	1	S	S	0.094	S	S	0.008	S	S	S	3	S	S	1.5	S	S	3	S	S	I
		UK23	1.5	S	S	0.125	S	S	0.012	S	S	S	2	S	S	1.5	S	S	2	S	S	I
	✧	UK27	1.5	S	S	0.094	S	S	0.012	S	S	S	4	S	S	3	S	S	3	S	S	I
C	0	UK5	128	R	R	0.125	S	S	0.008	S	S	S	8	S	S	>256	R	R	2	S	S	S
		UK17	128	R	R	0.125	S	S	0.008	S	S	S	6	S	S	>256	R	R	2	S	S	S
D	☆	UK3	2	S	S	0.094	S	S	0.016	S	S	S	4	S	S	2	S	S	4	S	S	I
E		UK9	2	S	S	0.19	S	S	0.012	S	S	S	6	S	S	4	S	S	4	S	S	I
	⬡	UK29	1.5	S	S	0.125	S	S	0.004	S	S	S	2	S	S	0.75	S	S	0.75	S	S	S
F	□	UK10	1.5	S	S	0.125	S	S	0.008	S	S	S	4	S	S	3	S	S	3	S	S	R
	✿	UK25	128	R	R	0.38	S	S	0.25	S	S	I	16	I	S	>256	R	R	6	I	S	R

[a]ENR, Enrofloxacin. [b]KF, Cephalothin. [c]As determined with PFGE. [d]MIC, Minimal inhibitory concentration; in µg/ml

5.5. Discussion

Results of the present study indicate that antimicrobial susceptibility profiles cannot be relied upon when distinguishing between relapsing or persisting infections and reinfections in cats with recurrent UTI and CRF. Changes in antimicrobial susceptibility profile were detected frequently within individual UPEC clones. Similar results have previously been reported in a case of monoclonal *E. coli* pyelonephritis and urosepsis in a human patient (Johnson et al., 1992). Changes in antimicrobial susceptibility patterns were also identified in UPEC causing persistent UTI in dogs (Drazenovich et al., 2004). In this study, an increase in the number of antimicrobials included in the antibiogram did not lead to better discrimination between relapses and reinfections, emphasising the unreliability of antibiograms for determining *E. coli* clonality. Etests® and disc diffusion testing yielded similar susceptibility categorisation results. A change in one or more MICs, identified with Etests®, may indicate reinfection, although NCCLS antibiogram categories predict a relapsing or persisting infection (e.g. the different clones from cat B (UK6 and UK23) or cat E (UK9 and UK29) in Table 5.2). However, MICs do not reliably distinguish between relapsing or persisting infections and reinfections, because *E. coli* clones may acquire antimicrobial resistance (e.g. the last isolate from cat A).

It is not surprising that changes in antimicrobial susceptibility patterns were frequently observed in this study. Antimicrobial resistance, in particular to β-lactams, is often transferred on mobile genetic elements, such as plasmids or transposons, which are acquired or lost readily (Lupski, 1987; Pfaller and Segreti, 2006). Changes in antimicrobial resistance are particularly often observed in patients treated with antimicrobials or in hospitals were antimicrobials are used frequently (Ball, 1986; Wagenlehner et al., 2005). In contrast to antimicrobial susceptibility testing, PFGE scans the complete genome of UPEC for mutational changes (Tenover et al., 1997). Thus, results obtained by PFGE typing are in general not influenced by changes in selection pressure associated with antimicrobial use. While changes in PFGE banding pattern may be observed when plasmids are acquired or lost, changes may be minimal (<4 bands difference between strains) and would probably lead to conclusions similar to those drawn in this study (Tenover et al., 1995). Results obtained by VFG profiling

were 100% in accord with results obtained by PFGE typing. When indistinguishable PFGE banding patterns were observed in UPEC strains, VFG profiles were also indistinguishable and when PFGE types differed between isolates, VFG profiles also differed. This finding further emphasises that UPEC strains with indistinguishable PFGE patterns have derived from a common ancestor very recently – that is, before VFG, which are commonly part of transferable genetic units (Dobrindt and Reidl, 2000; Hacker and Carniel, 2001), could be lost or acquired. Despite the agreement between VFG profiling and PFGE typing in this setting, VFG profiling by multiplex PCR assay cannot be used to distinguish epidemiologically related or unrelated UPEC *per se*. This is because VFG profiles may be indistinguishable in epidemiologically different UPEC strains (as shown in chapter 4). Furthermore, it is possible that VFG profiles differ in UPEC strains with indistinguishable PFGE banding patterns if (i) VFG are not detected because point-mutational changes to primer binding sites decrease the affinity of PCR primers; or (ii) loss or acquisition of VFG is not associated with detectable changes in PFGE banding pattern (e.g. if VFG are on small plasmids).

PFGE indicated that CRF-affected cats with multiple diagnoses of UTI suffered from persistent infections or relapses considerably more often than is appreciated using antimicrobial susceptibility profiles. Individual *E. coli* clones may persist for periods of a year or more despite treatment with appropriate antimicrobials (as assessed by antimicrobial susceptibility testing) and intermittently demonstrated urine sterility (data not shown).

Interestingly, one cat in this study was diagnosed with a relapsing or persisting infection with one *E. coli* clone after an intervening infection with a different *E. coli* clone. It remains to be determined whether *E. coli* clones that cause relapsing or persisting infections primarily persist in the external environment, the cat's gastrointestinal tract or, as has recently been shown in mice, in biofilms inside epithelial cells of the urinary tract (Justice et al., 2004; Mulvey, 2002; Schilling and Hultgren, 2002). Importantly, Schilling et al. (2002) demonstrated viable UPEC in the bladder tissue of experimentally-infected mice despite appropriate antimicrobial treatment of UTI. Periodic efflux of UPEC was demonstrated and associated with evident relapse. Mulvey et al. (2001) reported that invasion of murine uroepithelial cells is mediated by

FimH, the adhesin of Type 1 fimbriae. In this study, the gene marker *fimH* was present in all feline UPEC clones that caused relapsing or persisting infections (Table 5.1). Thus, it is possible that some of the relapsing or persistent UTI observed here were caused by UPEC that effluxed from uroepithelial cells of affected cats. Conversely, UTI may have been caused by UPEC that resided in close proximity to the urinary tract and possessed certain VFs that enabled them to regain entry into the urinary tract. In humans, *papG II*, *papG III*, *afa/dra, iutA* and *iha* have been suggested as predictors for same-clone recurrences of UTI (Foxman et al., 1995; Johnson et al., 1999; Johnson et al., 2001b). These VFs did not appear to be of importance in the 4 clones isolated from cats with relapsing or persisting UTI (Table 5.1). However, larger studies are needed to assess the role of these and other VF in the pathogenesis of relapsing or persisting *E. coli* UTI in cats.

UTI in cats frequently occurs without any clinical signs and without abnormalities on urine sediment evaluation and complete blood count (Bartges and Barsanti, 2000; Lulich and Osbourne, 2002). Only 2/18 of the UTIs in this study were associated with clinical signs. In the absence of clinical signs, early recognition of UTI may be achieved by regular urine cultures as part of the assessment of the health status of CRF cats (Lulich and Osborne, 2004). To prevent possible sequelae associated with UTI, antimicrobial treatment of any UTI, including clinically silent UTI, is currently recommended (Bartges, 2005; Kelly et al., 1979a; Kelly et al., 1979b, Ling, 2000). Conversely, ABU is not always treated in humans, particularly not in elderly patients (as discussed by Nicolle, 2000a). Although ABU may lead to the development of symptomatic UTI, morbidity and mortality do not decline with antimicrobial therapy of ABU (Nicolle et al., 1987). On the contrary, antimicrobial treatment may be harmful, due to the occurrence of adverse effects or emergence of antimicrobial resistance. Similar studies are needed to understand the aetiopathogenesis of clinically silent UTI in cats and to show whether antimicrobial therapy is beneficial.

The development of resistance to fluoroquinolones after several 3- to 6-week marbofloxacin treatment periods was observed in one isolate that caused persistent or relapsing UTI over a period of 14 months. Failure to resolve the persistent or relapsing infection and subsequent development of fluoroquinolone resistance may have been caused by sub-therapeutic antimicrobial levels at the site of infection due to poor tissue perfusion, deep-seated possibly intracellular infection, or poor owner compliance. The fluoroquinolone-resistant isolate also showed concurrent development of *in vitro* resistance to β-lactam antimicrobials (ampicillin, cefuroxime and cephalothin). This finding was surprising, because the animal was not known to have been treated with β-lactam antimicrobials. Post-collection development of resistance is not likely to have occurred, because contact between this *E. coli* clone and β-lactam antimicrobials or transmissible genetic elements was avoided. Antibiograms and PFGE were run in duplicate from separate subcultures of the original isolate. Identical results were obtained each time. Hence, it is suspected that this *E. coli* clone acquired resistance to 2 distinct classes of antimicrobial *in vivo*, possibly by a mutational upregulation of a chromosomal efflux pump with broad substrate specificity (Poole, 2000) or by acquisition of a plasmid-borne gene that confers a decrease in susceptibility to quinolones, cephalosporins and β-lactams (Mammeri et al., 2005).

Recent studies reported sharing of indistinguishable UPEC clones in the gastrointestinal tract or urinary tract of humans and cats living in a close relationship to each other (Manges et al., 2004; Murray et al., 2004). Spread of UPEC between individuals has also been demonstrated in mice populations (Schilling and Hultgren, 2002). This study demonstrated for the first time that individual UPEC clones can cause a UTI in more than one feline member of a household. The UPEC clone that caused a UTI in each of the 2 cats from one household was probably transferred from one cat to the other. Alternatively, both cats acquired the UPEC clone from an independent source. After transmission or acquisition of the UPEC clone, it overcame host defences in each of the cats and caused UTI in both cats.

5.6. Conclusion

In the presence of concurrent disease, treatment of UTI may be particularly rewarding, but can be challenging. When UPEC persist or cause relapsing UTI, long-term antimicrobial treatment is often indicated (Senior, 2000). It is at present recommended even when no clinical signs of UTI are observed (Bartges, 2005). When UPEC persists despite long-term therapy with apparently appropriate antimicrobials, *in vivo* antimicrobial resistance of UPEC should be considered. In this study, PFGE was successfully applied to discriminate UPEC causing relapsing or persisting UTI from UPEC causing reinfection. The application of PFGE in a clinical setting may allow identification of persisting or relapsing UTI quickly, thus enabling the optimisation of management of UTI. Ultimately, this may enhance overall treatment success.

Chapter 6

Naturally-Occurring Bacteriophages Lyse a Large Proportion of Canine and Feline Uropathogenic *Escherichia coli* Isolates *in Vitro*

6.1. Abstract

Here, the results of a preliminary study on the feasibility of phage therapy to combat canine and feline *E. coli* UTI are reported. The ability of 40 phages, isolated from processed sewage, to cause bacterial lysis was tested on 31 canine UPEC, 22 feline UPEC and 7 faecal *E. coli* strains. Each of the 40 phages displayed a unique lysis profile. The mean number of UPEC strains lysed by an individual phage was surprisingly high at 21.2/53 (40%, range 17% to 72%). In total, 50/53 (94%) of the UPEC strains could be killed by one or more of the phages. Ten phages could each lyse ≥ 51% of the UPEC strains. UPEC strains were more readily lysed than were faecal strains (P=0.001). Surprisingly, feline UPEC showed a significantly higher susceptibility to phage than did canine UPEC (P=0.001). Canine and feline UPEC differed significantly in the possession of VFG markers *papA, papEF* and *papG III.* These markers were overrepresented in feline UPEC. However, the presence or absence of these VFG markers did not influence susceptibility of UPEC to phage lysis. Electron microscopy and DNA sequencing of 5 phages with a broad host range revealed that 4 phages belonged to the exclusively lytic T4-like genus, while one phage displayed morphologic similarity to the temperate phage P2. Overall, these results indicate that a large majority of canine and feline UPEC are susceptible to lysis by naturally occurring phages. Although these *in vitro* results may not translate into *in vivo* efficacy, phages show promise as therapeutic agents for treatment of canine and feline *E. coli* UTI and, perhaps, other diseases caused by closely related *E. coli* strains.

6.2. Introduction

UPEC is the most important infectious cause of urinary tract disease in dogs, cats and people (Foxman, 2002; Ling, 2000; Russo and Johnson, 2003). UPEC infections, once diagnosed, are usually managed with antimicrobial therapy (Bartges, 2005). However, antimicrobial resistant forms of UPEC have emerged and pose problems in many parts of the world (Cohn et al., 2003; Cooke et al., 2002; Manges et al., 2001; Pfaller and Segreti, 2006; Sanchez et al., 2002; Warren et al., 2001). Even UPEC that are susceptible to antimicrobials *in vitro* may persist in an infected host or in its immediate environment, despite lengthy antimicrobial treatment (Drazenovich et al., 2004; Russo et al., 1995; Schilling et al., 2002; Seguin et al., 2003). In dogs, persistence or relapse of UTI has recently been shown to occur more often than had previously been realised (Drazenovich et al., 2004). This thesis has shown that cats also suffer from relapsing or persisting UTI more often than previously anticipated (chapter 5). When reinfection or relapse is recognised, antimicrobial therapy is often adjusted. However, it is sometimes difficult to cure patients with intractable UTI. Therapy with phages – viruses that can infect and kill bacteria – may be used to supplement or substitute antimicrobial therapy (Alisky et al., 1998). Phage therapy of human UTI dates back to early last century (cited by Chanishvilli et al., 2001 and Raettig, 1958). In more recent years, phages have successfully been used to resolve antimicrobial resistant UTI in humans (Perepanova et al., 1995; Slopek et al., 1987; Weber-Dabrowska et al., 2000a). These clinical studies are encouraging, but the feasibility of phage therapy for management of canine and feline UTI remains to be investigated.

In this study, the hypothesis was tested that a variety of phages able to lyse canine and feline UPEC can readily be found in the environment. The ability of collected phages to cause lysis *in vitro* was tested on 31 UPEC strains isolated from dogs, 22 UPEC strains isolated from cats and 7 faeces-derived *E. coli* strains, from dogs and cats. When lysis profiles suggested that phages were promising candidates for an *in vivo* trial, phages were subjected to further morphological and genetic studies, using EM and DNA sequencing.

6.3. Experiments and Results

6.3.1. Phage Isolation

Phage isolation was attempted from processed sewage water* using the double-layer plating technique (Ackermann and DuBow, 1987a). Twenty-one canine and 21 feline UPEC strains, cultured from cystocentesis-derived urine samples of different NZ animals with suspected UTI†, were used as propagation strains. Plaques were observed on 40 of the 42 UPEC strains after overnight incubation. No plaques formed on two canine UPEC strains.

6.3.2. High Susceptibility of UPEC to Phage

The ability of each phage to cause lysis was tested on each of the 42 UPEC used for phage propagation (see above) and a further 10 canine UPEC strains and one feline UPEC strain, cultured from cystocentesis-derived urine of individual NZ dogs or cats. The double-layer plating technique or modified macroplaque technique described in detail in chapter 2.6 were applied in this experiment.

Each of the 40 phages had a unique, distinctive host range. Phages lysed a mean of 21.2/53 (40%) UPEC strains (range 17%-70%, median 38%). The 10 phages with the broadest host range each lysed more than 27/53 (≥51%) of the UPEC strains (Figure 6.1). Twelve UPEC strains (5 canine and 7 feline) were lysed by all of these 10 phages. In total, 49/53 (92%) UPEC strains were lysed by one or more of the 10 phages with the broadest host range. All but 3 of the 53 UPEC strains (6%) could be killed by at least one of the 40 phages in the collection. These 3 UPEC strains (2 strains that yielded no plaques after initial incubation with sewage and one additional strain) originated from dogs.

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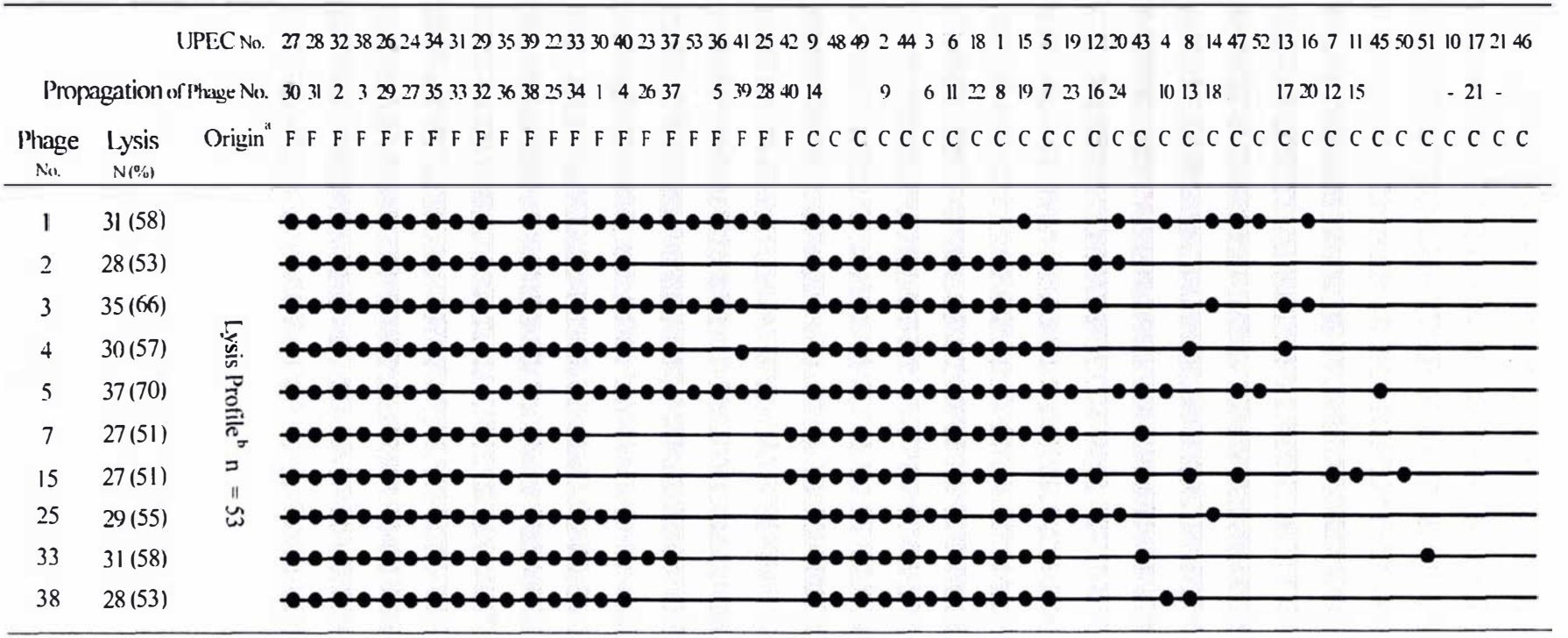
* Processed sewage: Chloroform extract of a polyethylene glycol precipitate of NaCl-enriched raw sewage waters – please refer to chapter 2.5 for the complete method.

† Please refer to appendix 8.4.1 for strain information

Figure 6.1 Lysis of 53 UPEC strains by the 10 phages with the broadest host range[x]



[x]: Lysis results of the remaining phages on UPEC strains and faecal *E. coli* strains are listed in the appendix 8.3.3.

[a]: Species UPEC strain was isolated from: C: canine, F: feline. [b]: Differences in lysis between 10 selected phages: ● indicates that lysis was observed, a space indicates that no lysis was observed. [c]: 53 UPEC strains were included in the illustrated lysis profiles of the 10 phages with the broadest host range.

6.3.3. Relatively Low Susceptibility of Faecal *E. coli* to Phage

Seven faecal *E. coli*, collected from droppings of 4 dogs and 3 cats without signs of UTI or gastrointestinal disease, were also tested with each of the 40 phages. The susceptibility of these faecal *E. coli* to phages was tested to help assess whether adverse effects related to lysis of commensal gastrointestinal *E. coli* should be anticipated in future therapeutic trials using phages. The 40 phages lysed on average 0.9/7 (13%) faecal *E. coli* strains (range 0-71%, median 14%). Lysis of faecal *E. coli* strains was significantly less likely than lysis of UPEC strains (Holm's adjusted z-test, P=0.001). The susceptibility of faecal *E. coli* to the 10 phages that lysed the most UPEC strains was similar to their susceptibility to the 30 other members of the phage collection. Of the 10 phages with the broadest UPEC host range, 7 (phages 3, 4, 5, 7, 15, 25 and 38) lysed 1/7 faecal *E. coli*, one (phage 33) lysed 2/7 faecal *E. coli* and 2 (phages 1 and 2) lysed 3/7 faecal *E. coli*. Compared to other faecal *E. coli* that were lysed by ≤6/40 phages, one faecal *E. coli* strain (Strain 135*) appeared particularly susceptible to the phages in the collection. Considering once again the 10 phages with the broadest UPEC host range, strain 135 was the target of 5 of the 7 phages that lysed only a single faecal strain and by both of those that lysed 3/7 faecal strains. Strain 135 was also lysed by 6 of the 30 other phages in the collection.

6.3.4. Susceptibility of Canine and Feline UPEC to Phage Lysis

In order to assess whether the species of the patient needs to be considered during future preparation of phage therapeutics, phage susceptibility of UPEC isolated from dogs was compared with the susceptibility of UPEC isolated from cats. On average, a given phage lysed 10.6/22 (48%) UPEC from cats (range 14-91%, median 41%) and 10.6/31 (34%) UPEC from dogs (range 10-58%, median 32%). UPEC from cats were significantly more likely to be lysed by the phages in the collection than were UPEC from dogs (Holm's adjusted z-test, P=0.001). The 21 phages that had been propagated on feline strains lysed on average 12/21 (57.1%) strains in the feline UPEC collection (median 57%; range 24-90.5%) in addition to the strain on which they were originally

* Please refer to appendix 8.3.3 for the complete lysis profile of this strain.

propagated. The phages originally propagated on feline UPEC lysed on average 11/31 (34.4%) canine UPEC (median 35.5%; range 9.7-58.1%). The 19 phages that had been propagated on canine strains lysed on average 7.9/22 (35.9%) strains in the feline UPEC collection (median 36%; range 14-64%). Similarly, phages propagated on canine UPEC lysed on average 9.5/30 (31.8%) canine UPEC (median 30%; range 20-50%) in addition to the strain upon which they were originally propagated. Overall, only 3 phages lysed ≥16/31 (51.6%) of all canine strains. These phages had been propagated on 2 canine strains (phages 11 and 15 lysed 16/31 (51.5%) canine UPEC each) and on one feline strain (phage 5 lysed 18/31 (58.1%) canine UPEC).

6.3.5. UPEC from Dogs and Cats shows Significant Differences in Surface-molecule Encoding VFG Markers

Urovirulence-associated cell surface components that may serve as phage receptors (Ackermann and DuBow, 1987a; Smith and Huggins, 1982) and species-specific differences in such components would be a possible explanation of the greater susceptibility of feline UPEC. Therefore, VFG profiling was used to assess genotypic similarities and differences of 9 surface-encoded virulence structures, namely *papA, papEF, papG II, papG III, sfaS, focG, ireA, iroN* and *kpsMT II*, in a sample of 30 canine and 15 feline UPEC that had been included in the phage lysis experiment. 39/45 (86.7%) UPEC possessed a unique VFG profile. A pair of feline UPEC (F94 and F140), a pair of canine UPEC (C79 and C81) and a pair consisting of one canine UPEC and one feline UPEC (F142 and C70) each shared an indistinguishable VFG profile (chapter 3, Table 3.2). These UPEC isolates are likely to be epidemiologically unrelated – they had been collected from 6 individual animals at different times*.

Each of the 9 VFG markers assessed was detected at least once in UPEC collected from each species (Table 6.1). The P-fimbrial subunit gene markers *papA, papEF* and *papG III* were significantly more often present in feline than in canine UPEC (Holm's adjusted Fisher's exact test, P=0.028). *PapA, papEF* and *papG III* were detected in all 6 isolates that shared a VFG profile with another UPEC strain.

* Two of these pairs had distinguishable PFGE banding patterns (chapter 3, Figure 3.4). The 3rd pair (F142, C70) was not assessed by PFGE analysis.

Table 6.1 Prevalence of VFG markers in UPEC isolated from dogs and cats

VFG marker	Canine UPEC (n=30) N (%)	Feline UPEC (n=15) N (%)	Significance value[a]
PapA	18 (60)	15 (100)	0.028*
PapEF	18 (60)	15 (100)	0.028*
PapG III	18 (60)	15 (100)	0.028*
PapG II	3 (10)	2 (13)	1
SfaS	2 (7)	4 (27)	0.354
FocG	14 (47)	11 (73)	0.354
IroN	22 (73)	15 (100)	0.19
IreA	5 (17)	8 (53)	0.096
KpsMT II	13 (43)	8 (53)	0.208

[a] As determined by Fisher's exact test, 2-tailed. Results were adjusted for problems of multiplicity using a Holm's step-down procedure. Significant differences are indicated in bold. * $P<0.05$.

6.3.6. Species-specific Differences in Susceptibility to Phage Lysis are not Explained by Differences in Surface-molecule encoding VFG markers

Stepwise binary logistic regression was used to assess whether inter-species differences in lysis by phage could be explained by inter-species differences in VFG profiles of UPEC*. The logistic regression models predicted that the presence or absence of *papA*, *papEF* and *papG III*, overrepresented in feline UPEC, did not significantly influence the ability of the 40 phages to lyse UPEC ($P>0.01$ for all phages). Thus, overrepresentation of these VFG in feline UPEC was probably not the reason why feline UPEC were more susceptible to phage.

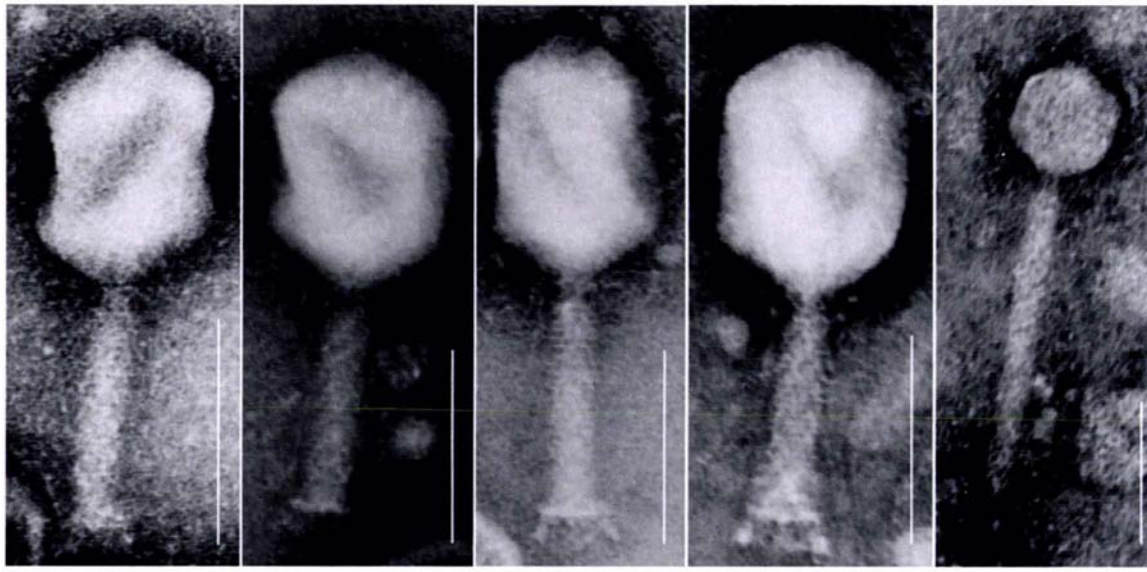
The analysis did, however, reveal a possible role of other VF in UPEC susceptibility to lysis by 3 of the phages: Phage 6 was significantly more likely to lyse UPEC that encoded the VFG marker *kpsMT II* ($P=0.004$, odd's ratio 10.1, 95% CI 2.1-49.6) or *sfaS* ($P=0.026$; odd's ratio 22.5; 95% CI 1.5-348.4). Similarly, lysis by phages 18 and 20 occurred significantly more often when the target UPEC encoded *kpsMT II* ($P=0.002$; odd's ratio 33.7; 95% CI 3.7-309.0 and $P=0.018$; odd's ratio 6.7; 95% CI 1.4-32.1, respectively).

* Please refer to chapter 2.9.4 for a detailed description of the method.

6.3.7. Characterisation of 5 Phages with a Broad Host Range

Five phages (1-5) that showed a broad host range, lysing ≥53% UPEC isolates (Figure 6.1), were characterised further by EM and sequencing of a tail tube glycoprotein gene*. EM revealed that all of these phages belonged to the order *Caudovirales* and the family *Myoviridae* (Büchen-Osmond, 2006). Phages 1-4 belonged to the morphotype A2 (Ackermann and Eisenstark, 1974) and had morphological similarity to lytic phage T4 (Figure 6.2; Büchen-Osmond, 2006).

Figure 6.2 Electron micrographs of phages 1-5 (from left to right)



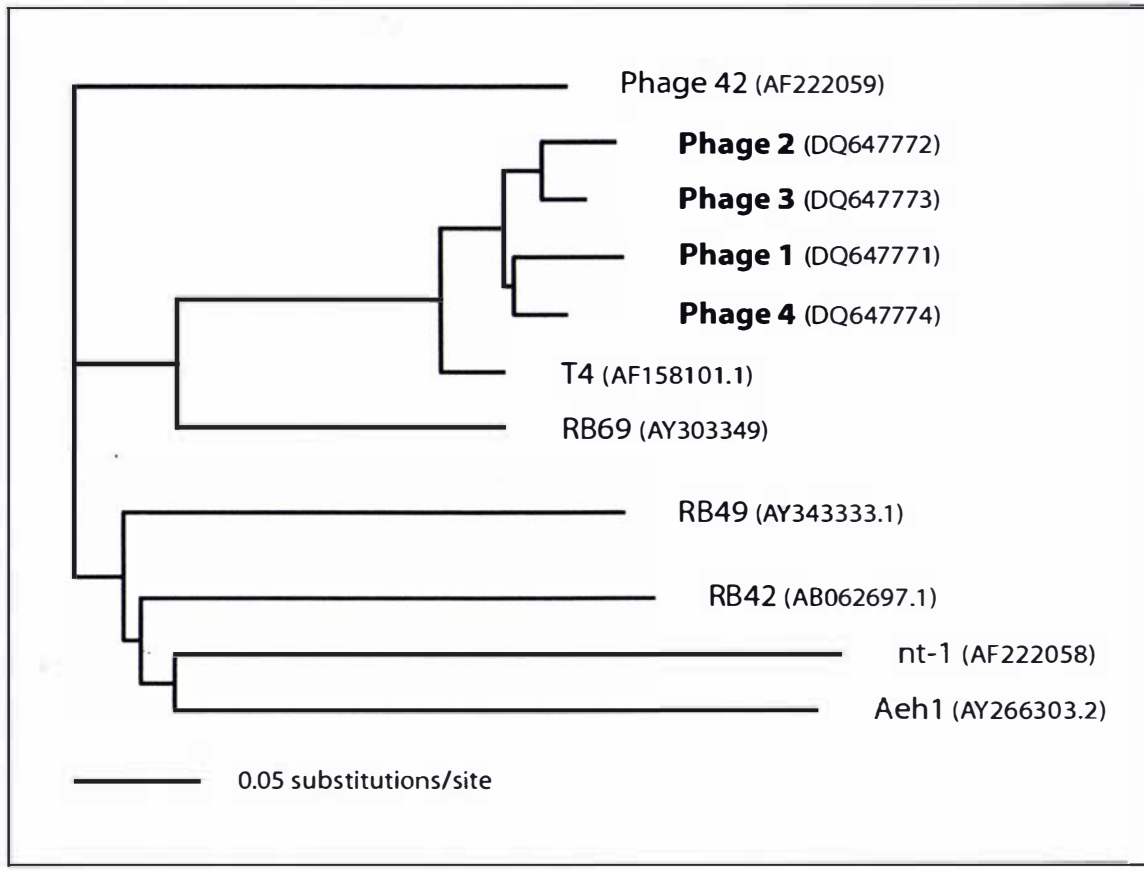
Size (nm)	1	2	3	4	5
Head[a]	117 x 87	121 x 91	117 x 87	114 x 87	66 x 63
Tail[b]	116 x 22	116 x 22	115 x 21	112 x 22	178 x 18

UA staining. The white bar indicates 100 nm.
[a]: Head size, apical measurement x side measurement.
[b]: Tail size, length x width.

These morphological findings were verified by sequence analysis. For each of the T4-like phages, a tail gene could be amplified using primers based on the T4 tail gene 18 (Tetart et al., 2001). All sequences were unique, but highly similar to each other (91% to 95%). When comparing the tail gene sequences with sequences of 7 phylogenetically well-characterised T4-like phages (Tetart et al., 2001), these phages clustered closely with others belonging to the T-even group (T4 and RB69; Figure 6.3).

* Please refer to chapters 2.7 and 2.8 for the methods of EM and DNA sequencing of the tail tube gene, respectively.

Figure 6.3 Comparison of phages 1-4 to phylogenetically well-characterised phages[a]



[a]: Previous phylogenetic characterisation of phages as reported by Tetart et al. (2001)
Accession numbers of gene sequences that were obtained from GenBank are given in brackets.

The morphology of phage 5 was dissimilar to the T4-like morphology of phages 1-4 (Figure 6.2). Therefore, phage 5 was not included in DNA sequencing of a tail tube glycoprotein specific for T-even phages (Tetart et al., 2001). Phage 5 belonged to the morphotype A1 (Ackermann and Eisenstark, 1974) and had a contractible tail sheath (EM image not shown). It had morphological similarity to temperate phages P2 and Mu (Büchen-Osmond, 2006). However, the head and tail measurements of phage 5 appeared distinct from those previously reported for P2 and Mu (Büchen-Osmond, 2006).

6.4. Discussion

This study shows that readily-obtained, naturally occurring phages are able to lyse a large proportion of the canine and feline UPEC strains in this collection, at least *in vitro*. Ten of the 40 phages obtained by making a single visit to a sewage treatment plant were able to kill ≥51% of a UPEC collection of 53 strains. This result is in marked contrast to a previously published study of *E. coli* UTI in children (Drulis-Kawa et al., 2002), in which only 14/44 phages lysed >15% of the UPEC strains studied. Only 3 phages studied by Drulis-Kawa et al. (2002) lysed >50% of the UPEC strains. Phages used in the previous study were selected from a hospital collection of *E. coli* phages that may not have been propagated on UTI-associated *E. coli* strains. It is possible that the selective propagation on UPEC improved overall lysis results in this study. However, it cannot be excluded that UPEC isolated from cats and dogs are, in general, more susceptible to phage lysis than are UPEC isolated from children.

No phage able to lyse all of the UPEC strains in the collection was found in this study. However, there were 10 phages that each lysed more than half of the UPEC strains. Each of them had a distinct lysis profile and, taken together, they lysed 92% of the UPEC strains. Hence, it may be possible to treat UTI caused by a large variety of UPEC strains with one preparation containing a mixture of phages with relatively broad host range. Previous applications of such combinations (so-called 'phage cocktails') in other disorders have been described as highly successful (reviewed in Kutter and Sulakvelidze, 2005). The use of a 'cocktail' may not only increase the proportion of UPEC strains that can be lysed and minimise the development of resistance to phages, but it may also allow recombination of attachment structures (Kutter et al., 2005; Tetart et al., 1998). Thus, new, "naturally recombinant" phages with an extended host range may be created.

One of the concerns when using naturally occurring phages is that these phages may have the ability transfer potentially deleterious genes to bacteria (Boyd and Brussow, 2002; Wagner and Waldor, 2002). These so-called transducing phages are almost exclusively temperate, that is, they have the ability to integrate their genome into the host bacterial genome (Birge, 2000). In addition to their transducing potential, temperate phages are considered inappropriate for phage therapy because these phages may not lyse bacteria consistently (Lwoff, 1953). In this study, EM and DNA

sequencing of a tail tube gene were applied to 5 phages with a promising lysis profile to investigate whether these naturally occurring phages are comparable to previously studied lytic or temperate phages. Four of these 5 phages were demonstrated to be T4-like (Büchen-Osmond, 2006) belonging to the T-even phage group (Tetart et al., 2001). Considering T4 phage biology, these 4 phages are very likely to be lytic and unable to integrate their viral genome into the host bacterial genome. Therefore, these T4 phages are considered appropriate candidates for future *in vivo* therapeutic trials and suitable candidates for inclusion in phage cocktails. A fifth phage with a very promising lysis profile that had a morphotype similar to temperate phage P2 is at present considered an inappropriate candidate for inclusion in therapeutic phage preparations. Inclusion in future phage cocktails may be considered if subsequent studies can show that this phage (i) lyses UPEC consistently; and (ii) does not encode deleterious traits.

Several previous studies have shown that phages readily cross physiological barriers and, irrespective of the administration method, distribute within virtually all body compartments (Biswas et al., 2002; Dubos et al., 1943; Geier et al., 1973; Reynaud et al., 1992; Smith and Huggins, 1982; Weber-Dabrowska et al., 1987). Therefore, it is likely that phages will encounter gastrointestinal commensal bacteria, even if phages are not administered orally. Recent studies in mice (Chibani-Chennoufi et al., 2004) and humans (Bruttin and Brussow, 2005) indicate that commensal bacteria susceptible to phages *in vitro* are *in vivo* largely resistant to orally administered phages. Nonetheless, to mimimise potential adverse effect on the commensal microflora, it was considered desirable to create a phage preparation that lyses few commensal *E. coli* strains *in vitro* and *in vivo*. Phages tested during this *in vitro* trial lysed significantly more UPEC strains than faecal *E. coli* strains. In fact, only one of the faecal strains tested showed a susceptibility to phage that was comparable to the average susceptibility of UPEC to phage. A likely explanation for this difference in susceptibility to lysis is that significantly more UPEC than faecal *E. coli* expressed the specific receptors necessary for phage attachment. The faecal strains were isolated from animals without signs of gastrointestinal disease or UTI and most of them were probably commensal inhabitants of the gastrointestinal tract. Commensal *E. coli* and UPEC commonly belong to different phylogenetic groups and faecal *E. coli* strains often lack VFGs that are present in UPEC (Johnson et al., 2003; Johnson and Russo, 2002a; Whittam et al., 1989). By propagation of phage on UPEC strains, a selection for phages that recognise receptors

specifically expressed by UPEC, but not by commensal (faecal) *E. coli*, may have been achieved in this study.

Feline UPEC strains were significantly more likely to be lysed by phages in the collection than were canine UPEC strains, despite the fact that phages were initially propagated on equal numbers of canine and feline UPEC strains. Feline UPEC were, on average, more susceptible to phage that had been propagated on feline UPEC than to phage that had been propagated on canine UPEC. In contrast, canine UPEC were, on average, equally resistant to phages that had been propagated on canine or feline UPEC. The relative resistance of canine UPEC to phages is unexplained by this study. One likely explanation is that surface properties of feline and canine UPEC of this collection differed and phage receptors were expressed to a greater extent by the feline *E. coli*. When UPEC from dogs and cats were compared, there were differences in 3/8 markers for genes that encode virulence determinants found on bacterial cell surfaces. While none of the three markers overrepresented in feline UPEC was directly linked to an increased probability of lysis, it is possible that further differences in surface properties of canine and feline UPEC in this collection existed and contributed to the observed differences in susceptibility to lysis. Another overlapping explanation is that a greater proportion of the canine UPEC strains had previously been exposed to phages similar to those in the collection. Hence, they may have naturally acquired resistance to some of the phages studied (Levin and Bull, 2004; Skurnik and Strauch, 2006). Dogs interact intensely and intimately with members of the same species and are perhaps more likely to ingest organic matter containing a variety of phage-resistant *E. coli*, than are cats. It is possible that phage-resistant *E. coli* are exchanged to a greater degree among dogs than among cats. Finally, it cannot be ruled out that the observed differences in susceptibility to phage lysis in UPEC from dogs and cats were influenced by subtle differences in the clinical characteristics of UTI in the patient groups. In humans, it has been shown that UPEC isolated from cases of pyelonephritis often differ from those isolated from simple cystitis (Johnson, 1998; Johnson et al., 2005b). Furthermore, UPEC isolated from immunocompromised people often express fewer VFs than those isolated from patients with an intact immune system (Johnson and Stell, 2000). Detailed information about the category of UTI (i.e. simple cystitis or pyelonephritis) and host immune response (i.e. intact or impaired) was not available in this retrospective study. Thus, it could not be determined conclusively whether (i) the

disease status of the source dogs and cats differed; (ii) selection of a certain kind of UPEC occurred; and (iii) selection of a certain kind of UPEC influenced the results concerning susceptibility to phage lysis.

Binary logistic regression predicted that 3 of the 40 phages assessed here (phages 6, 18 and 20) could lyse significantly more strains with the VFG marker *sfaS* or *kpsMT II* than strains without these VFG markers. This may indicate that these phages adsorb to S-fimbrial subunits (*SfaS*) or to parts of group 2 polysaccharide capsules (*Kps II*) during the infectious process. *E. coli* phages have previously been shown to attach to such structures (Ackermann and DuBow, 1987a; Scholl et al., 2001; Smith and Huggins, 1982). If phages 6, 18 and 20 indeed attach to fimbria or capsules, they may be particularly useful candidates for phage therapy of UTI caused by UPEC that express these VFs. This is because bacterial strains that subsequently acquire resistance to these phages will often show mutations (including complete deletions) of the particular VFG (Park et al., 2000; Smith and Huggins, 1982). Thus, therapeutic use of such phages may lead to the emergence of phage-resistant mutants that are concomitantly less virulent than the parental strain.

The association between susceptibility to lysis and the presence of a VFG marker was observed indirectly, by statistical means, and will need to be confirmed in subsequent studies that assess the receptor specificity of phages by direct, phenotypical or genotypical means.

6.5. Conclusion

In conclusion, this study showed that a diversity of phages able to lyse a majority of clinically-relevant canine and feline UPEC isolates, at least *in vitro*, can readily be found in nature. Phage therapy of canine and feline UTI – and of other serious diseases caused by UPEC strains and their close relatives – may therefore be feasible. Given the interesting differences in phage susceptibility between dog and cat-derived UPEC strains, different phage preparations may need to be investigated for use in dogs and cats. Further work is needed to investigate the clinical significance of these findings.

Chapter 7

General Discussion

7.1. Exploring the Pathotypic Traits of UPEC

UPEC are defined as *E. coli* that are capable of causing urinary tract disease. In most cases, these *E. coli* ascend from the distal urethra to the bladder, ureters and kidneys (Bartges, 2005). While UPEC often predominate in the gastrointestinal tract at the time of diagnosis of UTI (Johnson et al., 1998a; Johnson et al., 2003; Yamamoto et al., 1997), they - and their close relatives causing other extraintestinal diseases - have been established as a group of pathogenic *E. coli* that is phylogenetically distinct from intestinal pathogenic *E. coli* and commensal *E. coli* (Johnson and Russo, 2005; Russo and Johnson, 2000). UPEC have also been demonstrated to encode a diversity of adhesins, toxins, siderophores, capsules and other VFs that are considerably less often present in commensal *E. coli* and intestinal pathogenic *E. coli* (as discussed by Johnson, 2003 and Russo and Johnson, 2000). By investigating the prevalence of VFs in UPEC isolated from certain patient populations, such as patients from a particular species or patients with concurrent diseases other than UTI, one may gain a better understanding of the aetiopathogenesis of *E. coli* UTI in these populations (Johnson and Russo, 2005). Possible reservoirs of UPEC may be determined. This knowledge may then lead to important strategies for disease prevention and management.

Hence, one major objective of this project was to build upon previous knowledge derived from North American and European studies regarding the presence and distribution of VFGs in UPEC isolated from dogs and cats (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001d; Yuri et al., 1998). The main focus was upon feline UPEC, because the VFGs of these UPEC had been studied to a much lesser extent or in much smaller sample populations than VFGs of canine and human UPEC (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Whittam et al., 1989; Wilson et al., 1988; Yuri et al., 1998).

By investigating the VFGs and clonal relationships of feline UPEC this project intended to (i) contribute to an optimisation of the management of intractable *E. coli* UTI in cats; and (ii) complement studies that compare UPEC from dogs, cats and humans with the aim of elucidating the risk of cross-species infection.

Early in the project, the opportunity arose to study 31 feline UPEC isolated from London cats. During a 2-year period, these 31 *E. coli* had been isolated from 20 CRF-affected cats – a population that is particularly predisposed to developing *E. coli* UTI (Barber et al., 1999; Mayer-Ronne et al., 2004). Five of these 20 cats had been diagnosed with multiple UTI during this 2-year period. A total of 17 UPEC strains had been isolated from these cats. The clonal relatedness, assessed by PFGE, and the VFGs of UPEC isolated from these 5 cats have been reported in this thesis. Such a longitudinal assessment of genotypic traits of UPEC that cause intractable, recurring UTI has not been achieved before. Fingerprinting by PFGE and urovirulence genotyping by multiplex PCR showed that two-thirds of the recurring *E. coli* UTI in CRF-affected cats were persisting or relapsing infections, as opposed to reinfections. UPEC clonality was not accurately diagnosed by evaluation of antibiograms, one of the clinical tools previously considered helpful in distinguishing between relapsing or persisting infections and reinfections (Drazenovich et al., 2004; Seguin et al., 2003). Furthermore, individual UPEC clones caused persistent or relapsing infections over a surprisingly long period of time (≥ 354 days), despite apparently successful antimicrobial therapy. These findings clearly demonstrate that whenever multiple UTIs are diagnosed in CRF-affected cats, a relapsing or persisting infection should be expected. The time-span between successive diagnoses and the antibiograms of UPEC isolated from these infections cannot be used reliably to distinguish between same-clone and different-clone recurrences.

The vast majority of *E. coli* that were associated with recurrent or persistent UTIs were isolated from cats that did not show any clinical signs associated with urinary tract disease. In all instances, antimicrobial therapy was started upon positive urine culture - whether clinical signs were present or not. Thus, clinicians followed the currently recommended treatment approach of initiating antimicrobial therapy whenever bacteria are isolated from parts of the urinary tract of cats that are normally considered to be sterile (Bartges, 2005). Antimicrobial treatment is considered necessary to prevent a potential pyelonephritis that could cause a rapid progression of CRF (Horcajada et al., 2004; Slotki and Asscher, 1982). In contrast to the treatment approach in small animal medicine, antimicrobial treatment of ABU in people - which may be considered equivalent to clinically silent UTI in cats - is not always initiated, particularly not in elderly people (Nicolle, 2000a). This is because antimicrobial treatment of ABU is not associated with a decrease in morbidity and mortality (Nicolle et al., 1987) and may cause antimicrobial-associated adverse effects or lead to antimicrobial resistance (Nicolle, 2006). Strikingly, similar observations were made in this study. Firstly, *E. coli* persisted or caused relapsing infections despite the prolonged use of antimicrobials. Secondly, persistent or relapsing infections were not associated with a sudden and rapid progression of CRF. Thirdly, antimicrobial resistance development was observed in a UPEC clone that persisted in a cat despite long-term antimicrobial treatment. Thus, observations made in this project suggest that antimicrobial treatment of clinically silent UTI in cats may not be advantageous. In fact, a presence of clinically silent *E. coli* UTI may be beneficial. In humans with neurogenic bladders after spinal cord injury, colonisation by ABU strains has not been shown to cause harm and successfully prevented UTIs caused by more virulent UPEC (Darouiche et al., 2005). Further studies are needed to investigate whether *E. coli* associated with clinically silent UTI in CRF-affected cats (i) are capable of causing a potentially life-threatening pyelonephritis and, therefore, should be eradicated from the urinary tract; or (ii) are harmless and, therefore, do not require antimicrobial therapy.

The reservoirs of UPEC that cause relapsing or persisting infections could not be established by the study described in chapter 5. In fact, urine sterility was not documented for any of the cats with multiple UTI after the end of each course of antimicrobials. Thus, it is possible that *E. coli* clones persisted in low numbers in the bladder lumen despite antimicrobial treatment. Alternatively, *E. coli* clones were eliminated from the bladder lumen during antimicrobial treatment and regained entry to the bladder lumen from one of the reservoirs upon discontinuation of antimicrobials. Suggested reservoirs for UPEC are (i) the uroepithelial cells of the infected patient (Mulvey et al., 2001; Schilling et al., 2001a); (ii) the gastrointestinal tract of the infected individual (Johnson et al., 1998a); (iii) the gastrointestinal tract of animals or people that are in contact with the affected patient (Murray et al., 2004); and (iv) the external environment. Further studies will be needed to assess which reservoirs are of particular importance in UTI of CRF-affected cats and how cats acquire UPEC from these reservoirs. It also remains to be elucidated which, if any, VFs enable feline UPEC to persist within the urinary tract or facilitate the re-entry of UPEC that reside in close proximity to the urinary tract. These VFs may subsequently become the target of interventions designed to prevent or manage undesirable relapsing or persisting *E. coli* UTIs (Russo and Johnson, 2006).

In chapter 4, a geographic variability of pathotypic traits in UPEC isolated from cats was demonstrated. UPEC isolated from UK cats differed significantly from UPEC isolated from NZ cats – both in regards to proportions of VFG and PFGE banding patterns. Subtle differences in patient disease status or a disproportion in the number of *E. coli* causing clinically silent UTI may have contributed to the observed dissimilarity of VFG profiles and PFGE banding patterns in these 2 UPEC populations. Nonetheless, the reported findings illustrate the heterogeneity of UPEC isolated from one species and indicate that the geographic origin of isolates should be considered when comparing VFG profiles or clonal traits of UPEC.

A total of 53 feline UPEC was subjected to urovirulence genotyping using a previously validated multiplex PCR assay (Johnson and Stell, 2000). This has been the largest population of feline UPEC incorporated into a genotypic study to date. Most of the 30 VFG markers that had been shown to be prevalent in canine and human UTI were present in feline UPEC. Moreover, a comparative assessment of UPEC from dogs, cats and people from NZ showed that most VFGs were present in similar proportions in the canine, feline and human UPEC isolates of the collection. These findings are in accord with previous findings (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2001a; Whittam et al., 1989; Wilson et al., 1988; Yuri et al., 1998). They indicate that most of the VFs that are considered important in the pathogenesis of canine and human UTI are also likely to be important in the pathogenesis of feline UTI. Thus, innovative strategies developed to prevent or manage *E. coli* UTI may be efficacious across species boundaries, if VFs are targeted that are predominant in UPEC isolated from cats, dogs and people (Johnson et al., 2003; Klemm, 1994; Russo and Johnson, 2006; Russo et al., 2003b). Results of this project indicate that the siderophore receptor IroN, suggested as a potential vaccine candidate by Russo et al. (2003b), may be such a target. Furthermore, the yersiniabactin receptor gene *fyuA*, which has been consistently found in a higher proportion of UPEC strains than faecal strains (Johnson et al., 2003; Johnson et al., 2001c), was present in a large proportion of canine, feline and human UPEC in this study. Therefore, the yersiniabactin receptor FyuA may be another potential vaccine candidate.

To complement previous studies that compare UPEC isolated from dogs, cats and people, VFG profiles of UPEC derived from NZ cats, dogs and humans were assessed. Previous studies that had compared the VFGs of feline, canine and human UPEC focused on relatively few VFs or incorporated only a few feline UPEC (Feria et al., 2001a; Feria et al., 2001b; Johnson et al., 2000a; Johnson et al., 2001c; Johnson et al., 2001d; Low et al., 1988; Whittam et al., 1989; Wilson et al., 1988; Yuri et al., 1998). Furthermore, comparisons conducted in these studies were purely descriptive or based on univariate statistical analyses. Building upon previous studies, a substantial number of feline UPEC (22) was included in the comparative analysis described in this thesis. Furthermore,

extended VFG profiles of UPEC from dogs, cats and humans were incorporated into discriminant analysis, principal component analysis and nearest neighbour analysis. The application of these multivariate methods allowed, for the first time, assessment of the degree of similarity of VFG profiles (as opposed to proportions of single VFG) within and between UPEC populations sourced from NZ cats, dogs and humans.

In agreement with previous studies (Feria et al., 2001b; Johnson et al., 2000a; Johnson et al., 2001d), up to one-third of UPEC from dogs, cats and people were found to have VFG profiles that were highly similar to each other, if not indistinguishable. However, VFG profiles of the majority of UPEC isolated from NZ cats differed significantly from VFG profiles of UPEC isolated from NZ humans. This finding is in contrast to results of previous studies. It suggests that some UPEC may predominantly infect hosts of a particular species and may therefore be of particular interest for researchers aiming to predict accurately the risk of zoonosis associated with UPEC. However, due to the very small number of epidemiologically distinct human UPEC included in the assessment, the statistical power of the analysis is low. Thus, the proposed 'species-specificity' of some UPEC will need to be confirmed or rejected in a future study that includes a larger sample population of UPEC isolated from humans living in NZ. Ideally, UPEC assessed in a future study should derive from patient populations that are matched for factors that may influence the VFG possession of UPEC, such as clinical signs (clinically silent UTI vs. UTI associated with clinical signs), immune status (compromised or intact), localisation of UTI (pyelonephritis or cystitis) and geographic source of patient samples. In this study, matching could not be performed because the clinical information available in this retrospective study was limited. This is seen as a further weakness of the comparative assessment of VFG profiles in UPEC isolated from NZ humans, dogs and cats. It is noteworthy to mention that a comparative analysis of VFG profiles of UPEC isolated from different parts of the urinary tract (i.e. pyelonephritis vs. cystitis) may be particularly difficult to achieve in dogs and cats. It would require the positive identification of *E. coli* in fluid or tissue samples taken directly from the kidney by invasive sampling methods, such as pyelocentesis or renal biopsy. This is not usually done as part of the diagnostic work-up. Nonetheless, a prospective

trial investigating the prevalence of VFGs in pyelonephritis- or cystitis-causing UPEC may help with the clinical management of UTI in dogs and cats, particularly if VFGs were to be identified that predict whether isolated UPEC are likely to cause clinically-evident pyelonephritis, cystitis or both.

The VFs of UPEC were studied using a validated multiplex PCR assay. This assay had previously been shown to be of similarly high sensitivity and specificity to probe hybridisation techniques (Johnson and Stell, 2000). The advantage of this method over other laboratory methods such as hybridisation techniques or phenotypic assays was the speed with which the presence of 30 VFG markers could be detected in *E. coli*. Thus, the multiplex PCR assay was considered an overall superior molecular epidemiological tool to screen 120 UPEC isolated from cats (53), dogs (56) and humans (11). A weakness of this study was that PCR reactions were not run in duplicate after initial optimisation, during which reproducibility of the PCR method was visually established. Thus, there is a chance that, despite analysis according to standards established during optimisation, markers that were encoded by some isolates were not identified. A PCR based approach has also the disadvantage that the functionality of genes and their expression *in vivo* cannot be determined. Furthermore, PCR amplification depends on the annealing of a primer to its corresponding intact primer binding site and mutations that do not affect function may affect this binding. However, Blanco et al. (1997) demonstrated that a very good correlation between detection of VFG markers and *in vitro* expression of VFs can be expected - at least for some VFs. Furthermore, UPEC with mutational changes of VFGs were thought to occur at similar frequencies in the different UPEC populations (Matic et al., 1997). Therefore, it was considered likely that conclusions drawn from a DNA sequencing study that would identify truly functional VFGs would be similar to conclusions drawn in this study.

The clonal relatedness of isolates was studied using PFGE. Next to DNA sequencing, PFGE is considered the typing method with the highest discriminatory power (Johnson and Russo, 2005; Tenover et al., 1997). The method was applied successfully to demonstrate the clonality of isolates derived from cats with multiple diagnoses of UTI. Furthermore, PFGE demonstrated the

clonal relatedness of 2 pairs of human UPEC derived from 4 different patients who visited one local hospital over a brief period of time. Thus, typing by PFGE was essential for interpreting the results reported in chapters 5 and allowed refinement of the interpretation of results reported in chapter 3. Beyond assessing clonality, PFGE has been described as being of limited use when evaluating the relatedness of different UPEC populations (Johnson and Russo, 2005). This was also apparent in this study. Even isolates with an indistinguishable VFG profile were found to share little more than 15% similarity in PFGE banding patterns. Other methods, such as multi-locus sequence typing or random amplified polymorphic DNA analysis will need to be applied in a further study to assess the phylogenetic relatedness of UPEC isolates studied here.

7.2. Exploring the Potential Use of Phages to Combat *E. coli* UTI

Another major objective of this project was to conduct a preliminary investigation on the use of naturally occurring phages in combating *E. coli* UTI in dogs and cats. Phages are viruses that, with few exceptions, have the ability to kill bacteria (as discussed by Calendar, 2006). In the past, phages have been used extensively to treat bacterial infections in humans, farm animals and laboratory animals (reviewed by Sulakvelidze and Barrow, 2005; Sulakvelidze and Kutter, 2005 and Summers, 2005). However, to my knowledge, this is the first study to investigate the potential use of phages as antimicrobial agents in companion animals.

An investigation of phage therapy for feline and canine UTI is particularly timely, because over the last decades canine and feline urinary *E. coli* have become increasingly resistant to front- and second-line antimicrobials (Cohn et al., 2003; Cooke et al., 2002; Drazenovich et al., 2004; Sanchez et al., 2002; Seguin et al., 2003; Warren et al., 2001). Furthermore, work carried out in this and other studies demonstrates a commonality of VFGs in feline, canine and human UPEC (Feria et al., 2001b; Johnson et al., 2001a; Johnson et al., 2000a; Johnson et al., 2001d; Low et al., 1988; Yuri et al., 1998). This has led to the suggestion that some feline or canine UPEC pose a potential health risk to humans (Johnson et al., 2001d). To avoid a potential acquisition of antimicrobial resistant feline or canine

UPEC by humans, more stringent controls may, in future, be applied to veterinarians' use of antimicrobials. If so, phages and other alternative antimicrobials may be used as antimicrobial agents in companion animal clinical practice. Phages may also prove useful in the treatment of intractable infections caused by UPEC and their close relatives. Canine discospondylitis, canine prostatitis and, as has been shown in this and other studies (Drazenovich et al., 2004; Seguin et al., 2003), persistent or relapsing UTIs in cats and dogs are such intractable infections that could potentially be resolved with phage therapy. This is because phages have the ability to multiply at the site of infection (as discussed by Kutter and Sulakvelidze, 2005). This secures a 'therapeutic level of antimicrobial agent' until the bacterial population is decimated.
Furthermore, phages have the ability to evolve and may adapt *in vivo* to infect bacterial strains that have developed resistance to original phage types (as discussed by Kutter and Sulakvelidze, 2005).

This study indicates that phage therapy of canine and feline *E. coli* UTI may be feasible. Forty phages able to infect and kill a large variety of canine and feline UPEC were isolated with ease from sewage water collected during a single visit to a municipal sewage plant. The lytic ability of these naturally occurring phages was subsequently tested on 53 feline and canine UPEC and 7 faecal *E. coli* from dogs and cats without signs of gastrointestinal disease. Results of this cross-lysis experiment indicate that the majority canine and feline UPEC are susceptible to naturally occurring phages. Phages were highly selective, lysing on average 40% of all UPEC strains. None of the 40 phages lysed all 53 UPEC. Thus, it is likely that a combination of phages, a so-called "phage cocktail" (Kutter and Sulakvelidze, 2005), may need to be used to achieve a high therapeutic efficacy. Compared with the use of a single phage, the use of a phage cocktail may also be advantageous in situations where the susceptibility of the infecting UPEC strain is not known. This will commonly be the case during initial treatment of UTI. Furthermore, the combination of phages that attach to different structures of a particular UPEC strain may decrease the chance of resistance development to phage (Kutter and Sulakvelidze, 2005; Scholl et al., 2001). The number of phages in a highly efficacious phage cocktail can be kept to a minimum by combining

different phages with a particularly broad host range. In this study, 10/40 (25%) naturally occurring phages were considered promising candidates based on their lysis profile. These phages lysed ≥27/53 (≥51%) UPEC individually and 49/53 (92%) UPEC in combination. It is possible that further phages that target the remaining UPEC strains can be found in the environment. The intrinsic resistance of some UPEC strains to all of the 40 phages emphasises the importance of susceptibility tests as part of any future phage therapy in companion animals. Such tests were already advocated by Felix d'Hérelle (reviewed by Summers, 1999) and have been an important component of human phage therapy in the Former Soviet Union and Poland (reviewed by Sulakvelidze and Kutter, 2005). Similar to antimicrobial susceptibility tests, phage susceptibility tests will help to avoid therapy failure by identifying phage resistant UPEC strains.

Interestingly, phages lysed UPEC to a significantly greater extent than faecal *E. coli*. It is possible that the high selectivity of phages for UPEC was achieved by propagating phages on UPEC strains. The observed *in vitro* resistance of faecal *E. coli* may be an indicator for the overall intrinsic resistance of the gastrointestinal flora to phages used here. Moreover, recent phage trials in mice and humans demonstrate that gastrointestinal *E. coli* that are susceptible to phage *in vitro* show a dramatic decline of susceptibility to the same phages *in vivo* (Bruttin and Brussow, 2005; Chibani-Chennoufi et al., 2004). Overall, this may indicate that adverse effects associated with phage-lysis of the gastrointestinal flora (e.g. bowel upsets, diarrhoea, vomiting) are unlikely to occur during phage treatment.

While the *in vitro* phage susceptibility tests conducted as part of this project indicate that phages may be useful antimicrobial agents, further *in vitro* and *in vivo* trials are necessary to confirm the potential of phages as antimicrobial agents for treatment of canine and feline *E. coli* UTI. Future *in vitro* trials may focus on establishing the infectivity of candidate phage in conditions similar to those found in the urinary tract of pets. *In vivo* pharmacokinetic studies in humans suggest that viable phages can be isolated from the urinary tract for at least 10 days after oral administration (Weber-Dabrowska et al., 1987). No such study has been

conducted in dogs or cats. However, an investigation of the infectivity of phages in pet urine may be essential because (i) the urine of cats has a different composition to human urine (Lees et al., 1979); (ii) previous *in vitro* studies have demonstrated that high concentrations of urea can cause a decline in viability of some phages (as discussed by Guttman et al., 2005); and (iii) the attachment rate of some phages has been shown to decline if certain cations, such as calcium or magnesium ions, are lacking (reviewed by Kutter et al., 2005).

Electron microscopy and DNA sequencing of tail tube genes conducted on 5 phages in this phage collection indicates that both temperate and lytic phages are to be expected in a phage population isolated from raw sewage. Temperate phages may not lyse bacteria consistently (Lwoff, 1953). Thus, these phages are rarely used in phage therapy (as discussed by Merril et al., 1996; Sulakvelidze and Kutter, 2005 and Summers, 2001). Temperate phages cannot be distinguished satisfactorily from lytic phages by phenotypic means (as discussed in Calendar, 2006). One indisputable method of identifying temperate phages is to screen the viral DNA for genes that encode proteins known to be involved in the integration of viral DNA into the host genome (so-called integrases) or in the excision of viral DNA from the host genome (so-called excionases). At the same time, the viral DNA of future therapeutic phages may be screened for the possession of deleterious genes, such as antimicrobial resistance genes or genes encoding toxins (reviewed by Boyd and Brussow, 2002; Calendar, 2006; Dobrindt and Reidl, 2000; Saunders et al., 2001 and Wagner and Waldor, 2006). If such genes were present in therapeutic phages and if they were to be transferred to *E. coli* during phage infection, they could enhance the pathogenicity of *E. coli* and worsen the clinical outcome (reviewed by Boyd and Brussow, 2002 and Wagner and Waldor, 2006). It can be expected that the vast majority of phages that carry genes encoding deleterious traits are temperate. These phages may acquire such genes during excision from the bacterial host genome and transfer it directly to the genome of the subsequently infected bacterial host (reviewed by Birge, 2000). However, it cannot be ruled out completely that lytic phages carry genes encoding deleterious traits. Rarely, such genes may be packaged into the capsid of lytic phages during phage assembly (reviewed by Birge, 2000). Thus, it is desirable to

screen all phages that are considered candidates for therapy for the presence of genes encoding deleterious traits.

A further interesting *in vitro* study would be to determine whether phages can be found that target specifically surface-exposed VFs of canine, feline and human UPEC. The statistical assessment of the correlation between presence or absence of VFG markers in UPEC and presence or absence of lysis by certain phages conducted as part of this project indicates that such phages may be found in nature. In fact, the positive statistical correlation between lysis by phages 6, 18 and 20 and the presence of VFG marker *kpsMT II* observed in this study may indicate that these phages recognise group II capsules. They may be similar to the K1 phages used for phage therapy by Smith and Huggins (1982). Phages attaching to VFs may be particularly useful for treating UTI caused by UPEC that express targeted VFs. This is because UPEC that subsequently develop resistance to these phages will often show mutations (including complete deletions) that may affect the functionality of targeted VFs (Park et al., 2000; Smith and Huggins, 1982). Thus, therapeutic use of such phages may concomitantly lead to the emergence of phage-resistant mutants that are less pathogenic than the parental UPEC strain.

Once the *in vitro* efficacy of phages is established, *in vivo* trials may be conducted that investigate how, how often and at what dose phages must be given to eliminate *E. coli* from the urinary tract. It may be most efficacious to administer phage locally, by injection into the bladder after collection of a urine sample by cystocentesis. In humans, UTI has been treated successfully by oral administration of phage (Slopek et al., 1987; Weber-Dabrowska et al., 2000a). This may also be feasible in dogs and cats. Furthermore, oral administration may be the preferred administration method if repeated phage administrations are required. Once the preferred administration methods and the dose of therapeutic phage preparations are known, the efficacy of phages may be determined in a double-blinded placebo controlled clinical trial.

7.3. Concluding Remarks

Work undertaken in this thesis illustrates the heterogeneity of UPEC from cats and dogs. Results of this project also indicate that the therapeutic use of phage in canine and feline medicine deserves to be explored further.

In view of rising bacterial resistance to antimicrobials and stiffening regulations regarding the use of antimicrobials by companion animal veterinarians, the efforts undertaken here to investigate alternatives to antimicrobials may prove to be of practical importance. Of clinical significance is also the revelation that recurring *E. coli* UTI in CRF-affected cats are more commonly caused by single UPEC clones than previously appreciated. The extended knowledge concerning the prevalence of VFs in feline UPEC should assist in determining which VFs are of particular importance in the pathogenesis of *E. coli* UTI in cats. This project also demonstrated a geographic variability of VFG in feline UPEC. Furthermore, it illustrated similarities and differences in VFG profiles of canine, feline and human UPEC. These findings may be of particular interest for researchers studying comparative aspects of UPEC and others seeking to determine which feline or canine UPEC are of particular zoonotic concern.

Appendix

8.1. Publications and Presentations Arising from this Project

8.1.1. Publications

1. Freitag, T., Squires, R.A., Schmid, J., Elliott, J., 2005, Feline uropathogenic *Escherichia coli* from Great Britain and New Zealand have dissimilar virulence factor genotypes. Veterinary Microbiology 106; 79-86
2. Freitag, T., Squires, R.A., Schmid, J., Elliott, J., Rycroft, A.N., 2006, Antibiotic susceptibility profiles do not reliably distinguish relapsing or persisting infections from reinfections in cats with chronic renal failure and multiple diagnoses of *Escherichia coli* urinary tract infection. Journal of Veterinary Internal Medicine 20; 245-249
3. Freitag, T., Squires, R.A., Schmid, J., Naturally-occurring bacteriophages lyse a large proportion of canine and feline uropathogenic *Escherichia coli* isolates *in vitro*. To be submitted for publication in "Research in Veterinary Science"

8.1.2. Presentations

Presented at the Annual Forum of the American College of Veterinary Internal Medicine in 2004

1. Freitag, T., Squires, R.A., Elliott, J., 2004, Antibiotic sensitivity profiles underestimate the proportion of relapsing infections in cats with chronic renal failure and urinary tract infection. Journal of Veterinary Internal Medicine 18; 387 (Abstract 10; oral presentation)
2. Freitag, T., Squires, R.A., Schmid, J., Elliott, J., 2004, Virulence genotypes of feline urinary *Escherichia coli* isolates from New Zealand and Great Britain differ. Journal of Veterinary Internal Medicine 18; 387 (Abstract 11; oral presentation)
3. Freitag, T., Squires, R.A., 2004, Discriminant analysis of extended urovirulence genotypes distinguishes human, canine, and feline urinary *Escherichia coli* isolates from New Zealand. Journal of Veterinary Internal Medicine 18; 437 (Abstract 196, poster presentation)

8.2. Literature Review

Table 8.1 Summary of previous studies comparing UPEC isolated from humans, dogs and cats

Comparative Study*	Subject	Important Results	Conclusions
Johnson et al. (2000a)	Extensive genotypic and phenotypic comparison of 75 human urosepsis isolates with 8 canine *papG III*+ urine/rectal *E. coli* pairs previously studied by Low et al. (1988)	*PapG III* and *papA* F48 peptide sequences of UPEC isolated from dogs and humans highly similar. (*PapG III* mediated MRHA† agglutination indistinguishable between species) one pair of clonally-related strains was isolated from different species (PFGE)‡ *PapG III* predominant *papG* allele in canine UTI 7/8 canine urine/rectal pairs indistinguishable (RAPD)§	Established that canine and human *papG III* variants are almost identical (previously believed to confer host-specificity). Some UPEC strains from dogs and humans may be clonally related (crossover-clones). Canine UPEC may reside in canine intestinal tract.
Johnson et al. (2001d)	Genotypic and phenotypic characteristics of 17 UPEC strains previously studied by Garcia et al. (1988a) were compared with ECOR** collection and 18 human ExPEC strains	Gene markers for MRHA adhesins, toxins (*hly, cnf1*) and siderophore receptors highly prevalent in canine strains, less prevalent in ECOR strains 71% of canine strains cluster in phylogenetic groups B2, D (RAPD) Canine and human strains clustered irrespective of species origin (RAPD, dendrogram cluster analysis) *PapG III* only *papG* allele detected in canine strains MRHA of PapG III from canine strains indistinguishable to PapG II/III controls, different to PapG I or S-fimbrial controls†† Association of VF with phylogenetic groups and with each other assessed	Extensive phylogenetic similarity of canine and human strains PapG III adhesin is not host-specific Garcia et al. (1988b)†† VF association indication of common chromosomal origin (PAI) VF that are present in several phylogenetic groups may be located on plasmids Indications - see conclusions of J.R. Johnson, P. Delavari et al. (2001b; below)

* Previous comparative studies not mentioned, as they display similar results, or their hypothesis was rejected in later studies: Wilson et al. (1988), Garcia et al. (1988) and Low et al. (1988).

† MRHA: Mannose-resistant haemagglutination is observed when fimbriae other than type 1 fimbriae are present

‡ PFGE: Pulsed Field Gel Electrophoresis

§ RAPD: Random Amplified Polymorphic DNA (phylotyping)

** ECOR collection: *E. coli* reference collection, "ECOR homepage": http://foodsafe.msu.edu/Whittam/ecor/

†† This particular finding differed to what was described by Garcia et al. (1988) and let to a different conclusion.

Comparative Study*	Subject	Important Results	Conclusions
Whittam et al. (1989)	Assessment of the phylogenetic variation of different *E. coli* (ExPEC and intestinal strains) by MLEE* (Selander et al., 1986) and serotyping	Low genetic diversity among strains that cause UTI UTI strains from different species cluster together, no substantial phylogenetic differences observed Serotypes correspond to clusters within phylogenetic groups	UTI of humans, dogs and cats may be caused by specific clones that can infect hosts irrespective of species
Johnson et al. (2001b)	Assessment whether VFGs of 38 strains from 5 different electrophoretic types (ET) differ consistently between species. Strains previously studied by Whittam et al. (1989)	Many discrete VFG profiles observed, no correlation to host species if VFG profile is similar (as assessed by visualisation of VFG matrix dendrogram) Overlap of VFG within one given ET – VFG present in dogs, cats and humans, not just one specific species Lack of clonal relatedness of isolates within a given ET (PFGE), likely due to collection of strains from geographically different areas	Likeliness of cross-species transmission due to extensive overlap of virulence determinants and phylogenetic similarity. Suggests intervention of transmission and scrutiny when using antimicrobials Also suggests knowledge of pathogenesis and VF studied in one host may equally apply to other hosts Alternative treatment methods may be protective independent of host species
Yuri et al. (1998) and Yamamoto et al. (1995)	Prevalence of gene markers detecting *pil* (Type 1 fimbriae), *pap, sfa, afa, hly, cnf1* and *aer* in 50 canine and 30 feline UPEC compared with prevalence of these VFG in 30 canine and 30 feline faecal strains from healthy animals (Yuri et al. (1998) Compared with human strains analysed by Yamamoto et al. (1995)	*Pap, sfa, hly* and *cnf1* highly prevalent in canine and feline (and human) UPEC strains Overall lower prevalence of these factors in faecal strains, but relatively prominent in feline faecal strains (no statistics given) *Pil* (Type 1 fimbriae) also common in faecal strains	Suggested that Type 1 fimbriae are not associated with pathogenicity Suggested that faeces of pets (particularly cats) could be a reservoir for UPEC that cause UTI human beings
Feria et al. (2000a)	Establishment of the prevalence of gene markers *pap, sfa, afa, hly, cnf1* and *aer* in UPEC isolated from 90 humans, 35 dogs and 5 cats from Portugal	Observation of species-specific differences in prevalence of gene markers *aer, cnf1, afa* and *sfa*. 12/40 pet strains and 1/90 human strains contained >4 VFGs, respectively.	Note: The study does not reveal how strains were collected, however, collections are mentioned again with more detail in Feria et al. (2001b) Population sizes differ significantly and statistics are not given *E. coli* adaptation to specific hosts is suggested Virulence factors may be co-located on PAI

* MLEE: Multi-Locus Enzyme Electrophoresis

Comparative Study*	Subject	Important Results	Conclusions
Feria et al. (2001a),	Prevalence and association of gene markers of *pap* (including *papA* alleles), *sfa*, *afa*, *hly*, *cnf1* and *aer* in UPEC isolated from 93 dogs and 25 cats from Portugal and the Netherlands	25 canine and 25 feline UPEC were haemolytic (100% *hly*). These strains also showed a high prevalence of *pap*, *sfa* and *cnf1*. one *afa* $^{+}$ canine strain, aer in less than 1/3 of strains and more common in 68 canine strains that were not selected for haemolysis Strains had up to 3 different *papA* alleles (F-phenotypes), very diverse population in regards to *papA*	VFG that have been detected in humans are present in cats and dogs Suggested that *pap, sfa, hly* and *cnf1* in these strains on PAI, since the occur in combination Suggested that *papA* diversity is a sign of phase variation or evolution associated with selective pressure applied by host immune system
Feria et al. (2001b),	Prevalence of gene markers for 3 papG alleles in *papEF*$^{+}$ UPEC isolated from 33 humans, 51 dogs and 22 cats from Portugal and the Netherlands	Identified *papG I* alleles that are not associated with J96-like strains *papG I* and *II* described in pets for 1st time *papG III* predominant in UPEC from cats (95%), dogs (72%) and people (45%) *papG II* not present in UPEC from cats, present in 23% of canine UPEC	UPEC isolated from dogs and cats share *papG* allele gene markers with human UPEC Suggested zoonotic potential of pet UPEC strains

Table 8.2 Summary of some studies conducted in the former Soviet Union before 2001

No.	Year	Author	Infection	Pathogen	Outcome	Ref.
1	1935	In: Review Krestovnikova	Bacterial dysentery	*Shigella* spp.	Prophylactic application of dry phage tablets in high risk communities	[1]
2	1938	Tsulukidze	Urinary tract infections and Paranephritis	*E. coli*, *Staphylococcus aureus*, *Streptococcus epidermidis*	Success rate: Acute cystitis: 100% Paranephritis: 100% Pyelonephritis: 80% Chronic cystitis: temporarily improvement	[1]
3	1940	Izashvili	Purulent infection	?	Marked activation of immune system (activation of neutrophils and phagocytosis) after phage treatment	[1]
4	1944	Vilfenson				
5	1942	Gvazava	Profound purulent dermatitis	?	Treatment with expired phage lysate Vaccination effect of lysed bacterial antigens? 71 patients in total Full recovery: 88%	[1]
6	1946	Vlasov, Artemenko	Chronic dysentery	?	30 patients in total Antibiotic treatment failed Clinical and colonoscopic evaluation Full recovery: 7%	[1]
7	1946	Moroz et al.	Chronic infected (war-) wounds	?	Phages were targeted against anaerobes and aerobes Full recovery: 13/15 patients Marked improvement: 2/15 patients	[1]
8	1947	? cited in Krestovnikova	Post-surgical pulmonary infection	*S. aureus*	2 groups: Antibiotics (A) Antibiotics and Phage (A+P) Recovery: A: 80% A+P: 93% Reinfection: A: 67% A+P: 24%	[1]

Table 8.2 Summary of some studies conducted in the former Soviet Union before 2001 ctd.

No.	Year	Author	Infection	Pathogen	Outcome	Ref.
9	1947	Kokin	Gas gangrene	*Clostridium* spp.	Decreased morbidity and mortality compared to other treatments Combined with anti-Staphylococcus and anti-Streptococcus phage, used as prophylactic treatment	1
10	1964	Chirakadze, Chanishvili	Test of intravenous preparations	*Staphylococcus* spp.	900 patients in total, 4 groups: Antibiotics Phage Antibiotics and Phage Phage and Immunstimulators Investigation of adverse effects of phage preparations: No significant adverse effects detected	1
11	1974	Chanishvili				
12	1983	Nadiradze				
13	1968	Babalova et al.	Bacterial dysentery	*Shigella* spp.	Successful prophylaxis	2
14	1970/ 1971	Solodovnikov et al.	Bacterial dysentery	*Shigella* spp.	Prophylactic treatment of children with dry polyvalent phage tablet with pectin Test group: 3212 children Placebo group: 3310 children Result: 2-3-fold lower incidence (depending on application frequency) of dysentery in test group	2
15	1970	Shvelidze	Profound dermatitis*	*Staphylococcus* spp.	Treatment after Penicillin, Biomicin, Streptomycin treatment had shown no effect 161 patients in total Full recovery: 95% Improvement: 4.3% No effect: 1.3% Relapse over 4 years: 8.5% (Cured in 2nd round)	1

Table 8.2 Summary of some studies conducted in the former Soviet Union before 2001 ctd.

No.	Year	Author	Infection	Pathogen	Outcome	Ref.
16	1974	Vepkhvadze	Mice infevted with *S. aureus*	*S.aureus*	Comparison of effectiveness of different treatments: Only antibiotics Only phages Combination of antibiotics and phages Best survival rate (75%) when antibiotics given 24 hours before phage	[1]
17	1974	Sakandelidze, Meipariani	Peritonitis, osteomyelitis, lung abscesses, postsurgical wound infections	*Staphylococcus* spp. *Streptococcus* spp., *Proteus* spp.	Antibiotic-resistant infections, 236 patients in total Success rate: 92%	[2, 3]
18	1978	Litvinova et al.	Intestinal dysbacteriosis after antibiotic treatment	*Proteus* spp., *E. coli*	Used in combination with bifidobacteria, successful treatment of 500 premature infants	[2, 3]
19	1978	Zhukov-Verezhnikov	Suppurative surgical infections	*Staphylococcus* spp. *Streptococcus* spp., *E. coli, Proteus* spp.	Specifically adapted phages were more efficient than commercial phage preparations	[2]
20	1980	Ioseliani et al.	Lung and pleural infections	*Staphylococcus* spp. *Streptococcus* spp., *E.coli, Proteus* spp.	Used in combination with antibiotics, successful treatment of all 45 patients	[2]
21	1981	Tolkacheva et al.	Bacterial dysentery in immuno-compromised patients	*E. coli, Proteus* spp.	Used in combination with bifidobacteria in 49 immunocompromised patients. Superiority over antibiotic treatment (kanamycin, polymyxin, ristomycin)	[2, 3]
22	1982	Meladze et al.	Lung and pleural infections	*Staphylococcus* spp.	2 groups (340 patients): Antibiotics (A); Success: 64% Phages (P); Success: 82%	[2]

Table 8.2 Summary of some studies conducted in the former Soviet Union before 2001 ctd.

No.	Year	Author	Infection	Pathogen	Outcome	Ref.
23	1984	Anpilov, Prokudin	Bacterial dysentery	*Shigella* spp.	Prophylactic application of dry acid-resistant coated phage tablets, given orally during rise of morbidity Comparison with placebo-group (calcium gluconate), double-blinded trial Success (no onset of infection): 75% (3 days interval between phage application) 67% (5 days interval)	1
24	1984	Agafonov et al.	Typhoid-paratyphoid infections	*Salmonella* spp.	Prophylactic application of dry acid-resistant coated phage tablets, given orally during rise of morbidity over 5-7 days	1
25	1984	Cherkasskja et al.	Septicaemia	*S.aureus*	Convalescence decreased from 7 to 3 days, when antibiotics were combined with phage	1
26	1984	Review: Samsygina, Boni	Dysentery Suppurative infections (sinusitis, otitis media, cholecystitis, osteomyelitis, empyema) Lung infections	*Shigella* spp., *E. coli*, *Proteus* spp.	Use of bacteriophage therapy in paediatrics	
27	1984	Martynova et al.	Stomatitis	*S. aureus*, *P.aeruginosa*	Normalisation of microflora observed, Immune system stimulation (IgA) observed	4
27	1985	Samsygina	Rhinitis, conjunctivitis, dermatitis	*S.aureus*, *S.epidermidis*, *Enterobacteriaceae*	3 groups: Success rate: Phage (P) 86% Antibiotics (A) 48% Antibiotics and Phage (A+P) 82%	1
28	1987	Cislo et al.	Chronically infected skin ulcers	*Pseudomonas* spp., *Staphylococcus* spp. *Klebsiella* spp., *Proteus* spp., *E. coli*	31 patients in total 74% success rate Minor adverse effects (eczema, pain, vomiting) observed in 6 patients	2

Table 8.2 Summary of some studies conducted in the former Soviet Union before 2001 ctd.

No.	Year	Author	Infection	Pathogen	Outcome	Ref.
29	1988	Pavlenishvili et al.	Selective decontamination in clinics	?	Described as considerably effective	1
30	1989	Kochetkova et al.	Postoperative wound infections (laryngitis, pharyngitis, oesophagitis, pneumonia, mastitis) in cancer patients	*Staphylococcus* spp.*Pseudomonas* spp.	2 groups (131 patients): Success rate: Antibiotics (A) 61% Phages (P) 82%	2, 3
31	1990	Mchedlidze	Intestinal dysbacteriosis	*P.aeruginosa*	Prophylactic application in young children	1
32	1991	Sakandelidze	Infectious allergic rhinitis, pharyngitis, dermatitis, conjunctivitis	*Staphylococcus* spp.*Streptococcus* spp., *E. coli, Proteus* spp., *Enterococcus* spp., *P. aeruginosa*	1380 patients in total 3 groups: Success rate: Antibiotics (A) 48% Phage (P) 86% Antibiotics and Phage (A+P) 83%	2, 3
33	1992	Bogovazova et al.	Skin- and nasal mucosa infections	*Klebsiella ozaenae and other Klebsiellae*	Successful treatment of all 109 patients with phage that had been safety-tested in mice.	2
34	1993	Miliutina, Vorotyntseva	Bacterial dysentery and salmonellosis	*Shigella* spp., *Salmonella* spp.	Used in combination with antibiotics. Treatment of salmonellosis with antibiotics and phage when antibiotics alone ineffective.	2, 3
35	1994	Kwarcinski et al.	Recurrent subphrenic abscess	*E. coli*	Case report, one patient. Successful treatment	2
36	1995	Perepanova et al.	Acute and chronic urogenital infections	*Staphylococcus* spp., *E. coli*, *Proteus* spp.	46 patients in total 92% marked clinical improvement 84% bacteriological clearance	2
37	1999	Stroj et al.	Cerebrospinal meningitis	*Klebsiella pneumoniae*	Successful treatment of a newborn after failure of antibiotic treatment, phages applied orally	2
38	2000	Giorkhelidze et al.	Chronic septicaemia	*Staphylococcus* spp. *E. coli*	Decrease in convalescence when phages and antibiotics applied in combination (compared with antibiotics alone)	1

Treatment of profound *staphylococcal* dermatitis was also described in 1936, 1947, 1954 and 1957 [1] Chanishvilli et al., 2001; [2] Sulakvelidze et al., 2001; [3] Alisky et al., 1998; [4] Sulakvelidze and Kutter, 2005

Table 8.3 The potential use of phages to treat UTI – Studies published from 1923-1939[a]

No	Year	Title	Language
1	1922	Courcoux, Philbert, Cordney. Un cas de pyélonéphrite gravidique traitée par le bactériophage de d'Hérelle. Bull. Mém. Soc. méd. des Hôp. (1922) 38:1151	French
2*	1923	Beckerich, A., Hauduroy, P. Le traitement des infections urinaires à colibacilles par le bactériophage de d'Hérelle. Bull. méd. (1923) 37:273	French
3	1924	Alphonsi. Guérison rapide de deux cas de pyélo-néphrite gravidique traités par le bactériophage de d'Hérelle. Bull. Soc. d'obstét. et de gyn. (1924) 13:573	French
4	1924	Arloing, F., Dufourt, Bouvier, Sempé. Traitement de la pyélonéphrite par le bactériophage d'Hérelle. Compt. rend. Soc. med. d'Hôp. (13 May 1924).	French
5*	1924	Arloing, F., Dufourt, Bouvier, Sempé. Traitement de la pyélonéphrite à colibacilles par le bactériophage d'Hérelle. Press. méd. (1924) 464	French
6*	1925	Frisch, B. Zur Behandlung der Koliinfektion des Harntraktes mit Bakteriophagen. Wien. klin. Wschr. (1925) 38:839	German
7	1925	Rohmer, P., Berg, V. Quelques remarques sur la pyélite du nourrison et son traitement au bactériophage. Bull. Soc. Pédiatr. Paris (1925) 23:498	French
8*	1925	Sickenga, F. N. Over den bacteriophag bij de colipyelitis van kinderen. Inaug.-Diss. Leiden (1925)	Dutch
9	1925	Larkum, N. Bacteriophagy in urinary infections. Proc. Soc. Am. Bact. (1925)	English
10*	1925	Zdansky, E. Versuche einer Bakteriophagentherapie bei Coliinfektionen der abführenden Harnwege. Wien. Arch. inn. Med. (1925) 11:533	German
11	1926	Dalsace, R. Applications du bactériophage d'Hérelle à la cure des infections urinaires. Journ. d'Urol. (1926) 21:123	French
12	1926	Dalsace, R. Le bactériophage de d'Hérelle; ses applications en thérapeutique urinaire. Presse méd. (1926) 458	French
13*	1926	Larkum, N. Bacteriophagy in urinary infection. Part I. The incidence of bacteriophage and of bacillus coli susceptible to dissolution by the bacteriophage in urines. Presentation of cases of renal infection in which bacteriophage was used therapeutically. Journ. Bacter. (1926) 12:203	English
14	1926	Larkum, N. Bacteriophagy in urinary infection. II. Bacteriophagy in the bladder. Journ. Bacter. (1926) 12:225	English
15	1926	Ravina, A. Traitement des infections urinaires par le bactériophage de d'Hérelle. Presse méd. (1926) 548	French
16	1928	Caldwell, J. A. Bacteriophagy in urinary infections following the administration of the bacteriophage therapeutically. Arch. int. Med. (1928) 41:189	English
17	1928	Caldwell, J. A. Bacteriologic and bacteriophagic study of infected urines. Journ. inf. Dis. (1928) 43:353	English
18*	1928	Eftimescu, G., Jonescu, J. M. [Bacteriophagy in the Treatment of Coli-Pyelonephritis.] Rev. Stünt. med. (1928) 17:736	Romanian
19*	1928	Petrova, V. [The treatment of chronic coli-cystitis with bacteriophage – a trial.] Z. sovrem. Chir. (1928) 3:297	Russian
20*	1930	Angelo, J. Sensibilité compare de 32 souches de collibacilles urinaires à la sérothérapie et à la bactériophagie spécifiques. Bull. Acad. Méd. (1930) 104:750	French
21	1930	Christiansen, H. [The use of phage for infections of the urinary tract.] Ugeskr. Laeg. (1930) 92:387	Danish
22	1930	Krueger, A. P., Faber, H. K., Schultz, E. W. Observation on the bacteriphage in infections of the urinary tract. Journ. of Urol. (1930) 23:397	English
23*	1930	Frisbee, F. C., MacNeal, W. J. The behavior of *Escherichia coli* and its specific bacteriophage in urine. The influence of environmental factors. Journ. inf. Dis. (1930) 46:405	English
24*	1930	Nyberg, C. [About the treatment of colibacillosis with bacteriophages.] Finska. Läk. sallsk. Hdl. (1930) 72:926	Finnish

[a] As referenced by Raettig, 1958 and Chanishvilli, 2001 An asterisk (*) indicates studies that may have focused primarily on *E. coli* UTI.

Table 8.3 The potential use of phages to treat UTI – Studies published from 1923-1939[a] ctd.

No	Year	Title	Language
25*	1931/ 1932	Cowie, D. M., Hicks, W. C. Observations on the bacteriophage III. The treatment of colon bacillus infections of the urinary tract by means of subcutaneous and intravesicle of bacteriophage filtrates. Detailed case reports. Methods for preparation of filtrates. Journ. Lab. a. Clin. Med. (1931) 17:681	English
26*	1931	Moltke, O. [The use of bacteriophage to treat coli-pyuria in children and adults] Ugeskr. Laeg. (1931) I:378.	Danish
27*	1931	Shitate, Y. Studies on the bacteriophage of Bacillus coli. I. The particular nature of the bacteriophage of the Bacillus coli from the urine. Orient. J. Dis. Infants (1931) 9:1	English
28*	1931	Shitate, Y. Studies on the bacteriophage of Bacillus coli. II. Pt. I. Experimental and clinical studies on the refined bacteriophage of bacillus-coli from the urine, with special reference to its therapeutic application in coli-cystitis. Orient. J. Dis. Infants (1931) 9:37	English
29	1932	Harper, P. A. Bacteriophagy in urinary infections. Yale Journ. of Biol. a Med. (1932) 4:366	English
30	1933	Hellström, J. [Treatment of urinary infections with bacteriophage]. Hygiea (Stockholm) (1933) 95:372	Swedish
31*	1933	Pikkarainen, J. Über Bakteriophagenbehandlung der chronischen Colipyelitis und die Eigenschaften der bei dieser Krankheit auftretenden Colistämme. Act. Soc. Med. fenn. Duod. A. (1933) 16:1	German
32	1935	Wehrbein, H. L., Nerb, L. Bacteriophage in the treatment of urinary infections. With an appendix on the technique of phage preparation. Amer. Journ. Surg. (1935) 29:48	English
33	1936	Berger, M. Ein Beitrag zur Bakteriophagentherapie der Cystitis. Zbl. f. Bakt. I. Orig. (1936) 137:360	German
34	1936	Frisch, B. Über das Phänomen der Bakteriophagie in der Urologie. Zschr. urol. Chir. u. Gynäk. (1936) 42, 199.	German
35	1938	Tsulukidze AP [Application of phages in urology] Urology XV(1938) (1):10-13	English?
36	1938	Lipska, I. [Colibacilli and their bacteriophages - selected from cases of cystitis and pyelitis.] Med doświadcz. (1938) 23:93	Polish
37*	1939	Bychowsky, L. A. [Bacteriophage treatment of cystitis caused by B. coli.] Inst. f. Mikrobiol. u. Epidemiol. (Kiew) (1939) 373	Russian

[a] As referenced by Raettig, 1958 and Chanishvilli, 2001 An asterisk (*) indicates studies that may have focused primarily on *E. coli* UTI.

The original documents are not indexed in online search engines such as Medline and CAB. The references have been obtained from the Deutsche Bücherei, Leipzig, Germany.

8.3. Raw Data of Experiments

8.3.1. Chapter 3

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people

Isolate No	Category	*papA*	*papC*	*papEF*	*papG II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*
C 10	1	+	+	+	-	+	+	-	+	-	+	+	+	-
C 13	1	+	+	+	+	+	+	-	+	-	+	+	+	+
C 14	1	+	+	+	+	+	+	+	+	-	+	+	+	-
C 15	1	+	+	+	-	+	+	-	+	-	+	+	+	-
C 28	1	-	+	-	-	-	-	-	-	-	+	-	-	-
C 29	1	+	+	+	-	+	+	+	+	-	+	+	-	-
C 34	1	+	+	+	-	+	+	-	+	-	+	+	-	-
C 37	1	+	+	+	-	+	+	+	-	-	+	+	+	+
C 51	1	+	+	+	-	+	+	-	+	-	+	+	+	-
C 56	1	+	+	+	-	+	+	-	+	+	+	+	+	-
C 57	1	-	-	-	-	+	+	-	-	-	+	+	-	+
C 69	1	+	+	+	-	+	+	-	-	-	+	+	+	-
C 70	1	+	+	+	-	+	+	-	+	-	+	+	+	-
C 77	1	-	-	-	-	-	+	-	-	-	+	+	-	-
C 79	1	+	+	+	-	+	-	-	-	-	+	+	+	-
C 80	1	+	+	+	+	-	-	-	-	+	+	+	-	-
C 81	1	+	+	+	-	+	-	-	-	-	+	+	+	-
C 82	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 83	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 90	1	+	-	-	-	+	-	-	-	-	+	-	+	-
C 91	1	+	+	+	-	+	+	-	-	-	+	+	-	-
C 92	1	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
C 10	1	-	-	+	-	-	-	-	-	-	+	+	+
C 13	1	+	-	+	-	+	-	-	-	-	+	+	+
C 14	1	+	-	+	-	+	-	-	-	-	+	+	+
C 15	1	+	-	+	-	+	-	-	-	-	-	+	+
C 28	1	-	-	+	-	-	-	-	+	+	+	+	-
C 29	1	+	-	+	-	-	-	-	-	+	-	-	+
C 34	1	+	-	+	-	-	-	-	-	-	-	+	+
C 37	1	+	-	+	-	+	-	-	-	-	-	+	+
C 51	1	+	-	+	-	-	-	-	-	-	-	+	+
C 56	1	+	+	+	-	+	-	-	-	-	+	+	+
C 57	1	+	+	+	-	-	-	-	-	-	+	+	-
C 69	1	+	-	+	-	-	-	+	-	-	-	-	+
C 70	1	+	-	+	+	-	-	-	-	-	-	+	+
C 77	1	-	-	-	-	-	-	-	-	-	-	+	-
C 79	1	+	-	-	-	+	-	-	-	-	-	-	+
C 80	1	+	+	-	-	-	-	-	-	-	+	+	+
C 81	1	+	-	-	-	+	-	-	-	-	-	-	+
C 82	1	+	+	+	-	-	+	-	+	-	+	+	-
C 83	1	-	-	-	-	-	+	-	-	-	-	-	-
C 90	1	-	+	+	-	+	-	-	+	+	+	-	-
C 91	1	+	-	+	-	+	-	-	-	-	-	+	+
C 92	1	+	+	+	-	-	-	-	+	+	+	+	-

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*papA*	*papC*	*papEF*	*papG II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*
C 100	1	+	+	+	-	-	+	-	+	+	+	+	+	-
C 101	1	+	+	+	-	-	-	-	+	+	+	-	-	-
C 115	1	-	-	-	-	-	+	-	+	-	+	+	+	-
C 120	1	-	-	-	-	-	+	-	-	-	+	-	-	-
C 128	1	-	-	-	-	-	+	-	+	-	+	-	-	-
C 138	1	+	-	-	-	-	+	-	+	-	+	+	+	-
C 145	1	+	+	+	-	+	+	-	+	+	+	+	+	-
C 146	1	-	-	-	-	-	+	-	+	-	+	+	+	-
C 147	1	-	-	-	-	-	+	-	+	-	+	-	-	-
C 148	1	+	+	+	-	+	+	+	-	-	+	+	+	-
C 149	1	+	+	-	-	-	-	+	-	-	+	-	-	-
C 160	1	+	-	+	-	+	+	-	+	-	+	+	-	-
C 161	1	+	+	+	-	+	+	-	-	-	+	+	+	-
C 168	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 169	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 170	1	+	+	+	-	+	+	-	+	-	+	+	-	-
C 171	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 172	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 173	1	-	-	-	-	-	-	-	-	-	+	-	-	-
C 174	1	-	-	-	-	-	+	-	+	-	+	-	-	-
C 183	1	-	-	-	-	-	+	-	+	-	+	-	-	-
C 184	1	+	+	+	-	+	+	-	+	-	+	+	+	-

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
C 100	1	+	+	+	-	+	-	-	-	-	+	-	+
C 101	1	+	+	+	-	+	-	-	-	-	+	-	+
C 115	1	+	-	+	+	+	-	-	-	-	-	+	+
C 120	1	+	+	+	-	-	-	-	-	-	+	+	+
C 128	1	+	-	+	-	-	-	-	-	-	+	-	-
C 138	1	+	-	+	-	-	-	-	-	-	+	+	+
C 145	1	+	-	+	+	-	-	-	-	-	-	+	+
C 146	1	+	-	+	-	-	-	-	-	-	-	+	+
C 147	1	+	-	+	+	-	-	-	-	-	-	+	+
C 148	1	+	-	+	-	+	-	-	-	-	+	-	+
C 149	1	+	-	-	-	-	-	-	-	-	-	-	-
C 160	1	+	-	+	-	-	-	-	-	-	+	-	+
C 161	1	+	-	+	-	-	-	-	-	-	-	-	+
C 168	1	+	-	-	-	+	-	-	-	-	+	+	+
C 169	1	-	-	+	-	-	+	-	+	-	+	+	-
C 170	1	+	-	+	+	+	-	-	-	-	-	+	+
C 171	1	-	+	+	+	-	+	-	+	+	+	+	-
C 172	1	-	+	+	+	-	+	-	+	+	+	+	-
C 173	1	-	-	-	-	-	+	-	-	-	+	-	-
C 174	1	+	-	+	-	+	-	-	-	-	+	+	+
C 183	1	+	-	+	-	-	-	-	-	-	-	+	+
C 184	1	+	-	+	+	+	-	-	-	-	-	+	+

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*papA*	*papC*	*papEF*	*papG II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*
C 185	1	+	+	+	-	+	+	-	+	-	+	+	+	-
C 20	2	+	+	+	-	+	+	+	-	-	+	+	+	-
C 23	2	+	+	+	-	+	+	-	+	-	+	+	+	-
C 32	2	-	-	-	-	+	+	-	-	-	+	-	-	-
C 33	2	+	+	+	-	+	+	-	+	-	+	+	+	-
C 47	2	+	+	+	-	+	+	-	+	+	+	+	+	-
C 48	2	-	-	-	-	-	+	-	+	-	+	-	-	-
C 50	2	+	+	+	-	+	+	-	+	-	+	+	+	-
C 55	2	-	-	-	-	-	+	+	-	-	+	+	-	-
C 72	2	+	+	+	+	+	+	-	+	+	+	+	+	+
C 73	2	-	+	+	-	-	-	-	-	-	+	-	-	-
C 76	2	+	+	+	-	+	+	-	+	-	+	+	+	-
F 24	1	+	+	+	-	+	+	-	-	-	+	+	+	-
F 31	1	+	+	+	-	+	+	+	+	+	+	+	-	-
F 35	1	+	+	+	-	+	+	-	+	-	+	+	+	-
F 45	1	+	+	+	-	+	+	-	-	-	+	+	+	-
F 60	1	+	+	+	-	+	+	-	+	-	+	+	+	-
F 64	1	+	+	+	-	+	+	-	-	-	+	+	+	+
F 84	1	+	+	+	+	+	+	-	+	-	+	+	+	-
F 94	1	+	+	+	-	+	+	-	+	-	+	+	-	-
F 121	1	+	+	+	-	+	+	-	+	-	+	+	+	-
F 139	1	+	+	+	-	+	+	+	-	-	+	+	+	-

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
C 185	1	+	-	+	-	-	-	-	-	-	-	+	+
C 20	2	+	-	+	-	+	-	-	-	-	+	+	+
C 23	2	+	-	+	+	+	-	-	-	-	+	+	+
C 32	2	+	-	+	-	+	-	-	-	-	+	+	+
C 33	2	+	-	+	-	+	-	-	-	-	-	+	+
C 47	2	+	+	+	-	+	-	-	-	-	-	+	+
C 48	2	+	-	+	-	-	+	-	-	-	-	+	+
C 50	2	+	-	+	+	+	-	-	-	-	-	+	+
C 55	2	+	-	+	+	-	-	-	-	-	+	+	+
C 72	2	+	+	+	+	+	-	-	-	-	+	+	+
C 73	2	+	+	+	-	-	-	-	+	+	+	+	-
C 76	2	+	-	+	+	-	-	-	-	-	+	+	+
F 24	1	+	-	+	-	+	-	-	-	-	-	+	+
F 31	1	+	+	+	-	+	-	-	-	-	-	+	+
F 35	1	+	-	+	+	+	-	-	-	-	-	+	+
F 45	1	+	-	+	+	+	-	-	-	-	-	+	+
F 60	1	+	-	+	+	+	-	-	-	-	-	+	+
F 64	1	+	-	+	-	+	-	-	-	-	-	+	+
F 84	1	+	-	+	+	-	+	-	-	-	+	-	+
F 94	1	+	-	+	+	-	-	-	-	-	-	+	+
F 121	1	+	-	+	-	-	-	-	-	-	-	+	+
F 139	1	+	-	+	-	+	-	-	-	-	-	-	+

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*papA*	*papC*	*papEF*	*papG II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*
F 140	1	+	+	+	-	+	+	-	+	-	+	+	-	-
F 141	1	+	+	+	+	+	+	-	+	-	+	+	+	-
F 142	1	+	+	+	-	+	+	-	+	-	+	+	+	-
F 143	1	+	+	+	-	+	+	+	+	-	+	+	+	-
F 144	1	+	+	+	-	+	+	+	+	+	+	+	+	-
F 26	2	+	+	+	-	+	+	-	+	-	+	+	+	-
F 30	2	+	+	+	-	+	+	-	-	-	+	+	-	+
F 42	2	+	+	+	-	+	+	+	+	-	+	+	+	-
F 53	2	+	+	+	-	+	+	-	+	-	+	+	+	-
F 54	2	-	-	-	-	-	+	+	-	-	+	+	-	-
F 61	2	+	+	+	-	+	+	+	-	-	+	+	+	-
F 62	2	+	+	+	-	+	+	-	-	-	+	+	+	-
H 1	NA	+	+	+	-	+	+	+	-	-	+	+	+	-
H 2	NA	+	+	+	+	+	+	+	+	-	+	+	+	-
H 3	NA	-	+	+	+	-	+	-	+	+	+	+	-	-
H 4	NA	+	+	+	+	+	+	-	+	+	+	+	-	-
H 5	NA	-	+	-	+	+	+	-	+	-	+	-	-	-
H 6	NA	+	+	+	+	+	+	-	+	-	+	+	-	-
H 7	NA	+	+	+	+	+	+	-	+	-	+	+	+	-
H 8	NA	+	+	+	+	+	+	-	+	-	+	+	-	-
H 9	NA	+	+	+	+	+	+	-	+	-	+	+	+	-
H 10	NA	-	-	-	-	-	+	-	+	-	+	+	+	-
H 11	NA	-	-	-	-	-	-	-	-	-	+	+	-	-

Table 8.4 VFG profiles of UPEC isolated from NZ dogs, cats and people ctd.

Isolate No	Category	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
F 140	1	+	-	+	+	-	-	-	-	-	-	+	+
F 141	1	+	-	+	-	-	-	-	-	-	-	-	+
F 142	1	+	-	+	+	-	-	-	-	-	-	+	+
F 143	1	+	-	+	+	+	-	+	-	-	-	-	+
F 144	1	+	+	+	-	-	-	-	-	-	-	+	+
F 26	2	+	-	+	+	-	-	-	-	-	-	+	+
F 30	2	+	-	+	-	+	-	-	-	-	-	+	+
F 42	2	+	-	+	+	+	-	-	-	-	+	+	+
F 53	2	+	-	+	+	-	-	-	-	-	-	+	+
F 54	2	+	+	+	-	-	-	-	+	-	+	+	-
F 61	2	+	-	+	-	+	-	-	-	-	-	+	+
F 62	2	+	-	+	-	+	-	-	-	-	-	+	+
H 1	NA	+	-	+	-	+	-	-	-	-	+	+	+
H 2	NA	+	-	+	-	+	-	-	-	-	+	+	+
H 3	NA	+	+	-	-	+	-	-	-	-	+	-	+
H 4	NA	+	+	+	+	+	-	-	-	-	+	+	+
H 5	NA	+	+	+	+	+	-	-	-	+	+	+	+
H 6	NA	+	+	-	-	+	-	-	-	-	+	+	-
H 7	NA	+	-	+	+	-	+	+	-	-	+	+	+
H 8	NA	+	+	-	-	+	-	-	-	-	+	+	-
H 9	NA	+	-	+	+	-	+	+	-	-	+	+	+
H 10	NA	+	-	+	-	+	-	-	-	-	+	+	+
H 11	NA	-	-	-	-	-	-	-	-	-	+	+	+

Table 8.5 PFGE Dice coefficient similarity matrix of UPEC from NZ

	C20	C29	C33	C34	C79	C80	C81	C82	C83	C90	C91	C92	C101	C115	C120	C128	C160	C162	F24
C20	-	15	38.1	9.8	36.8	22.9	22.9	27.8	21.1	21.1	14.6	17.6	29.4	39	26.3	19.4	38.9	21.1	27.8
C29	15	-	26.3	43.2	23.5	32.3	32.3	25	17.6	11.8	37.8	0	20	27	17.6	14.8	18.8	11.8	6.3
C33	38.1	26.3	-	5.1	44.4	30.3	30.3	41.2	27.8	27.8	35.9	12.5	18.8	46.2	27.8	13.8	29.4	22.2	35.3
C34	9.8	43.2	5.1	-	17.1	31.3	31.3	12.1	28.6	0	21.1	12.9	25.8	15.8	17.1	7.1	36.4	17.1	12.1
C79	36.8	23.5	44.4	17.1	-	20.7	0	33.3	18.8	18.8	22.9	14.3	28.6	28.6	31.3	16	26.7	12.5	33.3
C80	22.9	32.3	30.3	31.3	20.7	-	30.8	22.2	48.3	20.7	25	8	32	50	13.8	9.1	22.2	6.9	14.8
C81	22.9	32.3	30.3	31.3	0	30.8	-	44.4	13.8	34.5	18.8	0	0	25	6.9	27.3	29.6	13.8	29.6
C82	27.8	25	41.2	12.1	33.3	22.2	44.4	-	20	46.7	12.1	15.4	7.7	18.2	20	34.8	28.6	20	35.7
C83	21.1	17.6	27.8	28.6	18.8	48.3	13.8	20	-	18.8	28.6	14.3	28.6	34.3	25	0	13.3	12.5	6.7
C90	21.1	11.8	27.8	0	18.8	20.7	34.5	46.7	18.8	-	11.4	14.3	0	34.3	31.3	16	26.7	25	53.3
C91	14.6	37.8	35.9	21.1	22.9	25	18.8	12.1	28.6	11.4	-	32.3	32.3	52.6	11.4	21.4	24.2	28.6	24.2
C92	17.6	0	12.5	12.9	14.3	8	0	15.4	14.3	14.3	32.3	-	33.3	25.8	14.3	38.1	15.4	21.4	23.1
C101	29.4	20	18.8	25.8	28.6	32	0	7.7	28.6	0	32.3	33.3	-	32.3	14.3	19	23.1	21.4	23.1
C115	39	27	46.2	15.8	28.6	50	25	18.2	34.3	34.3	52.6	25.8	32.3	-	28.6	7.1	36.4	22.9	36.4
C120	26.3	17.6	27.8	17.1	31.3	13.8	6.9	20	25	31.3	11.4	14.3	14.3	28.6	-	16	20	25	20
C128	19.4	14.8	13.8	7.1	16	9.1	27.3	34.8	0	16	21.4	38.1	19	7.1	16	-	17.4	16	34.8
C160	38.9	18.8	29.4	36.4	26.7	22.2	29.6	28.6	13.3	26.7	24.2	15.4	23.1	36.4	20	17.4	-	20	42.9
C162	21.1	11.8	22.2	17.1	12.5	6.9	13.8	20	12.5	25	28.6	21.4	21.4	22.9	25	16	20	-	40
F24	27.8	6.3	35.3	12.1	33.3	14.8	29.6	35.7	6.7	53.3	24.2	23.1	23.1	36.4	20	34.8	42.9	40	-
F31	23.5	20	25	45.2	35.7	24	32	23.1	7.1	14.3	19.4	16.7	25	25.8	7.1	9.5	38.5	28.6	38.5
F35	40	29.3	60.5	4.8	30.8	33.3	22.2	32.4	41	35.9	38.1	17.1	17.1	47.6	25.6	12.5	32.4	20.5	27
F45	33.3	31.3	23.5	42.4	33.3	22.2	14.8	21.4	20	26.7	36.4	7.7	15.4	30.3	20	8.7	50	26.7	35.7
F53	40.9	20	42.9	29.3	26.3	28.6	17.1	27.8	42.1	21.1	19.5	17.6	23.5	29.3	15.8	6.5	27.8	21.1	22.2
F60	25	16.7	42.1	10.8	29.4	32.3	12.9	18.8	41.2	29.4	48.6	26.7	33.3	59.5	23.5	7.4	12.5	29.4	25
F64	10.8	24.2	28.6	35.3	19.4	28.6	21.4	6.9	25.8	12.9	47.1	37	22.2	52.9	19.4	8.3	34.5	19.4	27.6
F84	35.9	17.1	43.2	16.7	30.3	20	13.3	19.4	36.4	30.3	22.2	13.8	27.6	27.8	48.5	15.4	12.9	6.1	12.9
F94	19.5	27	30.8	31.6	28.6	31.3	31.3	30.3	45.7	40	52.6	19.4	6.5	47.4	28.6	7.1	36.4	22.9	30.3
F121	43.2	24.2	40	23.5	25.8	7.1	28.6	34.5	6.5	32.3	47.1	14.8	7.4	35.3	12.9	16.7	48.3	25.8	34.5
F139	15.4	11.4	5.4	38.9	36.4	20	6.7	19.4	24.2	36.4	16.7	27.6	20.7	5.6	36.4	23.1	12.9	24.2	32.3
F140	30	22.2	26.3	27	11.8	25.8	12.9	18.8	35.3	23.5	21.6	40	40	21.6	41.2	29.6	12.5	17.6	25
F141	25.6	11.4	27	22.2	36.4	13.3	6.7	32.3	24.2	42.4	38.9	34.5	41.4	33.3	12.1	15.4	32.3	30.3	38.7
F142	30	16.7	31.6	10.8	23.5	19.4	32.3	31.3	17.6	70.6	16.2	13.3	0	32.4	35.3	22.2	25	23.5	43.8
F143	20.5	17.1	32.4	16.7	24.2	6.7	13.3	19.4	12.1	36.4	16.7	34.5	27.6	33.3	36.4	30.8	19.4	48.5	45.2
F144	27.8	25	35.3	18.2	33.3	51.9	14.8	21.4	33.3	40	18.2	15.4	23.1	36.4	26.7	8.7	28.6	40	57.1
H1	48.8	10.8	30.8	21.1	28.6	6.3	18.8	18.2	28.6	17.1	15.8	12.9	19.4	21.1	40	7.1	12.1	22.9	12.1
H6	37.2	20.5	34.1	15	54.1	17.6	23.5	22.9	16.2	16.2	10	12.1	30.3	20	32.4	20	28.6	27	28.6
H7	55	11.1	52.6	21.6	41.2	32.3	19.4	43.8	35.3	35.3	32.4	20	26.7	48.6	29.4	7.4	37.5	23.5	31.3
H8	35.6	14.6	27.9	28.6	35.9	16.7	22.2	21.6	15.4	15.4	4.8	17.1	22.9	9.5	30.8	18.8	21.6	35.9	27
H9	52.4	15.8	50	25.6	38.9	30.3	18.2	41.2	33.3	33.3	30.8	18.8	25	46.2	27.8	6.9	35.3	22.2	29.4

Table 8.5 PFGE Dice coefficient similarity matrix of UPEC from NZ ctd.

	F31	F35	F45	F53	F60	F64	F84	F94	F121	F139	F140	F141	F142	F143	F144	H1	H6	H7	H8	H9
C20	23.5	40	33.3	40.9	25	10.8	35.9	19.5	43.2	15.4	30	25.6	30	20.5	27.8	48.8	37.2	55	35.6	52.4
C29	20	29.3	31.3	20	16.7	24.2	17.1	27	24.2	11.4	22.2	11.4	16.7	17.1	25	10.8	20.5	11.1	14.6	15.8
C33	25	60.5	23.5	42.9	42.1	28.6	43.2	30.8	40	5.4	26.3	27	31.6	32.4	35.3	30.8	34.1	52.6	27.9	50
C34	45.2	4.8	42.4	29.3	10.8	35.3	16.7	31.6	23.5	38.9	27	22.2	10.8	16.7	18.2	21.1	15	21.6	28.6	25.6
C79	35.7	30.8	33.3	26.3	29.4	19.4	30.3	28.6	25.8	36.4	11.8	36.4	23.5	24.2	33.3	28.6	54.1	41.2	35.9	38.9
C80	24	33.3	22.2	28.6	32.3	28.6	20	31.3	7.1	20	25.8	13.3	19.4	6.7	51.9	6.3	17.6	32.3	16.7	30.3
C81	32	22.2	14.8	17.1	12.9	21.4	13.3	31.3	28.6	6.7	12.9	6.7	32.3	13.3	14.8	18.8	23.5	19.4	22.2	18.2
C82	23.1	32.4	21.4	27.8	18.8	6.9	19.4	30.3	34.5	19.4	18.8	32.3	31.3	19.4	21.4	18.2	22.9	43.8	21.6	41.2
C83	7.1	41	20	42.1	41.2	25.8	36.4	45.7	6.5	24.2	35.3	24.2	17.6	12.1	33.3	28.6	16.2	35.3	15.4	33.3
C90	14.3	35.9	26.7	21.1	29.4	12.9	30.3	40	32.3	36.4	23.5	42.4	70.6	36.4	40	17.1	16.2	35.3	15.4	33.3
C91	19.4	38.1	36.4	19.5	48.6	47.1	22.2	52.6	47.1	16.7	21.6	38.9	16.2	16.7	18.2	15.8	10	32.4	4.8	30.8
C92	16.7	17.1	7.7	17.6	26.7	37	13.8	19.4	14.8	27.6	40	34.5	13.3	34.5	15.4	12.9	12.1	20	17.1	18.8
C101	25	17.1	15.4	23.5	33.3	22.2	27.6	6.5	7.4	20.7	40	41.4	0	27.6	23.1	19.4	30.3	26.7	22.9	25
C115	25.8	47.6	30.3	29.3	59.5	52.9	27.8	47.4	35.3	5.6	21.6	33.3	32.4	33.3	36.4	21.1	20	48.6	9.5	46.2
C120	7.1	25.6	20	15.8	23.5	19.4	48.5	28.6	12.9	36.4	41.2	12.1	35.3	36.4	26.7	40	32.4	29.4	30.8	27.8
C128	9.5	12.5	8.7	6.5	7.4	8.3	15.4	7.1	16.7	23.1	29.6	15.4	22.2	30.8	8.7	7.1	20	7.4	18.8	6.9
C160	38.5	32.4	50	27.8	12.5	34.5	12.9	36.4	48.3	12.9	12.5	32.3	25	19.4	28.6	12.1	28.6	37.5	21.6	35.3
C162	28.6	20.5	26.7	21.1	29.4	19.4	6.1	22.9	25.8	24.2	17.6	30.3	23.5	48.5	40	22.9	27	23.5	35.9	22.2
F24	38.5	27	35.7	22.2	25	27.6	12.9	30.3	34.5	32.3	25	38.7	43.8	45.2	57.1	12.1	28.6	31.3	27	29.4
F31	-	17.1	38.5	23.5	13.3	29.6	13.8	25.8	44.4	27.6	13.3	6.9	20	20.7	30.8	32.3	18.2	26.7	22.9	25
F35	17.1	-	32.4	48.9	58.5	31.6	40	33.3	36.8	15	29.3	35	29.3	25	32.4	28.6	31.8	48.8	21.7	51.2
F45	38.5	32.4	-	27.8	18.8	20.7	19.4	42.4	62.1	25.8	18.8	45.2	31.3	19.4	42.9	6.1	17.1	43.8	10.8	41.2
F53	23.5	48.9	27.8	-	45	32.4	30.8	34.1	32.4	20.5	40	35.9	20	30.8	38.9	29.3	37.2	55	26.7	52.4
F60	13.3	58.5	18.8	45	-	36.4	34.3	37.8	24.2	28.6	33.3	45.7	27.8	34.3	25	32.4	30.8	55.6	19.5	52.6
F64	29.6	31.6	20.7	32.4	36.4	-	25	52.9	20	12.5	30.3	25	18.2	25	20.7	11.8	11.1	30.3	5.3	28.6
F84	13.8	40	19.4	30.8	34.3	25	-	27.8	31.3	41.2	57.1	35.3	51.4	35.3	32.3	44.4	31.6	51.4	30	48.6
F94	25.8	33.3	42.4	34.1	37.8	52.9	27.8	-	35.3	22.2	16.2	27.8	37.8	22.2	24.2	21.1	20	37.8	23.8	35.9
F121	44.4	36.8	62.1	32.4	24.2	20	31.3	35.3	-	25	18.2	31.3	30.3	18.8	27.6	29.4	11.1	48.5	10.5	45.7
F139	27.6	15	25.8	20.5	28.6	12.5	41.2	22.2	25	-	40	41.2	51.4	29.4	38.7	22.2	21.1	34.3	25	32.4
F140	13.3	29.3	18.8	40	33.3	30.3	57.1	16.2	18.2	40	-	28.6	38.9	40	37.5	27	30.8	38.9	34.1	36.8
F141	6.9	35	45.2	35.9	45.7	25	35.3	27.8	31.3	41.2	28.6	-	28.6	41.2	32.3	11.1	36.8	62.9	25	59.5
F142	20	29.3	31.3	20	27.8	18.2	51.4	37.8	30.3	51.4	38.9	28.6	-	34.3	43.8	21.6	20.5	33.3	14.6	31.6
F143	20.7	25	19.4	30.8	34.3	25	35.3	22.2	18.8	29.4	40	41.2	34.3	-	45.2	38.9	47.4	28.6	40	27
F144	30.8	32.4	42.9	38.9	25	20.7	32.3	24.2	27.6	38.7	37.5	32.3	43.8	45.2	-	18.2	34.3	43.8	27	41.2
H1	32.3	28.6	6.1	29.3	32.4	11.8	44.4	21.1	29.4	22.2	27	11.1	21.6	38.9	18.2	-	30	32.4	28.6	30.8
H6	18.2	31.8	17.1	37.2	30.8	11.1	31.6	20	11.1	21.1	30.8	36.8	20.5	47.4	34.3	30	-	30.8	77.3	29.3
H7	26.7	48.8	43.8	55	55.6	30.3	51.4	37.8	48.5	34.3	38.9	62.9	33.3	28.6	43.8	32.4	30.8	-	24.4	94.7
H8	22.9	21.7	10.8	26.7	19.5	5.3	30	23.8	10.5	25	34.1	2.5	14.6	40	27	28.6	77.3	24.4	-	27.9
H9	25	51.2	41.2	52.4	52.6	28.6	48.6	35.9	45.7	32.4	36.8	59.5	31.6	27	41.2	30.8	29.3	94.7	27.9	-

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Table 8.6 VFG profiles of UPEC isolated from UK cats

Isolate No	*papA*	*papC*	*papEF*	*papG II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*
UK 1	-	-	-	-	-	+	-	-	-	+	-	-	-
UK 4	+	+	+	-	+	+	-	+	-	+	+	+	-
UK 5	+	+	+	-	+	+	+	-	-	+	+	+	-
UK 6	-	-	-	-	-	+	+	-	-	+	+	+	-
UK 7	+	+	+	-	+	+	+	-	+	+	+	+	-
UK 8	-	-	-	-	-	-	-	-	-	+	-	-	-
UK 9	-	-	-	-	-	+	+	-	-	+	-	-	+
UK 10	-	-	-	-	-	-	-	-	-	+	+	-	-
UK 11	+	+	+	-	+	+	+	-	-	+	+	+	-
UK 13	-	-	-	-	-	+	+	+	-	+	+	+	-
UK 14	-	-	-	-	-	-	-	-	-	+	-	-	-
UK 15	-	-	-	-	-	-	-	-	-	+	-	-	-
UK 19	+	+	+	-	+	+	-	+	-	+	+	+	-
UK 20	-	+	-	-	+	+	+	+	-	+	+	+	-
UK 21	-	-	-	-	-	+	+	+	-	+	+	+	-
UK 22	+	+	+	-	+	+	-	+	-	+	+	+	-
UK 24	-	-	-	-	-	-	-	-	-	+	-	-	-
UK 25	-	-	-	-	-	-	-	-	-	+	-	-	-
UK 27	-	-	-	-	-	+	-	-	-	+	+	+	-
UK 29	-	-	-	-	-	+	-	+	-	+	-	-	-
UK 30	+	+	+	-	+	+	-	+	-	+	+	+	-
UK 31	+	+	+	-	+	+	+	+	-	+	+	+	-

Table 8.6 VFG profiles of UPEC isolated from UK cats ctd.

Isolate No	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
UK 1	+	-	+	-	+	-	-	-	-	-	+	+
UK 4	+	-	+	-	-	-	-	-	-	-	+	+
UK 5	+	-	+	-	+	-	-	-	-	+	+	+
UK 6	+	-	+	-	+	-	-	-	-	-	+	+
UK 7	+	+	+	+	-	-	-	-	-	-	+	+
UK 8	+	+	+	-	+	-	-	+	+	+	+	-
UK 9	+	+	+	-	+	-	-	+	+	+	+	+
UK 10	-	-	+	-	+	-	-	-	+	+	+	-
UK 11	+	-	+	-	+	-	-	-	-	+	+	+
UK 13	+	-	+	-	+	-	-	+	-	+	+	+
UK 14	-	-	+	-	-	-	-	-	+	+	+	-
UK 15	+	+	-	+	+	-	-	-	+	+	+	-
UK 19	+	-	+	-	+	-	-	+	-	+	+	+
UK 20	+	-	+	-	+	-	-	+	+	+	+	-
UK 21	+	-	+	+	+	-	-	-	+	-	+	+
UK 22	+	-	+	-	+	-	-	-	-	-	+	+
UK 24	-	+	+	-	+	-	-	+	+	+	-	-
UK 25	+	+	-	-	-	-	-	+	+	+	+	-
UK 27	+	-	+	-	+	-	-	-	-	-	+	+
UK 29	+	+	+	-	+	-	-	+	+	+	+	-
UK 30	+	-	+	-	+	-	-	+	+	+	+	+
UK 31	+	+	+	-	-	-	-	+	-	+	+	+

Table 8.7 VFG profiles of UPEC isolated from NZ cats

Isolate No	*papA*	*papC*	*papEF*	*papG II*	*pap G III*	*sfa/foc*	*sfaS*	*focG*	*iha*	*fimH*	*hlyD*	*univcnf*	*cdtB*
NZ 24	+	+	+	-	+	+	-	-	-	+	+	+	-
NZ 31	+	+	+	-	+	+	+	+	+	+	+	-	-
NZ 35	+	+	+	-	+	+	-	+	-	+	+	+	-
NZ 45	+	+	+	-	+	+	-	-	-	+	+	+	-
NZ 60	+	+	+	-	+	+	-	+	-	+	+	+	-
NZ 64	+	+	+	-	+	+	-	-	-	+	+	+	+
NZ 84	+	+	+	+	+	+	-	+	-	+	+	+	-
NZ 94	+	+	+	-	+	+	-	+	-	+	+	-	-
NZ 121	+	+	+	-	+	+	-	+	-	+	+	+	-
NZ 139	+	+	+	-	+	+	+	-	-	+	+	+	-
NZ 140	+	+	+	-	+	+	-	+	-	+	+	-	-
NZ 141	+	+	+	+	+	+	-	+	-	+	+	+	-
NZ 142	+	+	+	-	+	+	-	+	-	+	+	+	-
NZ 143	+	+	+	-	+	+	+	+	-	+	+	+	-
NZ 144	+	+	+	-	+	+	+	+	+	+	+	+	-

Table 8.7 VFG profiles of UPEC isolated from NZ cats ctd.

Isolate No	*fyuA*	*iutA*	*iroN*	*ireA*	*kpsMT II*	*kps MT III*	*rfc*	*cvaC*	*iss*	*traT*	*ompT*	*malX*
NZ 24	+	-	+	-	+	-	-	-	-	-	+	+
NZ 31	+	+	+	-	+	-	-	-	-	-	+	+
NZ 35	+	-	+	+	+	-	-	-	-	-	+	+
NZ 45	+	-	+	+	+	-	-	-	-	-	+	+
NZ 60	+	-	+	+	+	-	-	-	-	-	+	+
NZ 64	+	-	+	-	+	-	-	-	-	-	+	+
NZ 84	+	-	+	+	-	+	-	-	-	+	-	+
NZ 94	+	-	+	+	-	-	-	-	-	-	+	+
NZ 121	+	-	+	-	-	-	-	-	-	-	+	+
NZ 139	+	-	+	-	+	-	-	-	-	-	-	+
NZ 140	+	-	+	+	-	-	-	-	-	-	+	+
NZ 141	+	-	+	-	-	-	-	-	-	-	-	+
NZ 142	+	-	+	+	-	-	-	-	-	-	+	+
NZ 143	+	-	+	+	+	-	+	-	-	-	-	+
NZ 144	+	+	+	-	-	-	-	-	-	-	+	+

Table 8.8 PFGE Dice coefficient similarity matrix of feline UPEC from UK and NZ

	UK 1	UK 2	UK 3	UK 5	UK 6	UK 7	UK 8	UK 10	UK 11	UK 13	UK 14	UK 15	UK 19	UK 20	UK 21	UK 22	UK 24	UK 25	UK 27
UK 1	-	22.9	40	32.4	34.3	32.3	43.2	33.3	36.4	31.3	25	32.3	28.6	32.3	36.4	18.8	22.2	27.8	48.6
UK 2	22.9	-	38.9	36.8	22.2	25	47.4	32.4	23.5	24.2	12.1	25	33.3	37.5	35.3	12.1	21.6	37.8	47.4
UK 3	40	38.9	-	63.2	61.1	31.3	42.1	27	29.4	30.3	24.2	31.3	44.4	25	35.3	24.2	32.4	37.8	52.6
UK 5	32.4	36.8	63.2	-	63.2	17.6	45	20.5	33.3	45.7	28.6	23.5	31.6	29.4	22.2	22.9	41	41	60
UK 6	34.3	22.2	61.1	63.2	-	18.8	36.8	16.2	35.3	42.4	18.2	25	27.8	12.5	23.5	30.3	21.6	43.2	36.8
UK 7	32.3	25	31.3	17.6	18.8	-	29.4	36.4	40	27.6	13.8	35.7	25	28.6	40	27.6	36.4	24.2	23.5
UK 8	43.2	47.4	42.1	45	36.8	29.4	-	35.9	27.8	22.9	34.3	52.9	42.1	29.4	44.4	11.4	30.8	35.9	50
UK 10	33.3	32.4	27	20.5	16.2	36.4	35.9	-	34.3	11.8	5.9	30.3	10.8	18.2	28.6	17.6	31.6	21.1	20.5
UK 11	36.4	23.5	29.4	33.3	35.3	40	27.8	34.3	-	25.8	38.7	20	23.5	33.3	25	32.3	51.4	22.9	38.9
UK 13	31.3	24.2	30.3	45.7	42.4	27.6	22.9	11.8	25.8	-	20	13.8	24.2	34.5	19.4	26.7	47.1	47.1	28.6
UK 14	25	12.1	24.2	28.6	18.2	13.8	34.3	5.9	38.7	20	-	34.5	30.3	20.7	32.3	26.7	35.3	17.6	40
UK 15	32.3	25	31.3	23.5	25	35.7	52.9	30.3	20	13.8	34.5	-	25	35.7	33.3	13.8	18.2	18.2	35.3
UK 19	28.6	33.3	44.4	31.6	27.8	25	42.1	10.8	23.5	24.2	30.3	25	-	18.8	52.9	0	43.2	32.4	31.6
UK 20	32.3	37.5	25	29.4	12.5	28.6	29.4	18.2	33.3	34.5	20.7	35.7	18.8	-	13.3	20.7	30.3	18.2	52.9
UK 21	36.4	35.3	35.3	22.2	23.5	40	44.4	28.6	25	19.4	32.3	33.3	52.9	13.3	-	0	34.3	22.9	27.8
UK 22	18.8	12.1	24.2	22.9	30.3	27.6	11.4	17.6	32.3	26.7	26.7	13.8	0	20.7	0	-	17.6	29.4	11.4
UK 24	22.2	21.6	32.4	41	21.6	36.4	30.8	31.6	51.4	47.1	35.3	18.2	43.2	30.3	34.3	17.6	-	21.1	35.9
UK 25	27.8	37.8	37.8	41	43.2	24.2	35.9	21.1	22.9	47.1	17.6	18.2	32.4	18.2	22.9	29.4	21.1	-	30.8
UK 27	48.6	47.4	52.6	60	36.8	23.5	50	20.5	38.9	28.6	40	35.3	31.6	52.9	27.8	11.4	35.9	30.8	-
UK 29	25	30.3	18.2	11.4	6.1	20.7	34.3	29.4	32.3	6.7	26.7	34.5	18.2	34.5	12.9	20	29.4	29.4	34.3
UK 30	41.2	22.9	45.7	32.4	45.7	25.8	43.2	16.7	42.4	25	43.8	32.3	57.1	12.9	42.4	12.5	33.3	33.3	37.8
UK 31	50	12.1	36.4	22.9	18.2	69	22.9	23.5	32.3	20	13.3	27.6	30.3	27.6	32.3	20	23.5	17.6	28.6
NZ 24	25.8	37.5	43.8	17.6	25	28.6	23.5	24.2	26.7	20.7	20.7	21.4	43.8	21.4	40	13.8	24.2	30.3	11.8
NZ 31	34.5	13.3	26.7	18.8	20	61.5	25	32.3	42.9	22.2	29.6	38.5	40	15.4	57.1	14.8	45.2	6.5	12.5
NZ 35	25	58.5	43.9	41.9	34.1	27	46.5	42.9	25.6	26.3	15.8	27	24.4	27	35.9	21.1	19	47.6	51.2
NZ 45	19.4	37.5	43.8	29.4	25	50	41.2	6.1	20	20.7	20.7	35.7	43.8	21.4	26.7	13.8	24.2	30.3	35.3
NZ 60	34.3	38.9	44.4	31.6	27.8	18.8	36.8	43.2	23.5	24.2	24.2	31.3	22.2	43.8	35.3	30.3	21.6	43.2	47.4
NZ 64	18.8	18.2	18.2	17.1	12.1	41.4	11.4	29.4	45.2	6.7	26.7	27.6	12.1	34.5	12.9	60	29.4	17.6	22.9
NZ 84	41.2	28.6	34.3	48.6	34.3	19.4	37.8	27.8	24.2	25	25	25.8	11.4	19.4	36.4	18.8	22.2	33.3	54.1
NZ 94	22.2	27	27	20.5	16.2	36.4	25.6	21.1	22.9	5.9	17.6	24.2	21.6	24.2	40	35.3	21.1	31.6	20.5
NZ 121	25	42.4	48.5	40	30.3	48.3	45.7	29.4	19.4	13.3	26.7	41.4	24.2	20.7	25.8	13.3	35.3	23.5	34.3
NZ 139	29.4	22.9	17.1	21.6	22.9	25.8	32.4	16.7	24.2	25	25	25.8	40	19.4	42.4	18.8	33.3	33.3	21.6
NZ 140	40	27.8	22.2	31.6	44.4	25	31.6	21.6	35.3	36.4	24.2	25	22.2	31.3	11.8	36.4	21.6	43.2	36.8
NZ 141	23.5	45.7	45.7	43.2	28.6	19.4	37.8	22.2	24.2	31.3	25	19.4	34.3	25.8	30.3	31.3	33.3	44.4	43.2
NZ 142	34.3	22.2	33.3	26.3	27.8	18.8	47.4	27	29.4	12.1	24.2	18.8	38.9	12.5	47.1	12.1	21.6	32.4	31.6
NZ 143	41.2	28.6	34.3	21.6	34.3	12.9	27	16.7	24.2	31.3	31.3	38.7	17.1	32.3	36.4	37.5	16.7	27.8	27
NZ 144	38.7	37.5	43.8	29.4	31.3	35.7	35.3	6.1	13.3	34.5	20.7	28.6	43.8	28.6	33.3	13.8	12.1	42.4	29.4

Table 8.8 PFGE Dice coefficient similarity matrix of feline UPEC from UK and NZ ctd.

	UK 29	UK 30	UK 31	NZ 24	NZ 31	NZ 35	NZ 45	NZ 60	NZ 64	NZ 84	NZ 94	NZ 121	NZ 139	NZ 140	NZ 141	NZ 142	NZ 143	NZ 144
UK 1	25	41.2	50	25.8	34.5	25	19.4	34.3	18.8	41.2	22.2	25	29.4	40	23.5	34.3	41.2	38.7
UK 2	30.3	22.9	12.1	37.5	13.3	58.5	37.5	38.9	18.2	28.6	27	42.4	22.9	27.8	45.7	22.2	28.6	37.5
UK 3	18.2	45.7	36.4	43.8	26.7	43.9	43.8	44.4	18.2	34.3	27	48.5	17.1	22.2	45.7	33.3	34.3	43.8
UK 5	11.4	32.4	22.9	17.6	18.8	41.9	29.4	31.6	17.1	48.6	20.5	40	21.6	31.6	43.2	26.3	21.6	29.4
UK 6	6.1	45.7	18.2	25	20	34.1	25	27.8	12.1	34.3	16.2	30.3	22.9	44.4	28.6	27.8	34.3	31.3
UK 7	20.7	25.8	69	28.6	61.5	27	50	18.8	41.4	19.4	36.4	48.3	25.8	25	19.4	18.8	12.9	35.7
UK 8	34.3	43.2	22.9	23.5	25	46.5	41.2	36.8	11.4	37.8	25.6	45.7	32.4	31.6	37.8	47.4	27	35.3
UK 10	29.4	16.7	23.5	24.2	32.3	42.9	6.1	43.2	29.4	27.8	21.1	29.4	16.7	21.6	22.2	27	16.7	6.1
UK 11	32.3	42.4	32.3	26.7	42.9	25.6	20	23.5	45.2	24.2	22.9	19.4	24.2	35.3	24.2	29.4	24.2	13.3
UK 13	6.7	25	20	20.7	22.2	26.3	20.7	24.2	6.7	25	5.9	13.3	25	36.4	31.3	12.1	31.3	34.5
UK 14	26.7	43.8	13.3	20.7	29.6	15.8	20.7	24.2	26.7	25	17.6	26.7	25	24.2	25	24.2	31.3	20.7
UK 15	34.5	32.3	27.6	21.4	38.5	27	35.7	31.3	27.6	25.8	24.2	41.4	25.8	25	19.4	18.8	38.7	28.6
UK 19	18.2	57.1	30.3	43.8	40	24.4	43.8	22.2	12.1	11.4	21.6	24.2	40	22.2	34.3	38.9	17.1	43.8
UK 20	34.5	12.9	27.6	21.4	15.4	27	21.4	43.8	34.5	19.4	24.2	20.7	19.4	31.3	25.8	12.5	32.3	28.6
UK 21	12.9	42.4	32.3	40	57.1	35.9	26.7	35.3	12.9	36.4	40	25.8	42.4	11.8	30.3	47.1	36.4	33.3
UK 22	20	12.5	20	13.8	14.8	21.1	13.8	30.3	60	18.8	35.3	13.3	18.8	36.4	31.3	12.1	37.5	13.8
UK 24	29.4	33.3	23.5	24.2	45.2	19	24.2	21.6	29.4	22.2	21.1	35.3	33.3	21.6	33.3	21.6	16.7	12.1
UK 25	29.4	33.3	17.6	30.3	6.5	47.6	30.3	43.2	17.6	33.3	31.6	23.5	33.3	43.2	44.4	32.4	27.8	42.4
UK 27	34.3	37.8	28.6	11.8	12.5	51.2	35.3	47.4	22.9	54.1	20.5	34.3	21.6	36.8	43.2	31.6	27	29.4
UK 29	-	25	6.7	20.7	22.2	21.1	34.5	30.3	33.3	6.3	47.1	33.3	25	18.2	31.3	24.2	12.5	13.8
UK 30	25	-	25	45.2	41.4	30	32.3	22.9	31.3	17.6	16.7	25	29.4	28.6	29.4	28.6	23.5	32.3
UK 31	6.7	25	-	27.6	44.4	26.3	41.4	18.2	40	37.5	35.3	33.3	25	30.3	12.5	36.4	25	48.3
NZ 24	20.7	45.2	27.6	-	38.5	27	35.7	25	27.6	12.9	30.3	34.5	32.3	25	38.7	43.8	45.2	57.1
NZ 31	22.2	41.4	44.4	38.5	-	17.1	38.5	13.3	29.6	13.8	25.8	44.4	27.6	13.3	6.9	20	20.7	30.8
NZ 35	21.1	30	26.3	27	17.1	-	32.4	58.5	31.6	40	33.3	36.8	15	29.3	35	29.3	25	32.4
NZ 45	34.5	32.3	41.4	35.7	38.5	32.4	-	18.8	20.7	19.4	42.4	62.1	25.8	18.8	45.2	31.3	19.4	42.9
NZ 60	30.3	22.9	18.2	25	13.3	58.5	18.8	-	36.4	34.3	37.8	24.2	28.6	33.3	45.7	27.8	34.3	25
NZ 64	33.3	31.3	40	27.6	29.6	31.6	20.7	36.4	-	25	52.9	20	12.5	30.3	25	18.2	25	20.7
NZ 84	6.3	17.6	37.5	12.9	13.8	40	19.4	34.3	25	-	27.8	31.3	41.2	57.1	35.3	51.4	35.3	32.3
NZ 94	47.1	16.7	35.3	30.3	25.8	33.3	42.4	37.8	52.9	27.8	-	35.3	22.2	16.2	27.8	37.8	22.2	24.2
NZ 121	33.3	25	33.3	34.5	44.4	36.8	62.1	24.2	20	31.3	35.3	-	25	18.2	31.3	30.3	18.8	27.6
NZ 139	25	29.4	25	32.3	27.6	15	25.8	28.6	12.5	41.2	22.2	25	-	40	41.2	51.4	29.4	38.7
NZ 140	18.2	28.6	30.3	25	13.3	29.3	18.8	33.3	30.3	57.1	16.2	18.2	40	-	28.6	38.9	40	37.5
NZ 141	31.3	29.4	12.5	38.7	6.9	35	45.2	45.7	25	35.3	27.8	31.3	41.2	28.6	-	28.6	41.2	32.3
NZ 142	24.2	28.6	36.4	43.8	20	29.3	31.3	27.8	18.2	51.4	37.8	30.3	51.4	38.9	28.6	-	34.3	43.8
NZ 143	12.5	23.5	25	45.2	20.7	25	19.4	34.3	25	35.3	22.2	18.8	29.4	40	41.2	34.3	-	45.2
NZ 144	13.8	32.3	48.3	57.1	30.8	32.4	42.9	25	20.7	32.3	24.2	27.6	38.7	37.5	32.3	43.8	45.2	

8.3.3. Chapter 6

Table 8.9 Raw Data Phage Lysis Experiment

Isolate No.	Published No.	Phage																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
C 10	1	-	+	+	+	+	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-
C 13	2	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	-	+
C 14	3	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	+
C 15	4	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
C 28	5	-	+	+	+	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-
C 29	6	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
C 34	7	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-
C 51	8	-	-	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-
C 56	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C 57	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 69	11	-	-	-	-	-	-	-	+	-	-	+	+	+	-	+	-	-	-	-	-
C 70	12	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	+
C 77	13	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
C 79	14	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+
C 80	15	+	+	+	+	+	-	+	-	+	+	+	+	+	-	-	-	-	-	+	-
C 81	16	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+
C 81	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 83	18	-	+	+	+	+	-	+	-	-	-	-	+	+	-	+	-	-	-	+	-
C 90	19	-	-	-	-	+	+	+	+	-	-	+	-	+	+	+	+	+	+	-	+
C 91	20	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-
C 183	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F 24	22	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	-	+	-
F 31	23	+	-	+	+	+	+	-	+	-	-	+	-	-	+	-	+	+	-	+	-
F 35	24	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-
F 45	25	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
F 60	26	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
F 64	27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
F 84	28	+	+	+	+	+	-	+	+	-	-	+	-	-	+	+	+	+	-	+	-
F 94	29	+	+	+	+	+	-	+	-	+	+	-	+	+	-	-	+	-	-	-	-
F 121	30	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 8.9 Raw Data Phage Lysis Experiment ctd.

Isolate No.	Published No.	Phage																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
F 139	31	+	+	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+
F 140	32	+	+	+	+	+	-	+	+	-	-	+	-	-	-	+	+	+	-	-	-
F 141	33	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
F 142	34	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
F 143	35	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
F 144	36	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
F 164	37	+	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-
F 165	38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F 166	39	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
F 167	40	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	+	-	-	+	-
F 181	41	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F 182	42	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-
C 168	43	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C 169	44	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	-	-
C 170	45	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 171	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 172	47	+	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-
C 173	48	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	-	+	+
C 174	49	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-
C 184	50	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-	+	-	-
C 185	51	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
C 186	52	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
F 196	53	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Faecal C1	97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Faecal C2	98	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Faecal C3	99	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Faecal C4	107	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-
Faecal F1	135	+	+	+	+	-	-	+	-	-	-	+	-	+	-	-	+	+	-	-	-
Faecal F2	136	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Faecal F3	137	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-

Table 8.9 Raw Data Phage Lysis Experiment ctd.

Isolate No.	Published No.	Phage																			
		21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
C 10	1	-	-	+	-	+	-	-	-	-	+	-	+	+	-	-	-	-	+	-	-
C 13	2	-	-	+	+	+	-	+	-	+	+	-	+	+	-	-	+	+	+	-	-
C 14	3	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	-	+	-	+
C 15	4	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
C 28	5	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-
C 29	6	-	-	+	-	+	+	+	-	+	+	-	+	+	-	-	+	+	+	-	-
C 34	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 51	8	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+
C 56	9	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
C 57	10	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
C 69	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
C 70	12	-	-	+	-	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
C 77	13	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
C 79	14	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
C 80	15	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
C 81	16	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
C 81	17	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
C 83	18	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-
C 90	19	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-
C 91	20	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
C 183	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F 24	22	+	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-
F 31	23	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+
F 35	24	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	-	-
F 45	25	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-
F 60	26	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
F 64	27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F 84	28	-	-	-	-	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	-
F 94	29	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-
F 121	30	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	-

Table 8.9 Raw Data Phage Lysis Experiment ctd.

Isolate No.	Published No.	Phage																			
		21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
F 139	31	-	-	+	-	+	+	+	-	+	+	-	-	+	-	-	+	+	+	-	-
F 140	32	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	-	-
F 141	33	-	-	-	-	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	-
F 142	34	+	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-	+	-	-
F 143	35	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
F 144	36	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	-	+	-
F 164	37	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+	-
F 165	38	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
F 166	39	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-
F 167	40	+	+	-	-	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+
F 181	41	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-
F 182	42	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
C 168	43	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	+	+	-	+	-
C 169	44	+	-	-	-	+	-	+	-	+	+	-	+	+	+	-	+	+	+	-	-
C 170	45	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 171	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 172	47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C 173	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
C 174	49	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-
C 184	50	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
C 185	51	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C 186	52	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
F 196	53	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	+
Faecal C1	97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Faecal C2	98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Faecal C3	99	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
Faecal C4	107	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Faecal F1	135	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
Faecal F2	136	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+
Faecal F3	137	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

8.4. Materials and Methods

8.4.1. *Escherichia coli* Strain Collection

Please refer to the end of this section for a list of terms/abbreviations only used in this table.

No	C	Strain	Species	VF PCR	PFGE	Phage	Date	Region	Source
1	1	C10	canine	X		propagation	15-02-00	PMR	Massey University
2	1	C13	canine	X		propagation	15-03-00	PMR	Massey University
3	1	C14	canine	X		propagation	06-04-00	PMR	Massey University
4	1	C15	canine	X		propagation	30-05-00	PMR	Massey University
5	1	C28	canine	X		propagation	11-03-02	WLG	PetVet Taita
6	1	C29	canine	X	X	propagation	14-03-02	PMR	Massey University
7	1	C34	canine	X	X	propagation	11-03-02	WLG	Paraparaumu Vet Centre
8	1	C37	canine	X			24-04-02	PMR	Massey University
9	1	C51	canine	X		propagation	03-05-02	AKL	Blockhouse Bay Vets
10	1	C56	canine	X		propagation	16-04-02	PMR	Vet Services Hawkes Bay
11	1	C57	canine	X		propagation	16-04-02	PMR	Massey University
12	1	C69	canine	X		propagation	19-06-02	PMR	Massey University
13	1	C70	canine	X		propagation	10-07-02	PMR	Stratford Vets
14	1	C77	canine	X		propagation	12-09-02	PMR	Massey University
15	1	C79	canine	X	X	propagation	27-09-02	PMR	Bush Vets
16	1	C80	canine	X	X	propagation	11-09-02	PMR	Animal Hospital PMR
17	1	C81	canine	X	X	propagation	06-09-02	AKL	Hillsborough Vets
18	1	C82	canine	X	X	propagation	29-08-02	PMR	New Plymouth Vet Group
19	1	C83	canine	X	X	propagation	19-04-02	PMR	Massey University
20	1	C90	canine	X	X	propagation	06-11-02	PMR	Massey University
21	1	C91	canine	X	X	propagation	09-11-02	PMR	Massey University
22	1	C92	canine	X	X		11-11-02	PMR	Massey University
23	1	C100	canine	X			20-04-03	PMR	Massey University
24	1	C101	canine	X	X		25-04-03	PMR	Massey University
25	1	C115	canine	X	X		20-05-03	PMR	Massey University
26	1	C120	canine	X	X		29-05-03	PMR	Massey University
27	1	C128	canine	X	X		14-06-03	PMR	Massey University
28	1	C138	canine	X			21-08-03	PMR	Alpha Scientific
29	1	C145	canine	X			01-10-03	HLZ	Alpha Scientific
30	1	C146	canine	X			17-09-03	HLZ	Alpha Scientific
31	1	C147	canine	X			18-09-03	HLZ	Alpha Scientific
32	1	C148	canine	X			17-09-03	HLZ	Alpha Scientific
33	1	C149	canine	X			10-09-03	HLZ	Massey University
34	1	C160	canine	X	X		12-11-03	PMR	Massey University
35	1	C161	canine	X	X		14-11-03	PMR	Massey University
36	1	C168	canine	X		cross-lysis	05-12-03	HLZ	Alpha Scientific
37	1	C169	canine	X		cross-lysis	06-12-03	HLZ	Alpha Scientific
38	1	C170	canine	X		cross-lysis	02-12-03	HLZ	Alpha Scientific
39	1	C171	canine	X		cross-lysis	19-12-03	HLZ	Alpha Scientific
40	1	C172	canine	X		cross-lysis	18-12-03	HLZ	Alpha Scientific
41	1	C173	canine	X		cross-lysis	09-12-03	HLZ	Alpha Scientific
42	1	C174	canine	X		cross-lysis	23-12-03	HLZ	Alpha Scientific
43	1	C183	canine	X		propagation	11-03-04	HLZ	Alpha Scientific
44	1	C184	canine	X		cross-lysis	24-01-04	HLZ	Alpha Scientific
45	1	C185	canine	X		cross-lysis	03-02-04	HLZ	Alpha Scientific
46	1	C186	canine			cross-lysis	06-04-04	HLZ	Alpha Scientific
47	2	C20	canine	X	X		15-01-02	AKL	Massey Heights Vets
48	2	C23	canine	X			15-01-02	PMR	Wanganui Vet Club
49	2	C32	canine	X			22-03-02	AKL	Dairy Flat
50	2	C33	canine	X	X		22-03-02	AKL	Sommerville
51	2	C47	canine	X			03-05-02	AKL	Hillsborough Vets
52	2	C48	canine	X			03-05-02	AKL	North Harbour Vet Hosp
53	2	C50	canine	X			03-05-02	AKL	Levin & Horowhenua Vet
54	2	C55	canine	X			19-03-02	PMR	North Harbour Vet Hosp
55	2	C72	canine	X			11-07-02	PMR	Stratford District Vets
56	2	C73	canine	X			06-05-02	WLG	Maidstone
57	2	C76	canine	X			16-07-02	AKL	North Harbour Vet Hosp

No	C	Strain	Age (years)	Sex	Breed	History	UT signs ?
1	1	C10	11	fs	Huntaway X	Polyuria/polydipsia, incontinence, had prednisone treatment	yes
2	1	C13	12	f	Labrador	Urine taken as part of workup, possible hyperadrenocorticis	?
3	1	C14	10	mc	Border Collie	Polyuria/polydipsia, incontinence, possible hyperadrenocorticism	yes
4	1	C15	8	mn	GSH/Rotti X	Polyuria/polydipsia, dysuria, possible hyperadrenocorticism	yes
5	1	C28	7	fs	Shetland SD	Renal insufficiency	?
6	1	C29	0.4	f	GSH	Recurrent UTI, previously *E. coli*	?
7	1	C34	0.2	f	Gold. Retriever	Recurrent UTI, dysuria and stranguria 2 days after treatment	yes
8	1	C37	10	fs	Corgi	Haematuria	yes
9	1	C51	8	fs		Haematuria	yes
10	1	C56	9	f	Pointer	Renal failure?	?
11	1	C57	0.75	m	Rough Collie	No history given	?
12	1	C69	0.3	m	GSH	Polyuria/polydipsia since birth	yes
13	1	C70	10	m	?	Haematuria, inappetence, pyrexia (39.8 deg C)	yes
14	1	C77	10	fs	Labrador	Initially dysuria and incontinence, had been on prednisone. Final diagnosis after euthanasia: transitional cell carcinoma	yes
15	1	C79	0.74	f	Mastiff	Haematuria, possible renal insufficiency	yes
16	1	C80	13	mn	Labrador	Incontinence, chronic weight loss	yes
17	1	C81	0.67	f	Border Collie	No history given	?
18	1	C82	14	fs	Cocker Spaniel	Recurrent UTI	?
19	1	C83	1.2	f	Gold. Labrador	Incontinence at rest and excitement	yes
20	1	C90				No history given	?
21	1	C91				No history given	?
22	1	C92	10	fs	Weimaraner	Renal insufficiency	?
23	1	C100				No history given	?
24	1	C101				No history given	?
25	1	C115				Has been receiving prednisone	?
26	1	C120	0.13	f	GSH	Pollakiuria, polyuria/polydipsia, haematuria	yes
27	1	C128			Foxterrier	Has been receiving prednisone	yes
28	1	C138	0.37	f	GSH	Pollakiuria, recurrent UTI	?
29	1	C145	12	f	Boxer	No history given	no
30	1	C146	8	fs	Bichon X	Clinically silent, post-treatment culture	?
31	1	C147	13	f	WHWT	Recurrent UTI, previously Proteus spp	?
32	1	C148	8	fs	Bichon	No history given	?
33	1	C149	13	mc	Border Collie	Hacmaturia, hyperadrenocorticism.	yes
34	1	C160	11	m	Collie	Diabetes mellitus, urine taken post-treatment for UTI	?
35	1	C161				File not found	?
36	1	C168	13	fs	Border Collie	Incontinence	yes
37	1	C169	11	m	Spaniel	Bacteriuria seen in free flow sample	?
38	1	C170	10	mn	Labrador	Diabetes mellitus	?
39	1	C171	10	fs	Labrador	Recurrent UTI, previously *Proteus* spp	?
40	1	C172	9	m	Husky	Urinating in house	?
41	1	C173	9	f	Bichon Frise	Renal failure	?
42	1	C174	12	fs	Crossbreed	Possible hyperadrenocorticism	?
43	1	C183	5	fs	Border Collie	Incontinence	yes
44	1	C184	4	m	Pekinese	No history given	?
45	1	C185	12	fs	Terrier X	Urolithiasis	?
46	1	C186	8	fs	GSH	Recurrent UTI	?
47	2	C20	12	mn	Labrador X	Recurrent UTI	?
48	2	C23	14	m	Rottweiler	Haematuria, no dysuria	yes
49	2	C32	15	fs	Crossbreed	Incontinence	yes
50	2	C33	0.25	fs	Weimaraner	Dysuria 3 weeks ago, had treatment with Clavulox, now lethargy and pyrexia (40.2 deg C)	?
51	2	C47	9	mn	Bedlington T	Previous UTI, hyperadrenocorticism	?
52	2	C48	10	fs	Husky	Odorous urine	yes
53	2	C50	19	mn	Terrier	Haematuria	yes
54	2	C55	11	m	Workdog	Haematuria at end of urination	yes
55	2	C72	NK	m	Rottweiler	Haematuria	yes
56	2	C73	6	mn	Labrador	Recurrent UTI, Diabetes mellitus	?
57	2	C76	11	fs	Border Collie	Pre-surgical urine sample	no

No	C	Strain	Sample collection	*E. coli* only?	Part of urinalysis					
					Colour	Turbidity	SG	WBC/hpf	RBC/hpf	bacteria/hpf
1	1	C10	cysto				1.01	<5	0-2	+++
2	1	C13	cysto	Certain			1.043	>50	-	++
3	1	C14	catheter	Certain			1.008	<5	-	+++ rods
4	1	C15	? catheter	Certain			1.007	20	<5	+++ rods
5	1	C28	cysto							
6	1	C29	cysto							
7	1	C34	cysto							
8	1	C37	cysto							
9	1	C51	cysto		yellow	hazy	1.015	20	20	+++
10	1	C56	cysto	Certain						
11	1	C57	cysto							
12	1	C69	cysto							
13	1	C70	free flow	Certain	orange	cloudy	1.014	30	250	
14	1	C77	catheter	Certain			1.017	>50	>200	+++
15	1	C79	cysto							
16	1	C80	cysto		yellow	hazy	1.011	<20	5	
17	1	C81	cysto						numerous	
18	1	C82	cysto		yellow	hazy	1.013	10	2	+++
19	1	C83	cysto	Certain			1.02	1	0-5	+
20	1	C90	cysto							
21	1	C91	cysto							
22	1	C92	cysto	Certain			1.021	>50	-	+++ rods
23	1	C100	cysto							
24	1	C101	cysto							
25	1	C115	cysto	Certain			1.024	>50	>200	+++
26	1	C120	cysto	Certain			1.01	>50	>200	+++ rods
27	1	C128	cysto				1.025	<10	<5	
28	1	C138	cysto	Certain			1.024	>20	>100	+++
29	1	C145	cysto	Certain						
30	1	C146	cysto	Certain	yellow	slight	1.032	<5	-	+++
31	1	C147	cysto	Certain						
32	1	C148	cysto	Certain	yellow	slight		5-10	-	+++
33	1	C149	cysto	Certain	yellow	clear		20-50	-	+++
34	1	C160	cysto	Certain			1.019	-	-	+++ rods
35	1	C161	cysto							
36	1	C168	cysto	Certain	yellow	slight	1.016	50-100	<5	++
37	1	C169	cysto	Certain						
38	1	C170	cysto	Certain	yellow	slight		<5	-	+++
39	1	C171	cysto	Certain	yellow	slight		5-10	-	+++
40	1	C172	cysto	No	yellow	slight	1.021	5-10	<5	++
41	1	C173	cysto	Certain	yellow	marked	1.016	>200	-	+++
42	1	C174		Certain	yellow	slight	1.014	50-100	<5	+++
43	1	C183	cysto	Certain	yellow	slight	1.026	<5	<5	+
44	1	C184	cysto	Certain	yellow	slight	1.015	<5	<5	+++
45	1	C185	cysto	Certain	brown	markes		20-50	>200	+++
46	1	C186	cysto	No	yellow	slight	1.027	10-20	5-10	+++
47	2	C20		No	yellow	hazy	1.038	5	-	+++
48	2	C23	?	No	brown	cloudy	1.014	10	600	+
49	2	C32		Certain	yellow	cloudy	1.025	<5	10	+++
50	2	C33	express	Certain	yellow	clear	1.007	20	-	+
51	2	C47	free-catch	Certain	yellow	clear	1.006			
52	2	C48	free-catch	Certain			1030	>20		+++ rods
53	2	C50		Certain	yellow	hazy	1.021	<5	5	++
54	2	C55	catheter						10	
55	2	C72		No	yellow	cloudy	1.015		300	+
56	2	C73	catheter							
57	2	C76		Certain			1.040	<10		++ rods

No	C	Strain	Species	VF PCR	PFGE	Phage	Date	Region	Source
58	1	F24	feline	X	X	propagation	23-01-02	AKL	Papatoetoe East Vet Centre
59	1	F31	feline	X	X	propagation	23-02-02	NPL	New Plymouth Vet Group
60	1	F35	feline	X	X	propagation	22-03-02	AKL	Mt. Wellington
61	1	F45	feline	X	X	propagation	03-05-02	AKL	Hillsborough Vets
62	1	F60	feline	X	X	propagation	30-04-02	PMR	Vetcare
63	1	F64	feline	X	X	propagation	04-05-02	PMR	Animal Hospital PMR
64	1	F84	feline	X	X	propagation	06-08-02	PMR	Animal Hospital PMR
65	1	F94	feline	X	X	propagation	11-02-03	PMR	Massey University
66	1	F121	feline	X	X	propagation	03-06-03	PMR	Massey University
67	1	F139	feline	X	X	propagation	30-09-03	HLZ	Alpha Scientific
68	1	F140	feline	X	X	propagation	01-10-03	HLZ	Alpha Scientific
69	1	F141	feline	X	X	propagation	24-09-03	HLZ	Alpha Scientific
70	1	F142	feline	X	X	propagation	11-09-03	HLZ	Alpha Scientific
71	1	F143	feline	X	X	propagation	06-09-03	HLZ	Alpha Scientific
72	1	F144	feline	X	X	propagation	19-09-03	HLZ	Alpha Scientific
73	1	F164	feline			propagation	12-06-03	HLZ	Alpha Scientific
74	1	F165	feline			propagation	11-11-03	HLZ	Alpha Scientific
75	1	F166	feline			propagation	12-08-03	HLZ	Alpha Scientific
76	1	F167	feline			propagation	23-12-03	HLZ	Alpha Scientific
77	1	F181	feline			propagation	14-04-04	HLZ	Alpha Scientific
78	1	F182	feline			propagation	07-04-04	HLZ	Alpha Scientific
79	1	F196	feline			cross-lysis	30-04-04	HLZ	Alpha Scientific
80	2	F26	feline	X			16-01-02	PMR	Wanganui Vet Club
81	2	F30	feline	X			22-03-02	AKL	Papatoetoe
82	2	F42	feline	X			03-05-02	AKL	Auckland Vet Services
83	2	F53	feline	X	X		03-05-02	AKL	Onewa Rd Vet Clinic
84	2	F54	feline	X			03-05-02	AKL	Mt. Wellington Vets
85	2	F61	feline	X			08-07-02	PMR	Stratford District Vets
86	2	F62	feline	X			28-09-14	PMR	Massey University

No	C	Strain	Age	Sex	Breed	History	UT signs ?
58	1	F24	NK			Recurrent UTI, post-treatment sample	?
59	1	F31	19	fs	Oriental	Recurrent UTI, now haematuria	yes
60	1	F35	12	fs	DSH	Pyrexia, inappetence	?
61	1	F45	14	fs	DSH	Haematuria	yes
62	1	F60	6	fs	Birman	Recurrent UTI	?
63	1	F64	9	fs	DSH	Pyelonephritis?, Haematuria, Pyuria	yes
64	1	F84	16	fs	Burmese	Recurrent UTI, possibly renal insufficiency, has been on prednisone.	?
65	1	F94	17	f	Burmese	Pollakiuria and stranguria, 1 negative culture after treatment	yes
66	1	F121	6	fs	DLH	Incontinence, possible damage pudendal nerve	yes
67	1	F139	18	fs	DSH	Polyuria/polydipsia, possible renal insufficiency, recurrent UTI	yes
68	1	F140	10	f	DSH	Bacteriuria noted	?
69	1	F141	17	f	DSH	Bacteriuria noted	?
70	1	F142	16	fs	Burmese	Urine sample as part of diagnostic work-up?	?
71	1	F143	14	fs	DMH	No history given	?
72	1	F144	12	f	DLH	Haematuria and stranguria	yes
73	1	F164	12	f	DSH	No history given	?
74	1	F165	19	fs	DSH	No history given	?
75	1	F166	5	fs	Siamese	Recurrent UTI, dexamethasone treatment	?
76	1	F167	1	fs	DLH	Haematuria and pollakiuria	yes
77	1	F181	15	f	DSH	Stranguria, dysuria	yes
78	1	F182	14	mn	Burmese	No history given	?
79	1	F196	13	mn	Russian Blue	No history given	?
80	2	F26	8 yr	fs	DSH	Recurrent UTI, stranguria	yes
81	2	F30	13 yr	fs	DLH	Chronic (?) UTI	?
82	2	F42	17 yr	fs	DSH	Recurrent UTI, stranguria, Has received prednisone.	yes
83	2	F53	8 yr	fs		Polycystic kidney diagnosed on ultrasound	?
84	2	F54		f	DSH	Dysuria	yes
85	2	F61	5 yr	f	DSH	Recurrent UTI	?
86	2	F62	11 yr	fs	Abyssinian	Dysuria and haematuria	yes

No	C	Strain	Sample collection	Crea-tinine	*E. coli* only?	Part of urinalysis					
						Colour	Turbidity	SG	WBC/hpf	RBC/hpf	bacteria/hpf
58	1	F24	cysto		Certain						
59	1	F31			Certain	pale	hazy	1.015	10		++
60	1	F35			Certain	straw	slight	1.009	10	-	+++
61	1	F45	cysto						5	>100	++
62	1	F60	cysto		Certain	pale	slight	1026	80		+
63	1	F64			Certain	orange	cloudy	1.017	50	150	+++
64	1	F84	cysto	130				1.02			+++
65	1	F94	cysto		Certain				>20	>100	+++
66	1	F121	cysto		Certain			1.022	<5		+++
67	1	F139	cysto		Certain						
68	1	F140	cysto		Certain						
69	1	F141	cysto		Certain			1.035			
70	1	F142	cysto		Certain	yellow	slight	1.02	10-20	-	+++
71	1	F143	cysto		Certain	orange	slight		<5	5-10	+++
72	1	F144	cysto		Certain						
73	1	F164	cysto		Certain			1.05			
74	1	F165	cysto		Certain						
75	1	F166	cysto		Certain			1.008			
76	1	F167	cysto		Certain						
77	1	F181	cysto		Certain	yellow	moderate		10-20	5-10	++
78	1	F182	cysto		Certain						
79	1	F196	cysto		Certain	pale	clear		5-10	<5	+++
80	2	F26	?			pale	hazy	1.025	3		+++
81	2	F30			Certain						
82	2	F42	free-catch		Certain			1.020			
83	2	F53			Certain						
84	2	F54			Certain	pale	slight	1.026	5	5	++
85	2	F61			Certain	pale	hazy	1.023	2	15	++
86	2	F62	free-catch		Certain						

No	C	Strain	Species	VF PCR	PFGE	Phage	Date	Region	Source
201	1	UK1	feline	X	X		File not found	London, UK	File not found
202	1	UK2	feline	X	X		06-01-1999	London, UK	Sale
203	1	UK3	feline	X	X		13-07-1999	London, UK	Mellor-ellis
204	1	UK4	feline	X	X		13-07-1999	London, UK	Sale
205	1	UK5	feline	X	X		8-10-1999	London, UK	Theobald
206	1	UK6	feline	X	X		8-10-1999	London, UK	Kimmins
207	1	UK7	feline	X	X		24-08-1999	London, UK	Ceylan
208	1	UK8	feline	X	X		28-09-1999	London, UK	Sale
209	1	UK9	feline	X	X		28-09-1999	London, UK	Mellor-Ellis
210	1	UK10	feline	X	X		26-10-1999	London, UK	Henry
211	1	UK11	feline	X	X		15-11-1999	London, UK	Burton
212	1	UK12	feline	X	X		21-12-1999	London, UK	Sale
213	1	UK13	feline	X	X		File not found	London, UK	File not found
214	1	UK14	feline	X	X		15-02-2000	London, UK	Driscoll
215	1	UK15	feline	X	X		15-02-2000	London, UK	Lessiter
216	1	UK16	feline	X	X		03-07-2000	London, UK	Sale
217	1	UK17	feline	X	X		23-03-2000	London, UK	Theobald
218	1	UK18	feline	X	X		18-04-2000	London, UK	Sale
219	1	UK19	feline	X	X		05-09-2000	London, UK	Ali
220	1	UK20	feline	X	X		File not found	London, UK	Ali
221	1	UK21	feline	X	X		09-08-2000	London, UK	Cunningham
222	1	UK22	feline	X	X		15-08-2000	London, UK	Hogg
223	1	UK23	feline	X	X		26-09-2000	London, UK	Kimmins
224	1	UK24	feline	X	X		File not found	London, UK	File not found
225	1	UK25	feline	X	X		17-10-2000	London, UK	Henry
226	1	UK26	feline	X	X		17-10-2000	London, UK	Sale
227	1	UK27	feline	X	X		11-07-2000	London, UK	Kimmins
228	1	UK28	feline	X	X		28-11-2000	London, UK	Sale
229	1	UK29	feline	X	X		26-02-2002	London, UK	Mellor-Ellis
230	1	UK30	feline	X	X		05-03-2002	London, UK	Cleere
231	1	UK31	feline	X	X		06-03-2002	London, UK	Thompson

No	C	Strain	Age	Sex	Breed	History	UT signs ?
201	1	UK1				file not found	?
202	1	UK2	15.2	fn	DSH	CRF and hyperthyroidism, recurrent UTIs, this one with clinical signs	yes
203	1	UK3	17	fn	DSH	Clinical signs of UTI	yes
204	1	UK4	15.3	fn	DSH	clinically silent UTI, same cat as UK 2	no
205	1	UK5	10.4	f	DSH	Hyperthyroid, developed mild CRF post treatment; had a pyometra, clinically silent UTI	no
206	1	UK6	12.4	fn	DSH	Hyperthyroid, develped CRF post-treatment, clinically silent UTI	no
207	1	UK7	unknown	fn	DSH	Hyperthyroid (post-thyroidectomy), clinically silent UTI	no
208	1	UK8	15.5	fn	DSH	clinically silent UTI, same cat as UK 2	no
209	1	UK9	18.3	fn	DSH	CRF post-treatment for hyperthyroidism, clinically silent UTI	no
210	1	UK10	17.5	fn	DSH	CRF and hypertension, recurrent UTI, clinically silent UTI	no
211	1	UK11	14	fn	DLH	CRF case which developed hyperthyroidism, clinically silent UTI	no
212	1	UK12	15.7	fn	DSH	Clinically silent UTI, same cat as UK 2	no
213	1	UK13					?
214	1	UK14	9.2	mn	DSH	Renal insufficiency; surgery for hyperthyroidism 3 years prior to presentation, clinically silent UTI	no
215	1	UK15	unknown	fn	DSH	Uncontrolled hyperthyroid - has bilateral thyroidectomy and still hyperthyroid, owner not able to control problem with neomercazole. Lethargy	?
216	1	UK16	15.9	fn	DSH	Clinically silent UTI, same cat as UK 2	no
217	1	UK17	11	f	DSH	Clinically silent UTI, same cat as UK 5	no
218	1	UK18	16	fn	DSH	Clinically silent UTI, same cat as UK 2	no
219	1	UK19	13.7	fn	DSH	Uncontrolled hyperthyroid, tried neomercazole and surgery, hypertensive and on amlodipine, Lethargy, no symptoms of UTI	no
220	1	UK20	13.7	fn	DSH	Lethargy, no symptoms of UTI	no
221	1	UK21	11	fn	DSH	Hyperthyroid, on neomercazole, clinically silent UTI	no
222	1	UK22	13	fn	DSH	Clinically silent UTI	no
223	1	UK23	13.5	fn	DSH	ClinicallysilentUTI, same cat as UK 6	no
224	1	UK24					
225	1	UK25	18.5	fn	DSH	Clinically silent UTI, same cat as UK 10	no
226	1	UK26	16.6	fn	DSH	Clinically silent UTI, same cat as UK 2	no
227	1	UK27	13.7	fn	DSH	Clinically silent UTI, same cat as UK 6	no
228	1	UK28	16.7	fn	DSH	Clinically silent UTI, same cat as UK 2	no
229	1	UK29	20.8	fn	DSH	Clinically silent UTI, same cat as UK 9	no
230	1	UK30	unknown	fn	DSH	Inappropriate urination during night	yes
231	1	UK31	15.2	fn	DSH	UTI with clinical signs	yes

No	C	Strain	Sample collection	Crea-tinine	Part of urinalysis					
					Colour	Turbidity	SG	WBC/hpf	RBC/hpf	bacteria/hpf
201	1	UK1	cysto							
202	1	UK2	cysto	183			1.018	≤5		
203	1	UK3	cysto				1.021			
204	1	UK4	cysto	-			1.019	≤10		
205	1	UK5	cysto	182			1.011	≤10		
206	1	UK6	cysto	67			1.019	≤5		
207	1	UK7	cysto	94			1.032	≤15		
208	1	UK8	cysto	137			1.018	>20		
209	1	UK9	cysto	220			1.027	≤10		
210	1	UK10	cysto	357			1.013	≤10		
211	1	UK11	cysto	204			1.023	≤20		
212	1	UK12	cysto	197			1.016	≤20		
213	1	UK13	cysto							
214	1	UK14	cysto	132			1.022	≤10		
215	1	UK15	cysto	-			1.022	>20		
216	1	UK16	cysto	333			1.015	≤20		
217	1	UK17	cysto				1.015	≤20		
218	1	UK18	cysto				1.015	≤20		
219	1	UK19	cysto	-			1.022			
220	1	UK20	cysto							
221	1	UK21	cysto	102			1.038	0 cond		
222	1	UK22	cysto				1.018	≤20		
223	1	UK23	cysto				1.018	≤20		
224	1	UK24	cysto							
225	1	UK25	cysto	347			1.016	≤10		
226	1	UK26	cysto				1.016	>20		
227	1	UK27	cysto				1.015	≤20		
228	1	UK28	cysto				1.015	≤20		
229	1	UK29	cysto	244			1.016	≤5		
230	1	UK30	cysto	201			1.014	≤10		
231	1	UK31	cysto				1.036	≤10		

No	C	Strain	Species	VF PCR	PFGE	Phage	Date	Region	Source
301	NA	H1	human	X	X		17-12-01	PMR	Medlab Central, PMR Hospital
302	NA	H2	human	X			17-12-01	PMR	Medlab Central, PMR Hospital
303	NA	H3	human	X			17-12-01	PMR	Medlab Central, PMR Hospital
304	NA	H4	human	X			17-12-01	PMR	Medlab Central, PMR Hospital
305	NA	H5	human	X			17-12-01	PMR	Medlab Central, PMR Hospital
306	NA	H6	human	X	X		17-12-01	PMR	Medlab Central, PMR Hospital
307	NA	H7	human	X	X		17-12-01	PMR	Medlab Central, PMR Hospital
308	NA	H8	human	X	X		17-12-01	PMR	Medlab Central, PMR Hospital
309	NA	H9	human	X	X		17-12-01	PMR	Medlab Central, PMR Hospital
310	NA	H10	human	X			17-12-01	PMR	Medlab Central, PMR Hospital
311	NA	H11	human	X			17-12-01	PMR	Medlab Central, PMR Hospital

No	C	Strain	Age	Sex	History	UT signs?	Sample collection
301	NA	H1	48	f	Cystitis	?	MSU
302	NA	H2	14.7	f	4 previous episodes of pyelonephritis, now review	?	MSU
303	NA	H3	83.4	f	geriatric patient from rest home - cystitis	?	MSU
304	NA	H4	8.4	f	Urine taken for screening purposes only - UTI not expected, diagnosed with mesenteric adenitis	no	MSU
305	NA	H5	0.5	f	Paediatric patient - no pyuria, but growth	?	MSU
306	NA	H6	49	f	General unwellness - suspect urosepsis	?	MSU
307	NA	H7	54	f	Cystitis	?	MSU
308	NA	H8	37	f	Cystitis	?	MSU
309	NA	H9	62.1	f	Low dose steroids at time of diagnosis, cystitis	?	MSU
310	NA	H10	25	f	Cystitis	?	MSU
311	NA	H11	26	f	Cystitis	?	MSU

Legend to the *E. coli* strain collection tables

Column	Explanation of terms used in the column[a]
C	Category, as defined in chapter 2.
VF PCR or PFGE	X: This isolate was included in VF PCR assay or PFGE.
Phage	Cross-lysis: This *E. coli* isolate was included in the lysis experiment Propagation: This isolate was used to propagate a phage type
Date	Date submitted to providing laboratory
Region	AKL: Auckland; HLZ: Hamilton; NPL: New Plymouth; PMR: Palmerston North; WLG: Wellington
Sex	f: female; fn: female neutered; m: male; mn: male neutered
Breed	DSH: Domestic Short Hair Cat; DMH: Domestic Middle Hair Cat; DLH: Domestic Long Hair Cat; Gold.: Golden; GSH: German Shepherd; Rotti: Rottweiler; SD: Sheepdog; T: Terrier; WHWT: West Highland White Terrier; X: crossbreed
UT-signs?	Signs of urinary tract disease, e.g. pollakiuria, dysuria, stranguria, haematuria present?; ?: unknown
Sample collection	cysto: antepubic cystocentesis; MSU: Mid-stream urine catch
E. coli only?	Was the culture pure (*E. coli* only) or was mixed growth observed?
Urinalysis	bacteria: +: occasional, ++ moderate, +++ numerous; hpf: high power field; RBC: red blood cells; SG: specific gravity; WBC: white blood cells

[a] in alphabetical order

8.4.2. Recipes

8.4.2.1. Basic Reagents

Tris-Cl (1 M, pH 8 or pH 7.5)

To obtain 100 ml of 1 M Tris-Cl, 12.1 g of Tris-base was dissolved in 80 ml of micro-filtered (MQ) water. Following, the buffer was adjusted to pH 8 with ca. 4.0 ml concentrated HCl (Sambrook and Russell, 2001). After an equilibration period of 5 minutes and a final pH check, MQ water was added to a total volume of 100 ml. The solution was autoclaved (liquid cycle) and stored at room temperature until use. 1 M Tris-Cl pH 7.5 was prepared accordingly, with the difference that the pH was adjusted to 7.5 with ca. 6.5 ml HCl (conc; Sambrook and Russell, 2001).

EDTA (0.5 M, pH 8)

To obtain 100 ml of 0.5 M EDTA solution, 18.6 g of Disodium EDTA was dissolved in 70 ml of MQ water. Following, the buffer was adjusted to pH 8 using solid NaOH. After an equilibration period of 5 minutes, MQ water was added to a total volume of 100 ml. The solution was autoclaved (liquid cycle) and stored at room temperature until use.

NaCl (5 M)

To obtain 100 ml of 5 M NaCl, 11.5 g of NaCl were dissolved in 80 ml of MQ water. After equilibration, MQ water was added to a total volume of 100 ml. The solution was autoclaved (liquid cycle) and stored at room temperature until use.

8.4.2.2. Buffer

Tris EDTA (TE) Buffer (10:1, pH 8)

Chemicals/Concentration	Preparation	Ref.
10 mM Tris-HCl, pH 8 1 mM EDTA, pH 8	1 ml of Tris-HCl 200 µl 0.5 M EDTA MQ H2O ad 100 ml Autoclaved (liquid cycle) and stored at 4°C until use	(Alley et al., 2002)

TBE Buffer

Stock solution (5x)	Preparation	Ref.
0.45 M Tris-borate 0.45 M Boric acid 10 mM EDTA	54 g Tris base 27.5 g Boric acid 20 ml 0.5 M EDTA (pH 8) MQ H2O ad 1000 ml Autoclaved (liquid cycle) and stored at room temperature until use	(Sambrook and Russell, 2001)
Working solution (0.5x)	100 ml 5x TBE MQ H2O ad 1000 ml Prepared freshly before each electrophoresis run, pH at room temperature: 8±0.5	

PETT IV Buffer

Chemicals/Concentration	Preparation	Ref.
1 M NaCl 10 mM Tris-Cl, pH 8 10 mM EDTA, pH 8	20 ml 5 M NaCl 1 ml 1 M Tris-HCl, pH 8 2 ml 0.5M EDTA, pH 8 MQ H2O ad 100 ml Autoclaved (liquid cycle) and stored at room temperature until use	(Alley et al., 2002)

Lysis Buffer (PFGE)

Chemicals/Concentration	Preparation	Ref.
50 mM EDTA 50 mM Tris-Cl, pH 8 1% Sodium lauryl sarcosine (SLS) 0.1% Proteinase K (Roche, Mannheim, Germany)	2 ml 0.5 M EDTA 1 ml 1 M Tris-Cl, pH 8 200 mg SLS 20 mg Proteinase K MQ H2O ad 20 ml Stored at -20°C until use	(Alley et al., 2002)

Restriction Buffer (PFGE)

For each plug, the 1.2x restriction buffer was prepared by mixing 12 µl 10x NE buffer 2 (Roche, Mannheim, Germany) with 10 µl 10x bovine serum albumin (New England Biolabs Inc., Ipswich, MA, USA) and 78 µl sterile MQ water (Alley et al., 2002). Fresh buffer was prepared immediately before use.

Cutting Buffer (PFGE)

The cutting buffer consisted of 1x restriction buffer, containing 30 units of the restriction enzyme *Xba*I (Roche, Mannheim, Germany; Alley et al., 2002). For each plug 10 µl 10x NE buffer 2 (Roche, Mannheim, Germany) were mixed with 10 µl 10x bovine serum albumin (New England Biolabs Inc., Ipswich, MA, USA), 1.5 µl *Xba*I (20 U/µl) and 78.5 µl sterile MQ water. Fresh buffer was prepared immediately before use.

SM Buffer (Phage Dilution and Storage)

For 1000 ml SM buffer 58 g NaCl and 2 g MgSO4 x 7 H2O were dissolved in 800 ml MQ water. 50 ml Tris-Cl (1 M, pH 7.5) was added. After an equilibration period during which all ingredients dissolved and diffused completely, MQ water was added to a total volume of 1000 ml (Sambrook and Russell, 2001). The buffer was autoclaved (liquid cycle), dispensed into 50 ml aliquots and stored at room temperature until use. Opened aliquots were discarded after use to avoid contamination with phage.

8.4.2.3. Dyes

5x SDS Dye

Chemicals/Concentration	Preparation	Ref.
1% Sodium dodecyl sulfate (SDS) 0.02% Bromophenol blue 20% Sucrose 5 mM EDTA, pH 8	100 mg SDS 2 mg Bromophenol blue 2 g Sucrose 100 µl 0.5 M EDTA, pH 8 MQ H2O ad 10 ml Stored at room temperature until use	

8.4.3. Primer sequences

8.4.3.1. Multiplex PCR Assay

Set	Gene marker		Sequence	Size of product (bp)
1	Mal X	F R	ggacatcctgttacagcgcgca tcgccaccaatcacagccgaac	925
	PapA	F R	atggcagtggtgtcttttggtg cgtcccaccatacgtgctcttc	717
	FimH	F R	tcgagaacggataagccgtgg gcagtcacctgccctccggta	508
	KpsMT III	F R	tcctcttgctactattcccccct aggcgtatccatccctcctaac	392
	PapEF	F R	gcaacagcaacgctggttgcatcat agagagagccactcttatacggaca	326
	IreA	F R	gatgactcagccacgggtaa ccaggactcacctcacgaat	254
	Ibe10	F R	aggcaggtgtgcgccgcgtac tggtgctccggcaaaccatgc	171
2	Univcnf	F R	atcttatactggatgggatcatcttgg gcagaacgacgttcttcataagtatc	1105
	FyuA	F R	tgattaaccccgcgacgggaa cgcagtaggcacgatgttgta	787
	Ironec	F R	aagtcaaagcaggggttgcccg gacgccgacattaagacgcag	667
	BmaE	F R	atggcgctaacttgccatgctg agggggacatatagccccttc	507
	Sfa/foc	F R	ctccggagaactgggtgcatcttac cggaggagtaattacaaacctggca	410
	AerJ	F R	ggctggacatcatgggaactgg cgtcgggaacgggtagaatcg	302
	PapG III	F R	ggcctgcaatggatttacctgg ccaccaaatgaccatgccagac	258
3	HlyD	F R	ctccggtacgtgaaaaggac gccctgattactgaagcctg	904
	Rfc	F R	atccatcaggaggggactgga aaccataccaaccaatgcgag	788
	OmpT	F R	atctagccgaagaaggaggc cccgggtcatagtgttcatc	559
	PapG I'	F R	ctactatagttcatgctcaggtc cctgcatcctccaccattatcga	479
	PapG I	F R	tcgtgctcaggtccggaattt tggcatcccccaacattatcg	461
	KpsMT II	F R	gcgcatttgctgatactgttg catccagacgataagcatgagca	272
	PapC	F R	gtggcagtatgagtaatgaccgtta atatcctttctgcagggatgcaata	205

Set	Gene marker		Sequence	Size of product (bp)
4	GafD	F R	tgttggaccgtctcagggctc ctcccggaactcgctgttact	952
	CvaC	F R	cacacacaaacgggagctgtt cttcccgcagcatagttccat	679
	H7	F R	acgatgcaggcaacttgacg gggttggtcgttgcagaacc	547
	Cdts	F R	gaaaataaatggaacacacatgtccg aaatctcctgcaatcatccagtta	430
	FocG	F R	cagcacaggcagtggatacga gaatgtcgcctgcccattgct	364
	TraT	F R	ggtgtggtgcgatgagcacag cacggttcagccatccctgag	290
	PapG II	F R	gggatgagcgggcctttgat cgggcccccaagtaactcg	190
5	G allele I	F R	ctgtaattacggaagtgatttctg actatccggctccggataaaccat	1183
	G alleles II/III	F R	ctgtaattacggaagtgatttctg tccagaaatagctcatgtaacccg	1060
	Iha	F R	ctggcggaggctctgagatca tccttaagctcccgcggctga	829
	Afa/dra	F R	ggcagagggccggcaacaggc cccgtaacgcgccagcatctc	594
	Iss	F R	cagcaacccgaaccacttgatg agcattgccagagcggcagaa	323
	SfaS	F R	gtggatacgacgattactgtg ccgccagcattccctgtattc	244
	KpsMT K1	F R	tagcaaacgttctattggtgc catccagacgataagcatgagca	153

8.4.3.2. Bacteriophage Tail Tube Glycoprotein 18

Primer Name	Sequence	Size of product (bp)
FT18-N2	ggtaaattccaatggggtccagctt	1144
FT18-C3	atgttaaacagacgacgaacgttaat	

8.4.4. Suppliers

8.4.4.1. Providers of Bacterial Strains

1. Liz Burrows
   Gribbles Veterinary Pathology, 840 Tremaine Ave, Palmerston North, NZ
2. Karen Cooper
   Gribbles Veterinary Pathology, 35 O'Rorke Rd, Auckland, NZ
3. Graham Young
   Gribbles Veterinary Pathology, 57 Sunshine Ave, Hamilton, NZ
4. Microbiology Unit (arranged by Liz Burrows)
   Gribbles Veterinary Pathology, Farm Rd, Lincoln University, Lincoln, NZ
5. Lynn Rogers and Hamish Mack
   Microbiology Laboratory, IVABS, Massey University, Palmerston North, NZ
6. Professor Jonathan Elliott, Dr. Andrew Rycroft
   Royal Veterinary College,
   Camden Campus, Royal College St, London, NW1 0TU, UK
7. Lynn Rogers
   Floor 1, Clinical Services Block, Palmerston North Hospital,
   50 Ruahine St, Palmerston North, NZ

8.4.4.2. Suppliers of Laboratory Material

Address	Material provided
Oxoid NZ Ltd 3 Atlas Place Mairangi Bay Auckland Ph: +64 9 478 0522	Microbact™ gramnegative identification system, Kirby Bauer Disc Diffusion test system
Merck Ltd 680 Tremaine Ave Palmerston North Ph: +64 800 42 62 52 Fax: +64 6 356 7311	Basic chemicals Dehydrated culture media Polyethylene glycol (MG 8000)
Fort Richards Laboratories Ltd 12 Huia Rd, PO Box 22172 Otahuhu Auckland Ph: +64 9 276 5569 Fax: +64 9 276 9883	Custom culture media (LB agar plates)
Global Science and Technology Ltd. 241 Bush Rd Albany Auckland Tel. 0064 9 443 5867/ 0064 800 734 100 Fax 0064 9 444 7314 www.globalscience.co.nz	Etest® (AB Biodisk, Solna, Sweden) Phase Lock Gel™ Heavy Tubes (Eppendorf South Pacific Pty. Ltd., North Ryde, NSW, Australia) PerfectPrep Gel Cleanup Kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia) Axygen PCR tubes and plates
Invitrogen™ Life Technologies NZ Ltd 18-24 Botha Road Penrose Auckland Ph: +64 800 600 200 Fax: +64 9 5793119 www.invitrogen.com	PCR primers (custom) Taq DNA polymerase Platinum® Pfx DNA polymerase High DNA mass ladder Low DNA mass ladder 100 bp ladder 1 kb plus ladder Acrodisc® syringe filter (Pall Corporation, East Hills, NY, USA) Phenol:chloroform:isoamylalcohol (25:24:1)
Biolab Ltd 244 Bush Rd Albany Auckland Ph: +64 9 980 6700 Fax: +64 9 980 6788 www.biolabgroup.com/nzl	DNeasy Tissue Kit and Qiaquick PCR purification kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia) New England Biolabs Inc. products (bovine serum albumin; PFGE markers) Plasticware (centrifuge tubes, tissue plates)
Bio-Rad Laboratories Pty Ltd. PO Box 571, Albany Auckland New Zealand. Ph: +64 508 805 500 Fax: +64 9 415 2284	Certified molecular biology agarose Low-melt preparative agarose Pulsed Field certified agarose

Address	Material provided
Raylab New Zealand Ltd. 2B, 151 Stoddard Rd Mt. Roskill Auckland Ph: +64 9 620 2176 Fax: +64 9 620 5700 Email: raylockett@raylab.co.nz	Plasticware (centrifuge tubes, tissue plates) Sterile pipette tips
Roche Diagnostics New Zealand Ltd 15 Rakino Way Mt. Wellington Auckland Ph: +64 800 652 634 Fax: +64 9 276 8917 www.roche-applied-science.co.nz	$MgCl_2$ Stock Solution dNTP Set *Xba*I restriction enzyme NE buffer 2

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