Klebsiella pneumoniae in New Zealand sea lions

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Abstract

Klebsiella pneumoniae has been circulating in New Zealand sea lions since the outbreaks during the breeding seasons of 2001/02 and 2002/03 in Sandy Bay, on Enderby Island, Auckland Islands. A large number of pups have since died from *K. pneumoniae* every year during the breeding season. In order to prevent and control this infection, baseline data including bacterial phenotype and genotype, geographic distribution of the pathogen, and the immune response to the pathogen, have to be established.

In this study, hypervirulent (HV) *K. pneumoniae* was isolated from different sources including New Zealand sea lion (NZSL) pups from different breeding sites, and characterised using a combination of biochemical, phenotypic tests, serological analysis and genotyping via whole genome sequencing. Isolates from pups, substrate samples from different breeding sites, a NZSL adult and birds, all had a close genetic relationship. The isolates have the same basic characteristics including a hypermucoviscous phenotype, serotype K2, and sequence type 86. This suggested clonality of this pathogen. The geographic distribution of the pathogen was found to be Enderby Island, Dundas Island, Campbell Island, and the Otago Peninsula (New Zealand mainland).

The isolates analysed were all susceptible to commonly used antibiotics, with the exception of ampicillin. The HV isolates from pups were able to utilise a wide panel of carbon and nitrogen sources and had activity in a wide range of pH from 4.5 to 10, supporting the ability of this pathogen to survive in diverse environments.

The findings in this thesis also suggest that the environment can be a reservoir for a short time period. For the long term, between breeding seasons, New Zealand sea lion adults and birds that live around the breeding site are potential reservoirs.

The HV isolates from pups were resistant to some innate immune responses, including serum killing ability, oxidative killing ability and phagocytosis by neutrophils and monocytes.

Overall, this study provided phenotypic and genotypic information on *K. pneumoniae* isolated from NZSL pups, as well as some information about innate immune responses to this pathogen, which can aid in the prevention and control of this infection.

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Abbreviations

°C	Degrees Celsius
×g	Times gravity
ATCC	American Type Culture Collection
bp	Base pairs
CDS	Coding Sequence
CSF	Cerebrospinal fluid
CFU	Colony forming unit
CI	Confidence interval
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ENA	European Nucleotide Archive
ESR	Institute of Environmental Science and Research
GC	Guanine-cytosine
h	Hour
HMV	Hypermucoviscous (phenotype)
HV	Hypervirulent (strain)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K. pneumoniae	*Klebsiella pneumoniae*
kbp	Kilobase pairs
LPS	Lipopolysaccharide

MAC	Membrane attack complex
mL	Millilitre
MLST	Multilocus sequence type
mm	Millimetre
NCTC	National Collection of Type Cultures (UK)
NGS	Next generation sequencing
NZ	New Zealand
NZSL	New Zealand sea lion
OMP	Outer membrane protein
P	*p*-value
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PM plates	Biolog phenotypic microarray plates
rMLST	Ribosomal multilocus sequence type
SAM	Sequence Alignment Map
SD	Standard deviation
SNP	Single nucleotide polymorphism
ST	Sequence type
Vol	Volume
µl	Microlitre

Publications

Microbiology Resource Announcements: Komkiew Pinpimai, Wendi D. Roe, Patrick J. Biggs, Keren E. Dittmer, Sarah A. Michael. Draft Whole-Genome Sequences of Five *Klebsiella pneumoniae* Isolates from the Subantarctic Islands of New Zealand. Microbiol Resour Announc Nov 2018, 7 (20) e01328-18; DOI: 10.1128/MRA.01328-18

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Glossary of terms as used in this thesis

Strain is an isolate or group of isolates that can be differentiated from other isolates of the same genus and species by either phenotypic or genotypic characteristics or both.

Classical *K. pneumoniae* is an opportunistic pathogen. The infection is most common in immunocompromised patients causing mainly pneumonia and urinary tract infections.

Hypervirulent *K. pneumoniae* can cause infections in heathy people causing liver abscesses with or without septicaemia, occasionally complicated with meningitis and ophthalmitis. Most of the isolates from these infections are hypermucoviscous phenotype.

Hypermucoviscous phenotype is defined as a "string test" positive. A positive string test is defined as the formation of viscous strings that extended vertically for more than 5 mm.

Pair-end refers to the two ends of the same DNA molecule

SAM (Sequence Alignment Map) is a file format to save alignment information of short reads mapped against reference sequences.

Introduction

The New Zealand sea lion (NZSL) is an endangered species endemic to New Zealand (NZ). It has been classified as 'nationally critical' under the NZ threat classification system (Baker et al., 2016) and "endangered" by the International Union for the Conservation of Nature (IUCN, 2015). The main breeding sites of NZSL are limited to Subantarctic areas, the Auckland Islands (Enderby, Dundas, and Figure of Eight Islands) and Campbell Island. In the 2001/02 and 2002/03 breeding seasons at Sandy Bay, Enderby Island, there were mass mortality events in pups caused by *Klebsiella pneumoniae* (Castinel et al., 2007a). After these events, *K. pneumoniae* seemed to be endemic in these animal groups (Roe et al., 2015). A number of pups die every year during the breeding season from *K. pneumoniae* (S. Michael, personal communication), and *K. pneumoniae* isolated from fatal infections had a hypermucoviscous (HMV) phenotype. The HMV phenotype is associated with hypervirulent (HV) strains that cause primary liver abscesses in humans (Shon et al., 2013). Unlike the classical *K. pneumoniae* that causes infection mostly in immunocompromised patients, HV *K. pneumoniae* can cause severe infection in young and healthy people (Paczosa and Mecsas, 2016). HV *K. pneumoniae* has been reported in animals including NZSLs (Roe et al., 2015), California sea lions (Jang et al., 2010), non-human primates (Soto et al., 2012; Twenhafel et al., 2008), birds (Davies et al., 2016), buffalos and cows (Osman et al., 2014).

A better understanding of the pathogen in terms of phenotype and genotype, how long the pathogen can survive in the environment, and the immune response to

the pathogen could help management decisions in controlling NZSL infections. In this study, the author aimed to:

- Define the genotypic and phenotypic characteristics of *K. pneumoniae* isolated from NZSL pups from Enderby Island (Chapter 3, 5)
- Determine whether the *K. pneumoniae* isolates have changed over time (Chapter 3, 5)
- Establish the geographic distribution of *K. pneumoniae* and investigate possible environmental reservoirs (Chapter 4)
- Investigate aspects of the host immune response to *K. pneumoniae* (Chapter 6)

Chapter 1

Literature Review

This chapter provides background information relevant to the study of *K. pneumoniae* in NZSL. The first part of this chapter summarises information on NZSL biology, NZSL breeding sites, and pinnipeds infectious diseases. The second part of this chapter provides information on *K. pneumoniae*: clinical importance in humans and animals, isolation and identification, hypervirulent strains, *K. pneumoniae* classification systems (including next generation sequencing technology), antibiotic resistance, and virulence factors. The second part also provides information on strategies that bacteria use to survive in the environment. The third part of this chapter provides an overview of the immune system, the immune response to extra-cellular bacteria and newborn immunity, and the possible immunisation of *K. pneumoniae.*

1.1 Sea lions

Sea lions are pinniped marine mammals that belong to the Family Otariidae, Subfamily Otariiae. They are categorised into five genera; Genus *Eumetopias* (Steller's sea lion), Genus *Neophoca* (Australian sea lion); Genus *Otaria,* (South American sea lion); Genus *Phocarctos* (New Zealand sea lion); Genus *Zalophus,* (California sea lion, Japanese sea lion, and Galapagos sea lion) (Berta, 2002). They are carnivores and their diet consists mostly of fish and cephalopods such as squid and octopus (Boness, 2009).

1.1.1 New Zealand sea lion

New Zealand sea lions (NZSLs), *Phocarctos hookeri*, are the only species in Genus *Phocarctos.* They are the world's rarest otariid and are endemic to New Zealand (Chilvers and Wilkinson, 2008). The NZSL population is estimated to be around 11,767 (95% CI: 10,790–12,923) (Chilvers and Meyer, 2017). Due to a continued marked decline in number, this species has been classified as 'nationally critical' under the NZ threat classification system (Baker et al., 2016) and "endangered" by the International Union for the Conservation of Nature (IUCN, 2015).

NZSLs have marked dimorphism, with males being larger than females. The adult males are 240–350 cm long and weigh 320–450 kg with a black/brown hair coat. Adult females are 180–200 cm long and weigh 90–165 kg with a cream hair coat (Gales, 2009). At birth the pups are 70–100 cm long and weigh 7–8 kg, with a dark brown hair coat (Gales, 2009). Male pups are born heavier than female pups (Chilvers et al., 2007). The maximum age of NZSL males is 23 years and 18 years for females. Information on the diet of NZSLs is limited. A study of the stomach contents of NZSLs (juveniles, lactating females, nonlactating females and males) caught by the squid fishery in summer/autumn from 1997-2006 in the Auckland Islands area showed that the diet comprises opal fish, rattail fish, arrow squid, octopus and red cod (Meynier et al., 2009).

Males are polygamous, having a harem of 12 to 25 females. NZSL females become sexually mature at 3-4 years old; however, while males mature at 5 years, they do not own their territories until 8-9 years of age (Gales, 2009). The gestation period of NZSLs is 12 months (Gales, 2009). When the pups are 10 – 14 days of age, the female sea lion starts leaving the pup in order to forage at sea, returning every 2-3 days to nurse the pup (Gales, 2009). The pups are nursed for 8-12 months, and they start

swimming at age 2-3 weeks (Gales, 2009).

From archaeological data, NZSLs were historically distributed throughout the coastal New Zealand mainland (Childerhouse and Gales, 1998). Subsistence and commercial hunting are the most likely causes of changes in distribution and number of NZSLs over the past century (Childerhouse and Gales, 1998). Now NZSLs have restricted breeding areas, which are mostly in the Subantarctic islands. Small groups breed on Stewart Island (16-32 pups per year) and the Otago Peninsula (five pups per year), New Zealand (IUCN, 2015; Robertson and Chilvers, 2011). The Subantarctic Auckland Islands and Campbell Island account for 71% and 29% of pup production, respectively (Maloney et al., 2012).

The Auckland islands (50° 45'S, 166° 10'E) are 450 km south of New Zealand. The islands are comprised of a large island surrounded by smaller islands, with the main NZSL breeding areas being on Dundas Island, Sandy Bay on Enderby Island, and on Figure of Eight Island. The temperature in the Auckland Islands ranges from highs of 10 - 20°C in summer, to highs of 4 - 10°C in winter (Peat et al., 2003). The islands are mainly composed of volcanic rock and most of the surface is covered with peat, except the sandhill area behind Sandy Bay on Enderby Island (Leamy and Blakemore, 1960). Streams on Enderby island are slow moving, with the brown-coloured water indicating the presence of organic material moving along with water. Most of the small streams are seasonally temporary (Weller, 1975), drying up over summer. The Auckland Islands have a high rainfall (2000 mm/year) and a high relative humidity (87%). There is a large diversity of birds on Enderby Island, such as Gibson's albatross (*Diomedea antipodensis gibsoni*), yellow-eyed penguins (*Megadyptes antipodes*), Subantarctic skuas

(*Stercorarius antarcticus*) and the Auckland Island shag (*Leucocarbo colensoi*) (Weller, 1975).

Campbell Island (52° 32' S, 169° 08' E) is 700 km south of New Zealand and 270 km southeast of the Auckland Islands. The island is mountainous, rising to over 500 metres. The surface of Campbell Island is covered with peat. Campbell Island has a cold, cloudy, wet and windy climate. The highest recorded temperature is 21.2°C and the lowest is -7.9 °C (https://cliflo.niwa.co.nz/). The average annual rainfall is 1,329 mm/year (https://cliflo.niwa.co.nz/). There is a large diversity of animals on Campbell Island including marine mammals such as elephant seals, NZSLs and fur seals, and birds such as penguin, albatross, giant petrel and mollymawk (Maxwell, 1970).

Outside of the breeding season, male NZSLs are found at haul-out sites in the Snares, Macquarie Island, and the New Zealand mainland. In contrast, female NZSLs mostly travel from beach to beach on the same island (Chilvers and Wilkinson, 2008). The NZSL breeding cycle starts in November, when mature males return to breeding areas and establish their territories (Cawthorn, 1993). Pregnant females arrive in early to mid-December, and form harems that have one dominant male. Pregnant females give birth within a week of landing. The majority of births occur between the 20th and 25th December, with all pups born before the third week of January. Oestrus occurs 7 to 10 days after females NZSLs give birth, therefore mating occurs between mid-December and mid-January. Dominant males leave the breeding sites at the end of January and harems are taken over by subdominant males. Mating does not usually happen in this period (Cawthorn, 1993).

Pup production on the Auckland Islands declined by 50% between 1989 and

2009 (Chilvers, 2010). Epizootic disease has been considered to be a possible contributory cause. Mass mortality in the 2001/02 to 2002/03 seasons caused by infection with *K. pneumoniae* killed up to 36% of pups at Sandy Bay on Enderby Island. Pups had lesions of polyarthritis, peritonitis, cellulitis/dermatitis, and meningitis (Castinel et al., 2007b). After these outbreaks the incidence of *K. pneumoniae* seemed to decline. However, on Enderby Island from 2006 to 2010, 58% of pups died from *K. pneumoniae* infection (Roe et al., 2015). In 2013, a pup from Otago (mainland NZ) also died from *K. pneumoniae* infection. This isolate, as well as *K. pneumoniae* isolates from NZSL pups that died on Enderby Island, presented as hypermucoid colonies on agar plates and had a positive string test, suggesting a hypermucoviscous phenotype (Roe et al., 2015). In humans, the hypermucoviscous phenotype is associated with the virulent form of human *K. pneumoniae* infection, which causes liver abscesses, septicaemia and meningitis (Shon et al., 2013).

The origin of *K. pneumoniae* in NZSLs is unknown. Since adult males move far away from breeding sites outside of the breeding season (Geschke and Chilvers, 2010), they are considered a possible vector, and may carry the pathogen from Otago Peninsula to the Subantarctic. However, there are no reports of HV *K. pneumoniae* being cultured from any cases of human infection in New Zealand.

1.1.2 Sea lion diseases

1.1.2.1 Viral diseases

Herpes viruses have been detected serologically in marine mammals worldwide. In California sea lions, Otarine herpesvirus-1 (OtHV1) is associated with urogenital carcinoma and OtHV3 has been reported to be associated with oesophageal ulcers and

B cell lymphoblastic lymphoma (Venn-Watson et al., 2012), while OtHV2 has been isolated from eye swabs from California sea lions without significant clinical signs (Venn-Watson et al., 2012).

Seal pox, a parapox virus, is a zoonotic disease. It infects pinnipeds, including seals and sea lions, as well as humans. Species susceptible to this infection include grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*), harp seals (*Pagophilus groenlandicus*), northern fur seals (*Callorhinus ursinus*), northern elephant seals (*Mirounga angustirostris*), California sea lions (*Zalophus californianus*), Steller sea lions (*Eumetopias jubatus*), and South American sea lions (*Otaria flavescens*) (Nettleton et al., 1995; Roess et al., 2011; Wilson and Poglayen-Neuwall, 1971). Seal pox causes nodular proliferative skin lesions on the head and flippers. Most infected animals recover within six weeks. It most commonly causes infection in animals less than one year of age that have concurrent disease or immunosuppression. It is transmitted to humans via broken skin (Roess et al., 2011).

San Miguel sea lion virus is a calicivirus that causes disease in sea lions, fur seals and pigs (Gelberg and Lewis, 1982; Smith et al., 1973). In pinnipeds, it is characterised by vesicular skin disease on the flippers. It can be isolated from opal-eye fish (*Girella nigricans*), which are believed to be a reservoir for this virus for marine mammals. The routes of infection include ingestion of infected fish or contaminated water, or direct contact with infected animals (Smith et al., 1973).

1.1.2.2 Bacterial disease

Leptospira spp. have been identified in California sea lions along the Oregon and California coasts, and *Leptospira interrogans* serovar Pomona is considered endemic in

this species (Gulland et al., 1996). The clinical signs of infection include depression, abdominal pain and fever. It may also cause abortion, and haemorrhages in the foetus and neonates (Smith et al., 1974).

Tuberculosis caused by *Mycobacterium* sp. has been reported worldwide in marine mammals (Cousins et al., 2003). It is an important disease due to the zoonotic potential. In the sea lion, *Mycobacterium* spp. have been isolated from NZSLs and Australian sea lions (Cousins et al., 2003; Lenting, 2017). The causal agent has been identified as *Mycobacterium pinnipedii* (Cousins et al., 2003). Mycobacterium causes disease in all ages and the main clinical sign is anorexia. Lung infection is observed at necropsy in most cases, suggesting the route of infection is likely via inhalation (Dunn et al., 2001).

Salmonella spp**.** have been isolated from otariids from several parts of the world. They have been isolated from healthy pups of the northern fur seal and California sea lion populations on San Miguel Island in California (Gilmartin, 1979). However, salmonellosis has also caused significant mortality in northern fur seal pups in Alaska (Jellison and Milner, 1958). *Salmonella* spp. generally cause gastrointestinal diseases in pinnipeds, characterized by lethargy, diarrhoea and haemorrhagic enteritis (Dunn et al., 2001)

Campylobacter spp. have been detected in stranded pinnipeds (Broman et al., 2000; Foster et al., 2004). In 1997/1998, *Campylobacter* spp. were suspected to be a cause of mass mortality in NZSLs on the Auckland Islands. The lesions in these cases were indicative of acute septicaemia, with necrotising vasculitis and haemorrhagic pneumonia. Similar lesions were also found in New Zealand fur seals a year later

(Duignan, 1998). However, confirmation of Campylobacter by culture or PCR has not been done.

Klebsiella spp. are commonly isolated from internal tissues of marine mammals (Baker and McCann, 1989; Hernández-Castro et al., 2005; Stroud, 1979; Vedros et al., 1982) and can also be found in the environment (Podschun and Ullmann, 1998). *Klebsiella pneumoniae* has been reported in California sea lions where it caused suppurative pneumonia, pleuritis and abscesses (Jang et al., 2010). In the 2001/2002 breeding season, *K. pneumoniae* caused marked mortality in NZSL pups, with lesions of polyarthritis, peritonitis, cellulitis/dermatitis and meningitis (Castinel et al., 2007b).

1.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a gram-negative, encapsulated, non-motile, lactose fermenting, facultative, rod shaped bacterium belonging to the genus *Klebsiella* and the family Enterobacteriaceae. *K. pneumoniae* is ubiquitous in nature. It can be found in environmental samples such as soil, plants, surface water and on medical devices, as well as on mucosal surfaces in humans and animals (Podschun and Ullmann, 1998).

K. pneumoniae has received a great deal of interest due to its ability to acquire multiple antibiotic resistance genes, as well as an increase in the prevalence of hypervirulent (HV) strains over the past few decades. Initially, *K. pneumoniae* was recognised as an opportunistic pathogen (classical strains) in hospital-acquired infections, where it causes infection of surgical sites as well as the lungs and urinary tract (Podschun and Ullmann, 1998; Shon et al., 2013). It is also responsible for pneumonia in community-acquired infections, particularly in alcoholic and diabetic patients (Carpenter, 1990). In the 1980s, a new invasive form (HV strain) of *K.*

pneumoniae infection emerged in Taiwan and spread to other parts of the world. It caused liver abscesses with septicaemia, occasionally complicated by meningitis and endophthalmitis (Fang et al., 2004; Shon et al., 2013). Although half of the patients with invasive infections are alcoholics or have diabetes mellitus, many patients infected by HV strains are young and healthy without a history of underlying disease (Paczosa and Mecsas, 2016; Shon et al., 2013), suggesting that these strains are primary pathogens. Most of the isolates, but not all, from invasive infections have the hypermucoviscous (HMV) phenotype (Fang et al., 2004; Shon et al., 2013).

At present, there are 20 species (including subspecies) in the genus *Klebsiella* (Brisse et al., 2006). *K. pneumoniae* contains three subspecies based on the clinical features of the disease each causes: *Klebsiella pneumoniae* subspecies *pneumoniae*, the type strain of which is ATCC 13883*; Klebsiella pneumoniae* subspecies *ozaenae,* with the type strain ATCC 11296; and *Klebsiella pneumoniae* subspecies *rhinoscleromatis,* the type strain of which is ATCC 13884 (Brisse and Verhoef, 2001). *K. pneumoniae* subsp. *ozaenae and K. pneumoniae* subsp. *rhinoscleromatis* are biotypes of *K. pneumoniae* subsp. *pneumoniae,* but have a reduced capacity to metabolise different substrates. There is no substrate utilised by subspecies *ozaenae* and *rhinoscleromatis* that is not utilised by subspecies *pneumoniae,* but the converse is not true (Brisse et al., 2006). Without adding pyrroloquinoline quinone, *K. pneumoniae* subsp. *pneumoniae* is able to oxidise glucose to gluconate, which is a unique ability of *K. pneumoniae* subsp. *pneumoniae* (Bouvet et al., 1989).

Based on *gry*A and *par*C gene analysis, *K. pneumoniae* can be categorised into three groups KpI, KpII, and KpIII. *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae* are in the KpI group, while *K. pneumoniae* subsp.

pneumoniae are in KpII or KpIII (Brisse and Verhoef, 2001). The genetic differences between the KpI, KpII, and KpIII groups are supported by differences in biochemical reactions (Brisse and Verhoef, 2001). For example, the KpII and KpIII groups are lactose and lysine decarboxylase-positive while *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* in KpI are not (Brisse and Verhoef, 2001; Farmer et al., 1985). Moreover, all strains in KpIII and half of KpII are unable to metabolise adonitol, while KpI are able to (Brisse and Verhoef, 2001).

1.2.1 Isolation and identification.

K. pneumoniae grow on normal agar such as nutrient agar, tryptic soy agar and blood agar, as well as differential media for *Enterobacteriaceae* such as MacConkey agar and eosin-methylene blue agar (EMB) (Brisse et al., 2006). *K. pneumoniae* colonies are dome-shaped and mucoid in appearance, and 3 - 4 mm in diameter after overnight incubation at 30°C or 37°C. Depending on strain and media composition, the colony may be sticky (Brisse et al., 2006). Most bacteria in this genus produce lysine decarboxylase, but not ornithine decarboxylase, and are positive on the Voges-Proskauer test (Edwards and Ewing, 1972). However, identification to species level in the *Klebsiella* genus is difficult using biochemical testing, as a number of biochemical tests have to be used and several species share a similar biochemical profile, with several exceptions in each species (Brisse et al., 2006).

Molecular methods have been applied to identify *Klebsiella* species. Unlike other bacteria, the 16S rRNA gene sequence is not useful for species identification in *Klebsiella* spp., as there is limited nucleotide variation between two species in this genus (Brisse and Verhoef, 2001). Identification and phylogenetic grouping based on housekeeping genes such as combination of *gryA* and *parC* (Brisse and Verhoef, 2001)

and *rpoB* (Drancourt et al., 2001) is more reliable. In order to identify *K. oxytoca*, the *pehX* gene involved in pectin degradation can be used as a target gene for a PCR assay (Kovtunovych et al., 2003). For *K. pneumoniae,* several other genes have been used to identify this species, such as *pho*E (Shannon et al., 2007), and *khe* (Babu et al., 2013). The *khe* gene has been used to differentiate between bacteria in the Enterobacteriaceae family (Babu et al., 2013) as well as in identification of *K. pneumoniae* in non-human primates (Soto et al., 2012; Soto et al., 2016; Whitehouse et al., 2010).

1.2.2 Hypervirulent (HV) *Klebsiella pneumoniae*

Most hypervirulent *K. pneumoniae* have a characteristic known as a hypermucoviscous (HMV) phenotype (discussed below). Many studies in the past named hypervirulent *K. pneumoniae* as hypermucoviscous *K. pneumoniae.* However, not all HMV *K. pneumoniae* cause invasive infection and not all hypervirulent strains are HMV phenotype (Catalán-Nájera et al., 2017). Hypervirulent (HV) *K. pneumoniae* has been recognised as a virulent strain causing invasive community-acquired infections (Shon et al., 2013). The invasive forms of *K. pneumoniae* infection have been reported to cause primary liver, prostate, bone, kidney, and lung abscesses or necrotising fasciitis with septicaemia and spread to other organs (Catalán-Nájera et al., 2017; Chiu et al., 1988; Fang et al., 2004; Keller et al., 2013; Lee et al., 2006; Lim et al., 2002; Liu et al., 2003; Pomakova et al., 2012). The majority of affected patients have liver abscesses with septicaemia, occasionally complicated with neuro-invasion such as meningitis and endophthalmitis (Fang et al., 2004; Shon et al., 2013). The invasive form of *K. pneumoniae* community acquired infection was first reported in Asia, followed by reports of a similar syndrome in other parts of the world including North America,

South America, the Caribbean, Europe, the Middle East, Australia, Africa and South Africa (Fang et al., 2004; Shon et al., 2013), but not NZ. The majority of HV isolates are serotypes K1 or K2, with fewer cases being K5 or non-K serotypes (Paczosa and Mecsas, 2016; Shon et al., 2013).

On an agar plate, most HV *K. pneumoniae* isolates appear as dome-shaped colonies with a hypermucoid appearance and are characterised by a positive string test, which is indicative of the hypermucoviscous phenotype (Fang et al., 2004). The string test is conducted by touching a standard bacterial loop to the surface of the colony and pulling it vertically. The test is positive when the bacterium can produce a string more than 5 mm in length (Fang et al., 2004; Russo et al., 2011). However, not all hypermucoid colonies are postitive on string test.

HV *K. pneumoniae* has been reported to be more resistant to innate immune responses including antimicrobial peptides, complement cascade and phagocytosis, compared to classical strains (Cox et al., 2015; Fang et al., 2004; Soto et al., 2016). This enhances the ability of the pathogen to infect hosts and to spread to other organs from primary infection sites.

HV strains of *K. pneumoniae* have significantly higher amounts of sialic acid than classical strains in their capsules (Lee et al., 2014). Sialic acid (Sia), also known as N-acetylneuraminic acid, is a monosaccharide that can be found on the surface of human cells, as well as on the cell surface of particular bacteria (Angata and Varki, 2002). Sia-binding receptors are highly expressed on immune cells (Varki and Angata, 2006). These receptors recognise sialic acid and send signals to inhibit inflammatory activity (Carlin et al., 2007). Bacterial sialic acid mimics human cell surface sialic acid to reduce

inflammatory responses (Carlin et al., 2007), including inhibition of neutrophil phagocytic activity (Lee et al., 2014). Bacterial sialic acid can also inhibit the activation of the complement alternative pathway by binding to factor H to prevent the binding of C3bi to the bacterial cell surface (Marques et al., 1992).

Most HV isolates possess either *rmpA* or *rmpA2* (regulator of mucoid gene A) genes (Catalán-Nájera et al., 2017; Paczosa and Mecsas, 2016; Shon et al., 2013). There are three variants of *rmpA* . *RmpA and rmpA2* (p-*rmpA*, p-*rmpA2*) are on plasmids while c-*rmpA* is on the chromosome (Hsu et al., 2011). *RmpA2* is an isoform of *rmpA* and shares 80% identity with *rmpA* (Wacharotayankun et al., 1993). In serotype K2 strains, *rmpA/A2,* but not c-*rmpA,* regulate capsular polysaccharide biosynthesis, while in *K. pneumoniae* NTUH-K2044 (serotype K1), only p-*rmpA* regulates capsular polysaccharide biosynthesis (Cheng et al., 2010; Hsu et al., 2011; Lai et al., 2003; Wacharotayankun et al., 1993). This suggests that *rmpA/A2* may work in a different way in each serotype.

In *K. pneumoniae,* the primary structure and biosynthesis mechanism of capsular polysaccharide is similar to group I capsular polysaccharide in *E. coli,* which is conducted by the Rcs phosphorelay system (Whitfield and Roberts, 1999). The Rcs system regulates transcription of the genes in capsular polysaccharide biosynthesis, and was first identified in *E. coli*. This system is found in other bacteria such as *Salmonella*, *Klebsiella*, *Shigella, Erwinia carotovora*, *Pantoea stewartii*, and *Proteus mirabilis* (Majdalani and Gottesman, 2005). In this system, *rcsC* acts as a sensor kinase, which is activated by autophosphorylation after a stimulation signal from the external environment. The signal then transfers to *rcsD,* which phosphorylates *rcsB,* which in turn interacts with *rcsA*. Then *rcsA* binds to capsular polysaccharide promotors, which activate capsule biosynthesis (Majdalani and Gottesman, 2005). *RmpA/A2* functions in

a similar way to *rcsA* in *E. coli*. *RmpA/A2* act as positive regulators for capsular polysaccharide biosynthesis (Nassrf et al., 1989). They code for the counterparts of the *RcsA*. The critical difference between *rmpA/A2* and *rcsA* is that *rmpA* is active at 37°C, but *rcsA* is degraded at this temperature by Lon protease, a mitochondrial ATP-dependent protease that plays multiple roles in bacteria including the production of polysaccharide (Goldberg et al., 1994). Therefore, with *rcsA* "extra-capsule" is produced only at low temperatures. This suggests that *rmpA/A2* positive *K. pneumoniae* that infect a host species in which the body temperature is 37°C, are able to produce more capsule, and are thus more virulent.

In *K. pneumoniae* CG43, *Fur*, a ferric uptake regulator protein that controls the intracellular concentration of iron in many bacteria, regulates capsular polysaccharide (CPS) synthesis via *rcsA* and *rmpA* but not *rmpA2* in an Fe(II)-dependent manner (Lin et al., 2011). This further highlights the different roles *rmpA2* has in capsular polysaccharide synthesis. *Fur* downregulates the expression of *rcsA* and *rmpA* when there are high levels of available iron (Lin et al., 2011), suggesting that available iron affects capsular polysaccharide synthesis.

HV strains are highly associated with the presence of large plasmids such as pK2044, pLVPK, pK2044-like and pLVPK-like (Struve et al., 2015). These plasmids possess *rmpA/A2* genes, which are associated with the hypermucoviscous phenotype, plus other virulence genes including iron-scavenging genes such as salmochelin and aerobactin (Chen et al., 2004). As iron is an essential element for bacterial growth, limited availability of iron in the host's body acts as a non-specific immune defence mechanism. Generally, the little available iron in plasma is bound to proteins such as transferrin. Therefore, in order to salvage iron from the host, bacteria secrete

siderophores, molecules that possess a higher affinity for iron than host transport proteins. *K. pneumoniae* can produce several siderophores, which vary in expression and virulence. Enterobactin is a common siderophore secreted by *K. pneumoniae* (Holt et al., 2015), and is encoded in the core genome. As enterobactin is a common siderophore, the host's immune system often develops strategies to prevent iron scavenging by the bacteria. For example, lipocalin-2, a siderophore-binding antimicrobial protein, is released during *K. pneumoniae* infection by several types of host cell including neutrophils (Chan et al., 2009) and binds enterobactin to inhibit bacterial growth (Goetz et al., 2002). Possession of additional siderophores such as salmochelin and aerobactin may enhance the virulence of HV strains compared with classical strains since they are able to scavenge iron more than classical strains (Paczosa and Mecsas, 2016; Russo et al., 2015).

1.2.3 Klebsiella typing systems

Klebsiella spp. show diversity at both the phenotype and genotype levels. Several methods, including phenotypic and genotypic approaches, have been applied to type this species in order to monitor the spread of pathogenic and multidrug resistant strains, with each typing method having its own strengths and weakness. In this section, I aim to review typing methods that have been regularly applied to *Klebsiella* spp., and discuss new methods that may provide more epidemiological information.

1.2.3.1 Serotyping

Serotyping is based on the reaction of antigens on the capsular surface of bacteria. Like other gram-negative bacteria, *K. pneumoniae* has a cell envelope, consisting of a thin layer of peptidoglycan surrounded by an outer membrane, and an

inner membrane (cytoplasmic membrane), which is a phospholipid bilayer that acts as a permeability barrier (Podschun and Ullmann, 1998). The inner side of the outer membrane is also phospholipid, but the outer side is mainly composed of lipopolysaccharide (LPS). LPS is also known as endotoxin and consists of three parts: lipid A, a core oligosaccharide and O antigen (Rosenfeld and Shai, 2006). Lipid A is responsible for toxicity, and for stimulating the host immune system via Toll-like receptor 4 (Clements et al., 2008). The core oligosaccharide is a linking structure between O antigen and lipid A. The O antigen consists of a polymer of oligosaccharide repeating units. There are 12 O antigens in *Klebsiella* spp., of which O1 antigen is most prevalent in human clinical isolates (Hsieh et al., 2012; Podschun and Ullmann, 1998). Outside the cell envelope, *K. pneumoniae* is covered by an acid polysaccharide capsule, which is responsible for the mucoid appearance. There are at least 79 capsular serotypes recognized to date (Pan et al., 2013). Since the determination of O antigen is hampered by the heat-stable capsule, it is difficult to classify *Klebsiella spp*., and the K antigen is used for serotyping. The traditional serological method allows individual sera to be absorbed with the cross-reacting K-antigens. Recently, a molecular method has been developed, where K antigen type is determined by sequencing the *wzi or wzc* locus, the genes of which are present on the capsule of *K. pneumoniae* (Brisse et al., 2013; Pan et al., 2013). Serotyping can also be done via whole genome sequencing (Wyres et al., 2015).

1.2.3.2 Biolog© phenotype microarrays

The Biolog© phenotype microarray system (Biolog, Hayward, CA, USA) is a system that was developed to determine the phenotype of bacteria by using a series of plates that contain different elements (Bochner, 2009). This system approaches

phenotype expression by using two basic properties of bacteria: utilisation of basic elements that are essential for growth, such as carbon and nitrogen sources, and survival under different conditions such as pressure, chemicals and temperature (Bochner, 2009). Instead of measuring bacterial growth, the Biolog© system measures cell respiration via tetrazolium dye reduction. A phenotype microarray plate is used, with 96 wells containing different environments for the bacteria. For example, PM1 plate has 95 wells containing different carbon sources and a negative control well. If the bacteria can metabolise the carbon source supplied in the well, the electron flow will lead to reduction of tetrazolium dye to produce a purple colour (Bochner and Savageau, 1977). This can be read by a spectrophotometer or Omnilog plate reader. The Omnilog plate reader is an incubator that has a charge-coupled device (CCD) camera to spectrophotometrically record the dye colour change in each well on a plate every 15 minutes. The Omnilog plate reader comes with software to record and analyse the colour change of each well, and produces data which can be further analysed using the opm package (Vaas et al., 2013) in RStudio program (RStudio Team, 2015).

The Biolog© phenotype microarray system has been previously used to investigate *Klebsiella spp.* Liao et al. (2001) conducted a study with *K. pneumoniae* MGH 78578, which demonstrated this isolate's ability to utilise a wide panel of carbon, nitrogen, sulphur, and phosphate sources. Blin et al. (2017) used Biolog© plates, PM1 (carbon sources), PM2A (carbon sources), and PM3 (nitrogen sources) to study the metabolic diversity of *Klebsiella* spp. from different sources. The results from this study suggested these isolates had the ability to metabolise a wide panel of carbon and nitrogen sources, with each isolate having a few different types of carbon and nitrogen sources that they could utilise. In addition, this study suggested that the ability to utilise

D- arabinose was more frequently found in HV strains. Taken together, the results of both the Liao and Blin studies show that *K. pneumoniae* isolates were able to utilise a wide panel of nutrients, which explains why *K. pneumoniae* can be found in diverse environments.

1.2.3.3 Multi-locus sequence typing (MLST)

Multilocus sequence type (MLST) is a typing method using DNA sequences of internal fragments of housekeeping genes (Ibarz Pavón and Maiden, 2009). MLST characterises strains by using allelic profiles of conserved housekeeping genes (normally seven). For each housekeeping gene, the sequence is assigned a unique number, combined into an allelic profile, and assigned a sequence type (ST). ST can be further analysed using eBURST (http:// eburst.mlst.net/) which categorises closely related STs, defined as clonal complexes (CCs). The allelic profiles can be compared with data available online. The allelic profiles can be obtained from DNA extracted directly from field materials, such as blood and CSF, as well as from bacteria that cannot be cultured, by PCR and sequencing seven housekeeping genes. MLST has been successfully applied to many bacterial species. However, in some bacteria, such as *Mycobacterium tuberculosis* and *Yersinia pestis* that have little variation in their genome, MLST-based typing is relatively uninformative (Ibarz Pavón and Maiden, 2009).

The *K. pneumoniae* MLST scheme was set up in 2005 using seven housekeeping genes including *mdh*, *infB*, *tonB*, *gapA*, *phoE*, *pgi*, and *rpoB* (Diancourt et al., 2005). The database is available at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html. It has been widely used to investigate local outbreaks and global dissemination patterns. STs can also be used to assess antimicrobial resistance. For example, most ST258 isolates are extended spectrum beta-lactamases (ESBL) producers (Paczosa and Mecsas, 2016).

1.2.3.4 Whole genome sequence typing

Comparative bacterial genomics may be used to identify virulence factors. Several draft and complete *K. pneumoniae* genomes have been published (Conlan et al., 2016; Liu et al., 2012; Wang et al., 2018; Wu et al., 2009a). The genome of *K. pneumoniae* ranges in size from 5.3 to 5.6 Mb (Holt et al., 2015). The first genome of *K. pneumoniae,* strain MGH 78578, was partly sequenced by the Washington University Genome Sequencing Centre using the whole genome shotgun approach (McClelland et al., 2000). This strain was isolated from the sputum of a 66-year-old male patient in 1994. It was compared to the *E. coli* K12 genome, which revealed 2423 common genes with an average of 82% nucleotide similarity (McClelland et al., 2000) Later, several clinical and environmental isolates were whole genome sequenced, including the hypermucoviscous phenotype *K. pneumoniae* strain NTUH-K204. A comparison among the clinical and environmental isolates of K. *pneumoniae* Kp13, MGH78578, Kp424, and NTUH-K2044 showed a conserved genome comprising 4,269 coding DNA sequences (CDSs) (Ramos et al., 2014). Another comparative genomics study between multi-drug resistant strains of multilocus sequence type (ST) 258 showed that ST258 isolates are two distinct genetic clades which is the result from an ~215 kbp region that have a number of SNPs (Deleo et al., 2014).

Although analysis of the genome of *K. pneumoniae* enables us to examine a number of features, such as the evolution of the bacterium over time, the presence of drug resistance genes, and the presence of virulence factors that may help explain the pathogenesis of the disease it causes, at present this method is not commonly used to type this organism. This is largely due to cost (genome analysis is still more expensive

when compared with other methods), as well as the bioinformatic skills that are required for analysis whole genome data.

1.2.3.4.1 Whole genome sequencing technology

Sequencing technology can be divided into first generation sequencing, second generation sequencing, known as next generation sequencing (NGS), and third generation sequencing. First generation sequencing, also known as Sanger sequencing or dideoxy DNA sequencing or chain termination, relies on dideoxynucleotides (ddNTP), a type of deoxynucleoside triphosphate (dNTP) that lack a 3′ hydroxyl group and have a hydrogen atom instead (Sanger et al., 1977). A primer is annealed to a single stranded DNA template, and then a DNA polymerase extends the primer using ddNTPs. The fragments are separated on polyacrylamide gel by electrophoresis (Sanger et al., 1977). The Sanger method is still used and is the gold standard for whole genome sequencing, but is time-consuming and expensive, even when automated. NGS and third generation sequencing are considered to be the best tools for whole genome sequencing. The difference between NGS and third generation is that for NGS, the DNA fragments have to be amplified before sequencing, but for third generation sequencing, single molecules can be directly sequenced (single molecule sequencing) (Bleidorn, 2016). In this review, the author will focus on NGS technology, which was used in this study. NGS can be done via several platforms, and each platform has advantages and limitations. NGS is based on two different sequencing chemistry processes: sequencing-by-synthesis and sequencing-by-ligation (Liu et al., 2012). Generally, the process of NGS involves breaking genomic DNA into small fragments (the length of the fragment depends on type of platform), then the DNA fragments are ligated with adapters that contain amplification and sequencing primers. After ligation, the DNA is amplified and

sequenced. NGS platforms include Roche 454 Pyrosequencer, Illumina, ABI/SOLiD, and Ion Torrent.

Roche 454 (Hoffmann-La Roche; Basel, Switzerland)

This platform uses sequencing by synthesis. It is a non-electrophoretic method that detects the release of pyrophosphate during nucleotide incorporation (Liu et al., 2012). This platform uses emulsion polymerase chain reaction to amplify the fragments and then pyrosequence them. The advantage of this platform is the speed (10 h from the start until finish) (Buermans and den Dunnen, 2014). The GS FLX Titanium series can generate 400-600 million base pairs per run with 400-500 base pair read lengths (Liu et al., 2012). However, the high cost of the reagents and a high error rate (Liu et al., 2012) are issues in this platform.

Ion Torrent (https://www.thermofisher.com/us/en/home/brands/ion-torrent.html)

This platform uses sequencing by synthesis. It is based on detection of H^+ that are released during the polymerisation of DNA. The length of DNA fragments used in this method is ~200 bp. One molecule of DNA is placed on a bead and adapter is added. The DNA is amplified by emulsion PCR. Then, each bead is placed into a single well of a slide. The slide is flooded with one dNTP, along with buffers and polymerase. In each well, the pH is determined by detecting the released H^+ ions. The change of the pH is used to determine the presence and quantity of that base.

ABI/SOLiD (van Dijk et al., 2014)

This platform is based on ligation sequencing. It uses DNA ligase for sequencing. This platform was developed in 2007 and works on the principle of genomic library

construction and ligation followed by sequencing. It uses DNA ligase for sequencing rather than DNA polymerase. The genome to be sequenced is randomly fragmented and then ligated to the adapter molecules; the adapter-attached molecules are then attached to agarose beads. The bead-captured DNA molecules are amplified using an oil-emulsion PCR. The amplified bead-captured DNA is anchored to a glass slide and flooded with fluorescent-labelled oligonucleotides. If there is complementarity between the template and the oligonucleotide, it is ligated and then two bases are detected at a time. Then the oligonucleotide is cleaved and the next round of ligation commences. Each time two new nucleotides are detected. The read length of this technique is 25 to 35; approximately 40 million beads can be sequenced. The sequencing output of this method is 2 to 4 Gb. Since each base is identified twice, the accuracy of this method is high.

Illumina (Solexa) (https://sapac.illumina.com/systems.html)

The Illumina platform is based on sequencing by synthesis. It uses bridge amplification. All enzymatic processes and imaging steps occur in a flow cell, which, depending on the specific Illumina platform may be partitioned into one (MiSeq), two (HiSeq2500) or eight (HiSeq2000, HiSeq2500) separate lanes. Firstly, the sample must be cleaved into short sections. The length of these sections will depend on the particular machine. The DNA fragments are ligated to adaptors and annealed to a slide using the adaptors. The DNA fragments are attached at one end on the surface, then bridge amplification is generated by hybridising the free end to other adapters on the surface. Subsequently, an isothermal amplification process results in a cluster of identical fragments that are then denatured for sequencing primer annealing. Amplified DNA fragments are subjected to sequencing-by-synthesis using 3′ end labelled

nucleotides. These nucleotides are fluorescently labelled with different colour dyes corresponding to the base. This system generates at least 3 Gb of data in a paired-end run (Ansorge, 2009).

1.2.4 Antimicrobial resistance

K. pneumoniae is receiving increased attention due to many reports of multiple antimicrobial drug resistant strains, particularly in classical *K. pneumoniae.* Most HV strains are susceptible to most antibiotics; recently however, resistant HV strains have also been reported (Li et al., 2013). This suggests that the treatment of HV *K. pneumoniae* infections may become more complicated in the future, if antimicrobrial resistant strains become more prevalent.

There are five important groups of antibiotics that *K. pneumoniae* has resistance to, including beta-lactams, aminoglycosides, quinolones, tigecycline and polymyxins (Navon-Venezia et al., 2017), with the most common one being the beta-lactam group. *K. pneumoniae* is inherently resistant to ampicillin due to a chromosomal gene, bla_{SHV} (Brisse et al., 2006; Chaves et al., 2001). In addition, *fosA* and *oqxAB* genes that result in low-level resistance to fosfomycin and quinolone have been found in the *K. pneumoniae* chromosome (Holt et al., 2015). However, most antimicrobial resistance (AMR) in *K. pneumoniae* is a result of horizontal gene transfer mainly via plasmids (Iredell et al., 2016). In *K. pneumoniae*, carrying more than one plasmid is not unusual, hence multidrug resistant strains are expected in this species. This has been shown in a number of reports of multidrug resistant strains in *K. pneumoniae* (Molton et al., 2013).

In beta-lactam resistance, two main mechanisms have been observed in *K. pneumoniae*. The first one involves production of extended spectrum beta-lactamases

(ESBLs) leading to resistance to cephalosporins and monobactams (Gupta et al., 2003). Many types of ESBLs have been reported and at least 50 variants have been documented in *K. pneumoniae* (Brisse et al., 2006), which differ geographically (Drawz and Bonomo, 2010). The most common types are SHV, TEM, and CTX. TEM and SHV types result from mutations in genes encoding normal plasmid-mediated SHV or TEM, changing amino acids that lead to changes in the active site of these enzymes (Brisse et al., 2006; Drawz and Bonomo, 2010; Falagas and Karageorgopoulos, 2009). In contrast, CTX-M ESBLs are encoded from plasmid genes that are transferred from chromosomal genes of environmental bacteria such as *Kluyvera* spp. (Drawz and Bonomo, 2010; Falagas and Karageorgopoulos, 2009). The CTX-M ESBLs are different from SHV or TEM ESBLs: they are more active against cefotaxime than other oxyimino-beta-lactam substrates (Brisse et al., 2006), while TEM and SHV ESBLs are more active against ceftazidime than cefotaxime. ESBL resistance has been reported in HV strains from China, most of which were CTX-M type (Zhang et al., 2016). Interestingly, CTX-M are the most common ESBL type in China in classical *K. pneumoniae* (Yu et al., 2007b), suggesting mobile genetic transfer between classical and HV *K. pneumoniae*. AmpC beta-lactamase is another ESBL type that has reported in *K. pneumoniae* (Brisse et al., 2006; Gupta et al., 2012). They are chromosomally encoded in some bacteria such as *Enterobacter* spp. and *Citrobacter* spp., but are plasmid encoded in *K. pneumoniae*. Strains that produce AmpC beta-lactamase are resistant to penicillin, cephalosporins in the oxyimino group (cefotaxime, ceftazidime, ceftriaxone), 7-alpha methoxy group (cefoxitin or cefotetan), and beta-lactamase inhibitors including clavulanate, sulbactam, tazobactam (Gupta et al., 2012). AmpC *K. pneumoniae* are generally susceptible to cefepime or the carbapenems; however, some strains that have lost outer membrane proteins can be resistant to cefepime or carbapenem (Shi et al., 2013).

Another mechanism of AMR involves production of carbapenemases, which lead to resistance to all beta lactams including the carbapenems. There are many types of carbapenem resistance that have been reported in *K. pneumoniae* such as KPC, NMD-1, MBL, IMP, OXA-48 and VIN (Yigit et al., 2008; Yong et al., 2009). The most prevalent type is KPC (Mathers et al., 2015). To date, at least 16 variants have been identified within the KPC type (Wang et al., 2014). The KPC is located in mobile transposon Tn*4401* and is present in many plasmids. The KPC resistance type is associated with ST258, and ST512 (ST258 derivative), but it has been widely reported in other strains as well. In contrast with classical strains, the ST258 clone is not the predominant clone of KPC *K. pneumoniae* in HV *K. pneumoniae* (Lee et al., 2017). It is notable that carbapenem resistance can be present without carriage of carbapenem resistance genes, as a consequence of altered cell permeability due to porin loss and overexpression of efflux pumps (Padilla et al., 2010; van de Klundert et al., 1988).

1.2.5 Virulence factors

1.2.5.1 Adhesins

Adhesins are protein structures on bacteria that mediate adhesion between bacteria and host cells, allowing infection. Each bacterium uses different types of adhesins to facilitate adhesion. For most bacteria in the *Enterobacteriaceae* family, fimbriae function as adhesins. *K. pneumoniae* produces two main types of fimbriae: type 1 fimbriae, a mannose-sensitive haemagglutinin, and type 3 fimbriae, a mannose-resistant hemagglutinin. Type 1 fimbriae consist of a protein block (Fim A) and an adhesin (Fim H) at the tip of the organelle (Schembri et al., 2005), and are generally found in *Enterobacteriaceae* species. Type 1 fimbriae have been established as an important virulence factor in urinary tract infections caused by *E. coli* and *K.*

pneumoniae (Connell et al., 1996; Struve et al., 2008). Type 1 fimbriae in *K. pneumoniae* are closely related to *E. coli* in terms of genetics and regulation (Struve et al., 2008). The expression of type 1 fimbriae is phase variable and can be switched off (Bjarke Olsen and Klemm, 1994), which may help bacteria to evade the host immune system in the later stage of infection as they can be recognized by leukocytes (Brisse et al., 2006). The host environment also affects type 1 fimbriae expression. During colonization and infection of the intestine and lungs, the *fim* genes were found to be unexpressed, but found to be expressed in the urinary tract (Struve et al., 2008).

Type 3 fimbriae are mannose-resistant and agglutinate only tannin-treated ox erythrocytes (Brisse et al., 2006; Podschun and Ullmann, 1998). They are encoded by a cluster of *mrk* genes, including the *mrk*A gene that encode the major fimbriae subunit and *mrk*D that encodes fimbrial adhesion (Struve et al., 2009). Type 3 fimbriae are present in most clinical isolates. They mediate binding of bacteria to human endothelial cells, epithelial cells of the respiratory tract, uroepithelial cells, and type V collagen (Brisse et al., 2006).

1.2.5.2 Capsular Polysaccharides

Klebsiella spp. have a prominent capsule (known as K antigen) consisting of acidic polysaccharide which is composed of four to six sugar repeat units. Non-carbohydrate groups may also exist in some capsular serotypes (Brisse et al., 2006). The capsule is responsible for protecting bacteria from phagocytosis by polymorphonuclear leukocytes, as well as preventing killing by serum factors (Podschun and Ullmann, 1998). In HV strains, most of them are HMV phenotype, which have extra capsule that may enhance the virulence of these strains. At least 79 capsular serotypes have been recognised so far (Pan et al., 2013), with K1 and K2 being the most common serotypes

present in human isolates (Mizuta et al., 1983; Shon et al., 2013). Different capsular serotypes show different degrees of virulence, and this may relate to the mannose content of the capsular polysaccharide. K7 and K21a serotype, have a mannose-α-2/3-mannose or L-rhamnose-α-2/3-L-rhamnose structure that is recognized by a lectin on macrophages. K2 does not contain this structure (Kabha et al., 1995; Ofek et al., 1993; Podschun and Ullmann, 1992), suggesting that capsular serotypes free from these structures should be more virulent since they are able to evade the host immune system.

1.2.5.3 Siderophores

Iron is an essential element for bacteria for several metabolic processes, as well as DNA synthesis. Bacteria obtain iron from their immediate environment, which is available in two forms: Fe^{2+} and Fe^{3+}. Fe^{2+} and Fe^{3+} exist in equilibrium. Fe^{2+} is soluble in water at pH 7. Bacteria acquire this form of iron by using a general divalent metal transport system. However, Fe^{3+} is the most common form in the bacterial habitat, since it is spontaneously generated by reaction of Fe^{2+} with oxygen.

In hosts, there is little free iron available, since most iron is bound to proteins such as transferrin. This is one of the defensive mechanisms that hosts use to impede growth of bacteria. On the other hand, pathogenic bacteria can secrete high-affinity iron chelators known as siderophores to obtain iron from the host. Several siderophores have been documented in *K. pneumoniae* including enterobactin, yersiniabactin, salmochelin, and aerobactin. Each of these has a different ability to scavenge iron. Enterobactin has the highest affinity for iron, followed by yersiniabactin, salmochelin, and aerobactin (Brock et al., 1991; Perry et al., 1999).

Enterobactin can be found in both classic and HV *K. pneumoniae*. Enterobactin is synthesised by the chromosomal *entABCDEF* gene cluster. The *fepABCDG* gene cluster encodes the proteins that facilitate its transport, and *fepA* encodes the uptake receptor. During *K. pneumoniae* infection, the expression of *fepA* is upregulated which suggests an upregulation of enterobactin epression. Hosts can neutralise enterobactin using lipocalin-2. Lipocalin-2 is a multifunctional protein secreted from several kinds of host cells, including neutrophils. Lipocalin-2 can bind enterobactin, which prevents uptake of iron by *K. pneumoniae* (Bachman et al., 2012).

Yersiniabactin is found in only 18% of classical *K. pneumoniae* clinical isolates, but is found in 90% of HV *K. pneumoniae* clinical isolates (Bachman et al., 2011). The activity of yersiniabactin is not inhibited by Lipocalin-2, but it cannot obtain iron that is bound to transferrin, which is the normal form of iron found in blood (Bachman et al., 2011).

Salmochelin is a c-glucosylated form of enterobactin. The gene cluster that encodes Salmochelin can be found either on the chromosome or on a plasmid (Hsieh et al., 2008). It is present in 2-4% of classical *K. pneumoniae* isolates, but is more prevalent in HV strains (El Fertas-Aissani et al., 2013). This form of siderophore cannot be bound by lipocalin-2, and so enhances colonisation by *K. pneumoniae* (Fischbach et al., 2006).

Aerobactin is a citrate-hydroxamate siderophore that is found mostly in HV *K. pneumoniae* and rarely found in classical strains (Russo et al., 2018). The aerobactin gene cluster is located on the same plasmid that possesses the *rmpA* gene (the gene that plays an important role in producing the hypermucoid capsule), This plasmid is mostly possessed by HV strains (Hsieh et al., 2008), and may be one reason why

hypervirulent strains are more virulent than classical strains. A *K. pneumoniae* pneumonic and subcutaneous mouse model suggested that in HV *K. pneumoniae*, aerobactin is essential for successful infection (Russo et al., 2014).

Several studies have attempted to show a correlation between type of siderophore and HV strain, but the evidence is not consistent (Holt et al., 2015; Russo et al., 2015; Struve et al., 2015). However, in general, HV strains seem to possess more than one type of siderophore.

1.2.6 *Klebsiella pneumoniae* in animals

Klebsiella pneumoniae was first reported in NZSLs as a cause of mass mortality in pups during the 2001/2002 to 2002/2003 breeding seasons at Sandy Bay, Enderby Island (Castinel et al., 2007a). At post-mortem, polyarthritis, peritonitis, cellulitis/dermatitis and meningitis were present in affected pups (Castinel et al., 2007b). The number of fatal *K. pneumoniae* infections seemed to decline in the years immediately after these mortality events (Castinel et al., 2007b). A total of forty *K. pneumoniae* isolates from the outbreak in 2001/2002 to 2002/2003 seasons, 25 isolates from 2003/2004 and 2004/2005 seasons, three *K. pneumoniae* isolates from three adult female NZSLs in 2004/ 2005, one *K. pneumoniae* isolate from an adult male NZSL that died on the Otago peninsula in 2004, three New Zealand human isolates (from Palmerston North Hospital), and seven isolates of *K. oxytoca* and two other *Klebsiella* spp. from 2001/2002 to 2004/2005, plus one *K. oxytoca* isolated from a pup before 2000/2001, were studied using pulsed-field gel electrophoresis (PFGE) of XbaI DNA macro restriction fragments (Castinel et al., 2007b). The results revealed that the *K. pneumoniae* pup isolates were genetically indistinguishable from each other, but different from three human isolates used in the study (Castinel et al., 2007b). The *K.*

pneumoniae isolates from adult female sea lions were different to each other, and differed from the pup isolates, while the isolate from the adult male NZSL was identical to the pup isolates (Castinel et al., 2007b). This result suggested that *K. pneumoniae* from the pups and the adult male from Otago may have come from the same common source. The antimicrobial susceptibility test and extended spectrum beta lactamase (ESBL)-testing showed the similarity of the NZSL isolates, and all the isolates were negative for ESBL resistance (Castinel et al., 2007b).

After the first outbreaks of *K. pneumoniae,* the pathogen seemed to become endemic within this animal group. From 2006/2007 to 2009/2010, *K. pneumoniae* was responsible for 58% of NZSL pup deaths on Enderby Island (Roe et al., 2015). Dead pups had lesions of fibrinosuppurative meningitis, subdural haemorrhage, septic arthritis, herniation and haemorrhage of the cerebellar vermis, lymphadenitis and cellulitis (Roe et al., 2015). Moreover, in 2013 one pup died from *K. pneumoniae* near the Otago breeding site on the New Zealand mainland. All the isolates tested had a hypermucoid colony type and were positive on string test, suggesting a hypermucoviscous phenotype (Roe et al., 2015). This phenotype is similar to the HV *K. pneumoniae* in humans that causes primary liver abscesses with septicaemia, complicated by meningitis and ophthalmitis (Fang et al., 2004; Shon et al., 2013). The isolates from NZSLs pup were capsular serotype K2 and contained the *rmp*A gene (Roe et al., 2015). *Rmp*A is the gene involved in expression of hypermucoviscous phenotype (Shon et al., 2013).

From 1997 to 2008, 27 California sea lions from the California coast died from *K. pneumoniae,* and 25 of them had lesions of fibrinopurulent pleuritis and suppurative bronchopneumonia (Jang et al., 2010). Another two cases had abscesses; one had liver abscesses and the other had abscesses in the subcutis and muscle over the shoulder

(Jang et al., 2010). The isolates from California sea lions had a hypermucoviscous phenotype by positive string test. They were capsular serotype K2 and contained the *rmp*A gene (Jang et al., 2010). PFGE analysis showed differences among the isolates suggesting the isolates were from multiple sources (Jang et al., 2010).

HV *K. pneumoniae* infection has been reported in African green monkeys in a United States (US) army medical research unit (Twenhafel et al., 2008). It caused abscesses in seven animals, mostly in the abdomen as well as in the lungs, cerebellum and skin. Bacteria in these cases were identified by using culture and biochemical methods, and were further characterized by capsular serotype using slide agglutination, which revealed they were of serotype K2. The string test was done to determine hypermucoviscous phenotype. An isolate from this study was genetically analysed showing it was *rmp*A^{+}/magA^{-} (Twenhafel et al., 2008). In addition, other nonhuman primates in that research unit, including rhesus and cynomolgus macaques, were HV *K. pneumoniae* positive without clinical signs. This suggests that nonhuman primates can be a reservoir of this pathogen. A wild African green monkey in St. Kitts, West Indies, also carried HV *K. pneumoniae* that was indistinguishable on random amplified polymorphic DNA (RAPD) fingerprint from a strain that caused multiple abscesses in African green monkeys in a US army medical research unit (Whitehouse et al., 2010). In captive African green monkeys in St. Kitts, West Indies, *K. pneumoniae* isolates were recovered from abscesses and from oral and rectal swabs; 17 isolates from 29 isolates were HV (Soto et al., 2012).

1.3 Survivability of bacteria in environment

In nature bacteria have to face stressful and often variable conditions such as limitations in nutrients and oxygen, variations in temperature, ultraviolet light,

antimicrobial substances, and pH changes. In soil, limitation of carbon sources is a common factor that can inhibit the growth of microorganisms (Aldén et al., 2001). Bacteria have to adapt in order to survive in these situations, and do this by triggering several complex regulatory networks which involve physiological, behavioural, and genetic changes. Several mechanisms have been examined including switching phenotypes, sporulation (gram positive bacteria), and phase variation.

1.3.1 Phase variation

Phase variation is a reversible process that enable bacteria to deal with rapid changes in their environment. There are five phases in the life cycle of a bacterial population. First is the lag phase; when bacteria enter a new environment they set up metabolic reprogramming in order to thrive in the new environment. The time needed for this period depends on several factors, including the bacterial species, changes that have occurred in the environment, and the length of time the bacteria have been in a starved condition (Pin and Baranyi, 2008). After bacteria adapt to the new environment, they start growing and dividing exponentially, a state known as the exponential or log phase. At some point the bacteria use up all the nutrient sources, and must enter the stationary phase. In this phase, cell number does not increase and there are two possible outcomes from this phase. First, bacteria may fail to adapt to the new conditions and enter the death phase, a situation that occurs in 90-99% of bacteria (Finkel, 2006). Dead bacteria release nutrients for the survivors. The second outcome is that bacteria are able to adapt themselves to the new conditions, and the survivors can live for months to years. This phase is called the long-term stationary phase (Finkel, 2006). For example, *E. coli* can stay in a prolonged stationary phase for up to 5 years when water is added to the culture media (Navarro Llorens et al., 2010). However,

while the five described phases of the bacteria cycle are seen in laboratory conditions, the time spent in each phase in nature is likely to be slightly different, and the exact timings are unknown.

The transition to long-term stationary phase is governed by sigma factors. These work at the transcriptional level, as sigma factors are a subunit of the holoenzyme RNA polymerase. Bacteria in this stationary phase have structures and functions that are adapted to the conditions in the environment. The cells become smaller and spherical when compared to bacteria in the log phase due to the processes of reductive division and dwarfing (Nyström, 2004). Reductive division is the process by which cells divide, but do not grow, thus gradually increasing their surface-area-to-volume ratios until they become spherical. Dwarfing is a process of self-digestion due to degradation of endogenous cell material, including cytoplasmic and outer membranes, by the bacteria (Nyström, 2004). The cell envelope undergoes rearrangement leading the cell to become more resistant to chemical and physical agents.

Bacteria may respond to prolonged starvation in several ways. Phenomena related to this situation include the growth advantage in stationary phase (GASP) phenotype, viable but nonculturable (VBNC) state, and stationary phase contact-dependent inhibition (SCDI).

1.3.2 GASP phenotype

Growth advantage in stationary phase (GASP) phenotype is defined by the ability of the aged cell to outcompete the young cell, and it occurs during prolonged stationary phase (Finkel, 2006). Using *E. coli* as a model, when a 10 day culture of *E. coli* in Luria broth is transferred into a culture of one-day old *E. coli,* the aged *E. coli* will increase in

number within two to three days, with a corresponding reduction in young *E. coli* (Finkel, 2006; Zambrano et al., 1993). After co-culture for seven to ten days, none of the younger *E. coli* remain (Finkel, 2006; Zambrano et al., 1993).

The expression of the GASP phenotype is a genetic change, not a physiological adaptation, which can be demonstrated as follows. First, bacteria with the GASP phenotype maintain this phenotype, even after several passages that pass through the log phase of growth. Second, the GASP phenotype can be constructed in young cells. In *E. coli,* GASP mutations have been recognized in *rpoS* gene, which encodes the alternative sigma (σ) factor RpoS or σ^S (Zambrano et al., 1993), the *Irp* gene which encodes a leucine-responsive protein, and the *ybeJ–gltJKL* cluster which encodes a high-affinity aspartate and glutamate transporter (Finkel, 2006). Although these genes are involved in different processes, all of them enhance the ability of bacteria to catabolise amino acids as a source of carbon and energy (Zinser and Kolter, 2004). The population of GASP phenotype bacteria in long-term stationary phase is stable, but not static (Finkel, 2006). Novel GASP mutations continue to occur with continued incubation which increases the genetic diversity of bacteria in the culture (Finkel, 2006).

1.3.3 Viable but nonculturable (VBNC) state

Viable but nonculturable state in bacteria is a state where bacteria cannot be grown in media, but are still alive (Oliver, 2000). This phenomenon can be induced by prolonged exposure to adverse environments including nutrient starvation, abnormal temperatures, salinity or pH, shifts in osmotic or oxygen concentration, and exposure to heavy metals and white light (Navarro Llorens et al., 2010; Oliver, 2010). Over 50 bacterial species may undergo this physiological response, including pathogenic bacteria such as *Mycobacterium tuberculosis*, *Francisella tularensis* and *E. coli* (including

EHEC strains) (Oliver, 2005). Bacteria in this state are different from viable culturable bacteria in morphology, physiology, cell wall and membrane composition, metabolism, gene expression, ability to resist physical and chemical change, adhesion properties and virulence (Li et al., 2014). Viable but nonculturable bacteria often present with dwarfing and changes in metabolism including nutrient transport, respiration rates, and macromolecule synthesis (Oliver, 2000). The ATP level of bacteria in this state is high and rapidly declines in dead and moribund cells (Beumer et al., 1992; Federighi et al., 1998). A number of genes such as *mobA, rfbE*, *stxl,* as well as 16S rRNA synthesis are still expressed in nonculturable cells of *E. coli* O157:H7 (Yaron and Matthews, 2002). In some species, once in favoured conditions again, the dormant bacteria can recover from this state. The molecular mechanisms behind this phenomenon are still unknown. The recovered bacteria are still pathogenic, which is important in human and animal medicine and in the food preservation industry, because they cannot be detected by routine culturing of samples (Navarro Llorens et al., 2010).

1.3.4 Stationary phase contact-dependent inhibition (SCDI)

After prolonged starvation, a phenomenon that has been observed in *E. coli* K-12 is for new strains to develop that appear to kill or inhibit growth of the parent strain. This is related to the *glg*C gene that encodes ADP-glucose pyrophosphorylase, a regulatory protein involved in glycogen synthesis. The underlying mechanism of this inhibition by SCDI is unknown; but glycogen overproduction derived from *glgC* mutant may activate an inhibitory function (Lemonnier et al., 2008). Although SCDI and GASP seem to share some characteristics, they are different both functionally and genetically (Lemonnier et al., 2008). The *Irp and rpoS* mutations involved in GASP are not present in SCDI.

1.3.5 Switching phenotype

Switching phenotype is one of the mechanisms that bacteria use to respond to stress in the environment. The molecular mechanisms of this phenomenon are unclear. In some bacterial species, the specific phenotypic switching mechanisms are associated with genetic changes, but in other species, the phenotypic switching mechanisms are not genetically related, as changes in epigenetic regulation have been recognised. This phenomenon can be random (stochastic switching) or stress inducible by external factors (responsive switching). Phenotype switching can change the expression of lipopolysaccharide, pili, and flagella, and this state can be reversible. A significant change can be observed in cell morphology; any alteration in colour, opacity or texture could be a sign of phenotype switching. Several bacteria such as *Burkholderia pseudomallei*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Haemophilus influenzae* have been reported to alter colony morphology (Sousa et al., 2011). The small colony variants (SCV) and the mucoid morphotypes are types of cell morphology that are well known examples of phenotype switching. Some of these phenotypes are related to increased virulence in bacteria including antimicrobial resistance, changes in metabolism, reduced immunogenicity, and persistence. SCV are associated with persistent and recurrent infections in humans, and device associated infections. SCV have been characterized as hyperpiliated and have an increased ability to form biofilms (Déziel et al., 2001). Moreover, SCV have been reported in bacteria with increased twitching motility, increased capability under stationary growth and autoaggregative traits. This switching phenotype is due to gene mutations. The study of SCV in *P. aeruginosa* revealed that a mutation in *wspF* led to growth of an autoaggregative SCV with defects in swimming, twitching and swarming abilities (D′Argenio et al., 2002)

The mucoid phenotype is another switching form that has been described, and is due to overproduction of exopolysaccharides. In *P. aeruginosa*, the mucoid phenotype is due to overproduction of alginate. *P. aeruginosa* has a mucoid phenotype that can develop during chronic lung infections, and is associated with a mutation in *mucA* (Sousa et al., 2011). The overproduction of exopolysaccharides protects the bacteria from host defence mechanisms and antibiotic treatment (Sousa et al., 2011).

1.3.6 Biofilm

Biofilm formation is a mechanism used by bacteria to protect themselves from a stressful environment. It involves the synthesis of extracellular polysaccharides (EPS), together with proteins and nucleic acids bound to the cell surface, and is distinct from the bacterial capsule both physically and chemically (Prakash et al., 2003). Biofilm-bacteria have changes in physiology, particularly in proteins involved in resistance to oxidative damage, exopolysaccharide production, phospholipid synthesis and membrane transport (Sousa et al., 2011). There are several factors that are involved in biofilm synthesis, including surface, nutrient and environment signals such as pH, oxygen and temperature changing (Święciło and Zych-Wężyk, 2013). With different stress conditions, the biofilm matrix can be different in the same bacterial species. For examples, biofilms growing in fast-moving water tend to form filamentous structures (Hall-Stoodley et al., 2004). The bacteria in a biofilm are different from free living bacteria by up/down regulation of a number of genes, which makes them have different growth rates with increased resistance to biocides, antibiotics, antibodies and attack by bacteriophages (Święciło and Zych-Wężyk, 2013). There are four steps in biofilm formation; the first is attachment to the surface, follow by microcolony formation, then formation of a three-dimensional structure and maturation, and finally detachment.

Bacterial biofilm can colonise any humid surface such as teeth (plaque on the teeth), river stones (slippery slime) and infectious tissue. The bacteria that form biofilms are difficult to eliminate with general cleaning procedures as they are resistant to heat, light, drying, and traditional cleaning products (Stiefel et al., 2016; Whittaker et al., 1984).

1.4 The immune system

The immune system is a host defence mechanism against pathogens and development of cancers. As this thesis is focussed on infectious disease, in this review I focussed on immunity against pathogens.

The immune system consists of complex networks of biochemical and cellular reactions. The immune response can be divided into innate and adaptive immunity. The innate immune responses are considered the first-line mechanism. They provide immediate reactions and function at birth. They are not specific to the pathogen and lack memory. The major components of the innate immune system are physical and chemical barriers, such as skin and mucosal epithelium, phagocytic cells such as neutrophils and monocytes, dendritic cells, natural killer (NK) cells, and circulating proteins such as complement proteins. Adaptive immunity is the second line mechanism. Adaptive immune responses take several days or weeks to become effective, but they are specific to the pathogen and have memory. Adaptive immune responses can recognise pathogens to which the host has been exposed. If the host is infected with the same pathogen for a second time, the adaptive immune system reacts more rapidly and effectively. There are two types of adaptive immunity: humoral and cellular immunity. Humoral immunity is mediated by B-cell lymphocytes which secrete antibodies. It is responsible for protecting against extracellular pathogens such as

extracellular bacteria. Cellular immunity is mediated by T lymphocytes, which protects against intracellular pathogens such as viruses (Murphy and Weaver, 2016).

Monocytes and neutrophils are phagocytic cells that circulate in the blood. They can be recruited to sites of infection rapidly. Once monocytes enter tissues and mature, they are called macrophages. Macrophages have several functions, including phagocytosis of antigens, and presentation of antigens to other immune cells in order to activate other defence mechanisms. Macrophages also produce cytokines and chemokines to enhance the inflammatory response.

Dendritic cells are present in most tissues, particularly in the epithelium and lymphoid organs. They specialise in capturing protein antigens and presenting peptides to T lymphocytes. Dendritic cells also stimulate the secretion of cytokines that enhance inflammation.

Natural killer cells play a role in viral and intracellular bacterial infections. Soluble proteins such as complement play an important role in innate immunity. They can be activated directly by microbes via the alternative and lectin pathways. Manose-binding lectin and C-reactive proteins promote phagocytosis by coating the microbes (Murphy and Weaver, 2016).

1.4.1 The immune response to extracellular bacteria

Once bacteria overcome the chemical and physical barriers of the body, they have to face other immune factors. In tissues, pathogens can be recognised by resident macrophages. Macrophages detect bacteria via pattern recognition receptors (PRRs) present on the macrophage cell membrane, which recognise pathogen-associated molecular patterns (PAMPs) present on the surface of the bacteria. PAMPs are

carbohydrates, polypeptides, and nucleic acids that are expressed by bacteria, viruses and parasites, but not host cells.

PAMPs activate the macrophages to phagocytose the microbe and to release cytokines and chemokines to stimulate and recruit other immune cells, such as neutrophils and monocytes from the blood to the infection site. The chemokines and cytokines released from macrophages initiate the inflammatory process that helps to fight against the infection. An increase in lymph flow carries the pathogens and antigen presenting cells to the nearby lymph nodes, to further initiate the adaptive immune response (Murphy and Weaver, 2016).

Local inflammation and phagocytosis can be activated by plasma proteins known as the complement system. During activation of the complement cascade, several molecules are produced. The main outcome is amplification of inflammation, leading to enhanced phagocytosis, chemoattraction (recruitment of neutrophils and macrophages), formation of the membrane attack complex (MAC, which causes microbial cell lysis), and opsonisation of the pathogen. The complement proteins, C1-C9, circulate in the blood in an inactive form (Murphy and Weaver, 2016). They can be activated by microbial molecules, antibodies, polysaccharides, and venoms (Kumar et al., 2014). The critical step of activation of the complement cascade is activation of C3. There are three pathways that lead to the complement activation: classical, alternative and lectin pathways. The classical pathway can be activated by antigen-antibody complex (Doorduijn et al., 2016). The complement protein C1q is cross-linked with antibody (IgG or IgM), allowing interaction with C2 and C4, forming classical C3 convertase (C4b2b). C3 convertase cleaves C3 into C3a and C3b. In the lectin pathway, the complement cascade is activated by the presentation of sugar structures on the

pathogen surfaces, initiating the formation of C3 convertase. In the alternative pathway, the complement cascade can be activated by microbial products such as lipolysaccharide (LPS) from gram-negative bacteria, as well as activated plasma proteins such as kallikrein and plasmin that can cleave C3 into C3b. C3b together with factor B and D, result in formation of the alternative pathway C3 convertase (C3bBb) (Doorduijn et al., 2016). C3b attaches to bacteria and some intracellular viral pathogens to promote phagocytosis (Berends et al., 2014). C3b also activates formation of C5 convertase to cleave C5 to C5a and C5b. C5b initiates formation of the membrane attack complex (MAC) (Berends et al., 2014). The MAC kills pathogens via pore formation in the pathogen cell wall (Berends et al., 2014). It specifically kills gram-negative, but not gram-positive, bacteria, which may be due to the different structure of the cell walls (K A Joiner et al., 1984). Gram-positive bacteria have a thick peptidoglycan layer that might prevent cell lysis by MAC (Berends et al., 2014).

Phagocytes such as neutrophils and macrophages are required to clear bacterial infection. Neutrophils mostly focus on extracellular bacteria, while macrophages focus on intracellular microorganisms. In *K. pneumoniae* infection, in order to eliminate the pathogen, neutrophils require myeloperoxidase and neutrophil elastase, as shown in a study using mice deficient in either myeloperoxidase or neutrophil elastase. The mice were revealed to be markedly susceptible to *K. pneumoniae* infection compared with wild type mice (Hirche et al., 2005). However, the step prior to elimination of the pathogen in neutrophils is also important. At the site of infection, neutrophils work better after opsonisation of bacteria with complement and antibody. Some strains of *K. pneumoniae* are able evade complement opsonisation (Doorduijn et al., 2016), suggesting antibody is required to enhance phagocytosis (Wu et al., 2009b).

1.4.2 Newborn immunity

In most animals the immune system is completely formed at birth and able to respond to pathogens immediately. However, the immune response in newborn animals to pathogens is different to adults in several aspects, because newborn animals have not been exposed to any pathogens. Newborn animals are able to use both innate and adaptive responses to fight against pathogens, but adaptive immunity is the primary response and takes more time than adults due to low antibody concentrations. Moreover, some immune components have immature functions (Basha et al., 2014; Tizard, 2013). Pathogen protection in newborns depends mostly on passive immunity. In pinnipeds, it has been suggested that immunity in pups is affected by the duration of nursing. Phocids have a short nursing period and can develop highly specific antibody responses after an antigenic challenge (Ross et al., 1994). In addition, there is evidence of synthesis of IgM in *utero* (Marquez et al., 2000). In contrast, otariids have longer nursing periods, and *de novo* immunoglobulin may take a longer time to synthesise. Serum IgG in pups of Galapagos sea lions (*Zalophus wollebaeki*) does not reach adult concentrations until one year of age (Brock et al., 2013). In addition, IgG levels of NZSL pups at 5 months is four times lower than in adults (Castinel et al., 2008).

In mammals, the thymus is the first lymphoid organ to develop, followed by the secondary lymphoid organs. B lymphocytes form after development of the spleen and lymph nodes, and antibodies are produced mostly in late fetal life. The immune system develops as a series of steps, where each step allows a greater response to the antigen. The cell-mediated immune response and antibody production develop at the same time. However, T-cell receptor diversity and cytokine production in the fetus and the neonate may be low due to the lack of exposure to antigens (Tizard, 2013).

The innate immune system consists of cellular and soluble components, as well as physical and chemical barriers of the body. Newborns have similar cellular components as adults including granulocytes (particularly neutrophils), monocytes, macrophages, dendritic cells (DCs), and natural killer (NK) cells. However, some of the cellular components are less efficient than in adults. Neutrophils are considered to be a front-line immune cell against pathogens. In humans, in the first 24-72 h of life the blood neutrophil count rapidly rises and then decreases to the normal range in the first week. As neutrophils function via adhesion mechanisms, neutrophils in newborns do not function properly due to the low expression of adhesion molecules such as L-selectin and CD11b/CD18 (Mac-1) (Anderson et al., 1991). Subsequently, decreased calcium influx, and impaired actin polymerization leads to reduced chemotaxis and diapedesis. Phagocytic ability of neutrophils is also immature at birth, but reaches adult levels 3 days after birth. The newborn human neutrophil surface has poor expression of TLR4 and also poor signalling through MyD88 and p38 pathways. In otariid pups, there are some fluctuations in immune protection depending on age (Brock et al., 2013; Keogh et al., 2010). In Steller sea lions (*Eumetopias jubatus*), high concentrations of leukocytes were observed shortly after birth, but decreased with age (Keogh et al., 2010).

1.5 Active and passive immunisation

Researchers are attempting to find ways to control multidrug resistant *K. pneumoniae*. Immunotherapy by either active or passive immunisation is a potential option. Active immunisation refers to a process whereby the body is exposed to antigens and subsequently generates an adaptive immune response (Baxter, 2007). This results in long term immunity. Passive immunisation refers to the process of giving

antibodies to protect against infection, but only cofers short-term protection (Baxter, 2007). Several studies have shown that in order to clear the pathogen from a host, better outcomes occur with adaptive immune responses. For *Klebsiella spp*., both active and passive immunisation have been developed based on five categories including capsular polysaccharide (CPS), lipopolysaccharide (LPS), siderophores, adhesins (pili, fimbriae and aggregative adhesins), and exotoxins, with each type of vaccine having its own limitations (Ahmad et al., 2012).

1.5.1 Capsular polysaccharide (CPS) vaccine

The capsule of *K. pneumoniae* is composed of extra-cellular polysaccharide, which is different in each strain (known as K antigens). Many studies in the past have focussed on using CPS as a vaccine epitope since CPS is immunogenic and nontoxic (Cryz et al., 1985). Vaccines developed from CPS gave protection against *K. pneumoniae* sepsis in squirrel monkey (*Saimiri sciureus*) (Postal et al., 1988) and gave a significant ($p < 0.01$) result in protecting against burn wound sepsis (Cryz et al., 1984), as well as pneumonia in mice (Cryz et al., 1986). A major problem with CPS vaccine, however, is that there are at least 79 different K antigens, with little cross-protection between groups. In order to create the best outcome, CPS-based vaccines should be multivalent to protect against the most common or important isolates. However, there is no correlation between capsular serotype and geographic area, which makes it difficult to develop a vaccine to provide full protection, even in a specific geographic area.

1.5.2 Lipopolysaccharide (LPS)

LPS, also known as endotoxin, is a component of the cell membrane of gram-negative bacteria. LPS consists of three structures: lipid A chain, oligosaccharide core, and O-polysaccharide repeated outer region (O-antigen). In *K. pneumoniae*, there are

only 8 known O antigens, with O-1 being the most prevalent. O antigens are therefore suitable candidates for epitopes in vaccine development. Similarity between core regions may allow cross protection between gram-negative bacteria, as shown by a liposomal complete-core LPS vaccine from *E. coli*, *P. aeruginosa* and *Bacteroides fragilis* that provided cross protection against *K. pneumoniae* O1, O2ab and O3 (Bennett-Guerrero et al., 2000). However, cross reactions against normal flora gram-negative bacteria in the body have to be considered. In addition, endotoxic reactions may occur when using LPS active immunisation (Lüderitz et al., 1966). This may be reduced by incorporation LPS of into a liposome (Chhibber et al., 2004).

On the other hand, passive immunisation using monoclonal antibodies prepared from LPS from *K. pneumoniae* O1:K2 showed good protection against endotoxic shock when mice were immunised before challenge with *K. pneumoniae* (Mandine et al., 1990). Another study using monoclonal antibodies prepared by stimulation with LPS from *K. pneumoniae* ST258 (multidrug resistant clone) showed an endotoxin neutralization effect in mice and rabbit (Szijártó et al., 2017; Szijártó et al., 2016).

1.5.3 Siderophores

As bacteria require iron to grow, each bacterium develops their own system to acquire iron from the host. The siderophore receptor proteins are an interesting target for vaccine development in *Klebsiella* spp. as they are highly conserved in this species, as well as in other coliform bacteria (Gorden et al., 2018; Miethke and Marahiel, 2007). This suggests that a siderophore receptor protein-based vaccine may be able to provide cross protection against all coliform bacteria. A siderophore receptor protein vaccine has been developed to prevent mastitis in cattle, and showed efficacy against *Klebsiella*

mastitis and coliform bacterial mastitis such as *E. coli* and *Enterobacter spp*. (Gorden et al., 2018).

1.5.4 Other vaccine epitopes

Other potential vaccine epitopes that have been studied including outer membrane proteins (Hussein et al., 2018), fimbriae proteins (Witkowska et al., 1997) and conjugated proteins (e.g. LPS with iron-regulated cell surface proteins) (Chhibber and Bajaj, 1995). Problems with vaccine development based on these epitopes are due to variation in the sub-types of protein epitopes which vary in each isolate. For example, if outer membrane proteins are used as a protein epitope, each isolate may possess different types of outer membrane protein. Moreover, there is no association between geographic areas and the type of outer membrane proteins (Holt et al., 2015), which makes it difficult to develop vaccines even for a specific area.

The main problem with development of vaccines or passive immunisation against *K. pneumoniae* to use commercially in humans is the variation of epitopes between each *K. pneumoniae* isolate. However, in the case of NZSL pups, specific immunity might be possible as the isolates are thought to be clonal. Further classification of the phenotype and genotype of *K. pneumoniae* affecting NZSL pups may allow establishment of a baseline for development of a vaccine.

Chapter 2

Origin and selection of samples used

The samples used in this thesis came from a variety of sources, species, locations, and times, and several were collected by other scientists and field staff and archived as part of other studies. Therefore, this chapter is included to describe how each type of sample was collected, and why certain samples were chosen for each experimental chapter.

2.1 Sample information

Annually during the New Zealand sea lion breeding season from December to early March, the New Zealand sea lion team visits Enderby Island. The primary purpose of these visits is to collect population and foraging data, but NZSL pups found dead are also recovered for necropsy as part of annual health surveillance. The necropsies are carried out by a veterinarian or a trained sea lion biologist. A set of tissue samples including, lung, heart, liver, spleen, kidney, adrenals, lymph nodes, and brain plus tissues from organs that have lesions are collected into 10% buffered formalin during the necropsies. Selected tissues, including brain, liver, and lung, along with swabs of exudates and samples of lesions, are collected into cryovials and stored in liquid nitrogen. In addition, during these trips scientists also visited Dundas Island from 13 - 18 January 2015, and Campbell Island from 20 – 26 December 2014. NZSL pups found dead

during these periods were recovered for necropsy, and tissues and swabs were collected as described above.

All samples were transported to Massey University, Palmerston North, New Zealand for processing. Frozen tissues and swabs were transferred to a -80°C freezer for long term storage. Formalin-fixed tissues were processed routinely for histology and evaluated by a veterinary pathologist. The necropsy reports, histological slides and histology reports are held at Massey University, Palmerston North, New Zealand, and were available to the author for review.

2.1.1 Sample nomenclature

The bacterial isolates in this present study were named in the same way as the code given to NZSL pups in the necropsy reports, whereby each necropsied pup is given a unique code to indicate the location (island), breeding season, and species. For example:

Animal code, E11/12_24Ph and D14/15_09Ph:

E11/12_24Ph	D14/15_09Ph
E = Enderby Island	D = Dundas Island
11/12 = 2011/2012 breeding season	14/15 = 2014/2015 breeding season
24 = 24th necropsy of that season	09 = 9th necropsy of that season
Ph = *Phocarctos hookeri* (New Zealand sea lion)	Ph = *Phocarctos hookeri* (New Zealand sea lion)

Samples from other species plus substrate samples were given a unique code to indicate the location (island), breeding season or year, and species or nature of sample;

Code explanation:

1. Island code: **C**14/15_10sub

   A = Auckland main Island

   C = Campbell Island

   D = Dundas Island

   E = Enderby Island

   S = South Island (New Zealand mainland)

2. Year code: C**14/15**_10sub

   14/15 indicates 2014/2015 breeding season.

3. Number code: E11/12_**10**sub

   10 indicates 10th substrate sample collected of that season

4. Animal and substrate ID: E11/12_10**sub**

   Ma = *Megadyptes antipodes* (yellow-eyed penguin)

   Sa = *Stercorarius antarcticus* (Subantarctic skua)

   Sub = Substrate

Other examples: E13/14_10sub and E14/15_17Sa

E13/14_10sub	**E14/15_17Sa**
E = Enderby Island	E = Enderby Island
13/14 = 2013/2014 breeding season	14/15 = 2014/2015 breeding season
10 = 10th sample of that season	17 = 17th sample of that season
sub = substrate	Sa = *Stercorarius antarcticus* (Subantarctic skua)

However, there was one sample that did not fall into this naming system. C14_9476 is an isolate from a rectal swab from a live healthy female adult sea lion (ID number 9476) from Campbell island. The sample was collected as part of an unrelated foraging study conducted on the island in August 2014.

Each *K. pneumoniae* isolate was given a specific number in order to make it easier to follow the same isolates in each chapter.

Table 2.2.1 Bacterial isolates used in different experiments in this study

Isolate designation	Bacterial isolates	Source	Clinical status	Chapter
Non-HV_Kp1	*K. pneumoniae* ATCC 700603	Human	Clinical	3,4,6
HV_Kp2	*K. pneumoniae* E02/03_112Ph	Sea lion pup	Post mortem[a]	3,4,5,6
HV_Kp3	*K. pneumoniae* E11/12_24Ph	Sea lion pup	Post mortem[a]	3,5,6
HV_Kp4	*K. pneumoniae* S13_04Ph	Sea lion pup	Post mortem[a]	3,5,6
HV_Kp5	*K. pneumoniae* D14/15_08Ph	Sea lion pup	Post mortem[a]	3,5,6
Non-HV_Kp6	*K. pneumoniae* C14/15_09Ph	Sea lion pup	Post mortem[b]	3,5,6
Non-HV_Kp7	*K. pneumoniae* E09/10_13Ph	Adult sea lion	Post mortem[c]	3,5,6
HV_Kp8	*K. pneumoniae* C14_9476Ph	Adult sea lion	Healthy	3,5,6
Non-HV_Kp9	*K. pneumoniae* E14/15_17Sa	Subantarctic skua	Healthy	3,5
HV_Kp10	*K. pneumoniae* E14/15_42Sa	Subantarctic skua	Healthy	5
HV_Kp11	*K. pneumoniae* E14/15_53Ma	Yellow-eyed penguin	Healthy	5
HV_Kp12	*K. pneumoniae*_E13/14_10sub	Substrate	-	3,5
HV_Kp13	*K. pneumoniae*_C14/15_17sub	Substrate	-	5

[a] These animals died from *K. pneumoniae* infection and samples were collected at post mortem examination
[b] This animal died from starvation and samples were collected at post mortem examination
[c] This animal died from trauma and samples were collected at post mortem examination

2.1.2 Analysis of necropsy and histology reports, and tissue selection

In 2001/02 and 2002/03, outbreaks of fatal *K. pneumoniae* occurred in NZSL pups at Sandy Bay, Enderby Island. In the following years, this pathogen appears to have become endemic in this population (Roe et al., 2015), and continues to cause pup mortality each year (Childerhouse et al., 2015). The aims of Chapter 3 were to

characterise *K. pneumoniae* isolated from NZSL pups, determine phenotypic variation among the isolates, and investigate any phenotype changes over time. The steps to achieve these aims were: identify suspected cases of fatal infection with *K. pneumoniae* from different years; isolate the bacteria from the tissues of suspected cases; then characterise these isolates using the methods described in Chapter 3.

For the first step, the author reviewed the 2002/2003, 2011/12 and 2013/14 necropsy reports of NZSL pups from Enderby Island to find suspected *K. pneumoniae* infection cases by using the following protocol.

- Necropsy reports were reviewed for phrases describing gross lesions that could be associated with *K. pneumoniae* infection including (Roe et al., 2015):
    - Subdural haemorrhage
    - Herniation and haemorrhage of the cerebellar vermis
    - Polyarthritis, joints containing suppurative exudate or pus
    - Lymphadenitis, enlarged lymph nodes
    - Presence of suppurative exudate, purulent exudate or pus in other organs

For cases identified as having possible *K. pneumoniae* infection, frozen tissues were cultured by the author according to the protocol in Chapter 3.2.2. Brain tissue or brain swabs were selected to culture first; if these were not available or if there was no bacterial growth on culture of these tissues, further tissues were selected for culture in the following order of precedence depending on availability: cerebrospinal fluid (CSF) swab or atlanto-occipital joint swab, other joint swabs or joint fluid, swabs from lesions,

lymph nodes, liver, and lung. If the tissues or swabs were not available, the case were removed from the list.

- In cases where the gross lesions did not lead to suspicion of *K. pneumoniae* infection, histology reports were reviewed for the following lesions as described by Roe et al. (2015) and Roe (2011):
    - Fibrinosuppurative to histiocytic meningitis, possibly with the presence of rod-shaped bacteria
    - Cerebral or cerebellar vasculitis, or perivascular cuffing with histiocytes and neutrophils
    - Suppurative or fibrinopurulent lymphadenitis, cellulitis, peritonitis, pneumonia, omphalitis, pleuritis, osteomyelitis, valvular endocarditis, nephritis

The tissues or swabs from cases that had microscopic lesions consistent with *K. pneumoniae* were further investigated with bacterial culture as described above and in Chapter 3.2.2.

2.2 New Zealand sea lion samples from the Auckland Islands, Campbell Island and Otago Peninsula

2.2.1 Enderby Island, Auckland Islands

After review of the gross necropsy and histology reports of pups that died during 2002/03, 2011/12 and 2013/14, tissues and swabs from cases that were identified as having possible *K. pneumoniae* infection were retrieved and processed for bacterial culture as described above and in Chapter 3.2.3. Details for each season are shown below. The isolates from the tissues were assigned to Chapter 3 to determine

the basic characteristics of the bacteria including Chapter 3.2.4 (string test), and 3.2.6 (identification of capsular serotype and presence/absence of *rmpA* gene).

A total of 115 Enderby Island NZSL pups from 2002/2003 were necropsied. A total of 55 cases were identified as having possible *K. pneumoniae* infection, but only 21 cases had tissues available for culture. *K. pneumoniae* was isolated from seven NZSL pups.

A total of 21 Enderby Island NZSL pups from 2011/2012 were necropsied, and 11 cases were identified as having possible *K. pneumoniae* infection. *K. pneumoniae* was isolated from six NSZL pups.

A total of 71 Enderby Island NZSL pup from 2013/2014 were necropsied. A total of 55 cases were identified as having possible *K. pneumoniae* infection. *K. pneumoniae* was isolated from 47 NSZL pups.

Although all the isolates from the pups were HV phenotype, serotype K2 and positive *rmpA* gene, there was variation of colony morphology and the length of the viscous string (phenotypic variation). In order to document the variation among isolates, an isolate from 2002/2003 (HV_Kp2) and an isolate from 2011/2012 (HV_Kp3) from the study in Chapter 3.2.3 were selected to use in further studies.

HV_Kp2 was cultured from the brain tissue of a male pup that was necropsied on 14th February 2003. HV_Kp3 was isolated from an atlanto-occipital joint swab from a female pup that was necropsied on 11st February 2012. Both isolates were used in the studies in Chapter 3.2.4 (string test), 3.2.7 (antimicrobial susceptibility), and 3.2.8 (phenotype microarray). Both isolates were included in genome study in Chapter 5, and

in Chapter 6, the immune response to *K. pneumoniae*, as being representative of HV strains. HV_Kp2 was used in Chapter 3.2.5 (Investigation of the expression of hypermucoviscosity due to available iron) and Chapter 4.2.2.1 (bacterial survivability), as representative of a HV strain.

2.2.2 Dundas Island, Auckland Islands

A total of eight pups were necropsied. The author reviewed the necropsy and histology reports using the protocol in Chapter 2.1.2, and five cases were identified as having possible *K. pneumoniae* infection. The tissues from these cases were further investigated by bacterial culture. *K. pneumoniae* was isolated from four cases. An isolate, HV_Kp5 (D14/15_08Ph), from this batch was selected for further study. This isolate was cultured from brain tissue and confirmed to be HV, serotype K2 and *rmpA* gene-positive using the protocols described in Chapter 3.2.3, 3.2.4, and 3.2.6.

2.2.3 Campbell Island

2.2.3.1 Dead NZ sea lions

Necropsy reports for 73 pups that died during 2014/15 were reviewed using the protocol in Chapter 2.1.2. A total of 36 cases were identified as having possible *K. pneumoniae* infection. *K. pneumoniae* was isolated from 5 NZSL pups. The bacteria were confirmed to be *K. pneumoniae* using the protocol in Chapter 3.2.3. All the isolates were positive on string test using the protocol in Chapter 3.2.4. A total of four isolates were positive for the *rmpA* gene using the protocol in Chapter 3.2.6. One isolate, non-HV_Kp6, cultured from brain tissue from a sea lion pup that died from starvation, was *rmpA*-negative but string test-positive. As a result of this different characteristic, this isolate was selected for further investigation and was included in Chapter 3.2.7 (antimicrobial susceptibility) and in the genome study in Chapter 5. In addition, non-

HV_Kp6 was included into the experiment in Chapter 6.2.1 (oxidative mediated killing assay).

2.2.3.2 Live animals

Rectal swabs and faecal samples were collected from 11 adult NZ sea lions on Campbell Island by the New Zealand sea lion research team in August 2014, as part of a project that was monitoring sea lion health and foraging habits. Sample collection was performed under permits granted by the New Zealand Department of Conservation (DOC) Animal Ethics Committee approvals: AEC52, AEC86, AEC157, AEC158, AEC174, AEC200, and AEC232, while animals were handled for other studies. The adult NZSLs were restrained in a net and sedated with 1 to 3 mL of Midazolam (Hypnovel, Roche Products Ltd, Auckland, New Zealand) intramuscularly. Anaesthesia was induced using Isoflurane (Forane®, Abbott Laboratories Ltd, Queenborough, UK) and oxygen via a mask. Faecal samples or rectal swabs were collected from each individual before they recovered from the anaesthetic procedure and were released. Rectal swabs were collected using transport media swabs with Amies agar gel without charcoal (COPAN, Brescia, Italy) or with sterile plain swabs (with cotton or rayon heads and plastic or wooden stems) in sterile plastic cryogenic vials, and faecal samples were collected directly into sterile plastic cryogenic vials. The samples were stored in liquid nitrogen for up to 12 weeks and then transported to Massey University, Palmerston North, New Zealand and stored in a -80°C freezer until processing.

One of 11 rectal swabs was positive for *K. pneumoniae*. This bacterium was confirmed to be HV *K. pneumoniae*, serotype K2 and positive *rmpA* gene using the protocols described in Chapter 3.2.3, 3.3.4 and 3.2.6. HV_Kp8 was included in Chapter

3.2.8 (antimicrobial susceptibility), the genome study in Chapter 5, and the experiment in Chapter 6.2.1 (oxidative mediated killing assay).

2.2.4 Otago Peninsula

A nine-day old female NZSL pup was found dead on Tomahawk Beach, Otago Peninsula on 23rd January 2013. The pup was transported to Massey University, Palmerston North, New Zealand for necropsy. A swab of joint fluid was collected and submitted to New Zealand Veterinary Pathology Ltd, (Palmerston North, New Zealand) for bacterial culture. *K. pneumoniae* was identified using two different types of biochemical test: API® 20E system (bioMérieux Australia, New South Wales, Australia) and BioLog (Biolog Inc., CA, USA). The API® 20E system test was performed by New Zealand Veterinary Pathology Ltd. The BioLog system test was performed by mEpiLab, Institute of Veterinary, Animal & Biomedical Sciences, Massey University.

The author used the PCR technique in Chapter 3.2.3 to confirm that this isolate (HV_Kp4) was *K. pneumoniae*. HV_Kp4 was confirmed to have a HMV phenotype, serotype K2 and be positive for the *rmpA* gene using the protocols described in Chapter 3.3.4 and 3.2.6. This isolate was used in the studies in Chapter 3.2.7, antimicrobial susceptibility, and 3.2.8, phenotype microarray. This isolate was included in genome study in Chapter 5 and Chapter 6, the immune response to *K. pneumoniae*.

2.3 Other vertebrate samples from the Auckland Islands

2.3.1 Birds from Enderby Island

The bacterial isolates from birds were from a separate study. The bacterial isolation and confirmation of *K. pneumoniae* using PCR were done by a PhD student (Sarah Michael) using the protocol described in Chapter 3.2.3. The author performed string

tests and PCR for the *rmpA* gene and determined the capsular serotype of these isolates using the protocol in Chapter 3.2.6. Samples from birds were collected by a PhD student (Sarah Michael) between 11th January and 26th March 2015 at Sandy Bay, Enderby Island, Auckland Islands. The samples were collected under permit 39915-FAU from the New Zealand Department of Conservation and the Massey University Animal Ethics Committee Approval 14/114.

2.3.1.1 Subantarctic skuas (*Stercorarius antarcticus*)

A total of 33 cloacal swabs and 15 swabs of voided faeces were collected from adult Subantarctic skuas. For sample collection, adult Subantarctic skuas were captured with a hand net and were restrained in a towel for cloacal sampling with a rayon swab (Copan Diagnostics, Murrieta, CA, USA). The swabs were transferred to a sterile cryovial and frozen in liquid nitrogen. All samples were transported in liquid nitrogen to Massey University, Palmerston North and were stored at -80^{o}C until processing.

The swabs were incubated separately in Luria broth at 37^{o}C for 24 h in aerobic conditions in sterile glass vials. Each vial was vortexed for 5 s before the sample swab was inoculated on chromogenic agar (CHROMagar™ Orientation, Fort Richard Laboratories, Auckland, New Zealand), which was then incubated at 37^{o}C in aerobic conditions. After 24 h, colonies consistent with *Klebsiella* spp. (metallic blue) were subcultured onto blood agar (Fort Richard Laboratories) and incubated at 37^{o}C for 24 h.

The bacteria were confirmed to be *K. pneumoniae* by PCR using the *khe* gene protocol described in Chapter 3.2.3. Five samples from skuas were *K. pneumoniae* positive, three isolates were non-HV strains and two were HV strains based on string test.

An isolate from a skua cloacal swab, HV_Kp10, that had an HMV phenotype, serotype K2 and was positive for the *rmpA* gene using the protocols described in Chapter 3.2.4 and 3.2.6 was randomly selected to include in the genome study in Chapter 5.

A non-HV isolate from skua faeces, non-HV_Kp9, was selected to study in Chapter 3 since this isolate was string test-positive on Macconkey agar, but string test-negative on blood agar, suggesting that the external environment might affect expression of the HV phenotype of this isolate. Therefore, this isolate was used in Chapter 3.2.4 (investigation of the expression of hypermucoviscosity due to available iron). In addition, this isolate was included in genome study in Chapter 5.

2.3.1.2 Yellow eyed penguins (*Megadyptes antipodes*)

For sample collection, adult yellow eyed penguins were caught by hand at the eastern end (Sand Dune) and the western end (Penguin Alley) of Sandy Bay Beach Enderby Island, Auckland Islands. A total of 60 cloacal swabs and 7 voided faecal swabs were collected from yellow eyed penguins. The birds were restrained in canvas bags for weighing, morphometric measurements and PIT (passive integrated transponder) tag implantation. If any birds voided faeces during handling, the faeces were collected and frozen in liquid nitrogen. All samples were transported in liquid nitrogen to Massey University, Palmerston North, where they were stored at -80^{o}C until processing.

Bacterial isolation was conducted as described above for subantarctic skua samples (Chapter 2.3.1.1). One sample (cloacal swab) was *K. pneumoniae* positive from 67 samples collected. This isolate, HV_Kp11 was confirmed to be HV *K. pneumoniae*, serotype K2 and positive *rmpA* gene using the protocols described in Chapter 3.2.3 and

3.2.6. Since there was only one *K. pneumoniae* isolate from the yellow eyed penguins, the isolate was included in genome study in Chapter 5 to compare with the other isolates from New Zealand in terms of genotype.

2.4 Substrate samples from sub-Antarctic islands

The substrate samples used in this study were collected by scientists during field visits for other studies. The author cultured these samples and determined characteristics of *K. pneumoniae* isolates as described below.

2.4.1 Enderby Islands

Substrate samples including water, mud, and sand from Enderby Island were collected over three time periods: 2013/2014 breeding season, August 2015, and 2015/2016 breeding season. The details of sample numbers, collection areas and sample types are described in Chapter 4.2.1.1. *K. pneumoniae* were isolated according to the protocol in Chapter 4.2.1.1. The isolates were confirmed to be HV *K. pneumoniae*, serotype K2 and positive *rmpA* gene using the protocol in Chapter 3.2.3, 3.2.4 and 3.2.6. One isolate from 2013/2014, HV_Kp12, was confirmed as HV phenotype, serotype K2 and *rmpA* gene-positive and was selected to include in Chapter 3.2.7 to determine antimicrobial resistant profiles. This isolate was also included in the genome study in Chapter 5.

2.5 Substrate samples from Campbell Island

Substrate samples including water, mud, and sand from Campbell Island were collected in the 2014/2015 breeding season. *K. pneumoniae* were isolated according to the protocol in Chapter 4.2.1.2. The details of sample areas and sample types are described in Chapter 4.2.1.1. The isolates were confirmed to be HV *K. pneumoniae*,

serotype K2 and positive *rmpA gene* using the protocol in Chapter 3.2.3, 3.2.4 and 3.2.6. An isolate, HV_Kp13, from these samples was included in the study in Chapter 5.

2.6 Other vertebrate samples

2.6.1 Marineland pinnipeds

Blood samples of Marineland pinnipeds were collected by veterinarians as part of routine health examinations of captive pinnipeds held at Marineland, Napier, New Zealand. The method of blood collecting is described below.

The blood was collected in November 2014 from two adult California sea lions (an 18-year-old male, and an 11-year-old male) and three adult NZ fur seals (a 12-year-old male, a 9-year-old female, and a 15-year-old female). The animals were sedated with Zoletil 100 (Virbac, Virbac New Zealand Limited, Auckland, New Zealand) by intramuscular injection (IM) and anaesthetised with isoflurane delivered by a mask (Attane, Bayer New Zealand Ltd, Auckland, New Zealand) prior to blood collection. Blood was collected from the lumbar extradural intravertebral vein into plain Vacutainer tubes (Becton-Dickinson, NJ, USA) for serum (California sea lions only) and Vacutainer tubes containing EDTA as an anticoagulant (Becton-Dickinson, NJ, USA) for whole blood (both California sea lions and NZ fur seals). The collection tubes were immediately stored on ice and processed within 24 h. The whole blood was used for the phagocytosis experiment in Chapter 6 within 24 h.

After arrival at the laboratory, the serum tubes were removed from ice and left at room temperature for 30 min to allow for clot formation prior to centrifuging for 15 min at 1500 g at 4°C. The supernatant (serum) was collected into 1.5 mL Eppendorf

tubes and kept at -80°C for three months prior to being used. The serum was used in the study in Chapter 6.

2.6.2 Dogs

Pooled serum was obtained from 40 dog serum samples, submitted for routine serum chemistry to New Zealand Veterinary Pathology Ltd, (Palmerston North, New Zealand) during April to September 2014. The serum was stored at -20 °C for eight months prior to use. The serum was used in the study in Chapter 6.

Blood was collected from three healthy dogs; two male golden retrievers (1 and 10- years-old) and a female golden retriever (1-year-old). The blood was collected from the external jugular vein into Vacutainer tubes containing EDTA as anticoagulant (Becton, Dickinson and Company, USA) and used in the experiment within 1 h of collection. Blood and serum collection were approved by the Massey University Animal Ethics Committee (Approval 14/48). The blood was used in the study in Chapter 6.

2.6.3 African green Monkeys

Genomic DNA of *K. pneumoniae* isolated from three African green monkeys with clinical disease due to *K. pneumoniae* were provided from Ross University, St. Kitts West Indies. These samples were included in genomic study in Chapter 5.

2.7 Reference samples

2.7.1 Human reference samples

The human reference isolates were purchased from the New Zealand Reference Culture Collection (Institute of Environmental Science and Research (ESR), Kenepuru Science Centre, Porirua, New Zealand).

- *Klebsiella pneumoniae* ATCC 700603 (recently renamed *Klebsiella quasipneumoniae* subsp. *similipneumoniae* ATCC 700603). This isolate, non-HV_Kp1, was isolated from urine of a hospitalized patient in Richmond, VA, USA, in 1994. It was confirmed resistant to ceftazidime and other oxyimino-β-lactam antibiotics and was used as a quality-control strain to detect extended-spectrum β-lactamases (ESBLs) in Enterobacteriaceae by the Clinical and Laboratory Standards Institute (CLSI). This isolate was used in Chapters 3, 4, and 6 of this study as a representative of non-HV pathogenic *K. pneumoniae* isolated from a human.
- *Escherichia coli* NCTC 12900; This isolate is serotype O157:H7, Shigatoxin negative which has similar phenotype as toxigenic strains of *E. coli* O157:H7. This strain was a non-pathogenic strain. In this thesis, it was used as a control of non-pathogenic bacterium in Chapter 6.
- *Escherichia coli* ATCC 25992; This strain is a multidrug resistant strain used as a control of antimicrobial drug susceptibility test as recommended by the Clinical and Laboratory Standards Institute (CLSI) protocol. This strain was used as a control for the antimicrobial susceptibility test in Chapter 3.2.7.

Chapter 3

Preliminary characterisation of *Klebsiella pneumoniae* isolates from New Zealand sea lions

3.1 Introduction

In the last decade, interest in *Klebsiella pneumoniae* has increased as it causes a wide range of infections in humans and animals with strains that contain multidrug-resistance genes. *K. pneumoniae* is a gram-negative bacterium that can be found in the environment, such as in soil and surface water, on medical devices, and on the mucosal surfaces of mammals including the gastrointestinal tract and oropharynx (Podschun and Ullmann, 1998), indicating that *K. pneumoniae* can survive in a diverse range of environments. In the past, *K. pneumoniae* ('classical' *K. pneumoniae*) has been recognised as a cause of hospital and community-acquired infection in humans causing mainly pneumonia and urinary tract infections. Most of these cases present in immunocompromised people (Paczosa and Mecsas, 2016; Shon et al., 2013). In the 1980s, a new invasive form of *K. pneumoniae* infection emerged causing liver abscesses with or without septicaemia, occasionally complicated with meningitis and ophthalmitis (Fang et al., 2004; Shon et al., 2013). This invasive form can present in immunocompetent people without underlying causes (Podschun and Ullmann, 1998; Shon et al., 2013). Most of the isolates from invasive infections have a characteristic hypermucoviscous (HMV) phenotype (Fang et al., 2004; Shon et al., 2013). Infection with HV *K. pneumoniae* is not limited to humans, and has been reported in animals including African green monkeys (*Chlorocebus aethiops*) (Soto et al., 2012), camels

(*Camelus dromedarius*) (Sharma et al., 2013), cattle (*Bos Taurus*) (Osman et al., 2014), buffalo (*Bubalus arnee*) (Osman et al., 2014), California sea lions (*Zalophus californianus*) (Jang et al., 2010) and New Zealand sea lions (NZSL; *Zalophus californianus*) (Roe et al., 2015).

K. pneumoniae was reported as the cause of mass mortality in NZSL pups in the 2001/2002 and 2002/2003 breeding seasons at Sandy Bay, Enderby Island, Auckland Islands (Castinel et al., 2007a). After these mass mortality events, *K. pneumoniae* seemed to become endemic in this population (Roe et al., 2015). Previous studies suggested that the mass mortality events were caused by a single clone of *K. pneumoniae* (Castinel et al., 2007b). Bacteria that are from a single clone, however, might show different phenotypes because bacteria can modify themselves by passive mutation and selection or alter gene expression in order to survive in different environments. Moreover, bacteria such as *K. pneumoniae* are able to acquire genetic mobile elements from other bacteria. From the outbreak of *K. pneumoniae* infection in 2001/2002 and 2002/2003 until the present time, *K. pneumoniae* that circulate in this animal group may have changed, for example to survive better in the environment.

In order to better understand *K. pneumoniae* infection in New Zealand sea lions, the aims of this study were to characterise *K. pneumoniae* isolated from NZSL pups and from other sources, to determine if there was any phenotypic variation among the isolates and investigate any phenotype changes over time.

3.2 Materials and Methods

3.2.1 Isolates used in this chapter

In order to make the experiments in this chapter clearer, the author lists the samples that were used in each experiment in Table 3.1. The details of each isolate were described in Chapter 2.

Table 3.1 Isolates used in each experiment

Experiment	non-HV_Kp1	HV_Kp2	HV_Kp3	HV_Kp4	non-HV_Kp7	HV_Kp8	non-HV_Kp9	HV_Kp12
3.2.4 String test length		x	x	x				
3.2.5 Investigation of the expression of hypermucoviscosity due to available iron	x	x					x	
3.2.7 Antimicrobial susceptibility	x	x	x	x	x	x		x
3.2.8 Phenotypic microarray	x	x	x	x				

3.2.2 Review of post mortem reports

In order to compare the preliminary characteristics of *K. pneumoniae* isolates from NZSL pups during the outbreaks (2001/2002, 2002/2003) with those from more recent cases, the post mortem reports from 2002/2003, 2011/2012 and 2013/2014 were reviewed according to the protocol in Chapter 2.1.2. Tissues and swabs from cases that were suspected to have *K. pneumoniae* infection were chosen to be investigated by bacterial culture. If the tissues or swabs were not available, the selected cases were removed from the list.

3.2.3 Culture of *K. pneumoniae* from archived samples and *K. pneumoniae* identification

Tissues or swabs from each case were selected to culture in the following order of precedence: either brain or brain swabs, cerebrospinal fluid swab, atlanto-occipital joint swab or fluid, other joint swab or fluid, wound or pus swab, lymph node, liver,

lungs, other available tissues or swabs. If there was no growth of bacteria from a selected tissue sample, the next tissue in the order above was used to culture.

The frozen tissue and swab samples were thawed at room temperature before inoculation onto MacConkey agar plates (Fort Richard, Auckland, NZ) and incubation at 37°C in aerobic conditions overnight. If a single colony-type or a mixed culture with a predominance (>80%) of one colony type was seen, the colony was subcultured onto blood agar (Fort Richard, Auckland, NZ) for further identification.

K. pneumoniae identification was performed using a PCR assay targeting the hemolysin gene, *khe*, (Babu et al., 2013) as follows. DNA extraction was performed according to the method described by Jang et al., (2009) with the following modifications. Briefly, an overnight-cultured bacterial colony was added to 300 µL of 2% Chelex and boiled for 15 min at 100° C, followed by centrifugation at 12,000 x g for 5 min, and the supernatant was collected. The assay was performed using the primer set 5' ATGAAACGACCTGATTGC 3' and 5' GATTGAGCGGGTAATAAATG 3' (Babu et al., 2013). The expected PCR product size is 400 bp. Each 25 µL PCR reaction mix contained 1X PCR buffer with 2 mM of $MgCl_2$, 2 units TAQ-Ti DNA Polymerase (Fisher Biotec, Australia), 0.2 mM dNTP, and 1 mM each primer. In all experimental runs, negative controls (water) were performed to check for the presence of contamination. The PCR conditions included an initial denaturation at 95°C for 2 min, followed by a 40 cycle amplification consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec. The PCR products were analysed on a 1.5% agarose gel (Invitrogen Corp., CA, USA) containing ethidium bromide and visualised under UV light on a transilluminator.

3.2.4 String test

Bacteria confirmed by PCR as *K. pneumoniae* were inoculated on blood agar and incubated at 37°C overnight. A standard bacteriological loop was used to stretch a viscous string from the colony. A positive string test was defined as the formation of viscous strings that extended vertically for more than 5 mm (Fang et al., 2004)

According to Castinel et al., (2007b), isolates from NZSL pups that died during the 2001/2002 to 2004/2005 seasons from Enderby Island were clonal, based on genetic profiles using pulsed-field gel electrophoresis (PFGE). However, while performing string tests in the current study, variation in the length of the viscous string and the size and morphology of the colonies were observed, suggesting phenotypic variation among these isolates. Three different isolates were therefore selected to more closely characterise these variations. An isolate from 2002/2003 (E02/E03_112Ph, HV_Kp2) was selected as representative of the mass mortality event as an early case isolate. An isolate from 2011/2012 (E11/E12_24Ph, HV_Kp3) was selected as representative of a recent case isolate. An isolate from a pup that died of *K. pneumoniae* on the Otago Peninsula (S13_04Ph, HV_Kp4) was selected as a representative of a different environment (details of isolates were described in Chapter 2.2.1 and 2.3.1). The morphology and the size of colonies of these three isolates was observed using 10 colonies per isolate. The length of the viscous strings was measured by using ten colonies of each isolate (measured once per colony). A standard bacteriological loop was put onto the colony and stretched until the string separated. The string test was recorded using a video camera. The length of the string was captured from the video recording by reading from the scale placed on the background

(Fig 3.1). The result was presented as mean ± standard deviation (SD) of the viscous strings of each isolate.

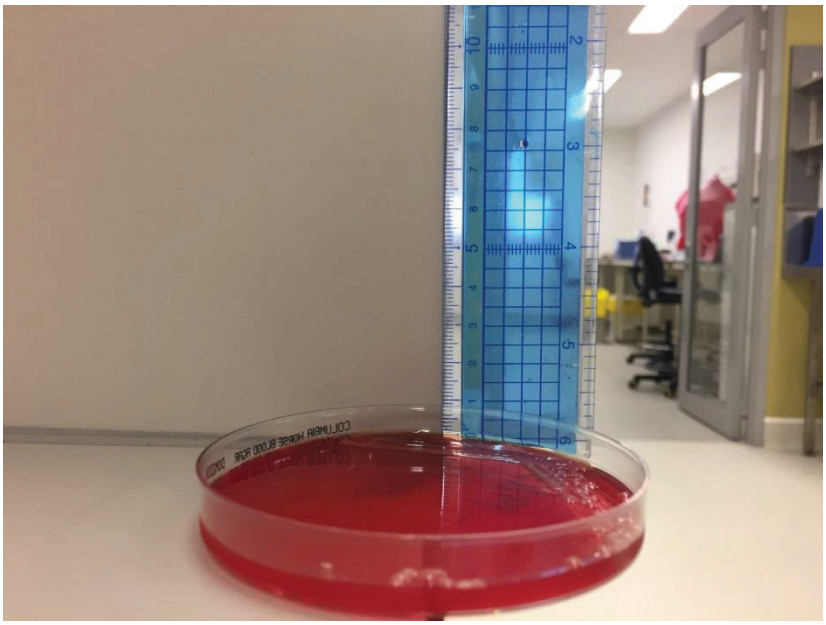

Figure 3.1 Scale for measurement of the length of viscous string
A picture from the video camera shows scale on background that was used to measure the length of viscous string from *K. pneumoniae*

3.2.5 Investigation of the expression of hypermucoviscosity due to available iron

During culture from stored NZSL tissues and swabs from birds from Enderby Island (as described in Chapter 2), an isolate from a faecal swab from a brown skua (non-HV_Kp9) was noted to be positive on string test when cultured on MacConkey agar. However, when subcultured onto blood agar, the same isolate was negative on string test. This characteristic of this isolate was different from non-HV isolates from a human (non-HV_Kp1) and a NZSL adult (non-HV_Kp7), which were consistently negative for the string test on both agars.

Cheng et al., (2011) showed that the availability of iron has an effect on the expression of the genes that control capsular polysaccharide synthesis, which is related to the hypermucoviscous phenotype in *K. pneumoniae*. I hypothesised that variability in string test results may be due to different levels of iron availability between blood and MacConkey agar. Accordingly, the following study was conducted.

Three isolates were used in this experiment: non-HV_Kp1, HV_Kp2 and non-HV_kp9 (details of isolates were described in Chapter 2.2.1, 2.3.1, 2.7.1). Non-HV_Kp1 was used as a non-HV isolate control. HV_Kp2 was used as a HV control. Non-HV_Kp9 was used as the isolate that can potentially switch phenotype.

Non-HV_Kp1, HV_Kp2 and non-HV_Kp9 (Table 2.1 in Chapter 2) were cultured at 37°C for 24 h on agars with different amounts of iron. The agars were prepared as follows:

- Nutrient agar powder, prepared according to the manufacturer's instructions (Oxoid LTD, Hampshire, England) and a final concentration of 1% EDTA (Sigma-Aldrich, St Louis, MO, USA) (a low iron concentration, less than 1.5 mg/100 g)
- Nutrient agar powder, prepared according to the manufacturer's instructions (a moderate iron concentration of 1.5 mg/100g)
- Nutrient agar powder, prepared according to the manufacturer's instructions and a final concentration of 10% horse blood (a high iron concentration, greater than 1.5 mg/100 g)

After incubation at 37°C for 24 h, the string test (described in Chapter 3.2.3) was performed on each isolate.

3.2.6 Investigation of the capsular serotype, *rmpA* genes using PCR

DNA extraction was performed according to the method described above (Chapter 3.2.2). The PCR assay was performed using primers for three targets: capsular type K1/*magA*, capsular type K2/*K2wzy*, and *rmpA*. The primer sequences are listed in Table 3.2. Separate reactions were set up for each primer pair. Each 25 µL PCR reaction mix contained 1X PCR buffer with 20 µM of $MgCl_2$, 2 units TAQ-Ti DNA Polymerase (Fisher Biotec, Australia), 0.2 mM dNTP, and 1 mM each primer. In all experimental runs, negative controls (water) were included to check for the presence of contamination. The PCR conditions included an initial denaturation at 95°C for 2 min, followed by a 40-cycle amplification consisting of denaturation at 95°C for 30 sec, annealing at 60°C (Capsular type K2, and *rmpA*) or 56°C (Capsular type K1) for 30 sec and extension at 72°C for 1 min. The PCR products were analysed on a 1.5% agarose gel (Invitrogen Corp., CA, USA) containing ethidium bromide and visualised under UV light on a transilluminator.

Table 3.2 Primer information for each target gene

Target	Primer	Sequence (5'–3')	Product size (bp)	Reference
Capsular type K1	*magA* F	GGTGCTCTTTACATCATTGC	1283	Fang et al. (2004)
	magA R	GCAATGGCCATTTGCGTTAG		
Capsular type K2	*K2wzy* F	GACCCGATATTCATACTTGACAGAG	641	Turton et al. (2008)
	K2wzy R	CCTGAAGTAAAATCGTAAATAGATGGC		
RmpA	*rmp*A F	ACTGGGCTACCTCTGCTTCA	516	Nadasy et al. (2007)
	*rmp*A R	CTTGCATGAGCCATCTTTCA		

3.2.7 Antimicrobial susceptibility

In order to investigate variation in antimicrobial susceptibility between isolates from different years, sources and locations, six isolates were selected as shown in Table 3.3 (details of isolates were described in Chapter 2).

The antimicrobial disk diffusion test was performed according to the CLSI (Clinical and Laboratory Standard Institute) M02-A12 protocol (CLSI, 2015) using seven antimicrobial agents: amoxycillin/clavulanic acid 30 µg, ampicillin 10 µg, ceftriaxone 30 µg, ciprofloxacin 5 µg, cefuroxime sodium 30 µg, gentamicin 10 µg, and sulfamethoxazole/trimethoprim 19:1 25 µg. The six *K. pneumoniae* isolates (Table 3.3) plus two controls, *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 purchased from New Zealand Reference Culture Collection (Institute of Environmental Science and Research (ESR), Kenepuru Science Centre, Porirua, New Zealand), were also included in the tests.

Colonies of overnight cultures of each isolate (Table 3.3) on a blood agar plate were mixed into 2.5 mL 0.85% w/v sterile saline (Fort Richard, Auckland, NZ) to a suspension density equivalent to 0.5 McFarland standard. For each isolate, plates were inoculated by dipping sterile cotton swabs (one swab per plate) into the tube containing 0.5 McFarland turbidity (approximately 1.5 x 10^8 CFU/mL) bacterial suspension and streaking the entire surface of a Muller-Hinton agar plate (Fort Richard, Auckland, NZ) in three directions, rotated approximately 60° each time. After 3 - 5 min, antimicrobial disks were placed on the plates, three to four antimicrobial disks per plate. The plates were incubated at 37°C for 24 h. The inhibition zone including the diameter of the disk was measured using a digital sliding calliper. The interpretation was performed

according to the interpretive standards of the Clinical and Laboratory Standards Institute (CLSI), M02-A12 protocol (CLSI, 2015), (Appendix A3.4)

Table 3.3 Bacterial isolates used in antimicrobial susceptibility test

Isolate designation	Bacterial isolates	Year	Source	Tissue/ substrate	String test
E. coli	*E. coli* ATCC 25992 (control)	1946	Human	-	-
Non-HV_Kp1	*K. pneumoniae* ATCC 700603 (control)	1994	Human	Blood	-
HV_Kp2	*K. pneumoniae* E02/03_112Ph	2002/2003	NZSL pup (post mortem) from Enderby Island[a]	Brain	+
HV_Kp3	*K. pneumoniae* E11/12_24Ph	2011/2012	NZSL pup (post mortem) from Enderby Island[a]	A/O joint swab	+
HV_Kp4	*K. pneumoniae* S13_04Ph	2013	NZSL pup (post mortem) from Otago Peninsula[a]	Wound swab	+
Non-HV_Kp7	*K. pneumoniae* E09/10_13Ph	2009/2010	NZSL adult from Enderby Island[b]	Tracheal lymph node	-
HV_Kp8	*K. pneumoniae* 9746	2013/2014	Healthy NZSL adultfrom Campbell Island[c]	Rectal swab	+
HV_Kp12	*K. pneumoniae* E13/14_10sub	2013/2014	Substrate sample from Enderby Island	Water	+

[a] These animals died from *K. pneumoniae* infection and samples were collected at post mortem examination.
[b] This isolate was from an unrelated study. The bacterium was isolated from the tracheal lymph node of a female adult NZSL that died from trauma.
[c] This isolate was from an unrelated study. The bacterium was isolated from a rectal swab of a healthy adult female NZSL from Campbell Island in 2014.
A/O = Atlanto-occipital

3.2.8 Phenotypic microarray

Three *K. pneumoniae* isolates (one early case from 2002/2003: E02/03_112Ph (HV_Kp2), one recent case from 2011/2012: E11/12_24Ph (HV_Kp3), and a pup that died at Otago, January 2013: S13_4Ph (HV_Kp4)) stored in glycerol stock from previous studies, plus *K. pneumoniae* ATCC 700603 (non-HV_Kp1), were cultured on blood agar plates at 37°C for 24 h. Details of isolates were described in Chapter 2.2. A single colony from each isolate was subcultured onto a new blood agar plate and incubated at 37°C for 24 h before testing.

3.2.8.1 Preparation of PM1, PM2A, PM3B, and PM10

The bacteria were tested using 96 well Biolog® Phenotype MicroArray (Biolog Inc., CA, USA) metabolic panels including carbon (PM1 and PM2A), nitrogen (PM3B), and pH sensitivity (PM10). Inoculation fluid-0a (IF-0a; Biolog Inc., CA, USA) was used for the carbon (PM1 and PM2) and nitrogen (PM3B), and IF-10 (Biolog Inc., CA, USA) for pH sensitivity (PM10). IF-0a fluid was prepared by adding 25 mL of sterile water into 125 mL of 1.2 x IF-0a (Biolog Inc., CA, USA); 12 mL of this was then transferred to a sterile test tube. The overnight cultures of each *K. pneumoniae* isolate were mixed with 12 mL of prepared IF-0a using sterile swabs to a final concentration 42%T (transmittance) as measured by the Biolog® Turbidimeter (Biolog Inc., CA, USA). Subsequently, 10 mL of 42%T cell suspension was added to 50 mL of IF-0a plus 0.72 mL of dye mix A (Biolog Inc., CA, USA), and 22 mL of this fluid was transferred into a sterile reservoir followed by inoculation into the carbon (PM1 and PM2) plates (100 µL/well). The remaining 38 mL of fluid had 760 µL of 50X sodium succinate/ferric citrate supplement added and was then inoculated into the nitrogen (PM3B) plate (100 µL/well). Another 150 µL of 42%T cell suspension was added to 25 mL of IF-10 plus 0.3 mL of dye mix A and inoculated into the pH sensitivity (PM10) plate (100 µL/well). All the steps were carried out in a laminar flow cabinet to maintain a sterile environment. A cell free negative control was included on each plate (PM1, PM2, PM3 and PM10). All plates were incubated at 37°C for 24 h in the OmniLog machine (Biolog Inc., CA, USA). All the assays were repeated at least three times separately. All the wells in the plates were cultured on blood agar to determine the growth ability after incubating in the OmniLog machine for 24 h.

The negative control well (A01) in PM1 was constantly positive. After consulting the manufacturer of Biolog® and doing literature search two methods were used to try

and solve the problem: using a lower cell density, and preventing volatile products from migrating from an adjacent well.

Lowering the cell density of the inoculum was aimed to reduce the effect of hypermucoviscous capsule, which mainly composed of polysaccharides. This may have provided an extra carbon source that could be metabolised by bacteria in the negative control well. As such, the bacterial suspension was diluted to concentrations of 1:10, 1:50, 1:100, 1:200, 1:500, and 1:1000.

PARAFILM®M sealing film (Bemis Company, Inc, WI USA) and Microplate Sealing Tape (Thermo Scientific, MA USA) was applied to the PM1 plate to prevent the migration of volatile products to adjacent wells. *K. pneumoniae* could potentially metabolise sugar and produce acetic acid. The volatile acetic acid could migrate to an adjacent well and act as a carbon source.

3.2.8.2 Data recording and statistical analysis

The metabolic activities were spectrophometrically recorded every 15 min by a charged coupled device (CCD) camera for 24 h in the OmniLog machine. Each well in these plates is designed to measure the ability of an organism to catabolise a particular metabolite. The positive reactions were visualised by a dye colour change. An increase in the metabolic response corresponds with a darkening of the colour. The camera captures the digital image of each plate and the computer transforms the colour change into quantitative values. The data can be accessed with OmniLog PM software (version 1.20.02, Biolog Inc., CA, USA). The data were exported from OmniLog PM software and analysed within the RStudio environment (Version 0.98.1103, R Development Core Team, 2013). The BioLog phenotypic microarray data was analysed

using the opm package (Vaas et al., 2013; Vaas et al., 2012). The function ci() in the opm package was used to calculate the significance of the activity in each well for the isolates using a 95% confidential interval.

3.3 Results

3.3.1 Case selection and culture of K. pneumoniae from archived samples

A total of 115 Enderby Island NZSL pups from 2002/2003 were necropsied. A total of 55 NZSL pups were suspected to have died of *K. pneumoniae* infection. Since the tissues from this year were used for several studies, only 21 cases had remaining tissue available to investigate with bacterial culture. *K. pneumoniae* was isolated from the tissues of seven of these NSZL pups (Appendix A3.1).

A total of 21 Enderby Island NZSL pups from 2011/2012 were necropsied. A total of 11 cases were further investigated with bacterial culture. *K. pneumoniae* was isolated from the tissues of six of these NSZL pups (Appendix A3.2).

A total of 71 Enderby Island NZSL pups from 2013/2014 were necropsied. A total of 51 cases were further investigated with bacterial culture. *K. pneumoniae* was isolated from tissues of 47 of these NSZL pups (Appendix A3.3).

All the isolates tested from Enderby Island NZSL pups were serotype K2, positive *rmpA* gene and hypermucoviscous phenotype (positive string test) (Fig 3.2, 3.3, 3.4). The full results of tissue culture and PCR can be found in Appendices A3.1, A3.2, A3.3.

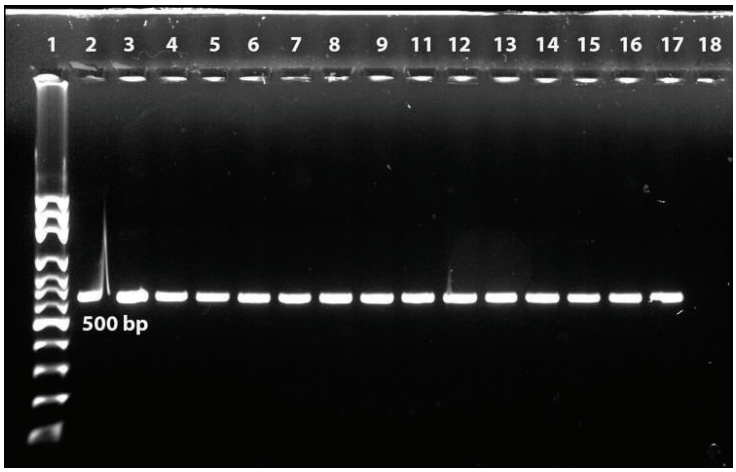

Figure 3.2 PCR result of K2 *wzy* gene

Positive PCR for the K2 *wzy* gene (641 bp). Lane 2 is a positive control, and lane 18 is a negative control. Lane 3 to lane 17 show positive results from the samples isolated in 2002/03 and 2013/2014 breeding season: E02/03_42Ph, E02/03_64Ph, E02/03_70Ph, E02/03_109Ph, E02/03_111Ph, HV_Kp2 (E02/03_112), E02/03_113Ph, E13/14_36Ph, E13/14_18Ph, E13/14_39Ph, E13/14_41Ph, E13/14_42Ph, E13/14_43Ph, and E13/14_44Ph, respectively.

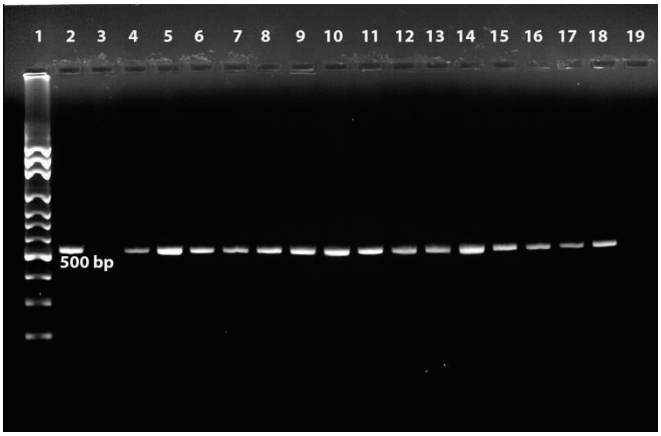

Figure 3.3 PCR result of *rmpA* gene

Positive PCR for the *rmpA* gene (516 bp). Lane 2 is a positive control, and lane 19 is a negative control. Lane 3, non-HV_Kp1 (*K. pneumoniae* ATCC 700603), shows a negative result. Lane 4 to 18 show positive results from the samples isolated in the 2002/03 to 2011/12 breeding season: E02/03_42Ph, E02/03_64Ph, E02/03_70Ph, E02/03_109Ph, E02/03_111Ph, HV_Kp2 (E02/03_112), E02/03_113Ph, E11/12_09Ph, E11/12_12Ph, E11/12_16Ph, E11/12_18Ph, E11/12_23Ph, E11/12_24Ph, E13/14_36Ph and E13/14_41Ph, respectively.

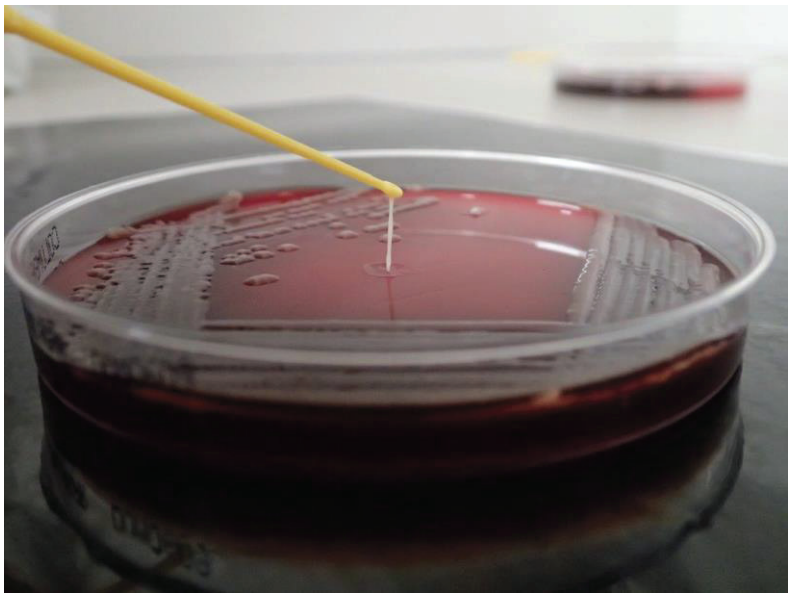

Figure 3.4 String test.
An example of a string test (a string of 5 mm or longer is defined as positive). The stretching of an HMV_Kp2 colony resulted in the formation of a string 18 mm in length.

3.3.2 String test

The three selected isolates for string test variation showed different lengths of viscous string and different colony morphology (Fig 3.5). The lengths of viscous string of HV_Kp2, HV_Kp3 and HV_Kp4 were 19.80 ± 2.14, 11.40 ± 1.26 and 13.40 ± 1.83 mm, respectively. Among the three isolates, HV_Kp2 had the biggest colony, 4.2 ± 0.3 mm in diameter with yellow transparency, while HV_Kp3 had the smallest colonies, 2.4 ± 0.2 mm in diameter. HV_Kp4 had a round mucoid white colony, 2.8 ± 0.2 mm in diameter.

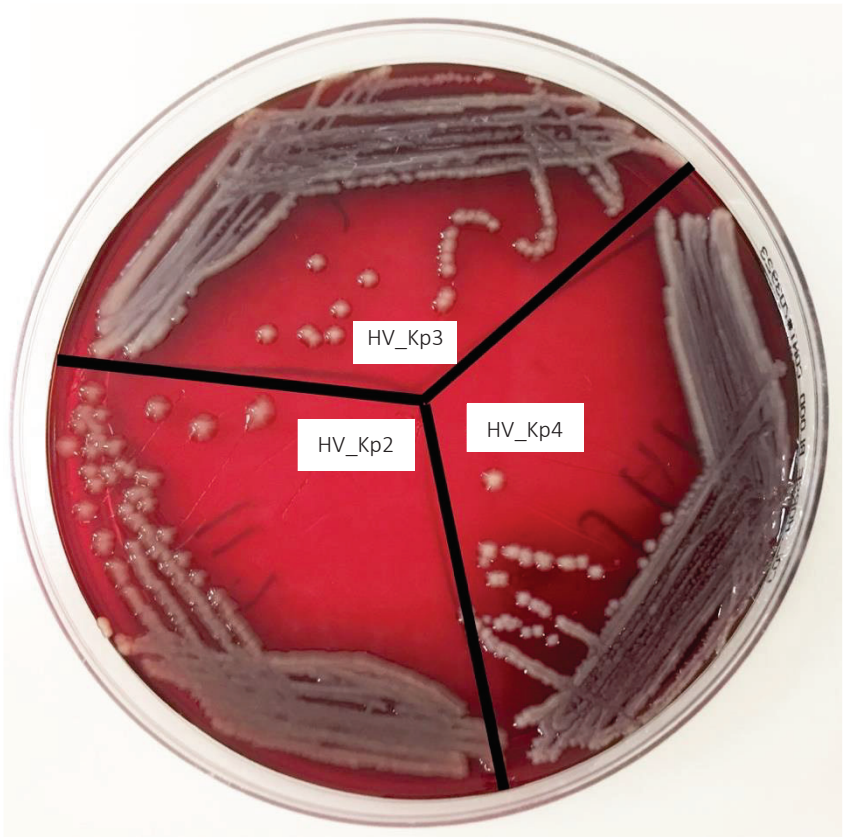

Figure 3.5 Three *K. pneumoniae* isolates on blood agar plate
A blood agar plate showing morphology differences between the isolates. Among three isolates, HV_Kp2 has the biggest colony with a yellow transparent colour. HV_Kp3 has the smallest colony with a white appearance. The size of colonies of HV_Kp4 are in the middle among these three isolates with a white appearance.

3.3.3 Phenotypic change due to availability of iron

After incubation at 37°C for 24h, HV_Kp2 colonies had a positive string test on agars with three different concentrations of iron. The non-HV_Kp1 and non-HV_Kp9 colonies were string test negative on agars with three different concentrations of iron.

3.3.4 Antimicrobial susceptibility

The selected isolates from NZSLs and the isolate from a substrate sample from Enderby Island (Table 3.1) were susceptible to six antimicrobial agents including amoxycillin/clavulanic acid (amoxy/clav), ampicillin, ceftriaxone, ciprofloxacin,

cefuroxime sodium, gentamicin and sulfamethoxazole/trimethoprim (sul/trimeth) 19:1.

They were resistant to ampicillin (Table 3.4, Fig 3.6).

Table 3.4 Antimicrobial susceptibility results. The isolates from NZSLs and the isolate from the substrate sample from Enderby Island were susceptible to all antimicrobial agents except ampicillin. The number represents the diameter of the inhibition zone (mm).

Isolate	Inhibition zone (mm)						
	Amoxycillin/ clavulanic acid	Ampicillin	Cefuroxime	Ceftriaxone	Gentamicin	Ciprofloxacin	Sulfamethoxazole/ Trimethoprim 19:1
HV_Kp2	24.3	7.9	23.7	33.3	23.5	32.1	24.9
HV_Kp3	25.6	6	28.9	37.5	23.4	35.3	23.6
HV_Kp4	24.1	9.6	26.2	36.8	23.9	32.9	24.6
Non-HV_Kp7	22.2	6	22.4	31.6	22.1	31	25.8
HV_Kp8	24.1	11.9	26.4	35.6	22.2	34.2	24
HV_Kp9	24.3	7.9	24.4	34.5	23.1	32.5	24.8
Non-HV_Kp1	16.6	6	12.1	21.1	14.1	25.1	9.9
Escherichia coli ATCC 25922	19	6	25	37.2	23.8	41.8	21.6

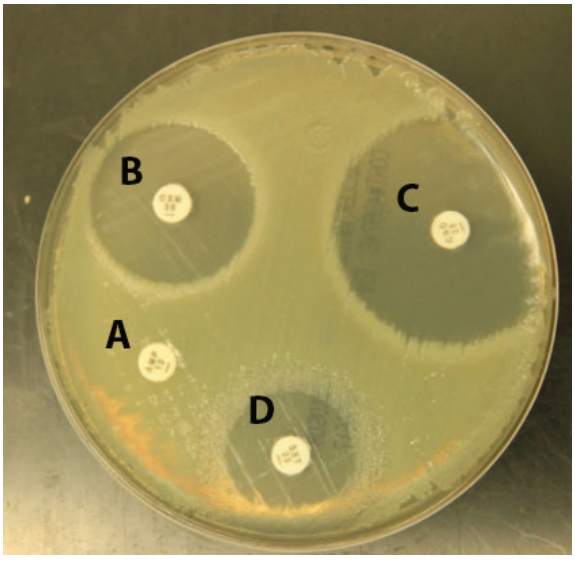

Figure 3.6 Antimicrobial sensitivity on Mueller Hinton using disk diffusion
Antimicrobial sensitivity of HV_Kp3: A = ampicillin, B = cefuroxime sodium, C = ciprofloxacin, D = sulfamethoxazole/trimethoprim

3.3.5 Phenotype microarray

After incubation in the Omnilog machine for 24 h, each well of every plate was inoculated onto blood agar to confirm the presence or absence of viable bacteria from the active (purple colour) and non-active (no colour) wells. Bacterial growth on agar was not observed from the wells with no activity (no colour), but from active wells.

The negative control well in PM1 was positive using the manufacturer's protocol. With the lower cell density inoculum method, the negative well still showed a positive reaction with 1:10, 1:50, 1:100, 1:200, and 1:500 dilutions. The negative control well tended to have decreased activity as bacterial density decreased, however the activity in other wells also decreased. At a concentration of 1:1000, no activity was observed in any well of the PM1 plate. For the other plates, PM2, PM3, and PM10, the negative control well was negative using the method described by the manufacturer.

PARAFILM®M sealing film and Microplate Sealing Tape were applied to the PM1 plate. The negative control well in PM1 was positive in both plastic sealing conditions.

3.3.5.1 Carbon source utilisation (PM1 and PM2)

Since there was a reaction for the negative control on the PM1 plate, the results from PM1 were excluded from the analysis. Non-HV_Kp1 were able to utilise 62 out of 95 available carbon sources on PM2 (Fig 3.7). HV_Kp2, HV_Kp3, and HV_Kp4 were able to utilise 58, 58 and 54 of the 95 carbon sources, respectively (Fig 3.6). There were four carbon sources including chondroitin sulphate C, D-tartaric acid, D-fucose and D,L-carnitine that only non-HV_Kp1 could utilise. Non-HV_Kp1 and HV_Kp4 were not able to utilise 2-deoxy-D-ribose and 5-keto-D-gluconic acid, while the other isolates were. Only HV_Kp4 was not able to utilise L-phenylalanine. The wells in which an isolate showed

phenotypic variation are shown in Table 3.5. The complete results for carbon source utilisation are shown as a heatmap in Fig 3.7.

Table 3.5 Utilisation of carbon sources in PM2A from three NZSL isolates and non-HV_kp1 (*K. pneumoniae* ATCC 700603). A positive result is (+), a negative (-) and (+/-) shows some replicates being positive and some negative.

Well number (Carbon source)	Non-HV_Kp1	HV_Kp2	HV_Kp3	HV_Kp4
A01 (Negative control)	-	-	-	-
A02 (chondroitin culfate C)	+/-	-	-	-
B09 (2-deoxy-D-Ribose)	-	+/-	+/-	-
B11 (D-fucose)	+/-	-	-	-
D09 (N-acetyl-D-glucosaminitol)	+/-	-	-	+/-
E12 (5-keto-D-gluconic acid)	-	+/-	+/-	-
F06 (quinic acid)	+	+	+	+
F11 (D-tartaric acid)	+	-	-	-
H02 (L-phenylalanine)	+	+	+	-
H05 (D,L-carnitine)	+	-	-	-
H07 (D,L-octopamine)	+	+/-	+/-	-

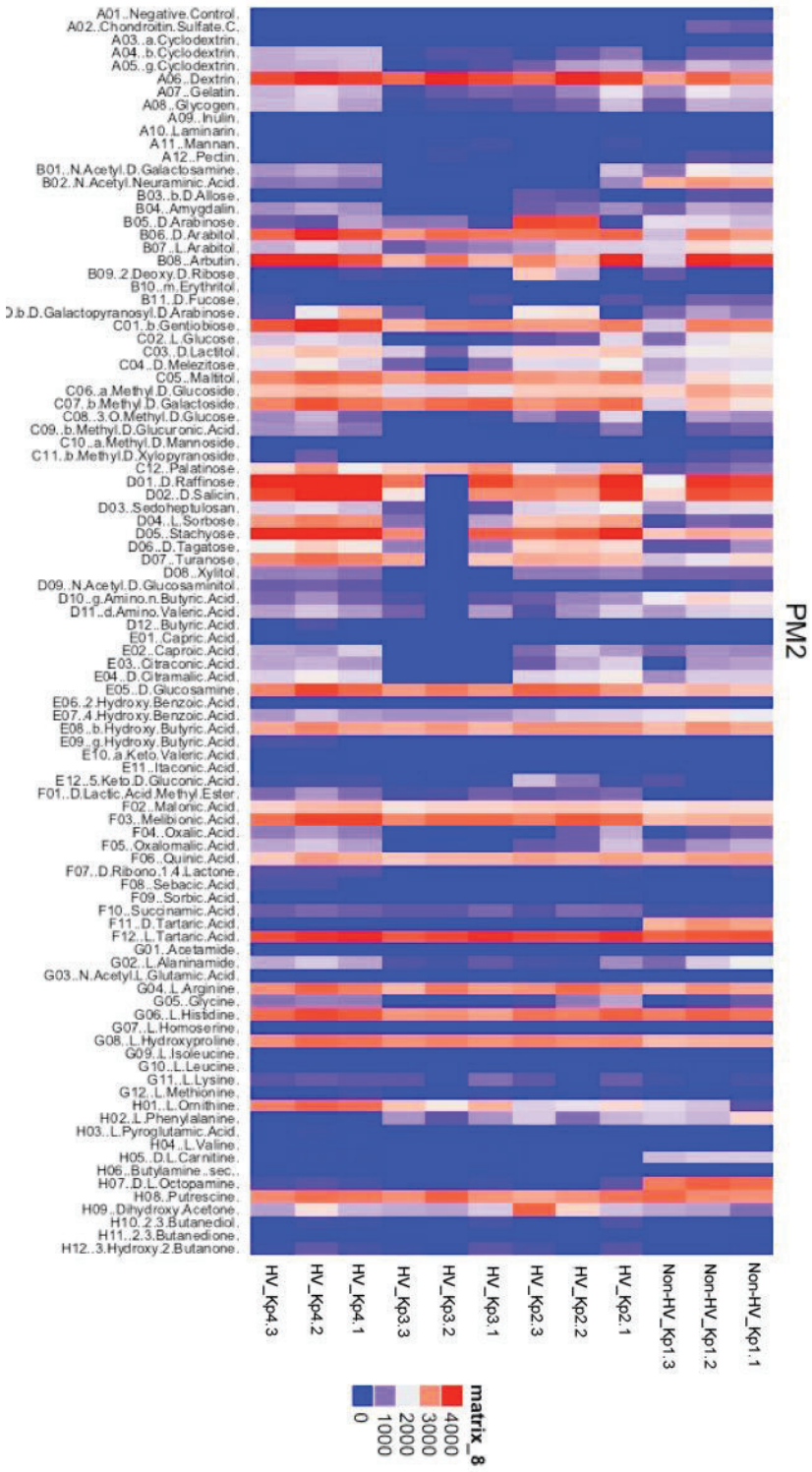

Figure 3.7 Heatmap of utilisation of carbon sources in PM2A

The heatmap of utilisation of carbon sources in PM2A (triplicate) from three NZSL isolates and non-HV_Kp1 (human isolate). Number after the isolate name represents the replicate. The isolate name identifies each row. The PM2A carbon source is identified in each column.

3.3.5.2 Nitrogen source utilisation (PM3B)

All four isolates tested showed the ability to utilise the same 42 nitrogen sources. Individually, non-HV_Kp1 was able to utilise 56 out of 95 available nitrogen sources. HV_Kp2, HV_Kp3 and HV_Kp4 were able to utilise 43, 44, and 47 out of the 95 nitrogen sources, respectively (Fig 3.8). Only non-HV_Kp1 was able to utilise L-cysteine, L-methionine, L-tryptophan, L-tyrosine, L-citrulline, tyramine, xanthine, parabanic acid and ε-amino-*N*-caproic acid. Only HV_Kp2 was not able to utilise glycylmethionine (gly-met) and only HV_Kp4 was unable to utilise L-phenylalanine. The wells in which an isolate showed phenotypic variation are shown in Table 3.6. The complete results for nitrogen source utilisation are shown as a heat map in Fig 3.8.

Table 3.6 Utilisation of nitrogen sources in PM3B from three NZSL isolates and non-HV_Kp1 (*K. pneumoniae* ATCC 700603). A positive result is (+), a negative (-) and (+/-) shows some replicates being positive and some negative.

Well number (Nitrogen source)	Non-HV_Kp1	HV_Kp2	HV_Kp3	HV_Kp4
A01 (Negative Control)	-	-	-	-
A02 (ammonia)	+	-	-	+/-
A11 (L-cysteine)	+	-	-	-
B06 (L-lysine)	+	-	-	+/-
B07 (L-methionine)	+/-	-	-	-
B08 (L-phenylalanine)	+	+	+	-
B12 (L-tryptophan)	+	-	-	-
C01 (L-tyrosine)	+	-	-	-
C09 (D-valine)	+	-	-	+/-
C10 (L-citrulline)	+	-	-	-
E03 (tyramine)	+	-	-	-
G01 (xanthine)	+	-	-	-
G04 (alloxan)	+	-	-	+/-
G06 (parabanic acid)	+	-	-	-
G09 (ε-amino-N-caproic acid)	+	-	-	-
H11 (gly-met)	+	-	+	+

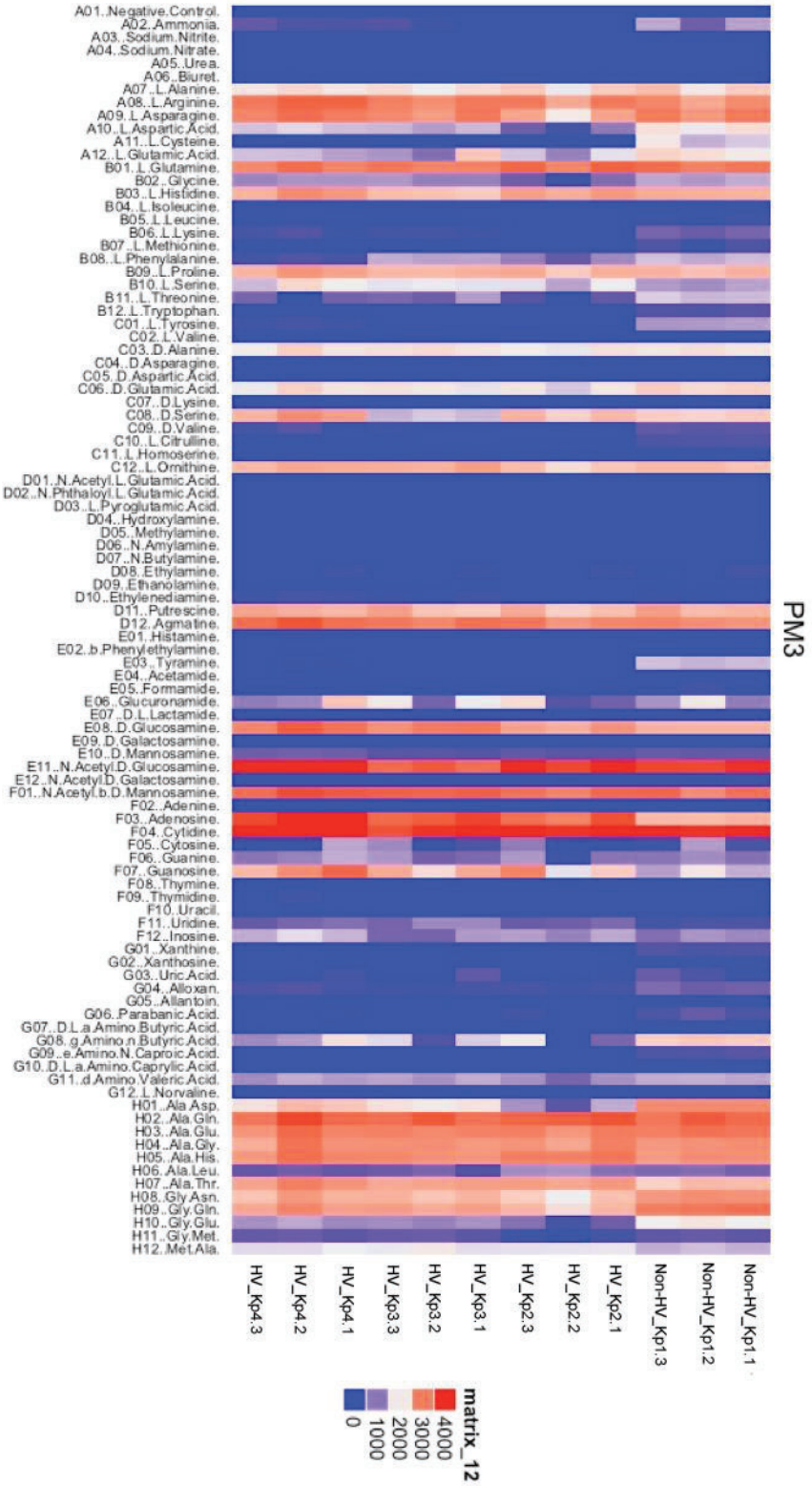

Figure 3.8 Heatmap of utilisation of nitrogen sources in PM3B

The heatmap of utilisation of nitrogen sources in PM3B (triplicate) from three NZSL isolates and non-HV_Kp1 (human isolate). Number after the isolate name represents the replicate. The isolate name to identifies each row. The PM3B nitrogen source is identified in each column.

3.3.5.3 pH sensitivity (PM10)

None of the isolates had activity at pH 3.5 or pH 4.0, but all were active from pH 4.5 to pH 10. Non-HV_Kp1 had activity in 84 of 96 different instances. HV_Kp2, HV_Kp3 and HV_Kp4 had activity in 81, 78, and 82 different instances, respectively. Only non-HV_Kp1 showed activity in L-isoleucine + pH 9.5 and L-leucine + pH 9.5. Only HV_Kp3 had no activity in L-methionine + pH 9.5. The activity of isolates at different pH's is shown in Table 3.7. The complete results for pH sensitivity are shown as a heatmap in Fig 3.9.

Table 3.7 pH sensitivity in PM10 from three NZSL isolates and non-HV_Kp1 (*K. pneumoniae* ATCC 700603). A positive result is (+), a negative (-) and (+/-) shows some replicates were positive and some negative.

Well number (Substrate)	Non-HV_Kp1	HV_Kp2	HV_Kp3	HV_Kp4
A01 (pH 3.5)	-	-	-	-
A02 (pH 4)	-	-	-	-
A03 (pH 4.5)	+	+	+	+
A04 (pH 5)	+	+	+	+
A05 (pH 5.5)	+	+	+	+
A06 (pH 6)	+	+	+	+
A07 (pH 7)	+	+	+	+
A08 (pH 8)	+	+	+	+
A09 (pH 8.5)	+	+	+	+
A10 (pH 9)	+	+	+	+
A11 (pH 9.5)	+	+	+	+
A12 (pH 10)	+	+	+	+
B01 (pH 4.5)	+	+	+	+
E10 (L-isoleucine + pH 9.5)	+	-	-	-
E11 (L-leucine + pH 9.5)	+	-	-	-
F01 (L-methionine + pH 9.5)	+	+/-	-	+/-

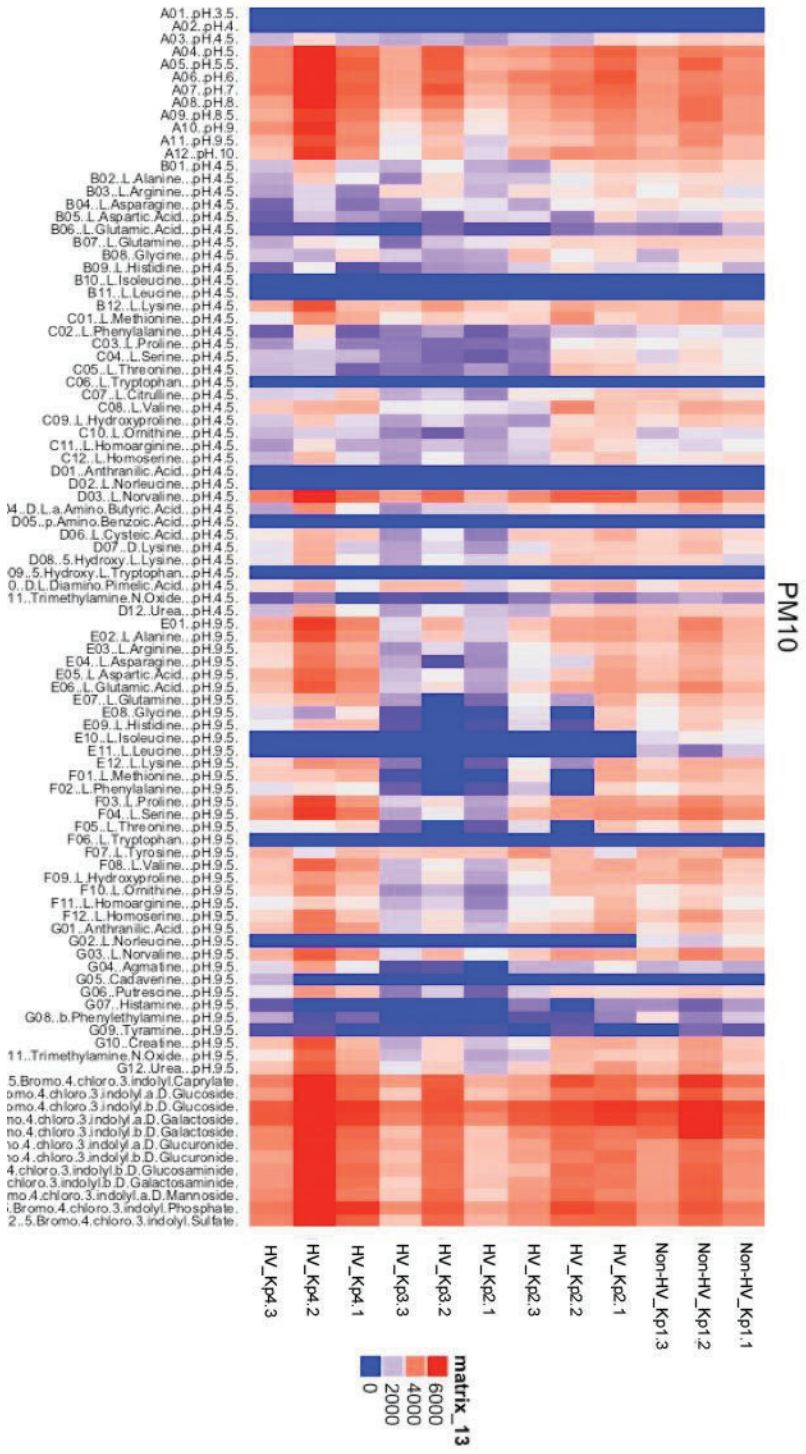

Figure 3.10 Heatmap of pH tolerance in PM10

The heatmap of pH tolerance in PM10 (triplicate) from three NZSL isolates and non-HV_Kp1 (human isolate). Number after the isolate name represents the replicate. The isolate name to identifies each row. The source is identified in each column.

3.4 Discussion

In this present study, the *K. pneumoniae* isolates from Enderby Island in 2002/2003, 2011/2012 and 2013/2014 were characterised. The isolates revealed the same basic characteristics, namely, a HMV phenotype, a K2 serotype and presence of the *rmpA* gene. This suggests that the *K. pneumoniae* that caused disease in 2011/12 and 2013/14 was a closely related strain to, or the same as, the strain that caused disease in 2002/3. The basic characteristics of NZSL pup isolates were consistent with isolates from the invasive form of *K. pneumoniae* infection in humans (Fang et al., 2004; Paczosa and Mecsas, 2016; Shon et al., 2013). These characteristics were also found in *K. pneumoniae* isolates from California sea lions (Jang et al., 2010).

A hypermucoviscous phenotype can be influenced by several factors including the presence of genes such as *magA, rmpA*/*A2*, and *rcsA*/*rcsB* (regulation of capsule synthesis A and B genes) as well as the external environment. In this present study, *rmpA* was found in the HV isolates from NZSL pups and all of these isolates were negative for *magA,* implying that *rmpA* controls the hypermucoviscous phenotype in these isolates. Initially, *magA* was reported to be associated with HV strains causing liver abscesses in humans (Fang et al., 2004), but further research pointed out that *magA* is restricted to serotype K1, as it is the serotype K1 allele of the *wzy* gene in the *cps* gene cluster (Fang et al., 2007). The *magA* gene has now been renamed to *wzy*_K1 (Fang et al., 2010). Accordingly, it was unsurprising that the NZSL pup isolates were *magA* negative because they were serotype K2.

K. pneumoniae infection is of concern in human medicine due to the ability of this bacterium to acquire multiple antibiotic resistance genes, particularly in “classical” (non-HV) strains. Most HV strains are susceptible to most antibiotics, except ampicillin,

to which *K. pneumoniae* is inherently resistant due to a chromosomal gene (bla_{shv-1}) (Davies et al., 2016), although evidence of multidrug resistance has been reported (Lee et al., 2017). A previous study showed that NZSL pup isolates from 2001/2 and 2002/3 were susceptible to cephalexin, cephalothin, cefuroxime, neomycin, and dihydrostreptomycin and were negative for extended-spectrum beta-lactamase (ESBL) production (Castinel et al., 2007b). From 2000 to the present, a number of *K. pneumoniae* antimicrobial resistant strains from humans, animals and the environment have been reported worldwide (Wyres and Holt, 2018), including non-HV isolates from humans in New Zealand (Howard et al., 2016; Williamson and Heffernan, 2014).

Most antimicrobial resistance in *K. pneumoniae* is a result of acquisition of antibiotic resistance genes by horizontal gene transfer, with the majority of these occurring via plasmid conjugation (Navon-Venezia et al., 2017; Wyres and Holt, 2016). This genetic transfer can happen over a short period of time, from several minutes up to several hours (Low, 2001). The genetic transfer can occur in human and animal hosts (Broaders et al., 2013), as well as in the natural environment (Lorenz and Wackernagel, 1994). Therefore, it is theoretically possible that NZSL pup isolates could have developed antimicrobial resistance between 2002/03 and 2012/13. In the present study, the author tested seven antibiotic drugs that are commonly used to treat gram-negative bacteria including ampicillin, amoxycillin/clavulanic acid, ceftriaxone, ciprofloxacin, cefuroxime sodium, gentamicin and sulfamethoxazole/trimethoprim. The result was uniform in all chosen isolates. They were susceptible to the antimicrobial drugs, with the exception of ampicillin. This shows that more recent isolates have the same antibiotic susceptibility profiles as the isolate from the outbreak in 2002/03. It is possible that the bacteria did not develop antibiotic resistance since antibiotics are

rarely used in NZSLs on Enderby and Campbell Islands, hence there is no selection pressure on the bacteria to develop antibiotic resistance. Furthermore, both islands are far from the New Zealand mainland and humans do not regularly visit these islands, which makes it less likely that waste products contaminated with bacteria that carry antibiotic resistance genes and bacteria travelling with humans could contaminate these islands (Iredell et al., 2016). On the other hand, the bacteria may have initially acquired antimicrobial genes or developed antimicrobial resistance ability, but with no selection pressure, these bacteria could revert to their original form (Moran, 2002).

The expression of different phenotypes among the same bacterial lineage can be caused by several factors; bacteria themselves, bacteriophages, and in response to the external environment (Smits et al., 2006). In this present study, some phenotypic characteristics varied within isolates, including colony morphology, the length of the viscous string and ability to metabolise different nutrients (phenotype microarray experiment). These isolates were from different years, different geographic locations, and from different pups (see Chapter 2), suggesting they had been in a different environment which might alter gene expression, result in mutations, phase variation or epigenetic variation affecting expression of colony morphology and capsule structure (Van Der Woude and Bäumler, 2004). Several environmental factors can affect expression of the HMV phenotype. High glucose concentrations can upregulate capsule production (Lee et al., 2016) while high iron availability can downregulate capsule production (Cheng et al., 2010; Lin et al., 2011). In the iron test of the present study, however, there was no observed change in the three isolates tested, which suggests that iron availability alone does not change hypermucoviscosity in these bacteria, and that there are other factors that might work together to influence capsule production.

In addition to environmental factors, genetic variation between isolates could also affect the expression of phenotype. This is further studied in Chapter 5.

The results of the phenotype microarray studies revealed the ability of HV *K. pneumoniae* to utilise a wide panel of substrates in different pHs, which is consistent with previous studies (Blin et al., 2017; Brisse et al., 2009; Liao et al., 2011), and with the fact that *Klebsiella* spp. can survive in diverse environments (Podschun and Ullmann, 1998). When compared with other bacteria such as *E. coli* and *Salmonella spp.,* which are mainly found in the intestinal tract of humans and animals, *K. pneumoniae* utilises a wider range of carbon and nitrogen sources (Liao et al., 2011).

In the present study, when a comparison was made between non-HV and HV isolates, a number of metabolite utilisation differences were observed, which is expected, because non-HV and HV isolates are genetically different (Blin et al., 2017; Elliott et al., 2016). There were a few metabolic differences among the HV isolates from NZSL pups, suggesting that each isolate had an individual ability to utilise nutrient sources even though they had the same basic characteristics. The external environment has influences on phenotype expression in bacteria (Smits et al., 2006). The isolates that were used in the phenotype microarray study were from different years and different geographic locations (Enderby Island and Otago region), and therefore had come from individual, distinct environments, which may explain the metabolic differences between them. Differences in phenotype expression of *K. pneumoniae* isolated at the same time, but from different organs was reported in humans where the isolates had similar genetic profiles using pulsed-field gel electrophoresis (Yu et al., 2015). It is possible that phenotypic differences between NZSL pup isolates in this study are solely due to differences in their environments leading to differential gene expression or epigenetic

changes, but the possibility of genomic differences between these isolates cannot be ruled out. This is investigated further in Chapter 5.

The results from the pH sensitivity plate (PM10) showed that three of three *K. pneumoniae* isolates and a reference *K. pneumoniae* ATCC 70063 (renamed as *K. quasipneumoniae subsp. similipneumoniae* ATCC 700603) (Brisse et al., 2014) isolate were unable to have activity at a pH below 4, but did have activity in the pH 4.5 to 10 range. In a previous study, *K. pneumoniae* was recovered from human gastric aspirates which have a pH of 3 (Graeme et al., 2005). The different results might be related to the different nutrients, microbial communities or *Klebsiella* strains between gastric aspirates and the substrate in the testing plate. The results of the present study support the ability of this bacterium to cause infection in a wide range of hosts. For example, *K. pneumoniae* can cause urinary tract infections in humans where the urine has a pH of 6 (Sakhaee et al., 1993), and can colonise the large intestine in mammals and birds where the pH ranges from 5.5 to 8 (Bitterman et al., 1969; Dierauf and Gulland, 2001; Fallingborg, 1999; Mabelebele et al., 2014; Stenkat et al., 2014). This finding is also consistent with the fact that this bacterium can be found in a diverse range of substrates that have different pHs, such as soil, surface water, sea water, and plants (Podschun and Ullmann, 1998).

In the phenotype microarray study, there was a degree of variability between replicates in a few wells. Variability between replicates has also been reported in *Pseudomonas aeruginosa* (Johnson et al., 2008) and *Campylobacter jejuni* (Sheppard et al., 2012). As gene expression is the principle of the phenotype microarray assay, the results can vary due to growth conditions, growth phase of the culture, and phase variation. Since the preparations of bacteria in every replicate of this study were

consistent, phase variation is a possible explanation of this result. Phase variation is a reversible, random and high frequency event that results in the turning on of one or more genes between individual cells of a clonal population (Van Der Woude and Bäumler, 2004). The mechanisms of phase variation include epigenetic methylation, state change, site specific recombination and simple sequence repeats (Van Der Woude and Bäumler, 2004). In *K. pneumoniae,* phase variation has been previously reported (Struve et al., 2008). Phase variation is usually random, but may be influenced by the environment (Van Der Woude and Bäumler, 2004). However, in this present study, as the author used the same conditions in every replicate, the role of different environmental factors can be eliminated, thus the result was likely from random events.

The main limitation of this chapter is that due to the cost of the different experimental techniques used, such as the phenotype microarray, only a small number of different isolates were used in each study. Therefore, results from these experiments should be interpreted with caution, and while reasons for for similarities and differences have been speculated, firm conclusions are not possible.

In the phenotypic microarray study the negative control well (A01) of PM1 (carbon plate) was positive when using a standard method according to the manufacturer's recommendation. There were two hypotheses from this result; one is that this may be due to their extra polysaccharide (the isolates used in this study were mucoid), and the other is related to volatile compounds such as volatile acetic acid produced by *K. pneumoniae* during metabolism of carbon sources (Bos et al., 2013) that can migrate to neighbouring wells. In this case, the postive reaction in the negative control well was not likely from the volatile compounds, as when the wells were sealed with impermeable material, the postive reaction in the negative control well was still

present. The second hypothesis that could explain these positives control wells is that the excessive polysaccharide produced by HV bacteria could act as an endogenous carbohydrate source. This hypothesis was tested by using a lower bacterial cell density method (dilution). The dilutions from 1:10, 1;100 and 1:500 continued to produce positive reactions on the negative control well with a reduced activity as bacterial density decreased. This suggests that the cell density might have an effect on the positive reaction in the negative control well. However, this also affected activities of bacteria in the other wells. When using the dilution 1:1000, the negative control well was negative with no reaction in any other wells, suggesting that the optimum bacterial density needs be further investigated. If the extracellular polysaccharide has an effect on this testing, removing the extracellular polysaccharide from the bacteria before testing is a possible method that should be investigated.

This study established the basic phenotypic characteristics of *K. pneumoniae* isolated from NZSL pups which have a hypermucoviscous phenotype, are capsular serotype K2, and are positive for the *rmpA* gene. The isolates were susceptible to most commonly used antibiotics, except for ampicillin. There were a few phenotypic variations among NZSL pup isolates from different years and places, suggesting that each isolate had individual utilisation abilities. The genetic profiles of these isolates were examined to see the relationship between phenotype and genotype in Chapter 5.

Chapter 4

Investigation of environmental reservoirs of *Klebsiella pneumoniae* at New Zealand sea lion breeding sites

4.1 Introduction

The New Zealand sea lion (NZSL), *Phocarctos hookeri,* is the world's rarest otariid and has been classified as endangered by the International Union for the Conservation of Nature (IUCN, 2015). The NZSL population is estimated to be around 11,767 (95% CI: 10,790–12,923) (Chilvers and Meyer, 2017). The main breeding sites are in the sub-Antarctic Auckland Islands (Enderby Island, Dundas Island and Figure of Eight Island) and Campbell Island, which account for 73% and 27% of pup breeding, respectively (Maloney et al., 2012). On Enderby Island, there have historically been two main pupping sites: Sandy Bay and Southeast Point, but there have been no pups born at Southeast Point since 2012/13 (Chilvers and Meyer, 2017). On the New Zealand mainland, there are small breeding colonies on Stewart Island and in the Otago region (Fig. 4.1). Around 16 - 32 pups are born per year on Stewart Island, and around 5 pups are born per year in Otago (Robertson and Chilvers, 2011).

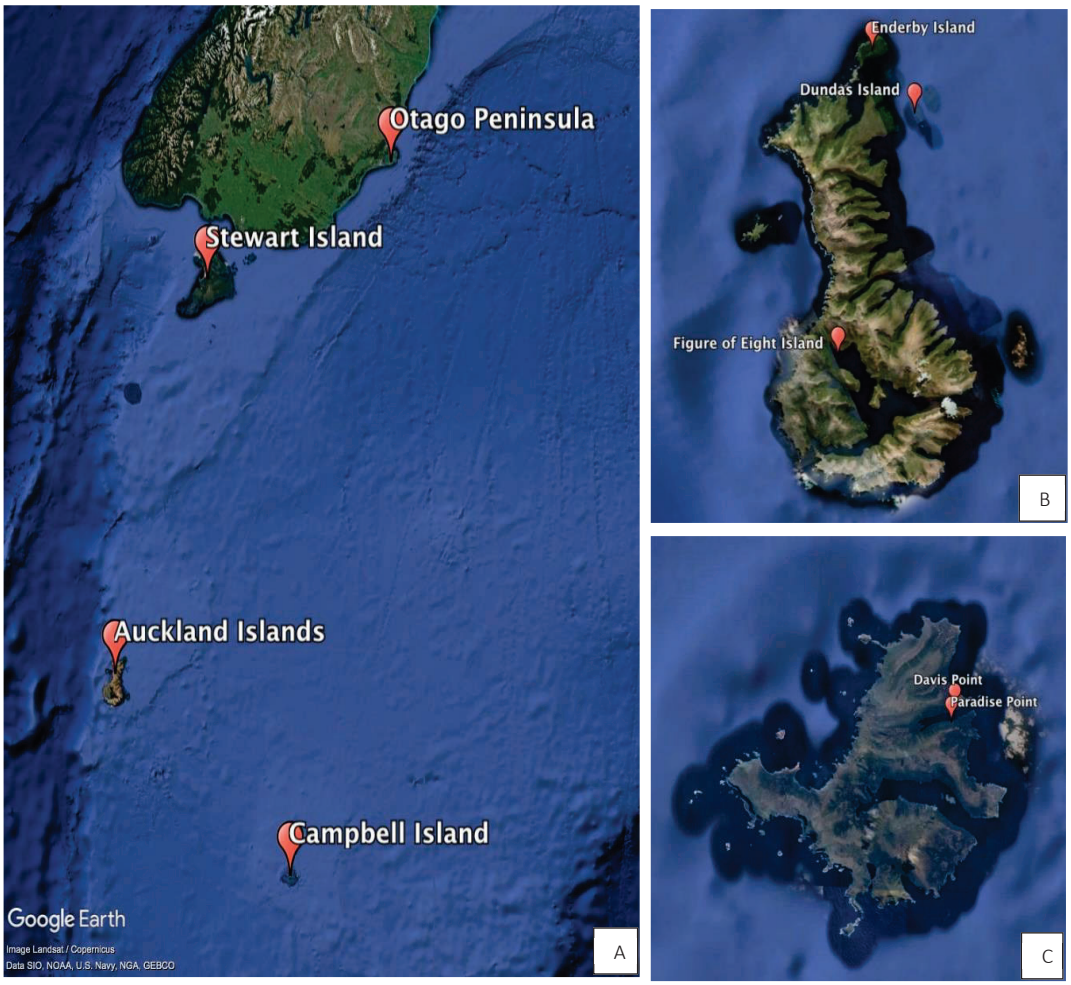

Figure 4.1 A maps of New Zealand and New Zealand sub-Antarctic Islands.
Maps of New Zealand and the New Zealand sub-Antarctic Islands, showing the distribution of NZSL breeding sites. A: present locations of Otago Peninsula, Stewart Island, the Auckland Islands and Campbell Island. B: presents locations of breeding sites at the Auckland Islands: Enderby Island, Dundas Island, and Figure of Eight. C: presents locations of breeding sites at Campbell Island: Davis Point and Paradise Point. Picture modified from Google Earth (Image @ 2018 DigitalGlobe)

The Auckland Islands (50° 45’ S, 166° 10’ E) are 450 km south of New Zealand. They have a different climate and environment from those of New Zealand mainland. The islands are mainly composed of volcanic rock and most of the surface is covered with peat, except the area behind Sandy Bay on Enderby Island which is sand hills (Leamy and Blakemore, 1960). The peat on Auckland Islands is acidic (pH 3.7 - 4.6) and has low base-saturation figures (44% to 19%) (Leamy and Blakemore, 1960). Water from the streams has a pH of approximately 5.0 – 5.5 (Leamy and Blakemore, 1960).

The streams on Enderby Island are slow moving, and most of the smaller streams are seasonal (Weller, 1975), as they develop after heavy rain and disappear within short periods of time (Leamy and Blakemore, 1960). Some ponds dry up if there are prolonged periods without rain (S. Michael, personal communication). This suggests that the streams or ponds that are present in summer may not necessarily be present in other seasons. However, for the past 5 years, the streams around the Sandy Bay breeding site have been present during the breeding season at the same location (S. Michael, personal communication). The climate has high humidity (87%), high rainfall (2000 mm/year) and high mean cloud cover resulting in a low rate of evaporation from the soil (Falla, 1948).

Campbell Island (52° 32' S, 169° 08' E) is 700 km south of New Zealand and 270 km southeast of the Auckland Islands. The island is mountainous, rising to over 500 metres. Like the Auckland Islands, the surface of Campbell Island is covered with peat. Campbell Island has a cold, cloudy, wet and windy climate. The highest temperature can be up to 21.2 °C and the lowest can reach -7.9 °C. The average annual rainfall is 1,329 mm/year (data retrieved from the National Institute of Water and Atmospheric Research, 2017).

The seasonal breeding cycle of NZSLs on Enderby Island starts with the arrival of adult males at the breeding areas in November, when they establish their territories. This is followed by the arrival of pregnant females in early to mid-December, who form harems and then pup within a week of their arrival. The mean pupping date is around 25th December and all pups are born by the third week of January. Females come into oestrus and are mated seven to ten days after pupping (mid-December to mid-January).

The dominant males start leaving the breeding site in the second week of January and the harems are then occupied by sub-adult males (Cawthorn, 1993).

K. pneumoniae was first reported in NZSL pups as a cause of mass mortality during the 2001/2002 and 2002/2003 breeding seasons at Sandy Bay on Enderby Island (Castinel et al., 2007a). After these outbreaks, *K. pneumoniae* appeared to become endemic in this population (Roe et al., 2015). All *K. pneumoniae* isolates cultured from pups that died between 2006 and 2010 showed a HV phenotype, had a K2 capsular serotype and contained the *rmpA* gene (Roe et al., 2015). Meningitis was present in over 50% of fatal *K. pneumoniae* cases, with infection of several other organs also common, including joints, the respiratory tract, and lymph nodes, suggesting spread of the pathogen via a haematogenous route (Roe et al., 2015).

The source of *K. pneumoniae* for NZSL pups is currently unknown, but it is possible that pups are exposed to the bacteria from an environmental source. The endemic nature of the infection further implies that either the bacteria can survive in the environment between breeding seasons, or that animal vectors are involved in transmission. In order to investigate the role of the environment in the pathogenesis of *K. pneumoniae* infection of NZSL pups, this study aims to:

1. Evaluate environmental samples collected from selected NZSL breeding sites for the presence of *K. pneumoniae*
2. Establish survival times of *K. pneumoniae* in substrate samples held at temperatures representing climatic conditions at Sandy Bay, Enderby Island.

4.2 Materials and Methods

4.2.1 Detection of *K.* pneumoniae in environmental samples from NZSL breeding sites

4.2.1.1 Sample collection

Environmental samples (sand, mud, and water) were collected from sites in the Auckland Island group, including Enderby Island, Dundas Island and Figure of Eight Island (Fig. 4.2), and from Campbell Island (Fig. 4.3). Sample collection from the Auckland Islands and Campbell Island was carried out by members of the NZSL research team during their field study seasons. These sites are unmonitored and uninhabited outside of these times. The samples were collected opportunistically since the study teams had other main responsibilities. Samples from the Auckland Islands were collected at four time points (see Table 4.1): 2013/2014 during the breeding season (December to early March); 2014/15 during the breeding season (three times: December, January, late February to March); 2016 during the winter season (August); and 2016/2017 during the breeding season (three times: December, February, March). More information on the sample sites can be found in Appendix A4.1.

The samples from Campbell Island were collected in 2014/2015 during the breeding season. Table 4.1 shows locations and sampling dates. More information on the sample sites can be found in Appendix A4.2. Sand and mud samples were collected using either a disposable scoop or a disposable stick, and placed into 2 mL sterile cryovials. Water samples were collected directly into 2 mL sterile cryovials.

Samples from the 2013/14 and 2016/17 breeding seasons were placed into liquid nitrogen within 48 h of collection, stored in liquid nitrogen until the team returned to the mainland, then transported to Massey University, Palmerston North. On

arrival at Massey University, the samples were stored at -80 °C for one to two weeks before processing. The samples were thawed at room temperature before processing. The samples from 2014/15 and 2016 (winter sample) were held at environmental temperatures, ranging from 3.9 to 14.5°C (for 3 months) and 0 to 10°C (for 23 days), respectively (NIWA, 2017) before being transported to Massey University, Palmerston North. On arrival at Massey University the samples were stored at 4°C for 2-3 days before processing.

The samples from Campbell Island were collected in 2014/2015 during the breeding season. Table 4.1 shows locations and sampling dates. More information on the sample sites can be found in Appendix A4.2. Sand and mud samples were collected using either a disposable scoop or a disposable stick, and placed into 2 mL sterile cryovials. Water samples were collected directly into 2 mL sterile cryovials.

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Table 4.1 Sample collection details at Auckland Island group (2013/2014, 2014/15 breeding season, 2016 August, 2016/2017 breeding season) and Campbell Island (2014/2015 breeding season)

Auckland Islands 2013/14 breeding season (n=17)			
Number of samples	Place	Sample type	Date of collection
2	Figure of Eight Island	mud, water	9th Jan 14
2	Dundas Island	mud, sand	20th Jan 14
1	Enderby, Southeast Point	water	30th Jan 14
1	Enderby, East Bay	sand	2nd Feb 14
11	Enderby, Sandy Bay	water, sand, mud	2nd - 11th Feb 14
Auckland Islands 2014/15 breeding season (n=46)			
Number of samples	Place	Sample type	Date of collection
10	Enderby, Sandy Bay	water, sand, mud	13th Dec 14
13	Enderby, Sandy Bay	water, sand, mud	11th-27th Jan 15
15	Enderby, Sandy Bay	water, sand, mud	28th Feb-3rd Mar 15
3	Figure of Eight Island	water, mud	9th-10th Jan 15
2	Dundas Island	mud, sand	17th-18th Jan 15
3	Dundas Island	mud, sand	14th Feb 15
Auckland Islands 2016 winter season (n=13)			
Number of samples	Place	Sample type	Date of collection
13	Enderby, Sandy Bay	water	7th-9th Aug 16
Auckland Islands 2016/17 breeding season (n=56)			
Number of samples	Place	Sample type	Date of collection
12	Enderby, Sandy Bay	water, mud, sand	12th Dec 16
1	Enderby, Teal Lake	water, mud	12th Dec 16
1	Enderby, Southeast Point	water, mud	12th Dec 16
1	Enderby, East Bay	water, mud	12th Dec 16
15	Enderby, Sandy Bay	water, mud, sand	1st-3rd Feb 17
1	Enderby, Teal Lake	water	1st Feb 17
1	Enderby, Southeast Point	water, mud	1st Feb 17
2	Enderby, East Bay	water, mud	1st-2nd Feb 17
16	Enderby, Sandy Bay	water, mud, sand	7th-10th Mar 17
3	Enderby, Teal Lake	water, mud, sand	7th-10th Mar 17
1	Enderby, Southeast Point	water	7th Mar 17
2	Enderby, East Bay	sand, water	7th-10th Mar 17
Campbell Island 2014/2015 breeding season (n=40)			
Number of samples	Place	Sample type	Date of collection
10	Paradise West	water, mud	20th Jan 15
10	Paradise East	water, mud	20th Jan 15
10	Davis Point Main Platform	water, mud	22nd Jan 15
10	Davis Point Bog	water, mud	26th Jan 15

Figure 4.2 Sample site at Auckland Islands
A; sample site at mud pool, Sandy Bay, Enderby Island, Auckland Islands, B; sample site at Dundas Island, Auckland Islands
Photo by Sarah Michael

Figure 4.3 Sample sites at Campbell Island
A; sample site at Davis Point (C19), Campbell Island, B; sample site at Paradise Point (C9), Campbell Island
Photo by A. K. Argandona

4.2.1.2 Substrate sample analysis

All samples were inoculated into Luria Broth and incubated at 37°C for 24 h, followed by four 10-fold serial dilutions in sterile PBS and plating of the serial dilutions onto CHROMagar^{TM} Orientation plates (Fort Richard, Auckland, NZ). Metallic blue colonies were subcultured onto blood agar for confirmation of bacterial species by PCR as follows. DNA extraction was performed according to the method described by Jang et al., (2009) with modifications. An overnight-cultured bacterial colony was added to 1 mL of 2% Chelex and boiled for 15 min at 100°C, followed by centrifugation at 12,000 x g for 5 min, and the supernatant collected.

The PCR hemolysin (*khe*) assay was performed to confirm *K. pneumoniae*, using the primer set shown in Table 4.2. Each 25 µL PCR reaction mix contained 1X PCR buffer with 2 mM of $MgCl_2$, 2 units Taq-Ti polymerase (Fisher Biotec, Australia), 0.2 mM dNTP, and 1 mM each primer. In all experimental runs negative controls (MilliQ water) were performed to check for the presence of contamination. The PCR conditions included an initial denaturation at 95°C for 2 min, followed by a 40-cycle amplification consisting of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 60 s. The PCR products were analysed on a 1.5% agarose gel (Invitrogen Corp., CA, USA) containing ethidium bromide and visualised under UV light on a transilluminator.

The pH measurements of substrate samples, including water from 'pup play pool' and mud from 'lower mud pool' collected from 1-3 February 2016/17, were performed using a pH meter (PHM220 Lab pH Meter, Radiometer Analytical SAS, France).

4.2.1.2.1 String test

The isolates that were subcultured onto blood agar and confirmed as *K. pneumoniae* underwent the string test for the HV phenotype. To perform the string test, a standard bacteriological loop was used to stretch a mucoviscous string from the colony. A positive string test was defined as the formation of a viscous string that extended vertically for more than 5 mm (Fang et al., 2004).

4.2.1.2.2 Capsular serotype and *rmpA* genes using polymerase chain reaction (PCR)

DNA from isolates confirmed as *K. pneumoniae* was further investigated for capsular serotype and presence of the *rmpA* gene using PCR. The PCR assay was performed using primers for three different targets, capsular type K1/*magA*, capsular type K2/K2*wzy*, and *rmpA* (see Table 4.2 for details). Separate reactions were set up for each primer pair. Each 25 µL PCR reaction mix contained 1X PCR buffer with 2 mM of $MgCl_2$, 2 units Taq-Ti polymerase (Fisher Biotec, Australia), 0.2 mM dNTP, and 1 mM of each primer. In all reactions, negative controls (water) were performed to check for the presence of contamination. The PCR conditions included an initial denaturation at 95°C for 2 min, followed by a 40-cycle amplification consisting of denaturation at 95°C for 30 s, annealing at 60°C (capsular serotype K2, and *rmpA*) or 56 °C (capsular serotype K1) for 30 s and extension at 72°C for 1 min. The PCR products were analysed on a 1.5% agarose gel (Invitrogen Corp., CA, USA) containing ethidium bromide and visualised under UV light on a transilluminator.

Table 4.2 Primers information for each target gene

Target	Primer	Sequence (5'–3')	Product size (bp)	Reference
Hemolysin	*khe* F	ATGAAACGACCTGATTGC	400	Babu et al. (2013)
	khe R	GATTGAGCGGGTAATAAATG		
Capsular type K1	*magA* F	GGTGCTCTTTACATCATTGC	1283	Fang et al. (2004)
	magA R	GCAATGGCCATTTGCGTTAG		
Capsular type	K2*wzy* F	GACCCGATATTCATACTTGACAGAG	641	Turton et al. (2008)
K2	K2*wzy* R	CCTGAAGTAAAATCGTAAATAGATGGC		
RmpA	*rmpA* F	ACTGGGCTACCTCTGCTTCA	516	Nadasy et al. (2007)
	rmpA R	CTTGCATGAGCCATCTTTCA		

4.2.2 Review of necropsy reports

The breeding site at Sandy Bay, Enderby Island has been monitored during the breeding season every year since 1994/1995. Information including dates of male and female adult NZSL arrival, pupping date, and the number of pups that were born and died on the island have been published (Cawthorn, 1993; Childerhouse et al., 2017; Chilvers and Meyer, 2017; Chilvers et al., 2007) and these, as well as personal communications (S. Michael, personal communication), were available to use in this study.

Every year during the field season, the breeding site is monitored twice daily and all dead pups are recovered for necropsy. In the 2013/2014 breeding season, the NZSL research team was on Enderby Island from 9th January to 9th March 2014. The necropsies performed during this time were conducted by a veterinarian, who wrote detailed reports of gross necropsy findings. These reports were reviewed by the author of this thesis, to identify pups with gross lesions consistent with *K. pneumoniae* infection, as described by Roe et al. (2015), including:

- Subdural haemorrhage
- Herniation and haemorrhage of the cerebellar vermis
- Suppurative polyarthritis
- Lymphadenitis, enlarged lymph nodes
- Presence of suppurative exudate in other organs

For cases identified as having possible *K. pneumoniae* infection, frozen tissues were cultured by the author according to the protocol in Chapter 3.2.2 to identify the infecting bacteria. Brain tissue or brain swabs were selected to culture first; if these were not available or if there was no bacterial growth on culture of these tissues, further tissues were selected for culture in the following order of precedence, depending on availability: cerebrospinal fluid (CSF), atlanto-occipital joint fluid or swab, other joint swabs or joint fluid, swabs from lesions, lymph nodes, liver, and lung. In cases where the gross lesions did not lead to suspicion of *K. pneumoniae* infection, histology slides that had been prepared earlier as part of other studies, were reviewed by the author. For histology processing, formalin-fixed tissues were embedded in paraffin. Sections were cut at 4μm and stained with haematoxylin and eosin (H&E). Slides were examined using a light microscope. The histology slides were reviewed for the following lesions as described by Roe (2011) and Roe et al. (2015).

- Fibrinosuppurative to histiocytic meningitis
- Cerebral or cerebellar vasculitis, or perivascular cuffing with histiocytes and neutrophils
- Suppurative or fibrinopurulent lymphadenitis, cellulitis, peritonitis, pneumonia, omphalitis, pleuritis, osteomyelitis, valvular endocarditis, or nephritis

Tissues or swabs from cases that had microscopic lesions consistent with *K. pneumoniae* were further investigated with bacterial culture as described above and in Chapter 3.2.2. Using these methods, pups that had died of *K. pneumoniae* infection were identified, and their dates of death established from necropsy records

For the 2016/2017 breeding season, the determination of dates of *K. pneumoniae* infection deaths was performed by Sarah Michael (PhD candidate) as part of her own studies, and details were provided to the author.

4.2.3 Survival of *K. pneumoniae* in soil and sea water

4.2.3.1 Retrieval of temperature data from National Institute of Water and Atmospheric Research (NIWA)

The daily maximum, minimum, and mean air temperatures (40 m above sea level) at NIWA Enderby Island weather station from 20th November 1991 to 31 July 2014 were retrieved from the National Institute of Water and Atmospheric Research (NIWA, 2017) (http://cliflo.niwa.co.nz/, agent Number 7340, and network number K92900) (Fig. 4.4). As the soil temperature for the 2013/2014 breeding season was not measured, air temperature data from Enderby Island in combination with soil temperatures recorded on Enderby Island from December 2003 to March 2004 (Castinel, 2006), and the known relationship between air and soil temperatures in the Subantarctic area, as determined by Boelhouwers, (2003), were used to estimate soil and sea water temperatures. The soil temperatures were calculated using the following equation (Castinel, 2006) (Fig. 4.4):

Y = soil temperature, X = air temperature

Minimum soil temperature: Y=0.4234*X + 7.9502

Maximum soil temperature: Y=0.2445*X + 10.318

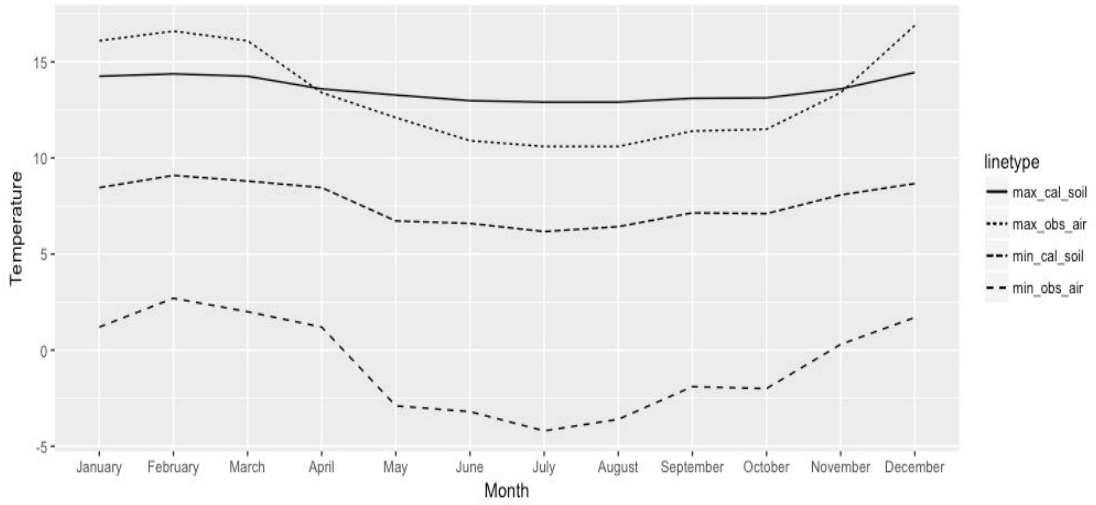


Figure 4.4 The temperature on Enderby Island
Mean maximum and mean minimum air temperature of each month at Enderby Island from 1991 to 2014. (Data retrieved from NIWA ((http://cliflo.niwa.co.nz/, agent Number 7340, and network number K92900)): maximum and minimum soil temperature of each month at Enderby Island (the data was calculated from for minimum soil (Y=0.4234*X+7.9502) and maximum soil temperatures (Y=0.2445*X+10.318), Y= soil temperature and X= air temperature

4.2.3.2 Bacterial strains, soil and water used in this study

A HV *K. pneumoniae* isolate cultured from the brain of NZSL pup HV_Kp2 that had already been cultured and stored as part of an earlier study in this thesis (Chapter 3), plus a non-HV *K. pneumoniae* human reference strain (non-HV_Kp1) purchased from New Zealand Reference Culture Collection (Environmental Science and Research (ESR), Kenepuru Science Centre, Porirua, New Zealand) were used in this study. The soil in this study was collected from a NZSL haul-out site in Surat Bay, Otago Peninsula. The soil was kept in plastic bags and stored at 4^{o}C until used. The sea water in this study was from Paraparaumu Beach, Kapiti Coast, New Zealand. The sea water was put directly into a 100 mL sterile bottle and stored at 4^{o}C for 2 days until use.

4.2.3.3 Laboratory procedures

Non-HV_Kp1 and HV_Kp2 were cultured in Luria broth separately at 37^{o}C for 24 h. The bacteria were centrifuged at 4000 x g for 10 min at 4^{o}C and washed three times with sterile phosphate-buffered saline (PBS). The cells were suspended in PBS to a final concentration of 10^{8} CFU/mL. The bacterial suspensions of each strain (5 mL) were put in either soil or water. Since *K. pneumoniae* can be found in soil and sea water, and *K. pneumoniae* can enter into a viable but non-culturable (VBNC) state (Byrd et al., 1991), the soil and sea water were autoclave-sterilised in order to eliminate *K. pneumoniae* and other bacteria that may have been present. However, since the sterilisation process can degrade organic materials, change the soil pH, and alter the microorganism community (Powlson and Jenkinson, 1976), which could affect the survival of *K. pneumoniae*, non-sterilised soil and sea water were also included in the study.

Inoculates were held in 10 g of autoclave-sterilised (120°C for 15 min at 15 psi) soil and 10 mL of autoclave-sterilised (120°C for 15 min at 15 psi) sea water. The same number of bacteria were also held in non-autoclaved soil and sea water that were confirmed free from *Klebsiella* spp. by culture. The inoculates were held under the following conditions: -4° C, 4° C, and 20° C for 2 months (representing survival within a breeding season), 5 months (midpoint of the experiment), and 9 months (representing survival between breeding seasons). For each condition, the experiments were done in triplicate. Bacterial survival was assessed using culture and confirmed by PCR as described Chapter 3.2.2. The pH measurement of soil (n=1) and sea water (n=1) was performed after 9 months using a pH meter (PHM220 Lab pH Meter, Radiometer Analytical SAS, France).

4.2.4 Statistical analysis

Statistical analyses were performed in RStudio (Version 0.98.1103). Logistic regression was used to analyse the results of the survivability test. Logistic regression was run using the glm () function in RStudio (glm () stat package, Marschner, 2011) with the following factors; sample type, time, strain, sterile and temperature (the following command line was used: "mod1 = glm (Pos ~ SampleType + Time + Strain + Sterile + Temp, data=dat, family="binomial")). The model takes the form:

P~Bernoulli(p_i) logit(p_i)

$$= \begin{cases} P(i) & \text{if } y=1 \\ 1-P(i) & \text{if } y=0 \end{cases}$$

4.3 Results

4.3.1 Detection of *K. pneumoniae* in substrate samples

4.3.1.1 Substrate samples from Auckland Islands in 2013/2014 breeding season (summer)

A total of seven from the 17 samples collected from the Auckland Island group in the 2013/14 breeding season were positive for growth of HV *K. pneumoniae* (Table 4.3, Fig. 4.5). The earliest collection date of a positive sample was 30th January 2014. This sample was from Southeast Point (Fig. 4.5A). All isolates were serotype K2 and positive for the *rmpA* gene.

Figure 4.5 Sampling locations on Enderby Island in 2013/14 breeding season
A: The main locations of samples collected on Enderby Island from 30th January - 11th February 2014: Sandy Bay, East Bay and Southeast Point. The purple balloons indicate areas that samples were positive. B: Location of samples collected near Sandy Bay from 2nd - 11rd February 2014; red balloons indicate areas that samples were negative; purple balloons indicate areas that samples were positive. The picture was modified from Google Earth (Image @ 2018 DigitalGlobe)

4.3.1.2 Substrate samples from Auckland Islands in 2014/2015 breeding season (summer)

All 46 samples collected during December 2014 to March 2015 were negative for *K. pneumoniae.*

4.3.1.3 Substrate samples from Auckland Islands in August 2016 (winter)

All 13 samples collected during August were negative for *K. pneumoniae.*

4.3.1.4 Substrate samples from Auckland Islands in 2016/2017 breeding season (summer)

Seven of 56 samples were positive for HV *K. pneumoniae.* All the isolates were serotype K2 and positive for the *rmpA* gene. Of the positive samples, four were from

water and three were from mud. Five of the positive samples were collected in February and two in early March. The two positive samples in March were collected from 'lower mud pool' and 'pup play pool' which were also positive in February (Fig. 4.6). The samples collected in December were all negative (Appendix A4.1). The pH of mud from 'lower mud pool' and water from 'pup play pool' were 5.5 and 5, respectively.

Figure 4.6 Sampling locations on Enderby Island in 2016/17 breeding season
Location of samples collected near Sandy Bay during 1st- 3rd February 2017 and 7th-10th March 2017; red balloons indicate areas that samples were negative (in both periods); purple balloons indicate areas that samples were positive on 1st-3rd February 2017, green balloons indicate areas that samples were positive on 1st-3rd February 2017 and 7th-10th March 2017.
The picture was modified from Google Earth (Image @ 2018 DigitalGlobe)

4.3.1.5 Substrate samples from Campbell Island in 2014/2015 breeding season

Five of the 40 samples (12.5%) from Campbell Island were positive for *K. pneumoniae* (Appendix A4.2). All 5 isolates were HV phenotype, serotype K2 and were positive for the *rmpA* gene.

Table 4.3 Substrate samples that were positive for *K. pneumoniae* in each season

Enderby Island, Auckland Islands 2013/14 breeding season			
ID Number	Place	Sample type	Date of collection
E13/14_1sub	Southeast point, Enderby Island	mud	30th Jan 14
E13/14_3sub	Pup paddle pool on sward, Sandy Bay, Enderby Island	water	2rd Feb 14
E13/14_4sub	Lower mud pool, Sandy Bay, Enderby Island	mud	2rd Feb 14
E13/14_8sub	Bush edge stream, Sandy Bay, Enderby Island	water	11th Feb 14
E13/14_9sub	Pup play pool, Sandy Bay, Enderby Island	water	11th Feb 14
E13/14_10sub	Skua lake, Sandy Bay, Enderby Island	mud	11th Feb 14
E13/14_13sub	Stream junction, Sandy Bay, Enderby Island	water	11th Feb 14
Enderby Island, Auckland Islands 2016/2017 breeding season			
ID Number	Place	Sample type	Date of collection
E16/17_22sub	Middle stream, Sandy Bay, Enderby Island	water	2nd Feb 17
E16/17_25sub	Lower mud pool, Sandy Bay, Enderby Island	mud	2nd Feb 17
E16/17_26sub	Upper mud pool, Sandy Bay, Enderby Island	mud	2nd Feb 17
E16/17_28sub	Pup play pool, Sandy Bay, Enderby Island	water	3nd Feb 17
E16/17_30sub	Skua lake, Sandy Bay, Enderby Island	water	3nd Feb 17
E16/17_43sub	Lower mud pool, Sandy Bay, Enderby Island	water	9th Mar 17
E16/17_46sub	Pup play pool, Sandy Bay, Enderby Island	mud	9th Mar 17
Campbell Island 2014/2015 breeding season			
ID Number	Place	Sample type	Date of collection
C14/15_6sub	Paradise East, Campbell Island	water	20th Jan 15
C14/15_11sub	Davis Point Main Platform, Campbell Island	water	22nd Jan 15
C14/15_17sub	Davis Point Bog, Campbell Island	mud	26th Jan 15
C14/15_18sub	Davis Point Bog, Campbell Island	mud	26th Jan 15
C14/15_19sub	Davis Point Bog, Campbell Island	water	26th Jan 15

4.3.2 Review of post mortem reports from Enderby Island

A total of 71 necropsy records from the 2013/14 season were reviewed, and 55 cases were identified as having possible *K. pneumoniae* infection. Of these, 47 pups (66%) were determined to have died from *K. pneumoniae* infection based on gross and histological findings, microbiological culture, and PCR. All the isolates were HV

phenotype, serotype K2 and were positive for the *rmpA* gene. (Appendix A3.3). The earliest fatal *K. pneumoniae* case from this season was found dead on 1st February 2014. Fig. 4.6 shows the distribution of pup deaths due to *K. pneumoniae* over the field season.

In the 2016/17 breeding season, the earliest fatal case of *K. pneumoniae* infection was found dead on 31st Jan 2017.

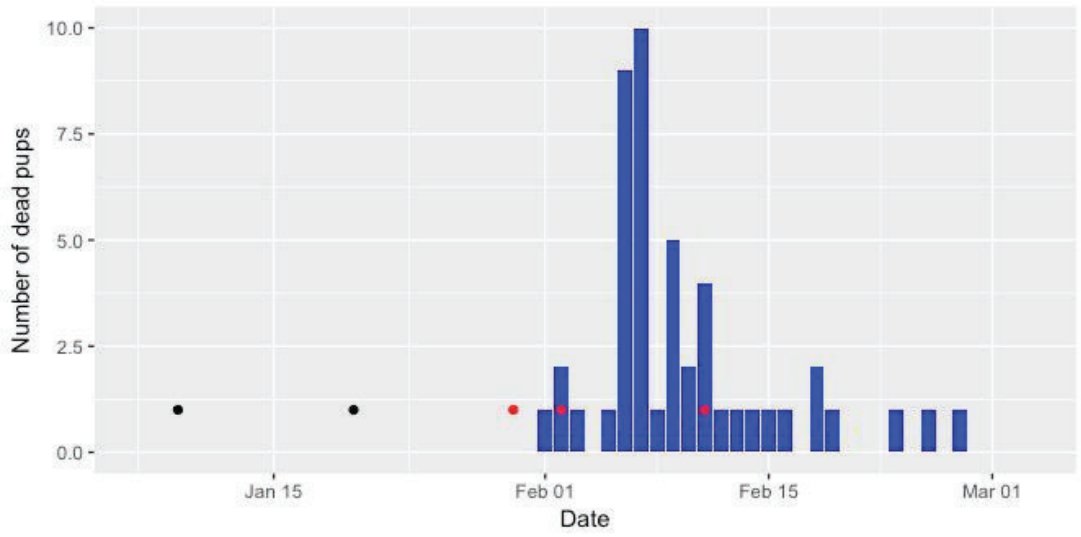


Figure 4.7 Distribution of pup deaths from *K. pneumoniae* in the 2013/2014 breeding season.
The black dots are the days that substrate samples were negative. The red dots are the days that substrate samples were positive.

4.3.3 Survival of *K. pneumoniae* in soil and sea water at different temperatures

At -4 and 4°C, HV and non-HV *K. pneumoniae* survived in soil for at least 5 months and survived in sea water for at least 2 months (Table 4.4). At 20°C, they survived in soil and sea water for at least 9 months (Table 4.4). There was no significant difference in survival between sterile and non-sterile soil, and sea water samples (p=1). At each temperature and time point, the number of HV isolate replicates that survived

was significantly higher than for the non-HV isolates (p=0.001). Both phenotypes survived significantly longer in soil than sea water (p=0.001) (Fig. 4.8).

The pH of soil and sea water after nine months of the experiment were 7.2 and 7.5, respectively.

Table 4.4 Survival results of *K. pneumoniae* at different temperatures.
The number represents the total positive samples from 3 replicates.

	Non-HV_Kp1		HV_Kp2	
	Sterile	Non-sterile	Sterile	Non-sterile
2 months				
Sea water/Temp.° C				
-4	0	1	2	2
4	1	1	3	1
20	3	3	3	3
Soil /Temp.° C				
-4	3	3	3	3
4	1	3	3	3
20	3	3	3	3
5 months				
Sea water/Temp.° C				
-4	0	0	0	0
4	0	0	0	0
20	3	3	3	3
Soil /Temp.° C				
-4	0	3	2	3
4	0	0	2	3
20	3	3	3	3
9 months				
Sea water/Temp.° C				
-4	0	0	0	0
4	0	0	0	0
20	1	0	1	0
Soil /Temp.° C				
-4	0	0	0	0
4	0	0	0	0
20	3	3	3	3

```
dat = read.csv("~/Desktop/stats.csv")
dat$Time = factor(dat$Time)
dat$Temp = factor(dat$Temp)
mod1 = glm(Pos ~ SampleType + Time + Strain + Sterile + Temp, data=dat, family="binomial")
summary(mod1)
```

```
##
## Call:
## glm(formula = Pos ~ SampleType + Time + Strain + Sterile + Temp,
##     family = "binomial", data = dat)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -2.07761  -0.07806   0.00000   0.09614   2.31195
##
## Coefficients:
##                  Estimate Std. Error z value Pr(>|z|)
## (Intercept)        3.7265     1.0226   3.644 0.000268 ***
## SampleTypewater   -4.6365     0.8983  -5.161 2.45e-07 ***
## Time5             -4.0221     0.9252  -4.347 1.38e-05 ***
## Time9            -24.3100  1406.2605  -0.017 0.986208
## Strainsealion      2.4472     0.6567   3.726 0.000194 ***
## Sterilesterile    -0.8751     0.6198  -1.412 0.157956
## Temp4             -0.8159     0.6602  -1.236 0.216540
## Temp20            23.5168  1406.2605   0.017 0.986658
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for binomial family taken to be 1)
##
##     Null deviance: 299.440  on 215  degrees of freedom
## Residual deviance:  75.907  on 208  degrees of freedom
## AIC: 91.907
##
## Number of Fisher Scoring iterations: 19
```

Figure 4.8 Statistics results from survival of *K. pneumoniae* in soil and sea water at different temperatures.

Time5 and Time9 represent five months and nine months respectively. Strainsealion represents the isolates from NZSL pups. Sterilesterile represents steriled substrate samples. Temp4 and Temp20 represent temperature 4°C and 20°C, respectively.

4.4 Discussion

Since the early disease outbreaks in 2001/02 and 2002/3, HV *K. pneumoniae* has become a common cause of death in NZSL pups on Enderby Island during the breeding season. However, reservoirs of the pathogen are currently unknown, and it is possible that pups are exposed to the pathogen from the environment. In the present study, HV *K. pneumoniae* was isolated from water and mud collected from Enderby (2013/14 and 2016/17) and Campbell (2014/15) Islands during the NZSL breeding season. The isolates

from substrate samples had the same basic characteristics as isolates from dead NZSL pups, with a HMV phenotype, serotype K2 and positive for the *rmpA* gene, suggesting that the pathogen that caused disease in NZSL pups was also present in the environment.

While the results suggest that *K. pneumoniae* isolates from pups and substrate samples are the same strain, it is unclear whether the environment is the origin of this pathogen, or is contaminated from other sources. If the environment is a long-term reservoir of infection, the pathogen should have been present whenever substrate samples were collected. In 2016/17 the Enderby Island samples were collected at three times: December, February and March. The areas that were positive in February and March were negative in the previous December. This indicates that the pathogen was not in the environment at the beginning of the season. Moreover, seven locations that were positive in 2013/14 including Southeast Point and six areas near Sandy Bay were negative when the samples were collected in December 2016/17, indicating that the pathogen does not persist for long periods at these sites. These findings suggest that the environment is not the main reservoir of infection, and that other sources are likely to be involved. NZSL pups, NZSL adults, birds that live around the breeding sites, and humans that visit the island are all potential sources of environmental contamination. However, NZSL pups cannot be a long-term reservoir since they move away after the breeding season (Cawthorn, 1993). Humans are also not likely to be a reservoir in this case as humans do not regularly visit this area and there is no evidence that HV *K. pneumoniae* has been isolated from humans in New Zealand.

For Enderby Island in 2013/14, the earliest date that a substrate sample was positive was 30th January, at Southeast Point. No pups have been born at this site since

the 2011/12 season (Childerhouse et al., 2017), and pups do not travel from Sandy Bay to this location until much later in the season, so the bacteria in this case could not have been shed into the environment by pups. NZSL adults, however, periodically visit Southeast Point during the breeding season (Department of Conservation, 2018), so are a potential source of shedding into the environment. In 2013/14, the year that a sample was positive at Southeast Point, there were five NZSL adults present at Southeast Point before 30th January, and three present on 30th January (Department of Conservation, 2018). As described by Argandoña (2017), HV *K. pneumoniae* has been isolated from the faeces of adult NZSLs, further supporting their potential role as reservoirs. Similarly, a variety of bird species are present on Enderby Island (Moore and McClelland, 1983), including at Southeast Point, and some have been shown in this present study (Chapter 2.2.3) and others (Stenkat et al., 2014) to be capable of harbouring *K. pneumoniae*.

Sandy Bay is the main Enderby Island breeding site. In 2013/14, there were no samples collected prior to the first pup death from *K. pneumoniae* on 1st February. Thus, it is not known when *K. pneumoniae* first appeared in the environment, but it is possible that the positive substrate samples found from 2nd February onwards were a result of bacteria being shed into the environment by sick or dying pups. In 2016/2017, the samples were collected at three times: December, February and March. The December samples were collected before pups started to die from *K. pneumoniae* infection (the first pup died on 31st January), and all the samples from this period were negative. All positive samples from this season were collected in February and March, after pups had begun to die from *K. pneumoniae*, and it is likely that at least some of this environmental burden is due to bacteria shed by pups. Although there are no precise data describing the time from infection to death in naturally acquired *K. pneumoniae*

infections, data from an experiment in mice using the oral route of infection showed that more than 80% of mice died of septic shock within one week of inoculation (Tu et al., 2009), while human infants infected via nasoduodenal tubing became bacteraemic in 24-96 hours (Donowitz et al., 1981). This suggests that, if the environment was the primary source of infection for pups dying early in the season, bacteria could be present for as little as one week before the first deaths. Due to the opportunistic nature of sampling for this study, however, substrate samples were not collected within this time period, so this could not be assessed.

Campbell Island is another large breeding site for NZSL outside the Auckland Islands. A high mortality rate of NZSL pups has been reported from this breeding site (Robertson and Chilvers, 2011) with the major causes being trauma, starvation, and drowning in rock pools and peat mires (Maloney et al., 2009). However, fatal bacterial infection was also found as a cause of death (Maloney et al., 2009). Although in that study, the culture results from necropsied pups did not reveal any *K. pneumoniae*, the presence of *K. pneumoniae* in Campbell Island substrate samples shown in this chapter suggests that *K. pneumoniae* could affect pups in the future. In addition, in the study in 2008 (Maloney et al., 2009), not all dead pups were necropsied, and not all tissues from necropsied pups were cultured, thus it cannot be concluded that none of the pups died from *K. pneumoniae.* Further study on the causes of death in pups on Campbell Island is required.

The fact that HV *K. pneumoniae* was found in the environment at both Enderby Island and Campbell Island, along with the previously published report of a fatal case of *K. pneumoniae* infection in a pup at Otago Peninsula (Roe et al., 2015), are further evidence of a reservoir host capable of transmitting infection between these locations.

NZSL adults travel between breeding sites (Geschke and Chilvers, 2010), and are a possible reservoir, hence could spread this pathogen to other breeding sites including Figure of Eight and Dundas Island, where the impact of *K. pneumoniae* on pup mortality is currently unknown. In the 2013/14 breeding season, all substrate samples collected from Figure of Eight and Dundas Island were negative. However, because these samples were collected early in the breeding season, we cannot be sure that this pathogen is truly absent from these breeding sites.

The presence of *K. pneumoniae* in the environment on Enderby Island from late January/early February onwards indicates that contaminated substrate could play a role in the pathogenesis of infection in pups within the breeding season. NZSL pups that died from *K. pneumoniae* between 2006 and 2010 presented with meningitis and the involvement of multiple other organs such as joints, the respiratory tract and lymph nodes, indicating haematogenous spread of bacteria within the host (neonatal septicaemia) (Roe et al., 2015). In animal species, bacteria can enter the blood stream of neonates through a variety of routes, including ingestion, inhalation, across the umbilicus or via open wounds (Zachary and McGavin, 2013). Similar routes are possible for NZSL pups. Since pups spend a lot of time playing in mud pools, often putting their heads below the surface and blowing bubbles (S. Michael, personal communication) they could acquire the pathogen by accidentally aspirating or swallowing contaminated water or mud. Similarly, substrate-borne contamination of the umbilicus within the first few days of life, or contamination of open skin wounds are other potential routes of infection.

The results of the substrate study show that *K. pneumoniae* was present in the NZSL environment during the breeding season, but do not support the hypothesis that

substrate could be a reservoir of infection between breeding seasons. Although *K. pneumoniae* can survive in the environment (Podschun and Ullmann, 1998), the survival duration is unknown for the Subantarctic area, and specific data for the HV strain present in NZSLs has not yet been published. The laboratory-based study in the second part of this chapter aimed to establish the survival duration of HV *K. pneumoniae* in soil and sea water at different temperatures. The results show that *K. pneumoniae* could survive in soil and sea water at -4, 4, and 20^{o}C for at least two months. At five months, they did not survive in either medium at -4 or 4^{o}C, however they could survive at 20^{o}C in both soil and sea water for nine months. While optimum growth of *K. pneumoniae* is at 30-37^{o}C (Brisse et al., 2006), the study presented here shows that this pathogen can also survive in the somewhat cooler environmental temperatures of a Subantarctic summer, in which the soil temperature on Enderby Island ranges from 8 to 15^{o}C (Fig. 4.4) for four months. However, unfortunately, the survival of *K. pneumoniae* between breeding seasons (nine months) cannot be determined because the soil temperatures on Enderby Island (6 to 14^{o} C) were not used in this study. The survival of *K. pneumoniae* at 20 ^{o}C for nine months suggests that this pathogen can survive in the environment for a long time if the pathogen is in an appropriate temperature. This further suggests that if there is a climate change in Subantarctic area, the situation of *K. pneumoniae* in NZSL pups might change.

This part of the study also shows that the number of triplicate cultures that survived at each temperature and time point was greater for HV isolates than for non-HV isolates. A comparative study of HV and non-HV *K. pneumoniae* strains shows that HV strains had a higher capacity for biofilm formation than non-HV strains (Wu et al., 2011). Biofilm has several functions, one of which is protection of bacteria from the

external environment (Balcázar et al., 2015; Hall-Stoodley et al., 2004), hence this ability might contribute to better survival of the HV isolates in the current study. Differences in survival could also be related to the different ability of isolates to utilise nutrient sources, as shown in Chapter 3. This might explain the findings in this part of the study, but also means that isolate survival time in the Subantarctic cannot be directly extrapolated from the current experimental study. The soil and sea water used in this study was sourced from the New Zealand mainland, and is likely to have different nutrient content than the environmental substrates at Subantarctic NZSL breeding sites. In addition, other factors such as microorganism community and pH may affect the survival of bacteria (Haruta and Kanno, 2015; Hobbie and Hobbie, 2013), therefore determination of site-specific survival times would require incubation in substrate material collected from breeding sites. The pH from substrate samples from breeding sites was more acidic (mud=5.5, water=5) than the substrate samples (soil=7.2, sea water 7.5) used in this study, which may also have affected bacterial survival. As the NZ Subantarctic Islands are legally protected heritage sites, and removal of substrate is illegal, such a study would likely need to be conducted in the field.

A further finding of this study is that substrate storage conditions may affect *K. pneumoniae* survival. In 2013/14 and 2016/17, the samples were stored in liquid nitrogen within 48 h after collection, while in 2014/15 and in 2016 (winter samples) they were held at ambient temperatures. Fourteen of 73 (19.2%) samples stored in liquid nitrogen were positive while 0/59 (0%) stored at environment temperatures were positive. Therefore, it is possible that the method of storage affected the viability of the pathogen possibly one to temperature fluctuations, although true absence of the pathogen cannot be definitively ruled out.

Summary

The results show that HV *K. pneumoniae* is present in the environment at NZSL breeding sites, including Enderby Island and Campbell Island, during the breeding season. The isolates of the substrate from these locations had the same basic characteristics as the isolates from NZSL pups. This suggests that the environment can be a reservoir of *K. pneumoniae* infection during the breeding season. The negative results from early breeding season (December) substrate samples in 2016/2017 indicate that the environment is not likely to be a reservoir between breeding seasons. Possible reservoirs between breeding seasons include adult sea lions, scavenging birds such as brown skuas, and giant petrels, and yellow eyed penguins. In vitro analysis of Klebsiella survival in different substrates and temperature conditions suggested the possibility that Klebsiella may persist in the environment of New Zealand's subantarctic islands during breeding seasons, however further work outside of the breeding season would be required to confirm this. In the following chapter the genomes of isolates from substrate samples from Enderby and Campbell Island are investigated and compared with isolates from NZSLs in order to see the genetic relationship between isolates.

Chapter 5

Genomic characterisation and genomic comparison of *Klebsiella pneumoniae* from New Zealand sea lions

5.1 Introduction

K. pneumoniae is a gram-negative bacterium that can be found in natural environments such as soil, surface water, sewage and plants, as well as in the hospital environment, such as on the surface of medical equipment (Paczosa and Mecsas, 2016; Podschun and Ullmann, 1998; Shon et al., 2013). *K. pneumoniae* can also be found on the mucosal surface of mammals i.e. the gastro-intestinal and respiratory tracts. It causes a wide range of infection in animals and humans (Paczosa and Mecsas, 2016). At present, two forms of *K. pneumoniae* infection have been described; *K. pneumoniae* (classical strains) act as an opportunistic pathogen causing infection in immunocompromised patients; *K. pneumoniae* (hypervirulent strain (HV)) act as a pathogen that can infect heathy people, causing liver abscesses (Shon et al., 2013). The invasive (HV) form of *K. pneumoniae* infection emerged in Taiwan and spread all over the world (Shon et al., 2013). At present (November 2018), HV *K. pneumoniae* has never been reported in humans in New Zealand. However, there have been a few cases reported in humans in Australia (Chang et al., 2013; Vandevelde and Stepanovic, 2014). HV *K. pneumoniae* has also been reported in animals such as NZSLs (Castinel et al.,

2007b; Roe et al., 2015), California sea lions (Jang et al., 2010) and African green monkeys (Soto et al., 2012).

During the breeding seasons (December to early March) in 2001/2002 and 2002/2003, there were mass mortalities of NZSL pups on Enderby Island due to *K. pneumoniae* (Castinel, 2006). After this event, this pathogen seemed to become endemic at this location (Roe et al., 2015). *K. pneumoniae* also was isolated from a NZSL pup that died at the Otago Peninsula, on the NZ mainland in January 2013 (Roe et al., 2015). The isolate from this pup had the same basic characteristics as the isolates from the dead pups on Enderby Island - HMV phenotype, serotype K2 and positive *rmpA* gene (see Chapter 3). Moreover, the results in Chapter 4 showed that during summer, HV *K. pneumoniae* could be isolated from substrate samples such as soil, mud or water collected from Enderby and Campbell Islands. In a separate study, HV *K. pneumoniae* was isolated from healthy birds that were around NZSL colonies during the breeding season (S. Michael PhD candidate, personal communication), which suggests that these birds might be a reservoir.

In order to investigate the relationships between these isolates, the genetic characteristics of these isolates need to be established. By using whole genome sequencing, the information from this approach can be linked to other information on bacteria such as phenotypes, virulence factors, metabolic pathways, and antimicrobial resistance. This is the baseline information required to begin considering prevention and control of the infection.

In this chapter, the author used Illumina MiSeq technology to perform whole genome sequencing on *K. pneumoniae* isolates from NZSL adults and pups, substrate

samples from sea lion habitation areas, and birds found around sea lion colonies, plus isolates from African green monkeys (see Chapter 2). The author also compared genomic information among the NZSL isolates and between NZSL isolates and other *K. pneumoniae* isolates from humans, African green monkeys and California sea lions.

5.2 Materials & methods

5.2.1 Bacterial isolates

Whole genome sequencing was performed on isolates from several sources including NZSLs, substrate samples and birds on Enderby Island, plus the isolates from African green monkeys (DNA provided from Ross University, St. Kitts, West Indies). Details of these isolates can be found in Chapter 2 and a summary of the isolates can be found in Table 5.1. All the isolates used in this chapter were string tested, and their capsular serotype and *rmpA* gene status determined using PCR according to the protocol in Chapter 3. The genome sequences from this study were deposited in GenBank under BioProject ID PRJNA485367 and PRJNA482675 and were published in Microbiology Resource Announcements: DOI: 10.1128/MRA.01328-18 and DOI: 10.1128/MRA.01270-18

Table 5.1 Details of the isolates and genomic DNAs used in the genome study

Isolate designation	Bacterial isolate	Source	Clinical status
HV_Kp2	*K. pneumoniae* E02/03_112Ph	Sea lion pup	Post mortem[a]
HV_Kp3	*K. pneumoniae* E11/12_24Ph	Sea lion pup	Post mortem[a]
HV_Kp4	*K. pneumoniae* S13_04Ph	Sea lion pup	Post mortem[a]
HV_Kp5	*K. pneumoniae* D14/15_8Ph	Sea lion pup	Post mortem[a]
Non-HV_Kp6	*K. pneumoniae* C14/15_9Ph	Sea lion pup	Post mortem[b]
Non-HV_Kp7	*K. pneumoniae* E09/10_13Ph	Adult sea lion	Post mortem[c]
HV_Kp8	*K. pneumoniae* C14_9476Ph	Adult sea lion	Healthy
Non-HV_Kp9	*K. pneumoniae* E14/15_17Sa	Subantarctic skua	Healthy
HV_Kp10	*K. pneumoniae* E14/15_42Sa	Subantarctic skua	Healthy
HV_Kp11	*K. pneumoniae* E14/15_53Ma	Yellow-eyed penguin	Healthy
HV_Kp12	*K. pneumoniae* E13/14_10sub	Substrate	-
HV_Kp13	*K. pneumoniae* C14/15_17sub	Substrate	-
M7_14_1026	*K. pneumoniae* M7_14_1026	African green monkey	Clinical
M11_0911	*K. pneumoniae* M11_0911	African green monkey	Clinical
M3_11_2499	*K. pneumoniae* M3_11_2499	African green monkey	Clinical

[a] These animals died from *K. pneumoniae* infection and samples were collected at post mortem examination.
[b] This animal died from starvation.
[c] This animal died from trauma.

5.2.2 DNA preparation and whole genome sequencing

DNA was extracted from 12 NZ isolates (Table 5.1) that had been previously cultured and were archived as glycerol stocks at -80°C. The genomic DNA from each isolate was prepared from overnight cultures on blood agar medium (Fort Richard, Auckland, NZ). The NucleoSpin® Soil kit (Macherey-Nagel, Germany) was used to extract genome-quality DNA as per the manufacturer's instructions (1.5 µg DNA, 20 ng/µL). A total of 15 genomic DNA samples, including 12 *K. pneumoniae* genomic DNA from NZ isolates plus 3 *K. pneumoniae* DNA from African green monkeys, were sequenced using an Illumina MiSeq (Illumina, San Diego, CA, USA) by New Zealand Genomics Limited (NZGL) at Massey Genome Service, Massey University, Palmerston North, New Zealand as 2 × 250 bp paired-end runs. The library for sequencing was prepared using an Illumina TruSeq DNA Library Preparation Kit V1 (Illumina, Scorsby, Victoria, Australia).

For sequencing, genomic DNA was fragmented, ligated with adaptors to both ends and amplified via PCR.

After sequencing and standard barcode demultiplexing, FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), SolexaQA++ (Cox et al., 2010) and fastQscreen (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) were used to check the quality of the data. Each sequence from the machine was mapped against the PhiX genome using Bowtie2 (Langmead and Salzberg, 2012), and any hitting PhiX sequences were removed from the SAM file. The data was reconstructed using the SamToFastq.jar program from the Picard suite (http://http://picard.sourceforge.net/), set using the default parameters. Then, the adaptors were removed through the "fastq-mcf" program (using the default parameters) from the ea-utils suite of tools (http://code.google.com/p/ea-utils/; version 1.1.2- 621).

5.2.3 Genomic assembly and annotation

The 15 samples from this study, plus 34 samples of *K. pneumoniae* isolated from humans (available at http://www.ebi.ac.uk/ena) (details of the samples can be found in Appendix A5.1) were assembled *de novo* using SPAdes in careful mode (version 3.10). QUAST (version 4.5; (Gurevich et al., 2013) (a tool for checking data quality) was used to evaluate and compare the genome assemblies and determine their GC content. The contigs of each isolate produced from SPAdes were annotated by Prokka using the default parameters (version 1.1.2; (Seemann, 2014)).

5.2.4 Genomic analysis

5.2.4.1 Multilocus sequencing typing (MLST)

Each genome in FASTA file were uploaded to Bacterial Isolate Genome Sequence Database (BIGSdb) servers (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) to analyse the sequence type of each isolate. Sequence homology matching was performed using BLAST (Altschul et al., 1997) with a word size of 15 and identity of 70% for DNA sequences. The *K. pneumoniae* MLST scheme used the following seven housekeeping genes (Diancourt et al., 2005):

- *rpoB* (beta-subunit of RNA polymerase)
- *gapA* (glyceraldehyde 3-phosphate dehydrogenase)
- *mdh* (malate dehydrogenase)
- *pgi* (phosphoglucose isomerase)
- *phoE* (phosphorine E)
- *infB* (translation initiation factor 2)
- *tonB* (periplasmic energy transducer)

5.2.4.2 Genes associated with virulence and antimicrobial resistance

Virulence genes were identified using in-house Perl scripts[1]. The sequences of the genes along with 1000-bp flanks on either side of the sequence were mapped with the reads of each isolate (49 isolates). The results were visualised and interpreted using Geneious (version 9.1.8). A list of virulence resistance genes and reference can be found in Appendix A5.2

[1] A/Prof Biggs wrote the computer code to perform the analysis, and the author analysed the results

In order to identify antibiotic resistance genes, the reads of each isolate were uploaded to Center for Genomic Epidemiology servers (http://www.genomicepidemiology.org/) using ResFinder 3.0 (Zankari et al., 2012). The threshold was set up at 90% identity with 60% minimum length.

5.2.4.3 rMLST

A total of 49 genome sequences of *K. pneumoniae* genomes, both from the present study (n=15) and those retrieved from http://www.ebi.ac.uk/ena and https://www.ncbi.nlm.nih.gov/genbank/ (n=34) (details of the sequences can be found in Appendix A5.1) were used to construct a tree. In-house Perl scripts[2] were used to extract the ribosomal protein subunit (*rps*) genes (Jolley et al., 2012) from the data generated from Prokka and to generate rMLST allelic profiles by BLAST against *rps* genes (53 genes). The best hit of each genome was located per *rps* gene. The rMLST alleles of the 49 genomes were converted into a distance matrix that counted of the number of loci that differed between each pair of isolates. The distance matrix was interpreted as a phylogenetic network using the Neighbor-Net algorithm and the result exported as a nexus file (Bryant and Moulton, 2004). SplitsTree (version 4.14.5; (Huson and Bryant, 2005) was used to visualise and interpret the association among the isolates.

5.2.4.4 Pan-genome, core-genome, and the presence and absence genes

A total of 22 from 49 genomes that were closely related to New Zealand isolates (12 isolates) based on rMLST clustering (Fig 5.3) were chosen for further study. The pan and core genome were calculated using GET_HOMOLOGUES (Contreras-Moreira and Vinuesa, 2013) in the default mode. The GET_HOMOLOGUES used the protein sequences of the predicted genes in fasta format to run BLAST searches. BLAST

[2] A/Prof Biggs wrote the computer code to perform the analysis, and the author analysed the results

searches in default mode was set as a minimum E-value of 10^{-5} with a minimum of 75% coverage in pairwise alignment. GET_HOMOLOGUES used three algorithms called BDBH, OMCL and COG to cluster the sequences. BDBH algorithm uses one sequence from the reference genome to grow clusters. The COG algorithm requires a triangle of reciprocal hits. The OMCL algorithm groups nodes in a BLAST graph to build clusters. The overlapping orthologous clusters identified by all three algorithms were defined as the organism's core genome. The pan-genome was calculated using OMCL clustering. OMCL was used to estimate the theoretical core and pan-genome size.

Roary (version 3.9.1) (Page et al., 2015) was also used to calculate the pan-genome (Tettelin et al., 2005), identifying the core and accessory genes by using annotated assemblies in GFF3 format previously produced by Prokka. The coding regions of each sequence were extracted and converted to protein sequences, filtered to removed partial sequences and iteratively pre-clustered with CD-HIT (Fu et al., 2012). BLASTP was used to perform an all-against-all comparison (percentage sequence identity 95%) on the sequences that had been already filtered. Then the sequences were clustered with MCL (Enright et al., 2002). The results from MCL and those pre-clustered from CD-HIT were merged together. The sequences were clustered based on the genes in the accessory genome. Roary constructed a gene presence/absence matrix, a multi-FASTA alignment of core genes using PRANK version 0.140603, and a tree based on the presence and absence of accessory genes among the isolates using FastTree (version 2.1.6) (Price et al., 2010).

The results from section 5.2.4.1 showed that HV isolates from NZ were the same ST. In order to compare the relationship between NZ isolates with human isolates that were the same ST, the ST genome data of these human isolates were retrieved from the

European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The genome data from HV isolates and the human isolates were clustered based on the pan-genome using Roary (version 3.9.1) (Page et al., 2015). Details of the human genome data can be found in Appendix A5.1.

5.2.4.5 Plasmid detection

The genomes that were closely related to the NZ isolates (n=22) from the rMLST clustering results were chosen for further analysis. The plasmids were identified using ARIBA (Antimicrobial resistance identification by assembly) methodology (Hunt et al., 2017). In brief, the plasmid references were clustered by similarity using CD-HIT and the reads were mapped to the reference sequences using minimap (Li, 2016). The reads that mapped to a reference were assigned to the cluster that the reference was grouped to. The fermi-lite algorithm (https://github.com/lh3/fermi-lite) was used to assemble a set of paired reads in each cluster independently. The assembly was compared to the closest reference sequence using the MUMmer package (Kurtz et al., 2004).

The 22 *K. pneumoniae* genomes were separately mapped to the full-length plasmid. Thirteen plasmids previously identified by the ARIBA methodology (Hunt et al., 2017), plus two publicly available plasmids: NC_005249.1 (HV *K. pneumoniae* CG43, serotype K2) and NC_006625.1 (HV *K. pneumoniae* NTUH-K2044, serotype K1) were separately mapped to the full-length plasmid sequences using Bowtie2 in both the "—very-sensitive-local" and "—very-sensitive" modes for local and global read mapping respectively. Default values were used for all other parameters. The percentage of reads mapped to the plasmids was produced by the "flagstat" option within samtools (Li

et al., 2009). R "pheatmap" package with hierarchical clustering was used to plot resulting mapping data (Kolde, 2018).

5.2.4.6 Pan-genome-wide association studies (pan-GWAS)

In order to find associations between the accessory genome and specified traits, and perform statistical analysis, Scoary (version 1.6.15; (Brynildsrud et al., 2016) was used to perform pan-genome-wide association studies (pan-GWAS) using default settings (p = 0.05). Two files were used as input: a gene presence/absence matrix (produced from Roary) and a trait file. Scoary first collapsed correlating gene content variants into a single unit. Fisher's exact test was performed to analyse gene and trait as a default of the program, followed by pairwide pairwise comparisons algorithm. In this pairwise comparisons algorithm, bionomial tests were performed to find the p value. Genes present in every isolate and the genes that were not present in any isolates were excluded.

In this study the traits analysed included NZSL fatal infection and NZSL carrier isolates, NZ HV and NZ non-HV isolates, NZSL fatal infection and California sea lion isolates, and NZSL fatal infection from Enderby Island and NZSL fatal infection from other places.

5.3 Results

5.3.1 General features of *K. pneumoniae* isolated from New Zealand

A total of 12 *K. pneumoniae* isolates from New Zealand were selected for whole genome sequencing. The basic characteristics were analysed before whole genome sequencing; 10 of the 12 isolates were string test positive, 9 from 12 isolates were serotype K2 (using PCR), and 9 of 12 were positive for the *rmpA* gene. The details of

each strain are summarised in Table 5.2.

K. pneumoniae isolated from New Zealand, including the isolates from NZSLs, birds from Enderby Island, and substrate samples from the NZSL habitation areas (Enderby and Campbell Island), were 5.3–5.8 Mb in length and had a GC content of 56.23%–57.48%. The number of tRNAs ranged from 81 to 89. The summary of genetic information for each strain can be found in Table 5.2.

Table 5.2 Genetic information of *K. pneumoniae* isolated from New Zealand

Isolate	Capsular serotype type	String test	Length (bP)	Contigs	%GC	rRNA	repeat region	sig_peptide	CDS	tRNA	tmRNA
HV_KP2	K2	+	5662712	730	56.23	10	0	433	7030	89	1
HV_KP3	K2	+	5672644	799	56.28	11	0	429	7374	88	1
HV_KP4	K2	+	5611619	624	56.43	10	0	435	6621	86	2
HV_KP5	K2	+	5353362	81	57.46	10	0	479	5008	81	1
Non-HV_KP6	Non-K1/1K2	+	5644618	105	57.06	10	1	492	5418	89	1
Non-HV_Kp7	Non-K1/1K2	-	5703228	97	57.01	8	1	500	5451	89	1
HV_Kp8	K2	+	5324629	93	57.46	10	0	480	4985	81	1
Non-HV_Kp9	Non-K1/1K2	-	5644626	116	57.05	10	1	489	5429	89	1
HV_Kp10	K2	+	5327210	94	57.5	11	0	482	4980	82	1
HV_Kp11	K2	+	5351349	91	57.46	10	0	483	5004	82	1
HV_Kp12	K2	+	5332787	85	57.48	10	0	480	4984	82	1
HV_Kp13	K2	+	5323133	99	57.46	9	0	481	4992	82	1

%GC = guanine-cytosine content, rRNA = ribosomal ribonucleic acid, sig_peptide= signal peptide, CDS= coding sequence, tRNA=transfer ribonucleic acid, tmRNA = transfer-messenger ribonucleic acid

5.3.2 Genomic analysis

5.3.2.1 Multilocus sequencing typing (MLST)

A total of nine isolates were ST 86, including all the HV isolates. Three isolates (non-HV_Kp6, non-HV_Kp7 and non-HV_Kp8) were an unidentified ST when first put on the BIGSdb database, suggesting a novel ST, with the following characteristics: *gapA* (14), *infB* (5), *mdh* (2), *pgi* (1), *phoE* (7), *rpoB* (1), *tonB* (23). The author submitted the novel ST to BIGSdb, and the novel ST was assigned the number ST2843 (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_isolates_public&page=query). Details for each isolate can be found in Table 5.3.

Table 5.3 MLST information for *K. pneumoniae* isolates from New Zealand sources

Isolate	gapA	infB	mdh	pgi	phoE	rpoB	tonB	ST
HV_Kp2	9	4	2	1	1	1	27	86
HV_Kp3	9	4	2	1	1	1	27	86
HV_Kp4	9	4	2	1	1	1	27	86
HV_Kp5	9	4	2	1	1	1	27	86
Non-HV_Kp6	14	5	2	1	7	1	23	2843 (new ST)
Non-HV_Kp7	14	5	2	1	7	1	23	2843 (new ST)
HV_Kp8	9	4	2	1	1	1	27	86
Non-HV_Kp9	14	5	2	1	7	1	23	2843 (new ST)
HV_Kp10	9	4	2	1	1	1	27	86
HV_Kp11	9	4	2	1	1	1	27	86
HV_Kp12	9	4	2	1	1	1	27	86
HV_Kp13	9	4	2	1	1	1	27	86

5.3.2.2 Plasmid detection

The 22 *K. pneumoniae* genomes were mapped with 15 known plasmids. Each

isolate revealed a different percentage of mapping with each plasmid. The colour that is shown in Fig 5.1 ranges from yellow to dark blue (0-5) and indicates the percentage of the reads from the isolates mapped to the plasmids being examined. The HV *K. pneumoniae* isolates show high similarity to the NC_005249 and NC_006625 plasmids belonging to HV *K. pneumoniae* CG43 and *K. pneumoniae* NTUH-K2044 respectively, by showing a blue to dark blue colour (indicating mapping percentages of 4-5) (Fig 5.1). The human isolates CAS688 and CAS689, and the California sea lion1 and California sea lion2 isolates showed mapping percentages of 3-4% to the NC_005249 and NC_006625 plasmids. The human isolate TG21905 and the African green monkey isolate (M3_11_2499) showed mapping percentages of 4-5% to the CP000648 and JN233704 plasmids belonging to *K. pneumoniae* MGH7578 and *K. pneumoniae* ST258 respectively.

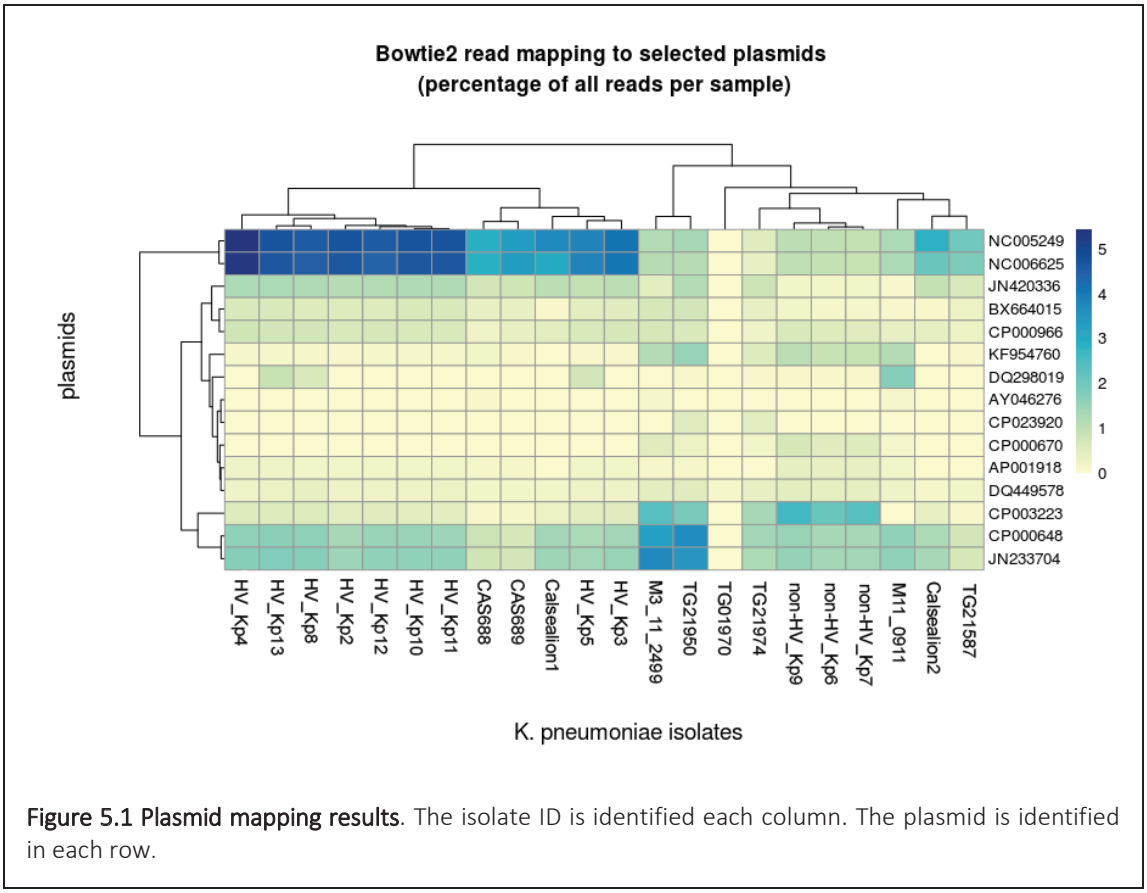


Figure 5.1 Plasmid mapping results. The isolate ID is identified each column. The plasmid is identified in each row.

5.3.2.3 Genes associated with virulence and antimicrobial resistance

The HV isolates from NZ carried the *rmpA* gene. All the serotype K1 isolates carried the *magA* and *rmpA* genes. *IucD* and *iucA* were also carried by the serotype K1 isolates. HV_Kp2, HV_Kp4, non-HV_Kp6, non-HV_Kp7, non-HV_Kp9 from New Zealand also carried *iucD* and *iucA*. *RcsA, rcsB*, *uge*, *iroN* and *mrkD* were carried by all of the isolates. *YbtS* was carried by most of the isolates from New Zealand except non-HV_Kp6 and non-HV_Kp9. The details of virulence genes of each isolate can be found in Fig 5.2.

For antibiotic resistance genes, all the isolates carried bla_{SHV} genes with different alleles. All the NZ isolates carried $bla_{SHV\text{-}1}$. All the serotype K1 isolates carried $bla_{SHV\text{-}36}$. California sea lion isolates and three human isolates carried $bla_{SHV\text{-}11}$. All the isolates in this study carried oqxA, oqxB, and fosA/fosA5.

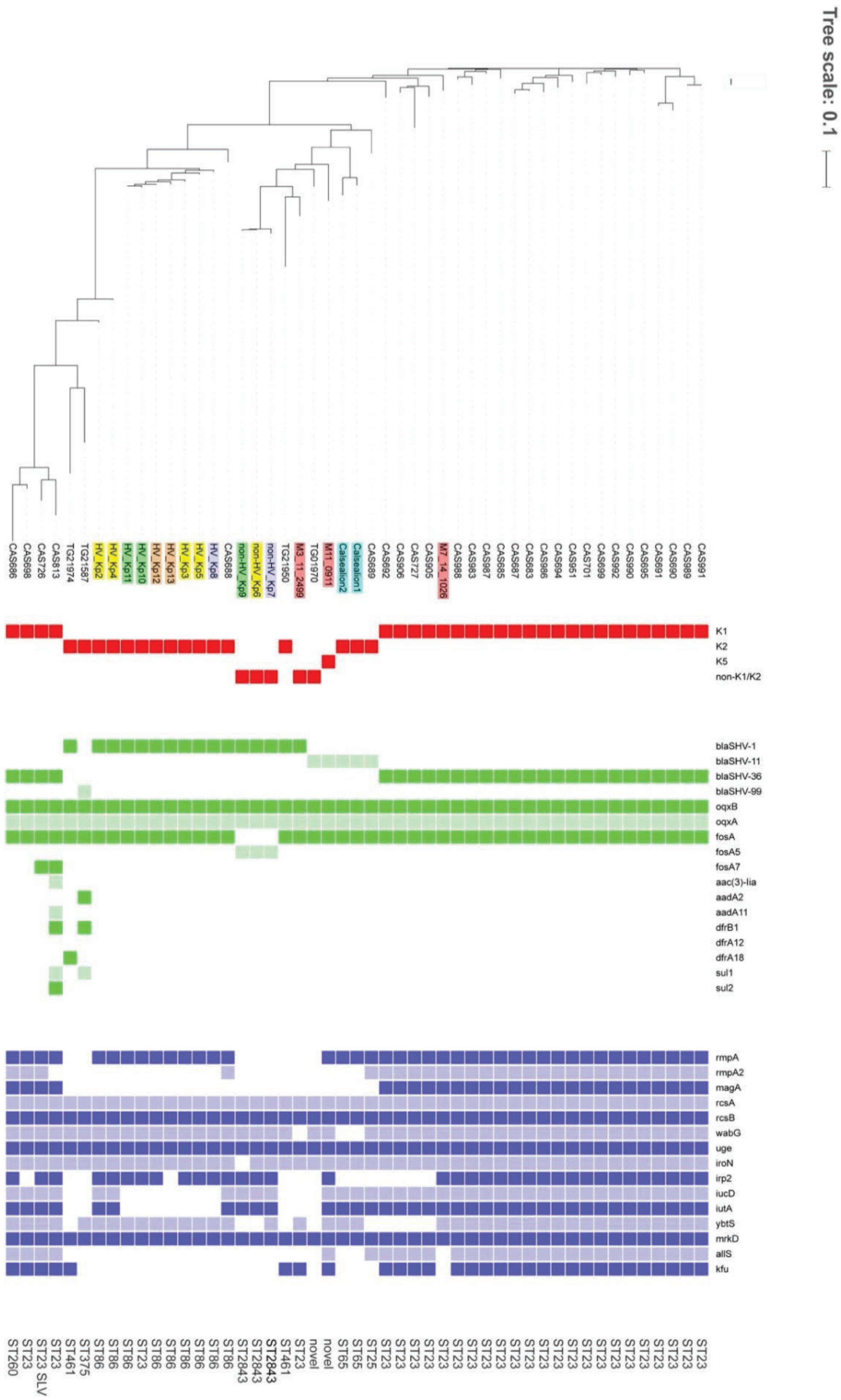


Figure 5.2 The presence of virulence and antibiotic resistance gene of 49 *K. pneumoniae* isolates.

The isolate background colour: red = African green monkey, blue = California sea lion, purple = NZSL adult, yellow = NZSL pup, green = bird, orange = substrate sample.

From the rMLST clustering, the isolates can be grouped into three major groups. All the serotype K1 from humans plus one African green monkey isolate, M7_14_1026 (serotype K1) were grouped together. Isolates from New Zealand (both HV and non-HV) plus the California sea lion isolates, six human isolates (five were serotype K2 and one was nonK1/K2) and one monkey isolate (nonK1/K2) were grouped together. The African green monkey isolate, M11_0911 (serotype K5), was separated from the others. The group of non-HV isolates plus M3_11_2499 were closer to the HV NZ isolates (serotype K2) than the serotype K1 isolates (Figure 5.3). The non-HV_Kp6 that was string test positive, but negative for the *rmpA* gene (in both PCR and genome sequencing), was grouped with the non-HV isolates from Enderby Island (non-HV_Kp7 and 9).

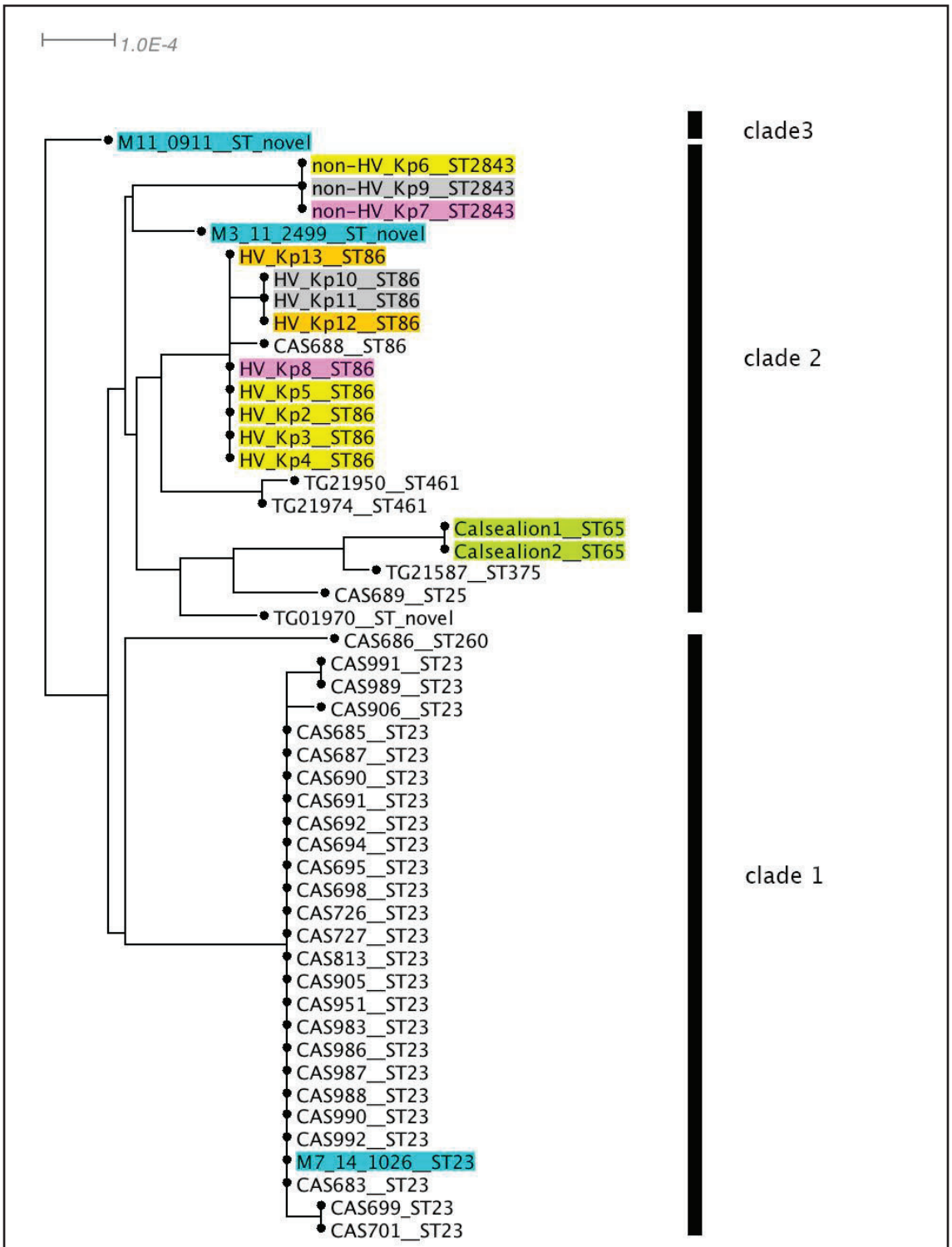


Figure 5.3 Dendrogram shows association of *K. pneumoniae* isolates using rMLST clustering.
The dendrogram can be divided into three clades. Clade 1 consists of one African green monkey isolates, M11 0911. Clade 2 consists of serotype K2 and serotype non-K1/K2 isolates (all the isolates from NZ plus human isolates). Clades 3 consists of all serotype K1 isolates (human isolates plus African green monkey isolate, M7_14_1025). The label backgrounds indicate source of the isolates as follow: transparency = human, green = California sea lion, blue = African green monkey, yellow = NZSL pup, pink = NZSL adult, orang= substrate sample, grey = bird.

In total, 22 isolates including New Zealand isolates and other isolates that were closely related according to rMLST profiles (Fig 5.3), were further investigated using GET_HOMOLOGUES. The pan-genome of these 22 *K. pneumoniae* isolates was calculated by GET_HOMOLOGUES using the OMCL and COG algorithms. The core-genome size was calculated using three algorithms: BDBH 3688, OMCL 3687, and COG 3697. The A total of 3660 consensus clusters were identified by all three algorithms (Fig. 5.4). The total number of gene clusters was 7859: cloud (2013, genomes <= 2), shell (1715), soft core (4131, genomes >= 20) and core (3663, genomes = 22) (Figure 5.5). OMCL was performed to estimate the theoretical core-genome and pan-genome size of *K. pneumoniae* (Fig. 5.6, 5.7). The estimate of theoretical pan-genome size tends to increase when adding more samples. The estimate of theoretical core genome size tends to decrease when adding more samples.

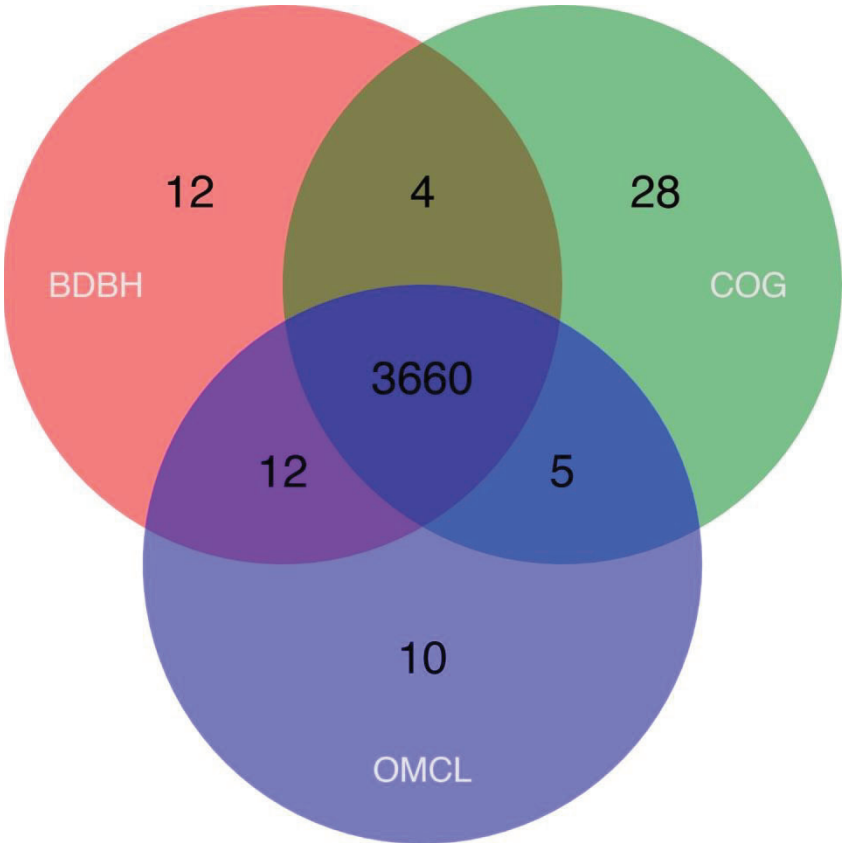


Figure 5.4 The core genome of 22 isolates of *K. pneumoniae*
The core genome of 22 isolates of *K. pneumoniae* calculated by three different methods including OMCL, BDBH and COG within GET HOMOLOGUES. Venn diagram showing the differences in calculated core size and shared content between the three results

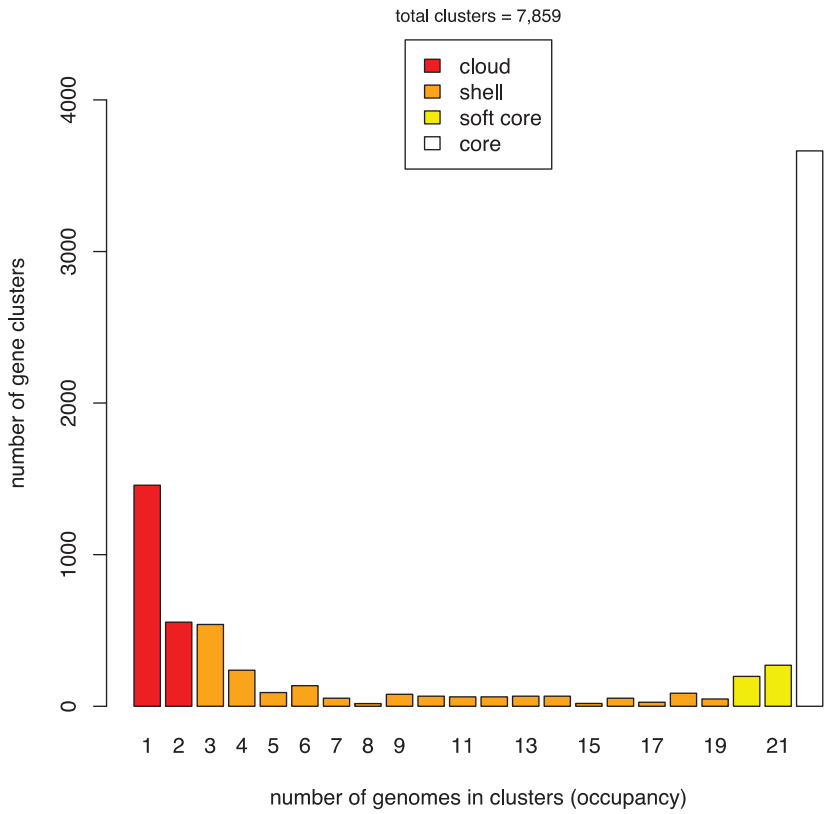


Figure 5.5 The pan-genome of 22 *K. pneumoniae*

The pan-genome of 22 *K. pneumoniae* was calculated by GET_HOMOLOGUES using OMCL and COG. A total number of gene clusters was 7859: cloud (2013, genomes <= 2), shell (1715), soft core (4131, genomes >= 20) and core (3663, genome s=22)

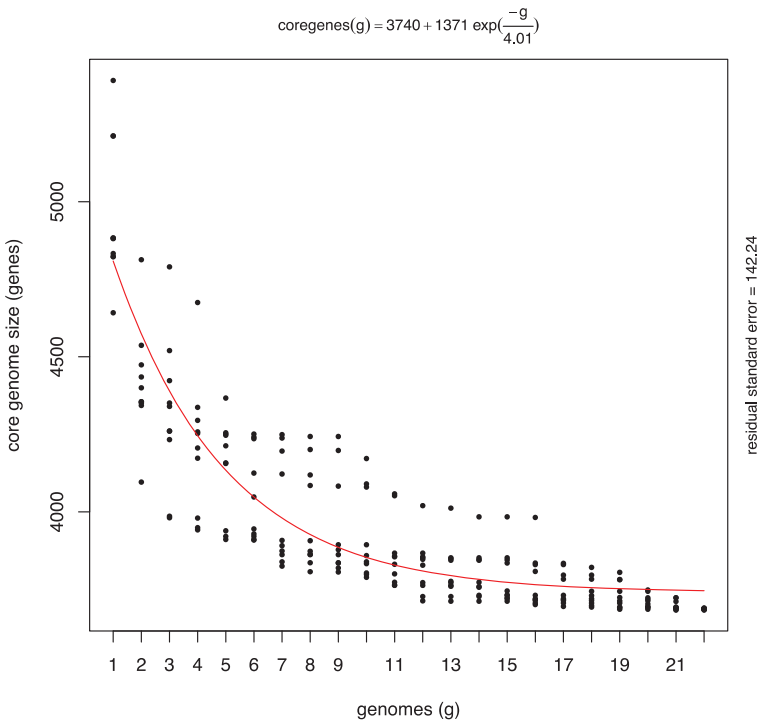


Figure 5.6 The estimation of theoretical core-genome size of *K. pneumoniae* using 22 isolates

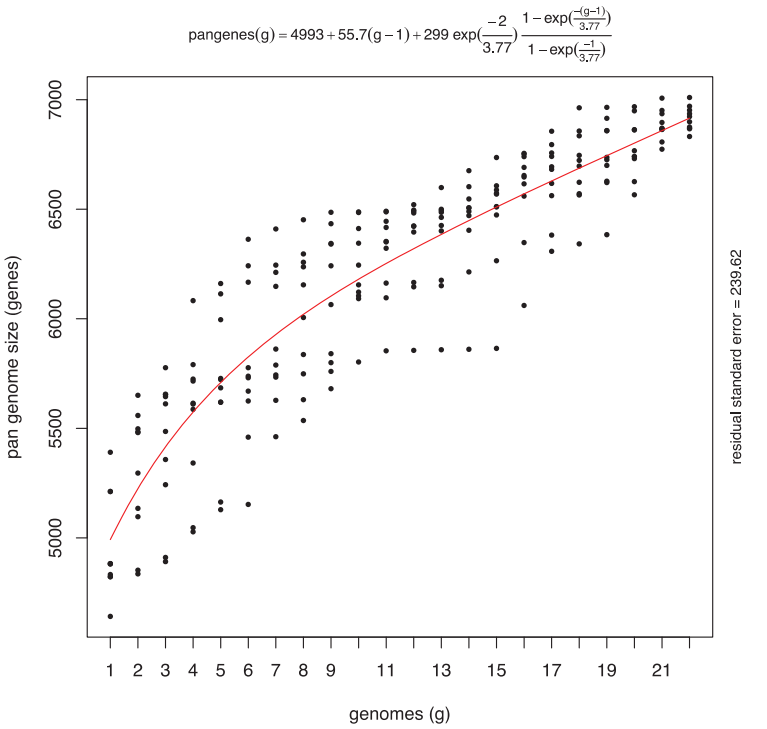


Figure 5.7 The estimation of theoretical pan-genome size of *K. pneumoniae* using 22 isolates

5.3.2.4 Protein and Gene Analysis

Roary was used to produce a pan-genome of the 22 *K. pneumoniae* isolates. Roary produced a total of 8,813 protein-coding gene sequence clusters. Roary produced an accessory binary tree based on the presence/absence status of gene clusters (Fig 5.8) that revealed 2 major groups. Group one consisted of all HV NZ isolates except HV_Kp10 and HV_Kp12. Group two consisted of the human isolates plus all non-HV NZ isolates and two HV NZ isolates (one from a substrate sample (HV_Kp12) and one from a skua (HV_Kp10)), and the California sea lion isolates.

Roary was as well used to produce a pan-genome of the 13 ST86 *K. pneumoniae* isolates. Roary produced a total of 8,253 protein-coding gene sequence clusters. Roary produced an accessory binary tree based on presence/absence gene (Fig 5.9). The isolates from humans were grouped separately from the NZ isolates (sea lion pups, substrate samples and birds).

Comparison of the common and unique genes in each set of isolates is shown in Table 5.8. Between HV NZSL isolates from fatal infections and from carriers, there were 4868 genes in common. The fatal infection group and carriage group had 303 and 13 unique genes respectively. Between HV and non-HV isolates, there were 4471 genes in common. The HV group and non-HV groups had 734 and 859 unique genes respectively. Between NZSL fatal infections and California sea lions, there were 4311 genes in common. The NZSL fatal infection group and California sea lion group had 859 and 370 unique genes respectively. Between NZSL fatal infection isolates from Enderby Island and NZSL fatal infection isolates from other places, there were 4962 genes in common. NZSL fatal infection isolates from Enderby Island and NZSL fatal infection isolates from other places had 71 and 138 unique genes respectively.

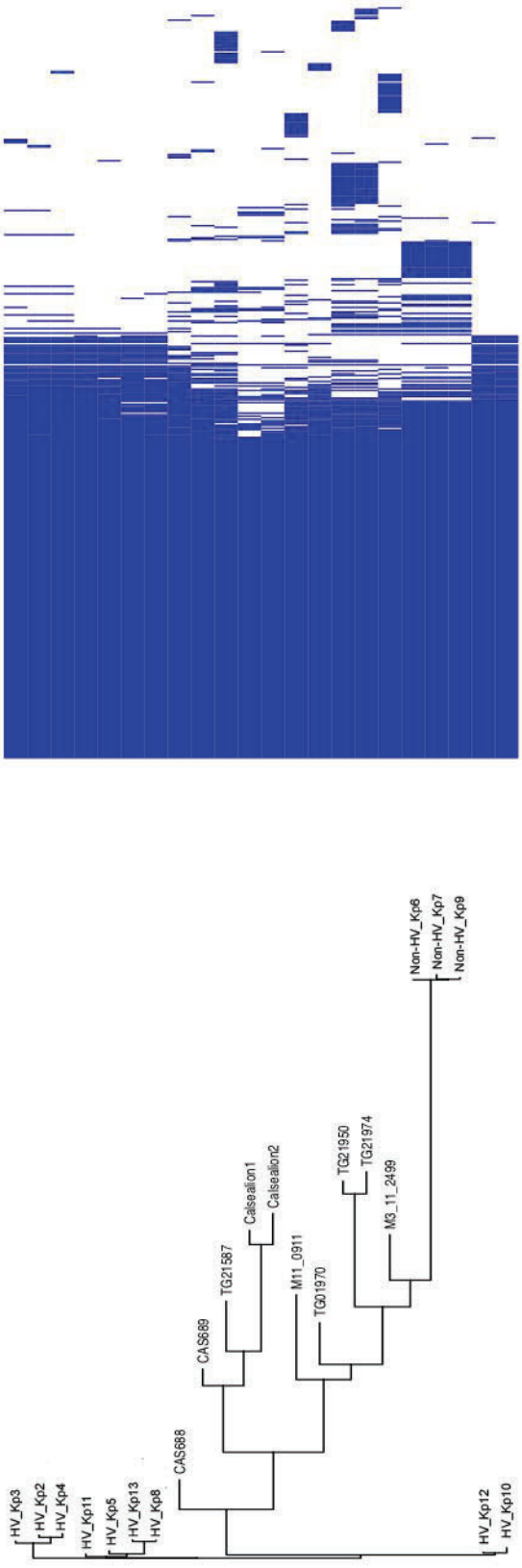


Figure 5.8 The accessory binary tree (22 isolates)

The accessory binary tree (22 isolates) and gene presence/absence (total 8,813 genes). All NZ HV isolates except HV_10 and HV_12 were grouped together. All the NZ non-HV isolates were grouped with human isolates plus HV_Kp10 and HV_Kp12

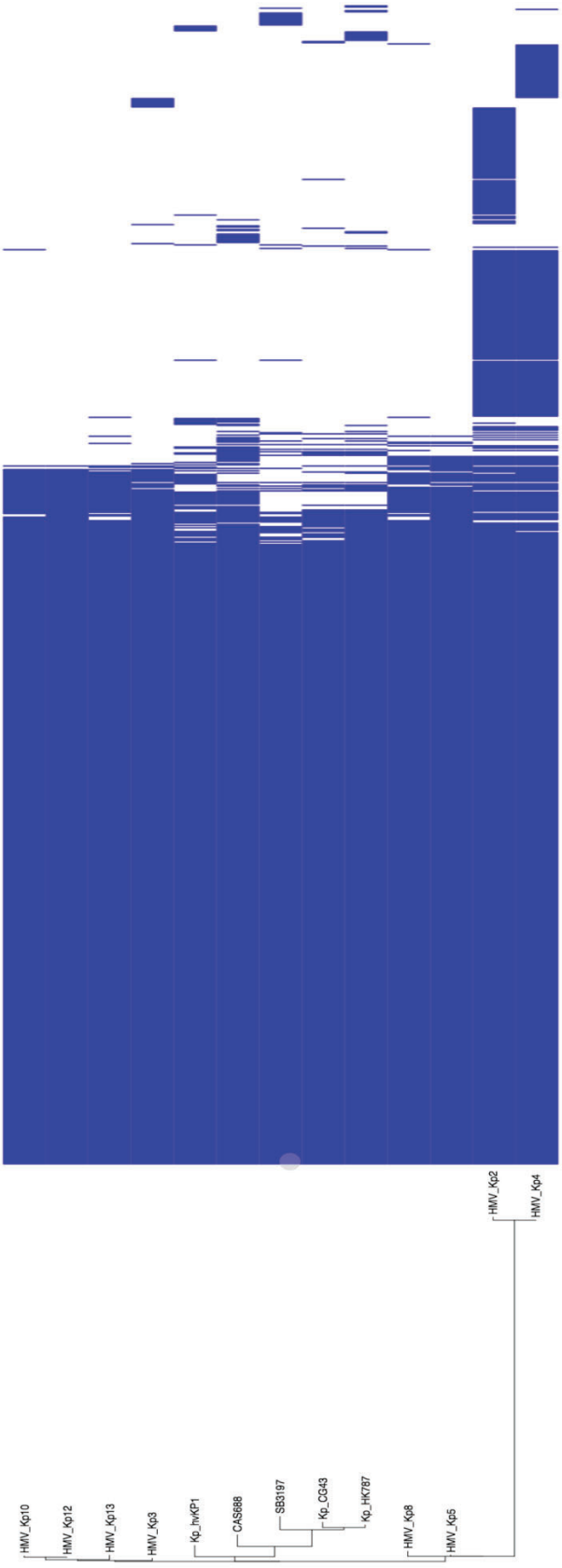


Figure 5.9 The accessory binary tree (ST 86) and gene presence/absence (total 8,253 genes). The isolates from humans were grouped together, but were separated from NZ isolates.

Table 5.4 Common genes and unique gene numbers of each group of the isolates

Source	Set A	Set B	Common genes	Unique genes of set A	Unique genes of set B
NZSL isolate	Fatal infection HV_Kp2,3,4,5	Carriage HV_Kp8	4868	303	13
NZ isolate	HV HV_Kp2,3,4,5,8,10,11,12,13	non-HV non-HV_Kp6,7,9	4471	734	859
NZSL, California sea lion isolate	Fatal infection NZSL HV_Kp2,3,4,5	California sea lion Calsealion1,2	4311	859	370
NZSL	Enderby Island HV_Kp2,3	Other place HV_Kp4,5	4962	71	138

5.4 Discussion

Here, by using rMLST clustering (Fig 5.3), the pup isolates from *K. pneumoniae* fatal infections were grouped together with other HV (*rmpA*$^{+}$) isolates from NZ, suggesting all the HV isolates were from a common source. This confirmed that the HV isolates found in other locations including the Otago Peninsula, Dundas Island and Campbell Islands were closely related. The results in this chapter also suggest potential reservoirs of this pathogen, including the environment, birds that live around the breeding site, and adult sea lions.

The NZ isolates plus three monkey isolates from St Kitts were further analysed with 34 other *K. pneumoniae* from humans retrieved from ENA and GenBank. The rMLST clustering clearly discriminated serotype K1 from the other serotypes. It grouped serotype K2 with the unidentified serotype isolates, suggesting the relationship of these isolates with serotype K2 isolates (Fig 5.3).

The New Zealand isolates were grouped in clade 2 (Fig 5.3). HV isolates were grouped together and the non-HV isolates were grouped together, but the HV and non-HV were separated from each other (Fig 5.3). This suggests that HV and non-HV isolates

are genetically distinct, and that the HV isolates are not a modified form of non-HV isolates or non-HV isolates are HV isolates that lost a plasmid. This was supported by the MLST study. MLST is a typing method using seven house-keeping genes which are highly conserved in bacteria (Ibarz Pavón and Maiden, 2009), with different bacterial species having different schemes. HV isolates and non-HV isolates were different ST; HV isolates were ST86; non-HV isolates were a new ST, namely ST2843. ST86 and ST2843 are not in the same clonal complex group.

The NZSL pup isolates (HV isolates) from Enderby Island were grouped together with the human sample CAS688 (Fig 5.3) that was serotype K2 and ST86, the same serotype and ST as the NZSL pup isolates. In a previous study, ST86 isolates from humans revealed a high degree of clonality (Struve et al., 2015), which raises the question as to whether NZSL pup isolates originate from human ST86 strains present in other parts of the world including Asia, North America and Europe (Struve et al., 2015). In order to establish this information, some of the available ST86s in the database (GenBank and ENA) were retrieved and analysed with the HV isolates from NZ. By using pan-genome clustering, the human isolates were clustered together, but were separated from NZSL isolates. However, this result cannot be used to eliminate the human origin of these isolates because clustering using pan-genome may be affected by mobile genetic elements (Medini et al., 2005).

Interestingly, by using pan-genome clustering (Fig 5.9), HV_Kp2, the isolate from Enderby Island from 2002/03, was closely related to HV_Kp4, the isolate from Otago (NZ mainland) from 2013. This suggests the possibility that HV *K. pneumoniae* that caused the deaths on Enderby Island may have come from NZ mainland or *vice versa*.

Alternatively, the isolates may have come from other geographic locations and become established on Enderby Island after visits by various shipping vessels or wildlife.

HV *K. pneumoniae* has been isolated from California sea lions (Jang et al., 2010) and these organisms show the same basic characteristics as the isolates from NZSL pups (Roe et al., 2015), suggesting they might have similar genetic characteristics. This further raises the question as to whether there is any genetic association between the bacteria and host species. In the MLST study, they were a different sequence type (ST); California sea lions were ST65 and NZSL pups were ST86 (Fig 5.2), suggesting no association between these two bacterial groups. This finding indicates that bacterial genetics is unlikely to be associated with host species in this case, giving further evidence of the ability of *K. pneumoniae* to infect a wide range of hosts.

Overall, the size of the genome from NZ isolates was 5.3 – 5.8 Mb in length with a GC content of 56.23-57.48%, which is similar to *K. pneumoniae* genomes previously studied (Caputo et al., 2015; Holt et al., 2015). In bacterial species, the genome size appears to be related to bacterial lifestyle (Moran, 2002; Weinert and Welch, 2017). Symbiotic bacteria have a smaller genome size than bacteria that live independently (Bobay and Ochman, 2017). When compared within a species, bacteria such as the pathogenic strains of *Streptococcus suis, E. coli* and *Shigella spp.,* have a smaller genome size than non-pathogenic strains, as pathogenic bacteria lose genes used to survive in the environment that they no longer use (Weinert et al., 2015; Weinert and Welch, 2017). However, in this present study, the relationship between genome size and the source of the isolates (i.e. dead and healthy sea lions, substrate samples or birds) was not observed. The was no relationship between genome size and HV (*rmpA*$^{+}$)

or non-HV (*rmpA*$^{-}$) isolates. However, due to the small sample size a conclusion cannot be drawn from this study.

Most of the HV strains possess either *magA* or *rmpA/A2* (Holt et al., 2015; Struve et al., 2015); later studies revealed that *magA* is limited to serotype K1 (Struve et al., 2005; Yeh et al., 2006). In this present study, the HV isolates were *rmpA* positive and *magA* negative, which were similar to other studies since the HV isolates in this study were serotype K2 (Holt et al., 2015; Struve et al., 2015). However, one isolate from a NZSL pup was HMV phenotype (positive string test), but was negative for both the *rmpA* and *magA* genes. A similar result was found in other studies concerning human isolates from China (Yu et al., 2015). This suggests that apart from *magA* and *rmpA* genes there are other genes or factors as yet unidentified that can enhance the expression of the HMV phenotype.

The isolates from clade1 and clade2 from the rMLST clustering were further investigated using pan and core genome analysis. The estimates of the core and pan-genome (Fig 5.6, 5.7) suggest that *K. pneumoniae* has an open genome, therefore adding additional strains to the analysis will lead to an increase in the number of genes in the pan-genome. This type of bacteria lives in multiple environments that have large microbial communities and have many ways to exchange their mobile genetic elements (Bobay and Ochman, 2017). This result is consistent with previous *K. pneumoniae* genome studies that have also shown that *K. pneumoniae* has an open genome (Holt et al., 2015). This corelated with nature of *K. pneumoniae*, which can be found everywhere. Moreover, in species with open genomes, there is a possibility of gaining new genes from the same species or others that can make them more virulent than in the past.

The HV isolates possess at least one large plasmid similar to pLVPK (NC_005249) that is possessed by HV *K. pneumoniae* CG43 (serotype K2). Most HV strains possess either the pLVPK or pNTUH-K2044 plasmids (Struve et al., 2015). These two plasmids are highly similar (Chen et al., 2004; Wu et al., 2009a). The plasmids may involve HV in several aspects. They possess *rmpA/A2* genes involved in the expression of the hypermucoviscous phenotype (Chen et al., 2004). They carry virulence genes such as aerobactin and salmochelin synthesis, plus other iron uptake regulation genes that have been suggested to be virulence factors in HV isolates (Chen et al., 2004). They also possess other genes that are involved in copper silver, lead, and tellurite resistance (Chen et al., 2004). Loss of this plasmid in *K. pneumoniae* serotype K2 results in a decrease of the virulence and loss of colony mucoidy (Lai et al., 2003). Classical *K. pneumoniae* (non-HV strains) does not appear to possess this kind of plasmid.

As iron is essential for bacterial growth, siderophores (iron chelating compounds) have been considered to be important virulence factors in pathogenic bacteria. Aerobactin, one type of siderophore, has been found to be highly associated with HV *K. pneumoniae* (Russo et al., 2018). Moreover, aerobactin has been proposed to be the essential virulence factor in *K. pneumoniae* (Russo et al., 2015). However, in this present study, not all of the isolates from fatal infections in sea lion pups possessed the *iucD* (encode aerobactin) and *iutA* (aerobactin receptor) genes, but all of the NZ non-HV isolates from carriers possessed *iucD* and *iutA* genes. A similar result was also found in human clinical isolates in this present study; not all possessed aerobactin associated genes (Fig 5.2). This suggests that not only siderophores are involved in the virulence of HV *K. pneumoniae*. Notably, the non-HV isolates in this study possess

several virulence genes that were in human clinical HV isolates (Fig 5.2), suggesting pathogenic potential.

Multi-drug resistance in HV *K. pneumoniae* strains has been described in many parts of the world. In this study, all the NZ isolates possessed the bla_{SHV}, *oqxA*, *oqxB* and *fosA* genes. These genes give resistance to ampicillin, quinolones (*oqxA* and *oqxB*) and low level of fosfomycin, respectively. However, only resistance to ampicillin was found in NZ isolates (Chapter 3). A difference between HV and non-HV NZ isolates was that HV isolates possessed *fosA,* while non-HV isolates possessed *fosA5*. *FosA* has been reported in bacteria in the family Enterobacteriaceae (Ito et al., 2017). The presence of this gene leads to reduced susceptibility or resistance to Fosfomycin, which has to be confirmed by an antimicrobial susceptibility test (Yang et al., 2017). In *K. pneumoniae,* both *fosA* and *fosA5* have been reported and *fosA/A5* are likely to be on the chromosome (Ito et al., 2017). *FosA5* shares only 69% amino acid sequence with *fosA* (Yang et al., 2017), suggesting that *fosA5* is not likely to originate from *fosA*. As the HV and non-HV NZ isolates possessed different alleles for *fosA* genes that are not related, this provides further support to the MLST and rMLST clustering results that HV and non-HV NZ isolates were not closely related.

Another interesting observation in this present study is that there were a number of different alleles of the bla_{SHV} genes in the isolates. Bla_{SHV} is a gene that can be found in gram-negative bacteria which encodes resistance to antibiotics in the beta-lactam group. They can be found either on the chromosome or a plasmid. Generally, *K. pneumoniae* possesses bla_{SHV} on their chromosome, which was also found in this present study, but bla_{SHV} genes located on plasmids have also been reported (Babini and Livermore, 2000). The isolates from NZ (ST86 and ST2483), the human CAS688

(ST86) isolate, the human ST461 isolate and the monkey isolate M3_11_2499 all possessed *bla*$_{shv-1}$. The *bla*$_{shv-1}$ in NZ isolates were functional as the isolates were resistant to ampicillin (Chapter 2), which is consistent with previous studies (Chaves et al., 2001; Rice and Bonomo, 2000). There was no pattern in the *bla*$_{shv}$ gene allele number based on geographic distribution or capsular serotype. However, a previous study of *bla*$_{shv}$ genes showed that *bla*$_{shv-1}$, *bla*$_{shv-11}$, *bla*$_{shv-36}$ and *bla*$_{shv-99}$ were categorised into different groups (Liakopoulos et al., 2016), suggesting a different origin. This supports the genome clustering analysis in the first part of this present study.

In the present study, the genetic characteristics of NZSL pup isolates have been described. Genetic analysis suggests a close relationship between HV isolates from different sources (NZSLs, birds and the environment). This further suggests possible reservoirs: adult sea lions, birds, and the environment. This also shows the geographic distribution of the pathogen: Enderby Island, the Otago region (NZ mainland), Dundas Island, and Campbell Island. The genetic analysis also suggests HV isolates and non-HV isolates were not from the same common sources. The origin of this pathogen cannot be established from this study. The HV isolates from NZ seem to group together, different from the other human isolates from the same ST based on using pan-genome clustering. However, a human origin cannot be excluded due to this result being affected by the horizontal transfer of mobile genetic elements.

Chapter 6

Immunity to *Klebsiella pneumoniae*

6.1 Introduction

Since the first outbreaks of *K. pneumoniae* in 2001/2002 and 2002/2003 in New Zealand sea lion (NZSL) pups at Sandy Bay, Enderby Island, *K. pneumoniae* seems to have become endemic in this animal group (Castinel et al., 2007b; Roe et al., 2015). The distribution of this infection is not limited to Sandy Bay. *Klebsiella pneumoniae* has also been isolated from a dead pup from the Otago peninsula on the New Zealand mainland in 2013, as well as from dead pups from Dundas Island and Campbell Island ((Roe et al., 2015); Chapter 2). As shown in Chapter 2, all examined *K. pneumoniae* isolates from NZSL pups have the hypermucoviscous (HMV) phenotype, the same phenotype that has been reported in humans with community-acquired infections, where it causes liver abscesses complicated by septicaemia known as hypervirulent (HV) *K. pneumoniae* (Fang et al., 2004; Shon et al., 2013). The HV *K. pneumoniae* phenotype also has been reported in California sea lions (Jang et al., 2010), African green monkeys (Soto et al., 2012; Twenhafel et al., 2008), camels (Sharma et al., 2013), buffalos and cows (Osman et al., 2014). One interesting point about this infection in NZSLs is that the disease caused by HMV *K. pneumoniae* has only been found in pups (Lenting, 2017; Roe et al., 2015), suggesting a difference in immune protection between adults and pups.

The immune system can be divided into innate and adaptive immunity. Innate immunity is the first line of defence that protects the body against pathogens and it can be functional after birth (Murphy and Weaver, 2016). It provides immediate reactions that are non-specific to the pathogen and does not require a memory of the invading organism. The major components of innate immunity are physical and chemical barriers, such as skin and mucosal epithelium, phagocytic cells such as neutrophils and monocytes, dendritic cells, natural killer (NK) cells, and circulating proteins such as antimicrobial peptides and complement proteins. While adaptive immunity is not functional at birth, the newborn animal utilises antibodies and other immune factors acquired from the mother's colostrum. Once the adaptive immune system is functional, it takes longer than the innate immune system (several days) to respond to a pathogenic challenge. Adaptive immunity is specific to the pathogen and exhibits memory (Murphy and Weaver, 2016).

In order to cause infection, a pathogen has to overcome the host's innate immunity. Each pathogen uses different mechanisms to evade the hosts innate immune responses followed by the pathogen evading or overwhelming the adaptive immune response. In *K. pneumoniae*, several studies have been performed to investigate how the immune system controls the infection and how the pathogen overcomes the immune response. Results from these studies have shown that the immunological reaction varies based on the *K. pneumoniae* strain (Paczosa and Mecsas, 2016; Shon et al., 2013; Xiong et al., 2015). Since the *K. pneumoniae* infection in NZSL is different from other species in terms of age of the animal affected (Castinel et al., 2007b; Jang et al., 2010; Twenhafel et al., 2008), the immune response to isolates from this species may be different from other *K. pneumoniae* isolates.

In this study, the author investigated a subset of innate immunological reactions, including phagocytosis, oxidative-mediated killing and serum-mediated killing, to *K. pneumoniae* isolated from NZSLs and studied these by comparing HV isolates and non-HV isolates. Since NZSLs live in remote areas making it difficult to obtain fresh blood samples, the experiments were performed with dog (*Canis lupus familiaris*), California sea lion (*Zalophus californianus*) and New Zealand fur seal (*Arctocephalus forsteri*) samples. The dog model was selected because dogs are closely related to sea lions (Denison et al., 1971; Higdon et al., 2007), and samples were readily available. California sea lions and NZ fur seals (Family Otariidae, same family as NZSLs) were selected as available representative pinnipeds.

6.2 Materials & Methods

6.2.1 Bacterial strains and culture conditions

Seven *K. pneumoniae* isolates from NZSLs (described in Chapter2, Table 6.1) plus a human reference strain (*K. pneumoniae* ATCC 700603 recently renamed as *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603) (Elliott et al., 2016) isolated from the urine of a hospitalised patient from Richmond, VA in 1994, and *E. coli* NCTC 12900, Federal Public Health, Austria, were used in this study (Table 6.1). *Klebsiella pneumoniae* ATCC 700603 and *E. coli* NCTC 12900 were purchased from the New Zealand Reference Culture Collection (Institute of Environmental Science and Research (ESR)), Kenepuru Science Centre, Porirua, New Zealand). All *K. pneumoniae* isolates and *E. coli* were grown from glycerol stock (kept at -80°C) on 5% sheep blood agar (Fort Richard Laboratories, Auckland, New Zealand) at 37°C overnight before performing further studies.

Table 6.1 Bacterial isolates used in different experiments in this study

Isolate designation	Bacterial isolates	Source	Isolate detail	String test	Serotype
E. coli	*E. coli* NCTC 12900 (used as a control)	Human	Shigatoxin negative	-	N/A
Non-HV_Kp1	*K. pneumoniae* ATCC 700603	Human	Clinical[a]	-	Non-K1,K2
HV_Kp2	*K. pneumoniae* E02/03_112Ph	Sea lion pup	Post mortem[b]	+	K2
HV_Kp3	*K. pneumoniae* E11/12_24Ph	Sea lion pup	Post mortem[b]	+	K2
HV_Kp4	*K. pneumoniae* S13_04Ph	Sea lion pup	Post mortem[b]	+	K2
HV_Kp5	*K. pneumoniae* D14/15_8Ph	Sea lion pup	Post mortem[b]	+	K2
Non-HV_Kp6	*K. pneumoniae* C14/15_9Ph	Sea lion pup	Post mortem[c]	+	Non-K1,K2
Non-HV_Kp7	*K. pneumoniae* E09/10_13Ph	Sea lion adult	Post mortem[d]	-	Non-K1,K2
HV_Kp8	*K. pneumoniae* 9476	Sea lion adult	Healthy[e]	+	K2

[a] Hospitalised human patient infected with *K. pneumoniae*
[b] These animals died from *K. pneumoniae* infection and samples were collected at post mortem examination
[c] This isolate was from a separate study. The bacterium was isolated from a NZSL pup that died from starvation (see Chapter 2)
[d] This isolate was from a separate study. The bacterium was isolated from the tracheal lymph node from a dead NZSL adult that did not die from *K. pneumoniae* infection
[e] This isolate was from a separate study. The bacterium was isolated from a rectal swab of a healthy adult female NZSL from Campbell Island in 2014

6.2.2 Animals and sample collection

6.2.2.1 California sea lions and NZ fur seal blood sampling

The blood used in this study was collected as part of routine health examinations of captive pinnipeds held at Marineland, Napier, New Zealand. The blood was collected by veterinarians in November 2014 from two adult California sea lions (an 18-year-old male, and an 11-year-old-male) and three adult NZ fur seals (a 12-year-old male, a 9-year-old female, and a 15-year-old female). The animals were sedated with Zoletil 100 (Virbac, Virbac New Zealand Limited, Auckland, New Zealand) intramuscular injection (IM) and anaesthetised with Isoflurane delivered by mask (Attane, Bayer New Zealand Ltd, Auckland, New Zealand) prior to blood collection. Blood was collected from the lumbar extradural intravertebral vein into plain Vacutainer tubes (Becton-Dickinson, NJ, USA) for serum (California sea lions only) and Vacutainer tubes containing EDTA as

an anticoagulant (Becton-Dickinson, NJ, USA) for whole blood (both California sea lions and NZ fur seals). The collection tubes were immediately stored on ice and processed within 24 h. The whole blood was used for the phagocytosis experiment within 24 h. The serum tubes were left at room temperature for 30 min to allow for clot formation, prior to centrifuging for 15 min at 1500 g at 4°C. The supernatant (serum) was collected into 1.5 mL Eppendorf tubes and kept at -80°C for three months prior to being use.

6.2.2.2 Canine blood sampling

Pooled serum was obtained from 40 dog serum samples, submitted for routine serum chemistry to New Zealand Veterinary Pathology Ltd, (Palmerston North, New Zealand) during April to September 2014. The serum was stored at -20 °C for eight months prior to use.

Blood was collected from three healthy dogs; two male golden retrievers (1 and 10 years old) and one female golden retriever (1-year-old). The blood was collected from the external jugular vein into Vacutainer tubes containing EDTA as anticoagulant (Becton, Dickinson and Company, USA) and used in the experiment immediately after collection. Blood and serum collection was approved by the Massey University Animal Ethics Committee (protocol 14/48).

6.2.3 Oxidative-mediated killing assay

This assay was performed with eight bacterial isolates (Table 6.1), plus *E. coli* as a control. The method was modified from Cox et al. (2015). The oxidative-mediated killing assay was performed using hydrogen peroxide (H_20_2) freshly prepared from 30 % H_20_2 (Sigma–Aldrich, St. Louis, MO, USA) into final concentrations of 4 mM and 8 mM using sterile Milli-Q filtered water. The bacteria (1 - 5 x 10^5 CFU/mL) were prepared

from an overnight culture. The number of bacteria was measured by adjusting the turbidity of a bacterial suspension to McFarland Standard No. 0.5 (approximately 1.5 x 10^8 CFU/mL). The bacterial suspension was serially diluted (dilution factor 10) in PBS three times to adjust the bacterial suspension to 1 – 5 x 10^5 CFU/mL. The concentration of bacteria in every batch of bacterial suspension was confirmed using five 10-fold serial dilutions and plating on blood agar in duplicate. The dilution that had 30-300 bacterial colonies per plate was used for counting.

For each bacterial isolate, triplicates of 1 mL of bacterial suspension in PBS were mixed with either 4 mM or 8 mM of H_2O_2 at a 1:1 vol/vol ratio, resulting in a final H_2O_2 concentration of 2 mM or 4 mM by volume. For the internal controls, 1 mL of bacterial suspension was mixed with 1 mL of PBS, also in triplicate. The mixtures were incubated at 37°C for 60 min and then transferred into an ice bath to stop bacterial growth. The colony count was determined using five 10-fold serial dilutions and plating on blood agar in duplicate. The dilution that had 30-300 bacterial colonies per plate was used for counting. This experiment was repeated on three separate occasions.

6.2.4 Phagocytic assay

This study was performed using California sea lion, NZ fur seal and dog blood. Due to the limited blood volume, the California sea lion and NZ fur seal blood were only used with the following isolates: non-HV_Kp1 and HV_Kp2 plus a control, *E. coli*. Canine blood was used with HV_Kp3 and non-HV_Kp7 in addition to the same isolates as used in California sea lion and fur seal blood.

To label bacteria with fluorescein isothiocyanate (FITC), bacteria from an overnight culture were washed with 40 mL of PBS and centrifuged at 3000 g for 15 min

three times, before incubation at 70°C for 60 min to kill the bacteria. The bacteria were then mixed with 0.1 mg/mL FITC in 0.10 M $NaHCO_3$ pH 9.0, and incubated for 20 min at room temperature in the dark. The labelled bacteria were washed in PBS three times to remove unbound FITC, and adjusted to a final concentration of 2 x 10^8 CFU/mL with PBS by adjusting the turbidity to McFarland Standard No. 1. FITC labelled bacteria were kept at -20°C until use.

The phagocytosis assay was performed using a flow cytometer (BD FACS Calibur, Becton, Dickinson and Company, USA). Whole blood (50 µL) and 25 µL of FITC labelled bacterial suspension were mixed in fluorescent-activated cell sorting (FACS) tubes (Becton, Dickenson and Company, USA) and incubated for 10, 30, 60 and 120 min at 37°C, at which point the samples were put into an ice bath to stop cell phagocytosis. 2 mL of FACS Lysing Solution (Becton, Dickinson and Company, USA) was added to the samples followed by incubation at room temperature for 15 min to lyse the red blood cells, then samples were centrifuged at 450 g for 15 min. The supernatant was removed and the cells fixed in 400 µL of 2% paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA), followed by mixing with 50 µL of trypan blue (0.4% in PBS (Sigma–Aldrich, St. Louis, MO, USA)). For each isolate, controls were prepared as follows: free non-quenched bacteria, free bacteria quenched with 50 µL trypan blue 0.4% in PBS, blood only, blood with bacteria on ice, and free quenched bacteria mixed with blood just prior to sample acquisition. Phagocytic activity was analysed using CellQuest software (Becton, Dickenson and Company, USA). Blood neutrophils and monocytes were gated by forward and side scatter characteristics (Fig. 4.1). For each sample, the target cells were acquired at 10,000 gated events if possible. To exclude neutrophils or monocytes that had bacteria bound externally, the lower threshold for positive-staining cells was

determined using a negative control consisting of fixed blood cells mixed with fixed, trypan blue-quenched bacteria.

Background phagocytosis levels were determined by incubating blood samples with each of the bacterial isolates on ice for 10 min, followed by lysis of the red blood cells as described above. Neutrophils and monocytes with fluorescence levels above the background phagocytosis tube were considered to be positive for phagocytosis.

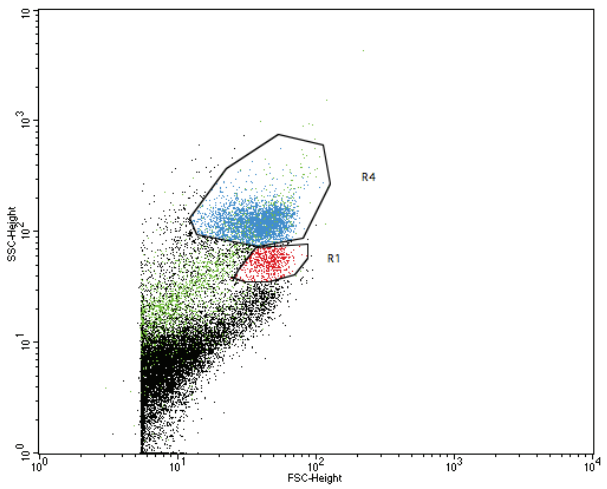


Figure 6.1 Dot plot with gating of leukocytes
Dot plot with gating of leukocytes, data derived from canine peripheral blood leucocytes using BD FACSCalibur and CellQuest software. Blood neutrophils and monocytes were gated on by forward and side scatter characteristics. The scatter defines two distinct populations. The region R4 has been drawn for neutrophils (blue). The region R1 has been drawn for monocytes (red).

6.2.5 Serum-mediated killing assay

The serum-mediated killing assay using canine serum was performed with eight bacterial isolates (Table 4.1), plus *E. coli* as a control. Due to the limited sample volume, serum mediated killing assays using California sea lion serum were performed with four bacterial isolates: non-HV_Kp1, HV_Kp2, HV_Kp3 and non-HV_Kp7, plus *E. coli* as a control.

The serum resistance assay was performed according to Cox et al. (2015) with modifications. Bacteria (1 – 5 x 10^4 CFU/mL) in PBS were prepared from overnight cultures, as described below. The number of bacteria was measured by adjusting the turbidity of a bacterial suspension to McFarland Standard No. 0.5 (1.5 x 10^8 CFU/mL). The bacterial suspension was serially diluted (dilution factor 10) in PBS four times to adjust the bacterial suspension to 1 – 5 x 10^4 CFU/mL. The concentration of bacteria in every batch of bacterial suspension was confirmed using five 10-fold serial dilutions and plating on blood agar in duplicate. The dilution that had 30-300 bacterial colonies per plate was used for counting.

Each selected bacterial suspension was mixed at a 1:1 (vol/vol) ratio with serum from healthy California sea lions or dogs. For canine serum, 1 mL of pooled serum was mixed with 1 mL of bacterial suspension in a sterile 5 mL glass bottle using aseptic technique, followed by incubation at 37°C. For an internal control, 1 mL of bacterial suspension was mixed with 1 mL of PBS, and then incubated at 37°C. For each bacterial isolate, three aliquots of each pooled serum sample (triplicate) and three internal controls were used. For California sea lions, due to limitations on serum volume, 50 µL of sea lion serum was mixed with 50 µL of bacterial suspension in a 96-well polystyrene sterile plate (Corning® CellBIND®, MA, USA) in triplicate.

At 0, 60 and 120 min, the samples were transferred into an ice bath to stop bacterial growth. A colony count was performed using five 10-fold serial dilutions and plating on blood agar in duplicate.

The experiment for each species was repeated on three separate occasions.

6.2.6 Statistical analysis

Data from the serum-mediated killing, oxidative-mediated killing and phagocytic assays were expressed as a percentage of mean ± standard deviation (SD) by adjusting the number using controls suspended in PBS as 100% bacterial survival, and evaluated using one-way ANOVA followed by the Bonferroni-type multiple t-tests using Prism 5 (version 5.0a) (GraphPad Software Inc., USA). All tests used a significance level of $p < 0.05$. Due to inadequate sample numbers, the phagocytic assay using California sea lion blood was not used in the statistical analysis.

6.3 Results

6.3.1 Oxidative killing assay

When incubated with 2 mM H_2O_2 for 60 min, the percentage survivals of HV_Kp2, HV_Kp3, HV_Kp4, HV_Kp5, non-HV_Kp6, and HV_Kp8, were higher than non-HV_Kp1 and non_HV_Kp7 ($p = 0.06$) (Table 6.2 and Fig. 2.2). With 4 mM H_20_2, the percentage survival of HV isolates were higher than non-HV isolates. However, only the percent survival of HV_Kp4 was significantly higher than the non-HV_Kp1 ($p = 0.0175$) (Fig. 6.2).

Table 6.2 Mean ± SD percentage survival of oxidative killing assay using H2O2: 2 mM, 4 mM.

Isolate	Mean percentage survival	
	2 mM	4 mM
E. coli	40.98 ± 10.54%	2.71 ± 1.86%
Non-HV_Kp1	59.38 ± 8.38%	16.03 ± 7.81%*
Non-HV_Kp7	59.87 ± 7.54%	30.16 ± 3.06%
HV_Kp2	75.21 ± 4.93%	55.33 ± 11.68%
HV_Kp3	62.20 ± 11.93%	35.54 ± 6.51%
HV_Kp4	97.70 ± 13.42%	61.63 ± 32.50%*
HV_Kp5	73.81 ± 7.93%	32.95 ± 6.65%
Non-HV_Kp6	90.87 ± 25.71%	29.95 ± 9.17%
HV_Kp8	72.85 ± 23.30%	32.31 ± 6.65%

* indicates significant difference within the same concentration of H_2O_2 (p = 0.0175) using one-way ANOVA followed by Bonferroni-type multiple t-tests

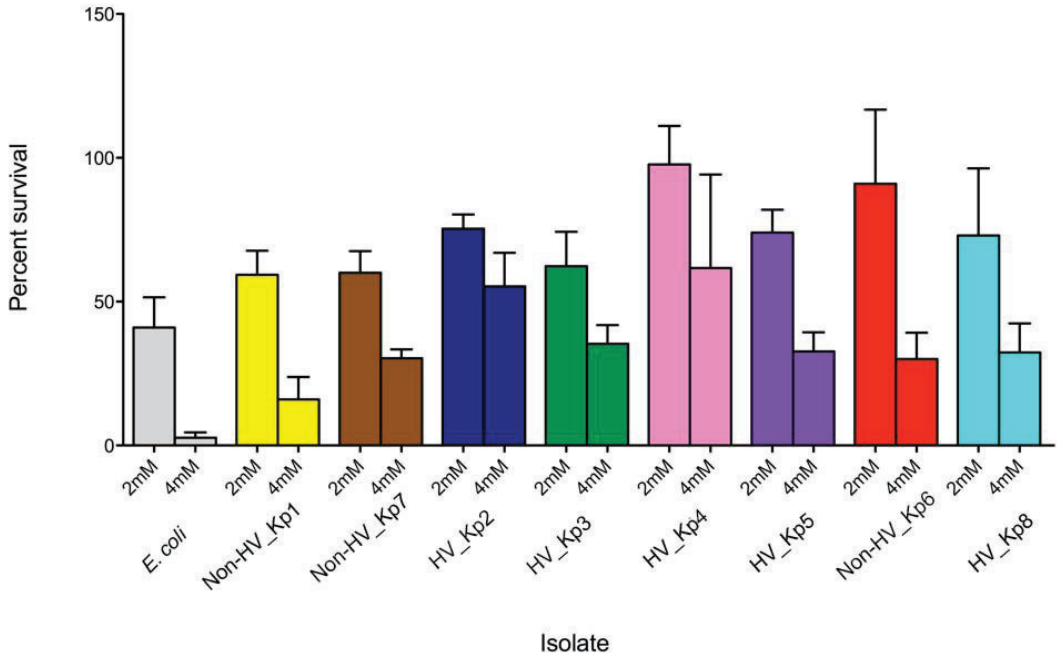


Figure 6.2 Percentage survival of bacteria in the oxidative-mediated killing assay

Mean ± SD percentage survival of bacteria in the oxidative-mediated killing assay using 2 and 4mM H_2O_2. At concentrations of 2 and 4mM H_2O_2, the mean percentage survival of non-HV_Kp1 and non_HV_Kp7 isolates was lower than all the HV *K. pneumoniaeand* and non-HV-Kp_6 isolates, but differences were not significant at 2mM (p = 0.06). At 4mM, the percentage survival of HV_Kp4 was significantly higher than non-HV_Kp1 (p = 0.0175). Percentage survival was calculated using controls suspended in PBS as 100% bacterial survival.

6.3.2 Phagocytic assay

6.3.2.1 California sea lion

The proportion of neutrophils from two California sea lions that actively phagocytosed of non-HV_Kp1 was higher than that of HV_Kp2 at 10, 30, 60 and 120 min. The mean phagocytosis of *E. coli* increased at each time point of 10, 30, 60 and 120 min. The mean phagocytosis of non-HV_Kp1 increased at 10 and 30 but not at 60 and 120 min (Fig. 6.3, Table 6.3).

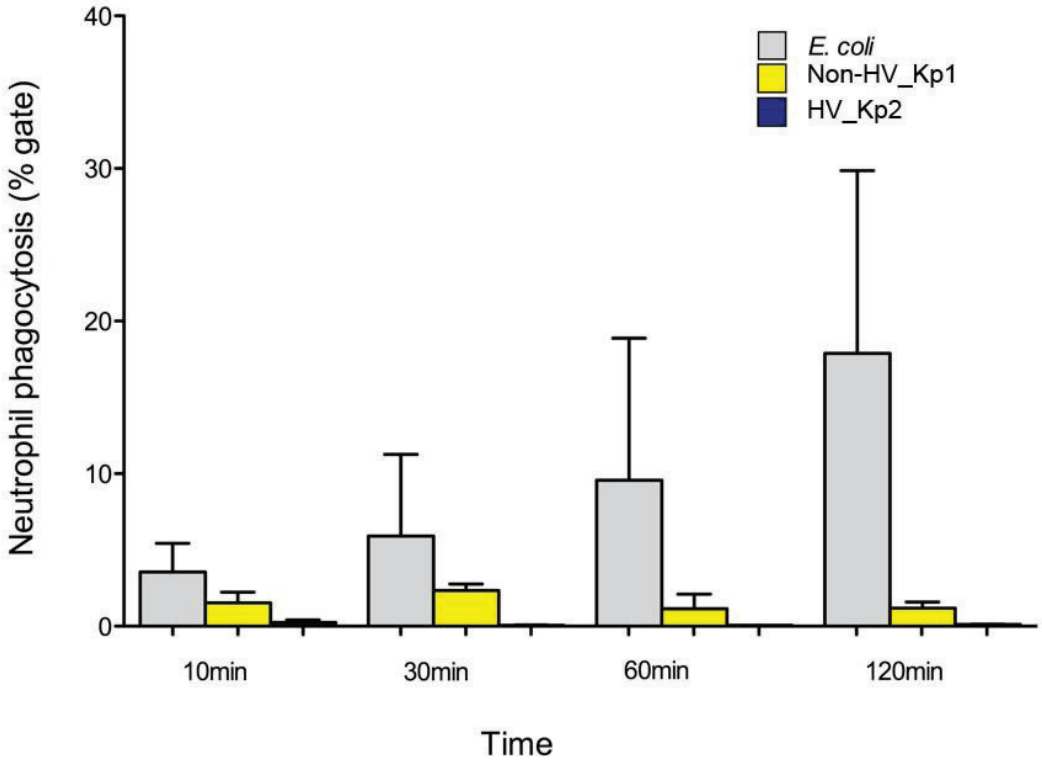


Figure 6.3 Phagocytosis graph of *E. coli*, non-HV_Kp1 and HV_Kp2 by neutrophils from California sea lions

Data are shown as mean +/- SD phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by neutrophils from two California sea lions at 10, 30, 60 and 120 min. At every time point, phagocytosis by neutrophils of non-HV_Kp1 was higher than HV_Kp2.

The mean phagocytosis of non-HV_Kp1 by monocytes from two California sea lions was higher than HV_Kp2 at each time point of 10, 30, 60 and 120 min. The mean phagocytosis of non-HV_Kp1 and HV_Kp2 increased at every time point. The phagocytosis of *E. coli* increased with every time point with the exception of 120 min (Fig 6.4, Table 6.3).

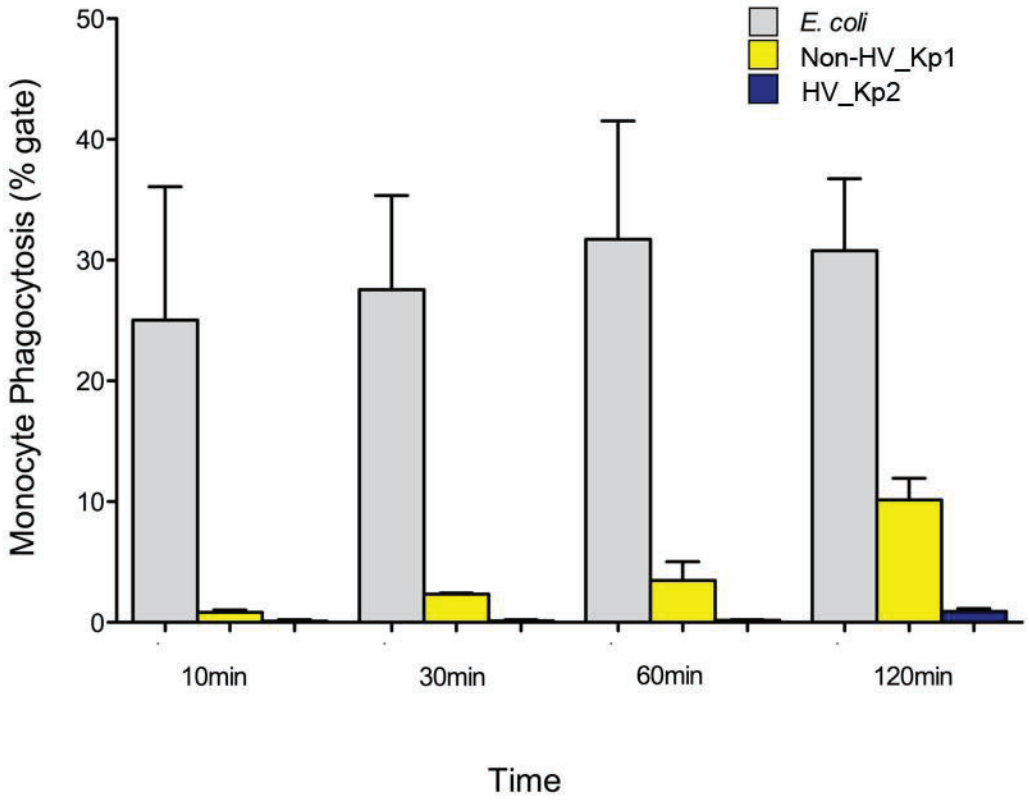


Figure 6.4 Phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by monocytes from California sea lions
Data are shown as mean +/- SD phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by monocytes from two California sea lions at 10, 30, 60, and 120 min. At every time point, phagocytosis by monocytes of non-HV_Kp1 was higher than HV_Kp2.

6.3.2.2 NZ fur seal

The mean phagocytosis of *E. coli* and non-HV_Kp1 by neutrophils from three NZ fur seals was higher than HV_Kp2 at 10, 30, 60, and 120 min. There was no significant difference between non-HV_Kp1 and HV_Kp2 at each time point (10 min: $p = 0.13$, 30 min: $p = 0.12$, 60 min: $p = 0.15$, 120 min: $p = 0.15$) (Fig 6.5, Table 6.3). However, fur seal neutrophils were consistently able to phagocytose non-HV_Kp1 more efficiently than HV_Kp2.

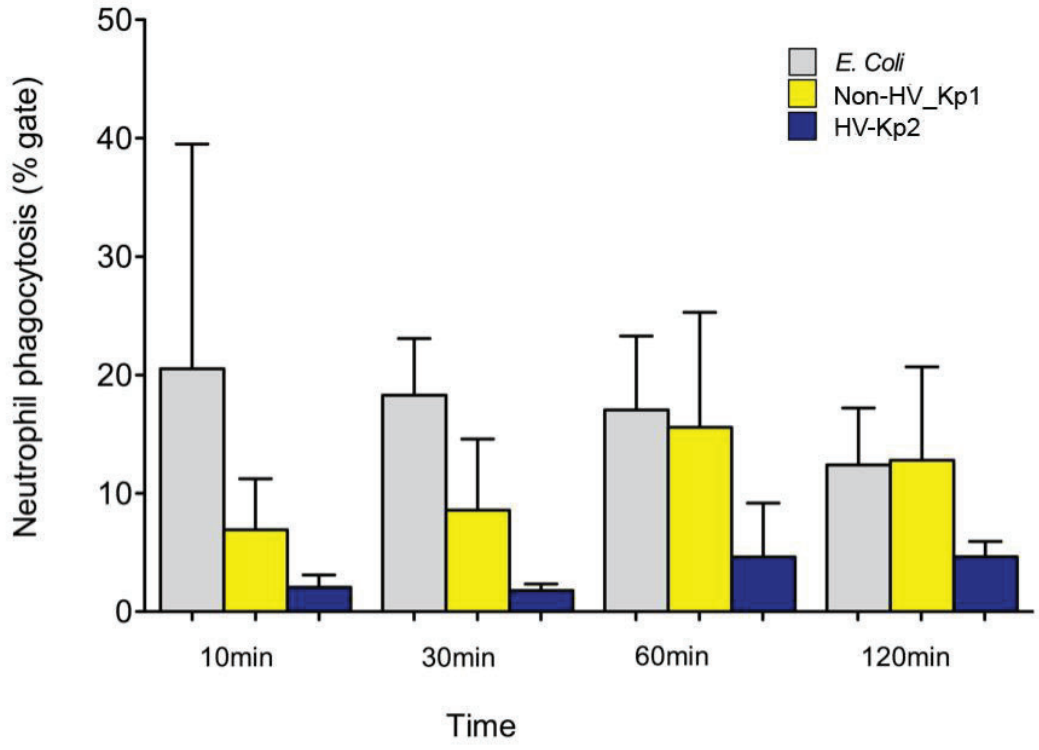


Figure 6.5 Phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by neutrophils from NZ fur seal
Data are shown as mean +/- SD phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by neutrophils from three NZ fur seal at 10, 30, 60 and 120 min. At every time point, phagocytosis by neutrophils of *E. coli* and non-HV_Kp1 was higher than HV_Kp2. There was no significant difference among the *K. pneumoniae* isolates at each time point (10 min: $p = 0.13$, 30 min: $p = 0.12$, 60 min: $p = 0.15$, 120min: $p = 0.15$)

The mean phagocytosis of *E. coli* and non-HV_Kp1 by monocytes from three NZ fur seals was significantly higher than HV_Kp2 at 30, 60 and 120 min, but not at 10 min (10 min: p = 0.11, 30 min p = 0.004, 60 min: p = 0.01, 120 min: p = 0.0003). The phagocytosis of non-HV_Kp1 increased at every time point (Fig 6.6, Table 6.3).

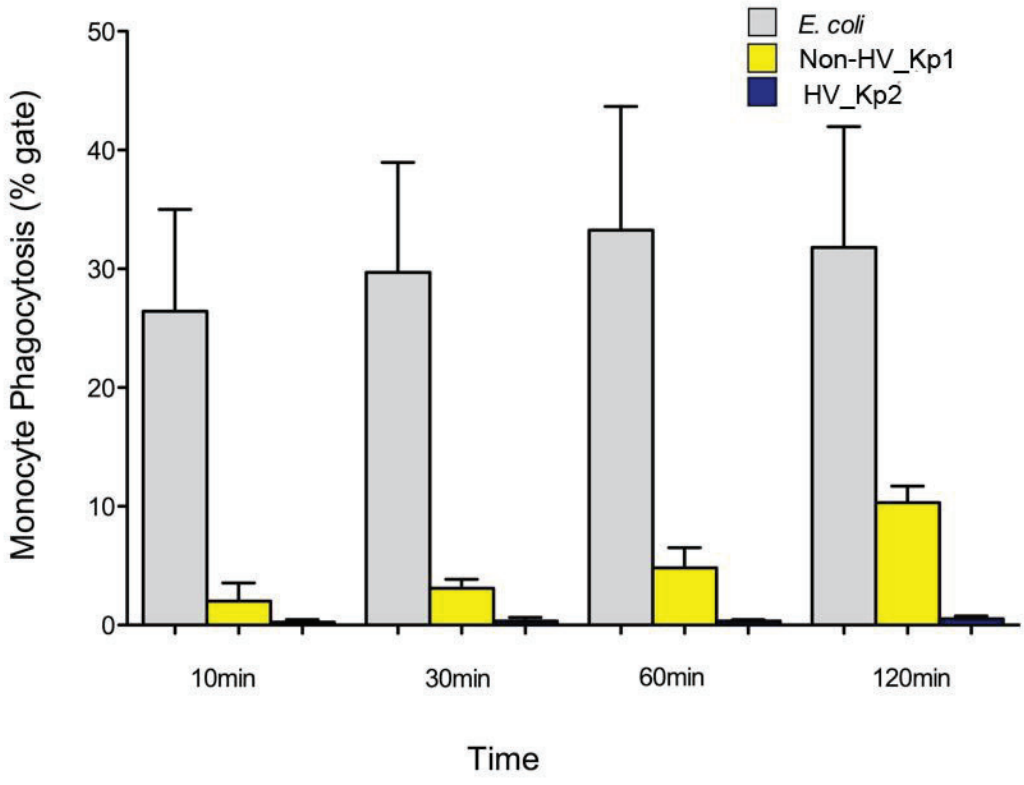


Figure 6.6 Phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by monocytes from NZ fur seals
Data are shown as mean +/- SD phagocytosis of *E. coli*, non-HV_Kp1 and HV_Kp2 by monocytes from three NZ fur seals at 10, 30, 60 and 120 min. At every time point, phagocytosis by monocytes of non-HV_Kp1 was significantly higher than HV_Kp2 at 30, 60 and 120 min, but not at 10 min (10 min: p = 0.11, 30 min: p = 0.004, 60 min: p = 0.01, 120 min: p = 0.0003).

6.3.2.3 Canine

The mean phagocytosis by neutrophils from three dogs of *E. coli*, non-HV_Kp1 and non-HV_Kp7 was higher than HV_Kp2 and HV_Kp3 at 10, 30 and 60, but not at 120 min. However, there was no significant difference among the *K. pneumoniae* isolates at each time point (10 min: p = 0.447, 30 min: p = 0.535, 60 min: p = 0.920, and 120: p = 0.671 min) (Fig 6.7, Table 6.3).

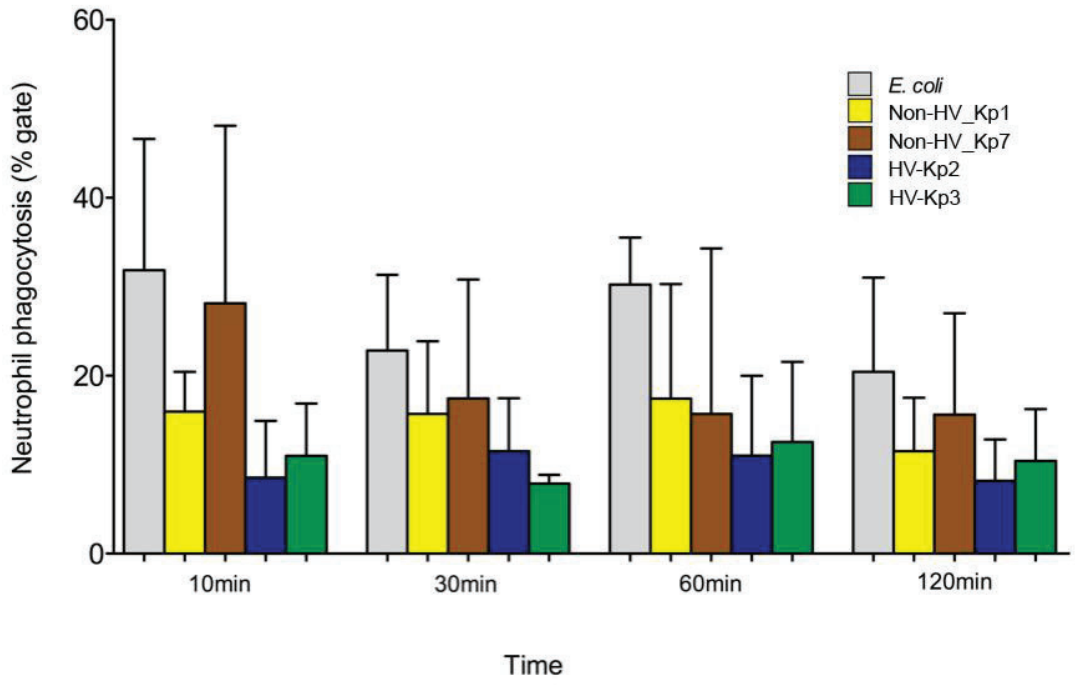


Figure 6.7 Phagocytosis of *E. coli*, non-HV_Kp1, non-HV_Kp7, HV_Kp2 and HV_Kp3 by neutrophils from dogs

Mean phagocytosis of non-HV_Kp1, non-HV_nonKp7, HV_Kp2 and HV_Kp3 by neutrophils from three dogs. Mean phagocytosis by neutrophils of non-HV *K. pneumoniae* isolates were higher than HV *K. pneumoniae* isolates at every time point except at 120 min. There was no significant difference among the *K. pneumoniae* isolates at each time point (10 min: p = 0.447, 30 min: p = 0.535, 60 min: p = 0.920, and 120 min: p = 0.671).

The mean phagocytosis of non-HV_Kp1 and non-HV_Kp7 by monocytes from three dogs was higher than phagocytosis of HV_Kp2 and HV_Kp3 at 10 and 30 min. There was no significant difference among the *K. pneumoniae* isolates at each time point (10 min: $p = 0.08$, 30 min: $p = 0.71$, 60 min: $p = 0.57$, 120 min: $p = 0.84$) (Fig 6.8, Table 6.3).

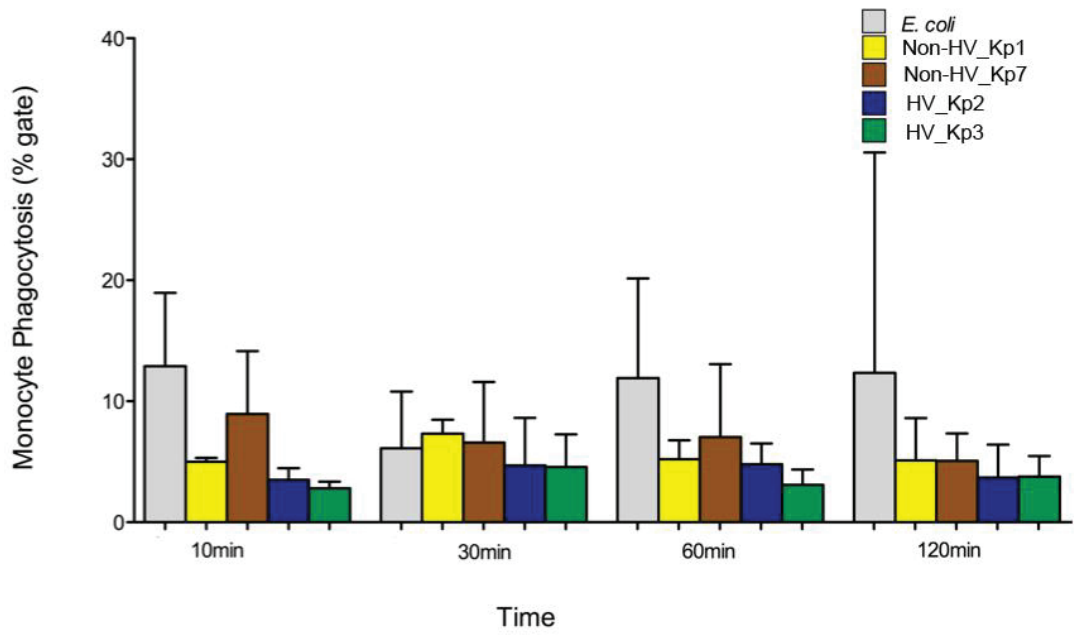


Figure 6.8 Phagocytosis graph of *E. coli*, non-HV_Kp1, non-HV_Kp7, HV_Kp2 and HV_Kp3 by monocytes from dogs

Mean phagocytosis of non-HV_Kp1, non-HV_Kp7, HV_Kp2 and HV_Kp3 by monocytes from three dogs. Mean phagocytosis by monocytes of non-HV *K. pneumoniae* isolates were higher than HV *K. pneumoniae* isolates at 10 and 30 min. There was no significant difference among the *K. pneumoniae* isolates at each time point (10 min: $p = 0.08$, 30 min: $p = 0.71$, 60 min: $p = 0.57$, 120 min: $p = 0.84$).

Table 6.3 Mean ± SD percentage phagocytosis (% gate) by neutrophils and monocytes using canine, California sea lion and fur seal blood. The same letter indicates a significant difference within the same incubation time.

Isolate	Mean ± SD percent phagocytosis (% gate)					
	10 min					
	Canine		NZ fur seal		Cal. sea lion	
	Neutrophil	Monocyte	Neutrophil	Monocyte	Neutrophil	Monocyte
E. coli	31.85 ± 14.77	12.9 ± 6.05	20.53 ± 18.98	26.42 ± 8.58	3.55 ± 1.87	25.03 ± 7.79
Non-HV_Kp1	15.95 ± 4.48	4.99 ± 0.32	6.94 ± 4.29	2.01 ± 1.53	1.54 ± 0.70	0.85 ± 0.19
HV_Kp2	8.53 ± 6.38	3.51 ± 0.96	2.05 ± 1.04	0.24 ± 0.22	0.23 ± 0.17	0.12 ± 0.11
HV_Kp3	10.97 ± 5.89	2.79 ± 0.56				
Non-HV_Kp7	28.14 ± 19.96	8.94 ± 5.19				
	30 min					
	Canine		NZ fur seal		Cal. sea lion	
	Neutrophil	Mono.	Neutrophil	Monocyte	Neutrophil	Monocyte
E. coli	22.82 ± 8.51	6.01 ± 4.6	18.31 ± 4.78	29.69 ± 9.27	5.92 ± 5.34	27.54 ± 7.79
Non-HV_Kp1	15.70 ± 8.17	5.00 ± 2.65	8.58 ± 6.00	3.09 ± 0.75[a]	2.34 ± 0.42	2.33 ± 0.10
HV_Kp2	11.50 ± 5.97	4.70 ± 3.92	1.77 ± 0.56	0.34 ± 0.31[a]	0.065 ± 0.02	0.13 ± 0.11
HV_Kp3	7.88 ± 0.98	4.55 ± 2.70				
Non-HV_Kp7	17.43 ± 13.39	6.58 ± 5.01				
	60 min					
	Canine		NZ fur seal		Cal. sea lion	
	Neutrophil	Mono.	Neutrophil	Monocyte	Neutrophil	Monocyte
E. coli	30.25 ± 5.28	11.92 ± 8.23	17.07 ± 6.22	33.25 ± 10.42	9.75 ± 9.29	31.72 ± 9.81
Non-HV_Kp1	17.42 ± 12.86	4.86 ± 2.09	15.56 ± 9.73	4.83 ± 1.69[b]	1.45 ± 0.95	3.47 ± 1.54
HV_Kp2	11.01 ± 8.98	5.12 ± 1.62	4.61 ± 4.58	0.33 ± 0.13[b]	0.07 ± 0.01	0.19 ± 0.05
HV_Kp3	12.55 ± 9.01	3.09 ± 1.25				
Non-HV_Kp7	15.70 ± 18.61	7.03 ± 6.04				
	120 min					
	Canine		NZ fur seal		Cal. sea lion	
	Neutrophil	Monocyte	Neutrophil	Monocyte	Neutrophil	Monocyte
E. coli	20.41 ± 10.58	12.34 ± 18.20	12.41 ± 4.82	31.80 ± 10.16	17.88 ± 11.97	30.87 ± 5.96
Non-HV_Kp1	10.16 ± 5.09	5.10 ± 3.49	12.78 ± 7.92	10.30 ± 1.39[c]	1.18 ± 0.41	10.51 ± 1.78
HV_Kp2	8.18 ± 4.66	6.03 ± 6.73	4.64 ± 1.27	0.91 ± 0.22c[c]	0.12 ± 0.01	0.35 ± 0.24
HV_Kp3	15.99 ± 10.43	8.09 ± 8.02				
Non-HV_Kp7	15.63 ± 11.39	5.07 ± 2.26				

When the different animal species are compared, the mean percentage phagocytosis by pinniped monocytes was higher than pinniped neutrophils, while the mean phagocytosis by canine neutrophils was higher than canine monocytes. The mean

phagocytosis by neutrophils of NZ fur seals was generally higher than California sea lions.

6.3.3 Serum-mediated killing assay

6.3.3.1 California sea lion

After 60 min incubation, only non-HV_Kp1 had decreased percentage survival when compared with the internal control. In contrast, other isolates from NZ sea lions including non-HV_Kp7, HV_Kp2, and HV_Kp3 had increased percentage survival when compared with their internal controls (Fig. 6.9, Table 6.4). There were significant differences between the percent survival of non-HV_Kp1 with non-HV_Kp7, HV_Kp2, and HV_Kp3 isolates at the 60 min time point (p=0.001). Similarly, after 120 min incubation, only non-HV_Kp1 had decreased percentage survival while other isolates had increased percentage survival when compared with their internal control. There was a significant difference between the percentage survival of non-HV_Kp1 with HV_Kp2 and HV_Kp3 at 120 min (p = 0.0021). When the percentage survival of non-HV_Kp1 was compared using time as a factor, after 120 min of incubation, the percentage survival was significantly less than with 60 min incubation (p = 0.0078). At 120 min, the percentage survival of non_HV_kp7 and HV_Kp3 were significantly greater than with 60 min incubation (p = 0.02, p = 0.035 respectively)

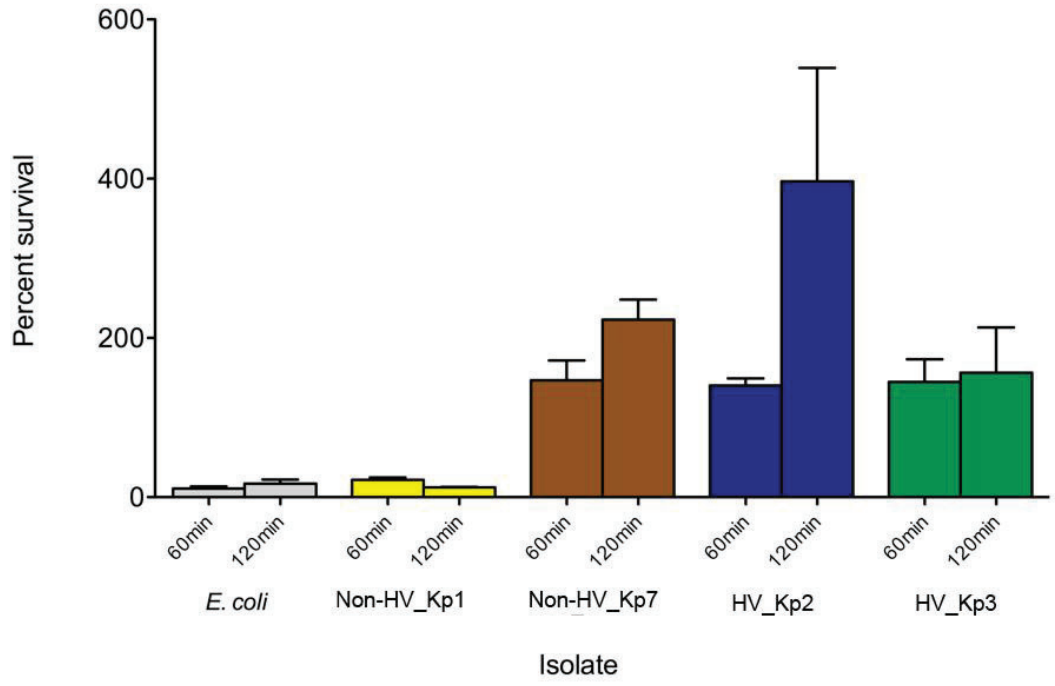


Figure 6.9 Percent survival of bacteria in serum resistance assay from California sea lion serum
Percent survival of bacteria in serum resistance assay from pooled California sea lion serum. At 60 and 120 min, non-HV_Kp1 showed decreased percentage survival while other isolates had increased percentage survival. At 60 min percentage survival of non-HV_Kp1 was significantly lower than non-HV_Kp7, HV_Kp2 and HV_Kp3 (p=0.0001). At 120 min, percentage survival of non-HV_Kp1 was significantly lower than HV_Kp2 (p=0.0021).

6.3.3.2 Dog serum

After 60 and 120 min incubation with dog serum, only non-HV_Kp1 had decreased percentage survival (Fig. 6.10, Table 6.4) when compared with the internal control. In contrast, other isolates from NZ sea lions including HV_Kp2, HV_Kp3, HV_Kp4, HV_Kp5, HV_non-Kp6 and HV_Kp7 had increased percentage survival when compared with their internal controls (Fig. 6.10). There were significant differences between percentage survival of non-HV_Kp1 with every other isolate at 60 min and 120 min ($p < 0.0001$ and $p < 0.0001$, respectively). When the percentage survival of non-HV_Kp1 was compared using time as a factor, the percentage survival at 120 min incubation was significantly lower ($p < 0.0001$) than at 60 min incubation. For other

isolates, after 120 min incubation, the percentage survival rates were significantly greater ($p < 0.0001$) than with 60 min incubation with dog serum.

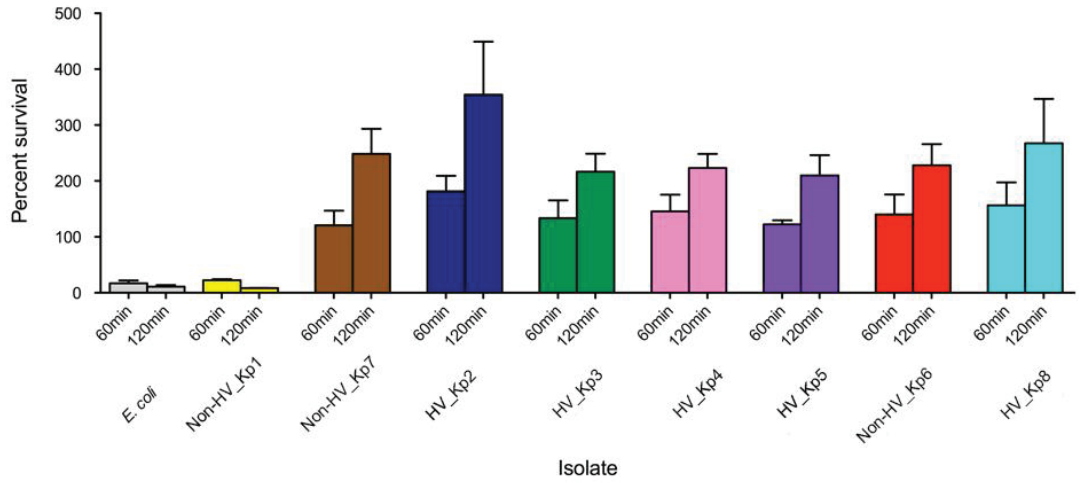


Figure 6.10 Percent survival of bacteria in serum resistance assay from dog serum
Percent survival of bacteria in serum-mediated killing assay from pooled dog serum. At 60 and 120 min, non-HV_Kp1 decreased percentage survival while other isolates increased the percentage survival. At 60 and 120 min, the percentage survival of non-HV_Kp1 was significantly lower than that of the other isolates ($p < 0.0001$, $p < 0.0001$ respectively).

Table 6.4 Mean percent survival ± SD of serum-mediated killing assay using canine and California sea lion serum. The same letter indicates significant difference within the same incubation time.

Isolate	Percent survival (canine serum)		Percent survival (sea lion serum)	
	60 min	120 min	60 min	120 min
E. coli	17.00 ± 5.19%	10.67 ± 2.88%	11.07 ± 2.89%	17.29 ± 5.19%
Non-HV_Kp1	22.33 ± 2.08%[a,b,c,d,e,f,g]	8.33 ± 0.58%[h,I,j,k,l,m,n]	21.64 ± 3.21%	12.28 ± 0.58%
Non-HV_Kp7	120.67 ± 26.10%[a]	248.33 ± 44.99%[h]	146.78 ± 24.91%	223.06 ± 25.23%
HV_Kp2	181.66 ± 27.53%[b]	354.33 ± 94.97%[i]	140.31 ± 8.96%	369.42 ± 142.48%
HV_Kp3	133.67 ± 31.79%[c]	216.67 ± 32.15%[j]	144.15 ± 28.54%	156.14 ± 56.89%
HV_Kp4	145.67 ± 29.93%[d]	223.33 ± 25.17%[k]	-	-
HV_Kp5	122.33 ± 7.50%[e]	210.00 ± 36.05%[l]	-	-
Non-HV_Kp6	140.33 ± 35.38%[f]	228.00 ± 38.03%[m]	-	-
HV_Kp8	156.33 ± 41.23%[g]	267.33 ± 79.11%[n]	-	-

6.4 Discussion

The results of this study show that HV *K. pneumoniae* isolates from NZSLs are more resistant to components of the innate immune system, including phagocytosis, serum-mediated and oxidative-mediated killing in pinnipeds and canines than non-HV *K. pneumoniae* and *E. coli* isolated from humans.

For extracellular bacteria, phagocytosis is an important part of the innate immune mechanism that clears bacteria from the body (Murphy and Weaver, 2016). In the study presented here, HV isolates tended to be more resistant to phagocytosis than non-HV isolates. A study using African green monkey blood revealed similar results, with HV isolates showing greater resistance to phagocytosis by monocytes than non-HV isolates (Cox et al., 2015). Greater resistance to phagocytosis by neutrophils of HV isolates than non-HV isolates was also observed in studies using mouse and human blood (Lee et al., 2014; Lin et al., 2004; Paczosa and Mecsas, 2016; Pomakova et al., 2012). Moreover, the results of the oxidative-mediated killing assay, the intracellular killing system in neutrophils, in the current study showed that HV isolates were more resistant to oxidative-mediated killing than some non-HV isolates. A similar result was found African green monkeys (Cox et al., 2015). All the data suggests that the HV phenotype assists the bacteria to evade phagocytosis and killing by the host immune system.

One explanation for this resistance to phagocytosis may be related to the fucose moities present in the capsule of HV strains (Wu et al., 2008). Fucose is a hexose deoxy sugar that can be found on the cell surface of mammals, plants and insects (Becker and Lowe, 2003), which the host immune system recognises as self and does not stimulate an immune response. The capsule of non-HV isolates contains a large amount of

mannose which can be recognised by macrophages (Pan et al., 2011; Wu et al., 2008), while HV isolates contain large amounts of fucose, which assists bacteria in avoiding recognition by the host immune system (Wu et al., 2008). The capsular fucose of HV isolates is converted from mannose using the *gmd* and *wcaG* genes, which can be found in most HV isolates (Wu et al., 2008). A mutation of *gmd* increased the attraction of bacteria to macrophage surfaces when injected intraperitoneally in a mouse model, indicating that the mutation of *gmd* gene increased the recognition of bacteria by macrophages (Pan et al., 2011).

When percent uptake between neutrophils and monocytes was compared among the three animal species used in the current study, differences were found. The percentage phagocytosis by monocytes was higher than neutrophils in California sea lions and NZ fur seals. In comparison, when using canine blood, the percentage of phagocytosis by monocytes was lower than neutrophils. While, due to limited sample numbers, we cannot draw definitive conclusions from these results, it appears that each host species might have a different immune response to this pathogen. A previous study showed that in order to clear *K. pneumoniae* from the body, the predominant type of phagocytic cells varies according to the strain of *K. pneumoniae* (Xiong et al., 2015).

In the serum mediated killing assay presented here, sera from dogs and California sea lions were not able to kill either HV or non-HV isolates from NZSLs. Moreover, all the isolates increased in number of bacteria over the time of the experiment when incubated with the serum. Similarly, in a study using human non-immunised serum incubated with *K. pneumoniae* isolated from hospitalised patients, the number of bacteria increased, whereas the number of bacteria decreased when

using serum from patients that had recurrent *K. pneumoniae* infections (Yeh et al., 2012). This suggests that *K. pneumoniae* was able to evade the humoral innate immunity in the blood, but were susceptible to specific antibodies. Studies using human and non-human primate serum revealed that HV strains were more resistant to serum-mediated killing than non-HV strains (Cox et al., 2015; Fang et al., 2004; Paczosa and Mecsas, 2016; Pomakova et al., 2012). This contrasted with the study presented here, as the non-HV isolated from an adult NZSL was also resistant to the serum killing assay at a similar degree to the other isolates.

Several studies show that the susceptibility of bacteria to serum killing is variable from strain to strain depending on the components of each strain, such as capsular serotype, LPS and outer membrane proteins (Cortés et al., 2002; Hsieh et al., 2013; Lee et al., 2014; Paczosa and Mecsas, 2016). One of the mechanisms in serum-mediated killing is the complement system. Several strains of *K. pneumoniae* have been reported to be able to avoid the complement-killing system by using their capsule, LPS and outer membrane proteins which vary in each strain (Doorduijn et al., 2016; Paczosa and Mecsas, 2016). *K. pneumoniae* are encapsulated with a thick acid polysaccharide capsule that contains repeat units of four to six sugars (Brisse et al., 2006). The capsule provides a barrier to the membrane attack complex (MAC), a product of the complement cascade that kills bacteria (Doorduijn et al., 2016). Moreover, in capsular serotype K2, which is found in the HV sea lion isolates in this current study (see Chapter 2), the sugar structure in their capsule lacks mannose, which is recognised by mannose-binding protein (Sahly et al., 2009). The lack of the mannose structure prevents complement activation via the lectin pathway (Doorduijn et al., 2016). Together with previous studies, the results of this current study suggest that the HMV phenotype may

not be the main factor in serum-mediated resistance, and other factors such as capsular type may be also be involved.

The results of this present study may partly explain why NZSL pups are more susceptible to HV *K. pneumoniae* than adults. In this study, an isolate from an apparently healthy sea lion (HV_Kp8, Table 4.1) was used and the immune response results were similar to the isolates from pups. The isolate from this adult sea lion showed the HMV phenotype, was of serotype K2 and was positive for the *rmpA* gene, similar to the isolates from dead pups, suggesting that healthy adult sea lions can carry HV *K. pneumoniae* strains that cause disease in pups. However, clinical disease due to HV *K. pneumoniae* has not been described in adult NZSLs (Lenting, 2017; Roe et al., 2015). This suggests a different immune response to *K. pneumoniae* between pups and adults.

Although the experiments in this study were limited in that NZSL blood samples were unavailable, the results using serum from other pinniped species and from dogs clearly show that HV isolates from NZSLs were more resistant to innate immune responses than some non-HV isolates in avoiding serum and oxidative-mediated killing as well as phagocytosis. Although the immune system in NZSL pups has never been fully studied, data from other pinnipeds shows that the immune system in pups is not fully developed in phagocytic function and immune cell proliferation (Frouin et al., 2010; Keogh et al., 2010). Therefore, maternal immunity is required to protect animals from infection. In mammals, maternal antibodies can be transferred via the placenta or via colostrum. NZSLs have an endotheliochorial type placenta (Carter and Enders, 2004), and about 5-10% of antibodies can be transferred via this route (Borghesi et al., 2014).

Most of the transfer of maternal antibodies to newborn carnivores, including pinnipeds, is therefore via colostrum (Burton, 1982).

Each breeding season, NZSL pups on Enderby Island begin to die from *K. pneumoniae* infection at approximately the end of January (Roe et al., 2015; see Chapter 5), when pups are around five to six weeks of age. It is possible that NZSL pups acquire antibodies from their mother, but by the age of five weeks, maternal antibodies may begin to drop and become inadequate to protect the pups from *K. pneumoniae,* while the maternal antibody concentrations are still too high to allow the pup's immune response to work (window of susceptibility) (Tizard, 2013). This hypothesis is not supported by a previous study, however, which found that anti-*Klebsiella* spp. antibodies were found in adult NZSL, but could not be detected in pups until seven weeks of age, indicating a failure of anti-*Klebsiella* spp. antibody transfer as well as a delay in development of an adaptive immune response to bacteria (Castinel et al., 2008). However, in the Castinel et al. (2008) study, only immunoglobulin G (IgG) was tested, and other classes of Ig such as IgM or IgA that might play an important role for clearing *K. pneumoniae* from the body were not measured. A study of acute respiratory distress syndrome (ARDS) in rats using *K. pneumoniae* showed that IgM-enriched polyclonal immunoglobulins reduced the number of bacteria in the blood by preventing translocation of bacteria from lung to blood (Lachmann et al., 2004).

A second potential reason that pups may die from *K. pneumoniae* at five to six weeks of age may be due to age-related changes in behaviour. At that age, pups start moving from sand on to grass, playing, and practicing swimming in the mud pools, which gives them a greater chance of becoming exposed to the pathogen in the environment (S. Michael, personal communication; see Chapter 4).

In order to control the disease, immunisation could be considered, and could be either passive or active. However, *K. pneumoniae* isolated from NZSL pups can avoid part of the innate immune reaction, which is a critical step in developing protection from infection using active immunisation (Murphy and Weaver, 2016), which suggests that active immunisation vaccination might not be the best option to protect NZSL pups against *K. pneumoniae*. Moreover, the presence of maternal antibody may interfere in the vaccination process. Providing passive immunity such as hyper-immune serum may be an alternative option. A study using monoclonal antibody derived from specific LPS types as a vaccine showed significant protection against *K. pneumoniae* ST258 when animals were dosed with monoclonal antibody intraperitoneally 24h before challenging in mouse and rabbit model (Babb and Pirofski, 2017; Szijártó et al., 2017). This suggests the theoretical possibility of passive immunisation to control and protect against this infection in NZSL pups. However, more studies of NZSL pup immunity, half-life of antibody, and response of this pathogen to antibody are required.

Further studies should be focused on assessing anti-*K. pneumoniae* antibodies in colostrum, serial detection of anti-*K. pneumoniae* antibody titres from sea lion pups by detecting all classes of immunoglobulin, and determining the type of immunoglobulin that is able to control HV *K. pneumoniae* infection. This will establish the presence/absence of anti-*K. pneumoniae* antibody in colostrum and pups, the ability of pups to absorb the antibody, and the half-life of maternal antibody which will help determine if immunising pups against *K. pneumoniae* is a valid option.

Summary

The study in this chapter aimed to investigate aspects of the innate immune system responses to *K. pneumoniae* isolated from NZSLs. The results of this study clearly show that HV *K. pneumoniae* isolates from NZSL pups were more resistant to selected components of the innate immune response of pinnipeds and dogs than the non-HV human isolate, including serum-mediated killing, neutrophil and monocyte phagocytosis, and oxidative-mediated killing. Even though the study was not conducted using NZSL blood samples, the findings suggest that susceptibility of NZSL pups to *K. pneumoniae* infection is at least partly due to the ability of *K. pneumoniae* to evade innate immune responses.

Chapter 7

General discussion

Fatal *K. pneumoniae* infection in NZSL pups is an ongoing problem since the outbreaks in 2001/02 and 2002/03. A large number of pups die from *K. pneumoniae* every year during the breeding season. As virulence and pathogenicity of *K. pneumoniae* vary depending on the isolate, in order to prevent or control this disease baseline information on the *K. pneumoniae* isolates causing infections in NZSL pup needs to be established. In this study, the author investigated phenotypic and genetic information, the geographic distribution, possible reservoirs and the immune response to the *K. pneumoniae* isolates causing infection in NZSL pups.

Characterisation of NZSL pup isolates

Most HV *K. pneumoniae* strains in humans are serotype K1 or K2 (Paczosa and Mecsas, 2016). The isolates that caused fatal infections in NZSL pups were hypermucoviscous phenotype, serotype K2, ST86, *rmpA*$^{+}$ which possess some virulence genes that have been reported from HV strains found in humans (Paczosa and Mecsas, 2016). They all were susceptible to common antibiotics used to treat gram-negative bacteria except ampicillin, which is generally found in *K. pneumoniae* as the bla_{shv} gene is in their chromosome. This finding is consistent with a previous study of *K. pneumoniae* in NZSL pups at Sandy Bay (Castinel et al., 2007b). This is also similar to other HV strains, in that most of them are sensitive to most antibiotics (Paczosa and Mecsas, 2016). However, ESBL-producing HV strains have been reported (Khaertynov et

al., 2017; Zhang et al., 2016), raising the possibility that the NZSL pup isolates might acquire drug resistance genes in the future. Notably, all *K. pneumoniae* in this study (including genomes retrieved from Genbank) possess antibiotic resistance genes other than the bla_{shv} gene including *FosA* and *oqxAB,* leading to low level resistance to fosfomycin and quinolones respectively. This is comparable with the findings of a study by Holt et al., (2015), showing that the *FosA* and *oqxAB* genes were in the core genome (Holt et al., 2015).

The isolates in this study were able to utilise a wide range of carbon and nitrogen sources similar to other *K. pneumoniae* studies (Blin et al., 2017; Henry et al., 2017). This is the first study to show activity of *K. pneumoniae* at different pHs, ranging from 4.5 to 10, which corresponds with their diverse habitat and the ability to cause infection in multiple organs. Between NZSL pup isolates, there was some variation in the type of carbon and nitrogen sources that each isolate could utilise. However, due to the small number of samples analysed, a conclusion as to if there was any evolution of the pathogen from the first outbreak until the present cannot be drawn from this study.

What is the origin of the *K. pneumoniae* strain causing fatal infection in NZSL pups?

From the pan-genome study using available published genome data from human ST86 isolates, the NZSL pup isolates grouped with each other, but separate from the human isolates. However, it cannot be concluded from this that the NZSL strain did not originate from humans, because using the pan-genome (core + accessory genomes), clustering is affected by gene loss or gene acquisition through horizontal gene transfer. If the NZSL pup strain came from a human source it may have acquired or lost genes in

response to the different environment. As far as the author is aware, there have been no reports of HV *K. pneumoniae* from humans in NZ. Australia is the nearest country that has evidence of HV *K. pneumoniae* in three human patients (Chang et al., 2013). On the other hand, there is a possibility that this HV strain could have emerged from a pre-existing classical *K. pneumoniae* strain by acquisition of virulence factors via horizontal gene transfer. If this is the case, there are two possibilities: this strain emerged from a non-HV human strain from the NZ mainland or somewhere else, or it emerged from a pre-existing strain that originated in the Auckland Islands. From the study of Castinel (2006) using PFGE, NZSL pup isolates were not genetically close to human isolates from Palmerston North hospital microbiology laboratory. However, it cannot be definitively concluded that the NZSL pup strains were not from NZ humans, since only three samples were used in that study and they were all from the same area. In order to clarify this, a more thorough study of NZ human isolates would have to be undertaken.

In the current study, non-HV *K. pneumoniae* was isolated from an adult sea lion and a bird (Chapter 2). By using rMLST clustering, they were grouped together and also grouped together with a non-HV isolate (non-HV_Kp6; C14/15_09Ph) from a pup that died from starvation. This pup isolate was positive on string test but negative for *rmpA*$^{+}$. Is it possible that the HV strain found in NZSL pups is a modified version of the non-HV strain that was isolated from a bird and a sea lion adult? This is unlikely since these are different ST types that are not closely related.

An unexpected finding in this study was that the non-HV (*rmpA*$^{-}$) isolates grouped together using rMLST clustering. There were three isolates in this group that came from different sources: a sea lion adult from Enderby island, a bird from Enderby Island and a sea lion pup from Campbell Island. This may suggest that *rmpA*$^{-}$ isolates are

clonal in sub-Antarctic areas. However, more samples are needed to confirm this hypothesis.

Geographic distribution of HV stain

In this study, the author was able to confirm that the isolates that cause fatal infections in NZSL pups on Enderby Island are the same strain that was isolated from a dead Otago pup (Roe et al., 2015). Moreover, the distribution of this strain is not limited to only two breeding sites, as the pathogen also has been isolated from Dundas and Campbell Islands. This suggests that close monitoring of pup mortality at other breeding sites needs to be conducted to determine whether deaths due to *K. pneumoniae* also occur at these sites.

The pathogen was also isolated from birds that live around the breeding sites, from soil and water at breeding sites, and from a non-clinical sea lion adult, suggesting that they have potential to be reservoirs. The environment could be a reservoir for a short time period only, as the pathogen was only found during the breeding season. To determine the long-term reservoir, further studies in birds and adult sea lions need to be undertaken. The pathogen is present at several breeding sites which are far from each other, and although Subantarctic skua can travel from the southern hemisphere to the northern hemisphere, they are more likely to stay near their colonies all year round (Higgins and Davies, 1996). In the non-breeding season, sea lion adults can travel far away from breeding sites to other Subantarctic islands and the NZ mainland (Geschke and Chilvers, 2010). There is a possibility that sea lion adults could spread the pathogen between locations.

Why are NZSL pups more susceptible to *K. pneumoniae*?

As far as the author is aware, fatal *K. pneumoniae* infection is found only in NZSL pups (Lenting, 2017; Roe et al., 2015). The *K. pneumoniae* that was isolated from a dead sea lion adult was not the cause of death in this animal, and was a different strain from the pup isolates (Chapter 5). There are several reasons why pups could be more sensitive to this pathogen: the pathogen itself, pup immunity and the burden of pathogen in the environment at the time that pups are infected.

In this study the isolates from NZSL pups, HMV phenotype, serotype K2, show resistance to some innate immune responses, which is similar to other HV *K. pneumoniae* that have the same structure (Paczosa and Mecsas, 2016). As the bacterial capsule assists *K. pneumoniae* in immune evasion, isolates with less capsule are more susceptible to complement-mediated and opsonophagocytic killing (Álvarez et al., 2000). The isolates from NZSL pups are HMV phenotype, which means that the amount of capsule is greater than classical *K. pneumoniae*. Thus, increased resistance to some innate immune responses was expected. In addition, the capsular serotype of the pup isolates is K2. Serotype K1/K2 are more resistant to immune responses than other serotypes (Paczosa and Mecsas, 2016). This is related to part of the surface of the K2 serotype capsule that presents sialic acid and mimics the sialic acid produced by the host, subsequently allowing the pathogen to evade immune detection. Moreover, unlike other serotypes, serotype K1/K2 lacks mannose, which makes them more resistant to macrophage uptake via mannose/lectin receptors (Álvarez et al., 2000).

The age that NZSL pups die of *K. pneumoniae* is approximately five to seven weeks (Roe et al., 2015). At that time, there may be an overlap between a sufficient number of pathogens in the environment that can infect the animals (discussed below)

and a period of low immune protection in the pups. Studies on other pinniped species show there are some fluctuations in immune protection depending on age of pups (Brock et al., 2013; Keogh et al., 2010). In Steller sea lions (*Eumetopias jubatus*), high concentrations of leukocytes were observed shortly after birth and decreased with age (Keogh et al., 2010). A decrease in total Ig with age is also observed in Galapagos sea lion pups (Brock et al., 2013). This is associated with decreasing maternal immunity. This time period is a window of susceptibility during which the protection of the passive immune response from the mother declines and the pup's own immune response is not fully functional. As the *K. pneumoniae* strain causing fatal infection in pups is resistant to some aspects of the innate immune response, together with low immune protection in pups and the burden of the pathogen in the environment, infection may occur without difficulty. As mentioned above, this pathogen can be isolated from rectal swabs from healthy NZSL adults (Argandoña, 2017), thus contamination of the environment can be expected. The results in Chapter 4 show that in the early breeding season (December to January) when adult sea lions have only just arrived, the pathogen was not isolated from the environment, suggesting that it may take time for a sufficient number of bacteria to build up in the environment to cause infection.

Study limitations and further studies

One limitation of this study was the small number and the distribution of samples that were tested. This might not reflect the whole picture of this pathogen. More samples from each breeding site should be added in the study of phenotype and genotype. Another limitation is that NZSLs live in remote areas, and it is therefore impossible to do some immune test studies such as phagocytic assays that require fresh

blood (24h collected). Instead, other species that were available nearby and were relatively closely related to NZSLs such as California sea lions and dogs had to be used.

As *K. pneumoniae* is able to acquire antimicrobial resistance genes, the study of antimicrobial susceptibility in the present isolates from NZSL pup should be updated annually.

Further research could include collection of oral or rectal swabs from live sea lion pups from one week old until the end of March (end of the field season) for whole genome sequencing, to compare with isolates from dead pups.

Whole genome sequence plus the phenotype microarray on isolates from humans in NZ in different area should also be conducted. This will give us a picture of phenotypes and genotypes of the *K. pneumoniae* that circulate in NZ. It would be interesting to see the genome comparison among non-HV isolates (*rmpA*$^{-}$) collected from different sources including substrate samples, sea lion pups, sea lion adults as well as animals that live around the breeding sites.

As NZSL pups live in an open environment, it is impossible to eliminate the pathogen from the environment, therefore to control infection increasing host immunity is an option. As the isolates from NZSL pups had similar genetic characteristics, it may be possible to develop a vaccine that could provide protection from this infection. Passive immunisation is recommended in this case since active immunisation might be disrupted by maternal antibody. Notably, the epitope that is selected to develop the vaccine would need to address the concern about cross reactions to microflora in pups that might occur as *K. pneumoniae* share some structures with other bacteria in the same family. Thorough studies of NZSL pup

immunity are also important, as well as the response of this pathogen to adaptive immune responses, the antibody half-life and the side effect of the antibody.

Summary

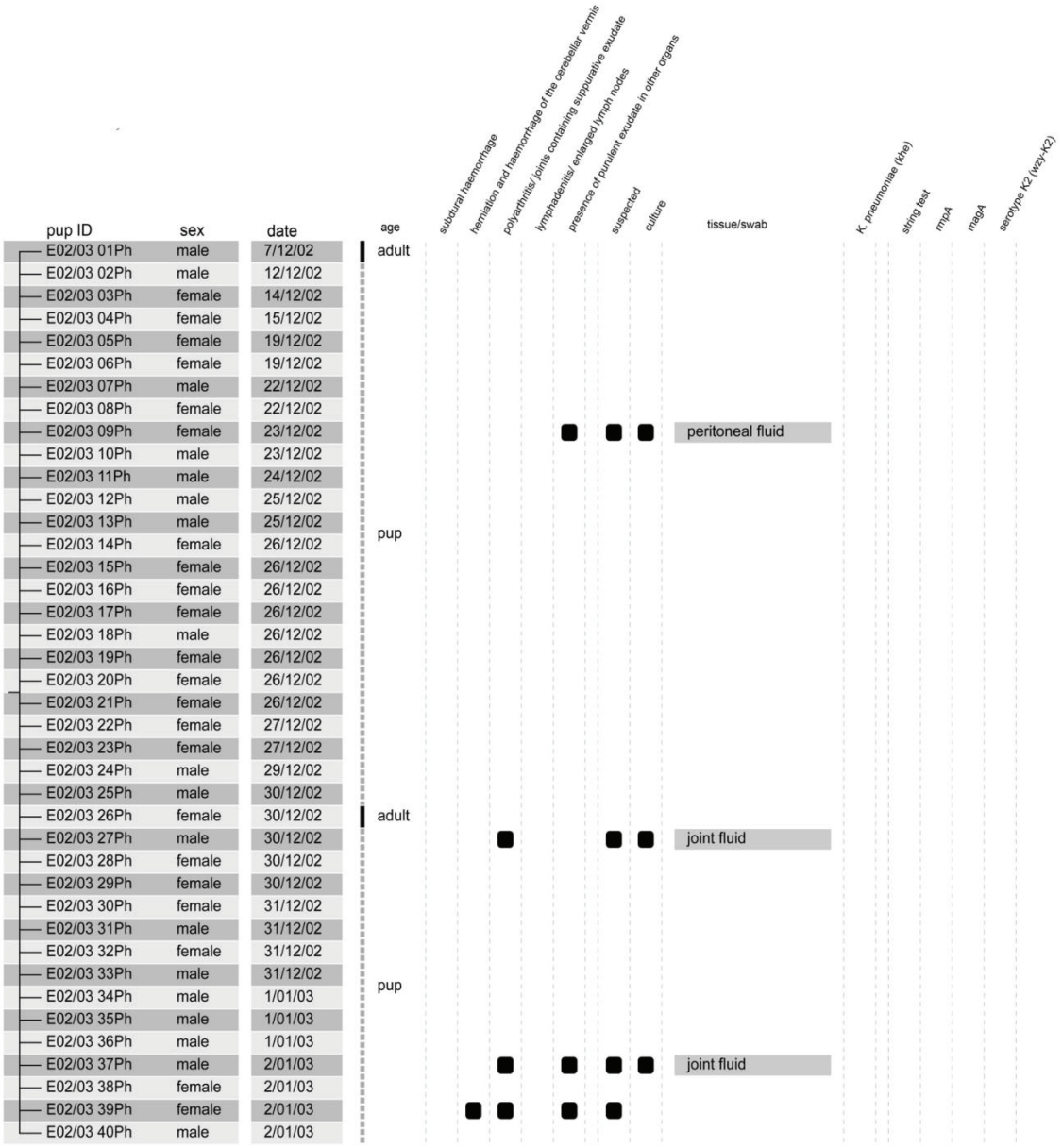
In this study, the author characterised the phenotype and genotype of *K. pneumoniae* isolated from NZSL pups from Enderby Island and compared them with other *K. pneumoniae* isolates (both HV and non-HV isolates), and studied some parts of innate immune response to this pathogen. The results provide baseline data to develop the prevention and the control of this infection, and identify areas within the scope of microbiology and immunology that require further research.

Appendices

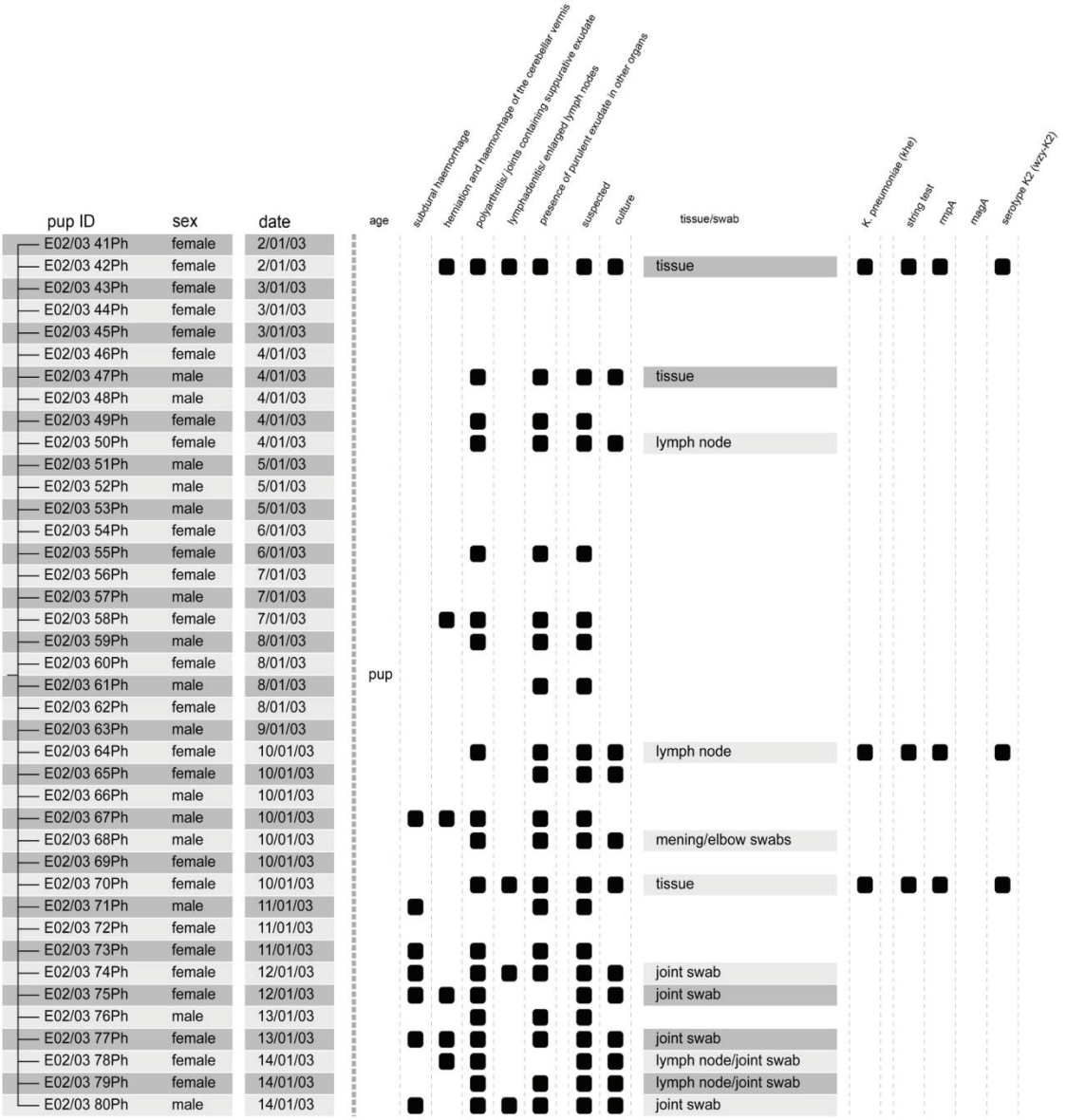
Supplementary material to Chapter 3

A3.1 Case selection and culture of *K. pneumoniae* from archived samples 2002/03 breeding season (1)

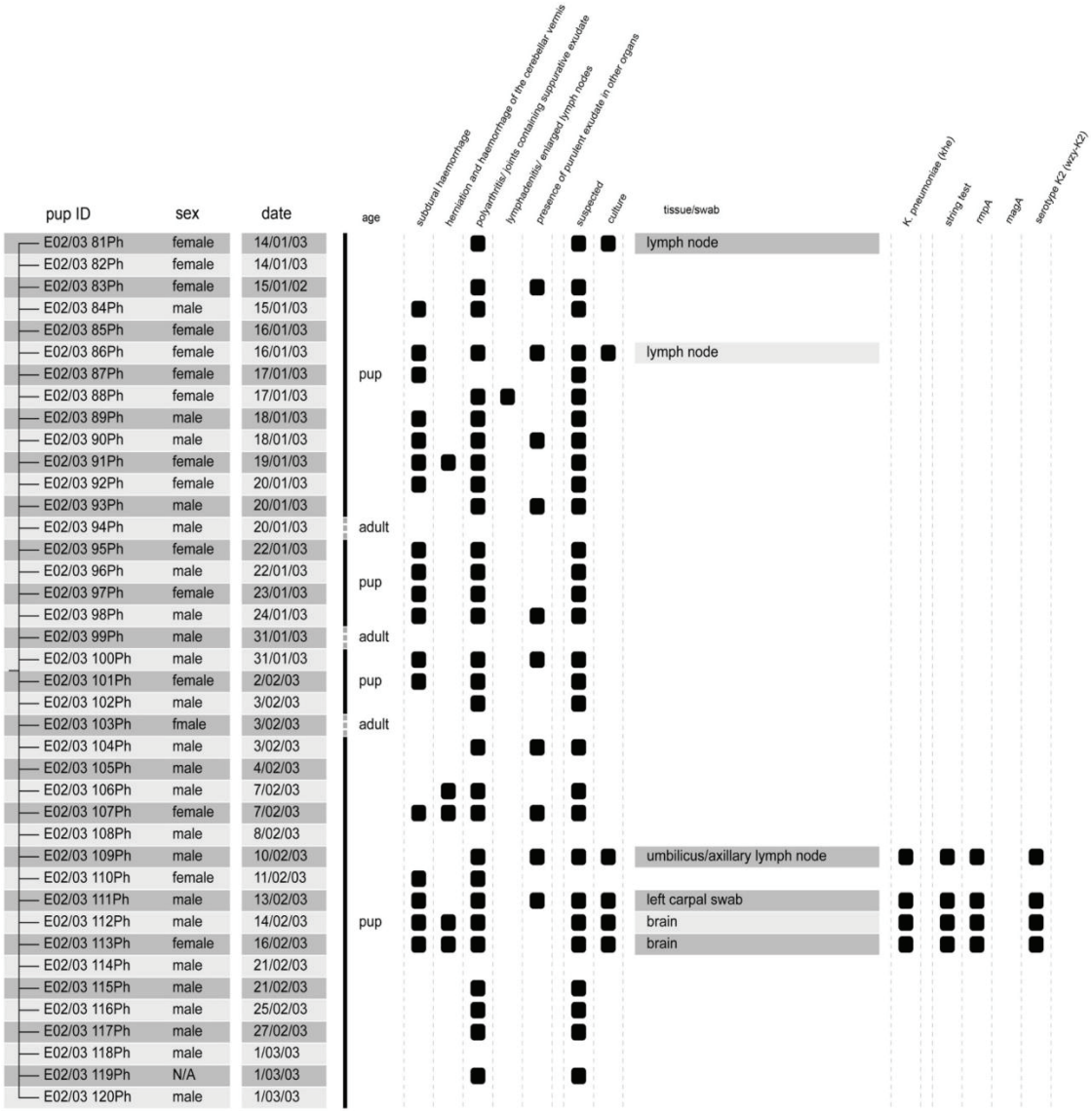
A total of 115 were necropsied and total of 55 NZSL pups were suspected to have died of *K. pneumoniae* infection. A total of 21 cases had remaining tissue available to investigate with bacterial culture. *K. pneumoniae* was isolated from seven of these NSZL pups.



A3.1 Case selection and culture of *K. pneumoniae* from archived samples 2002/03 breeding season (2)

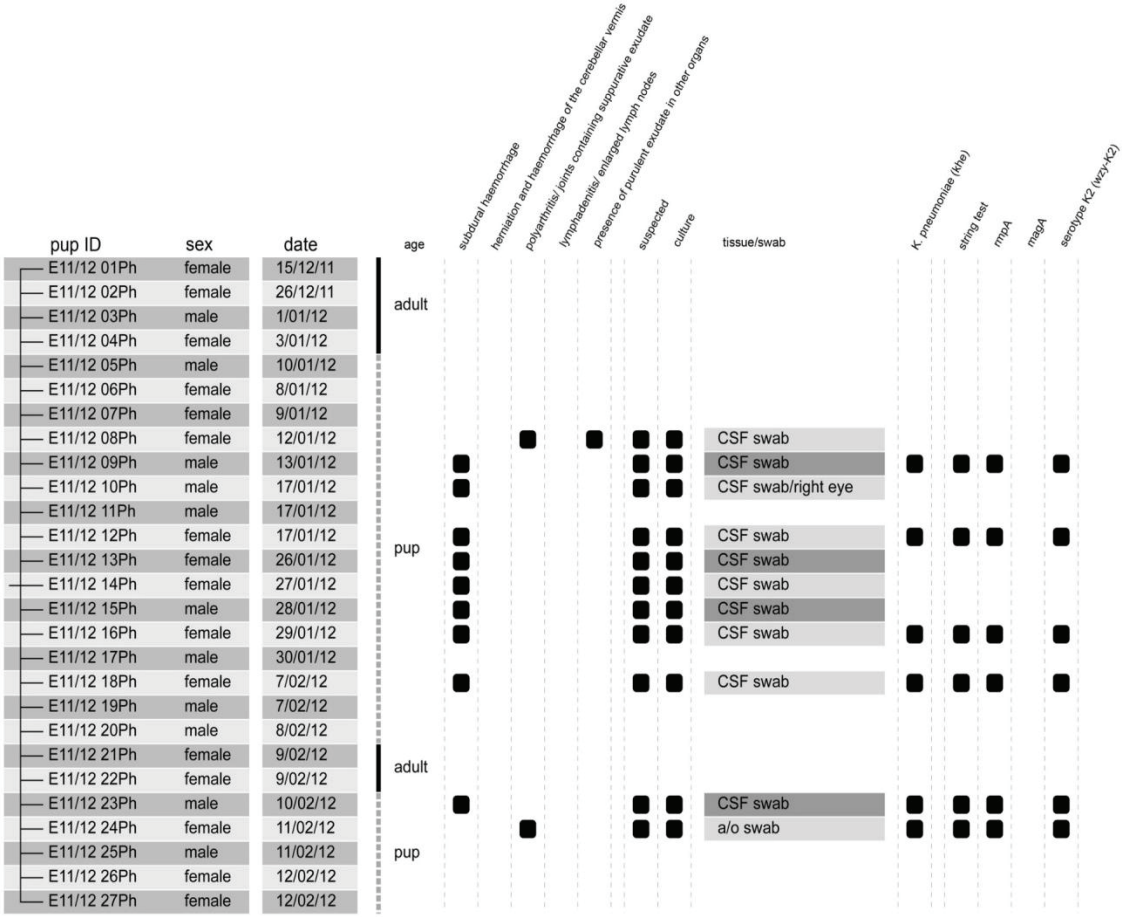


A3.1 Case selection and culture of *K. pneumoniae* from archived samples 2002/03 breeding season (3)



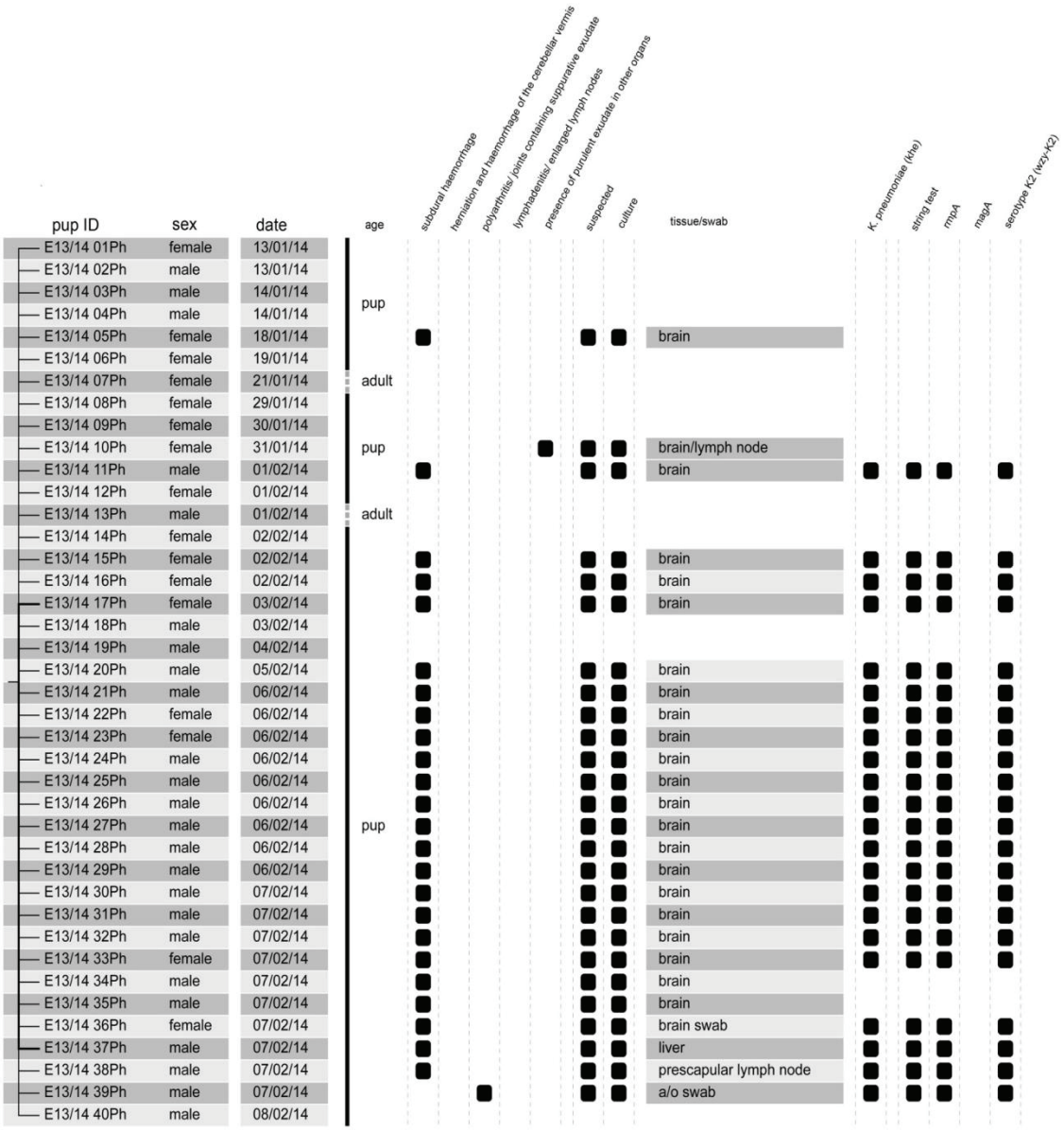
A3.2 Case selection and culture of *K. pneumoniae* from archived samples 2011/12 breeding season

A total of 21 NZSL pups from 2011/2012 were necropsied. A total of 11 cases were further investigated with bacterial culture. *K. pneumoniae* were isolated from tissues of six of these NSZL pups.

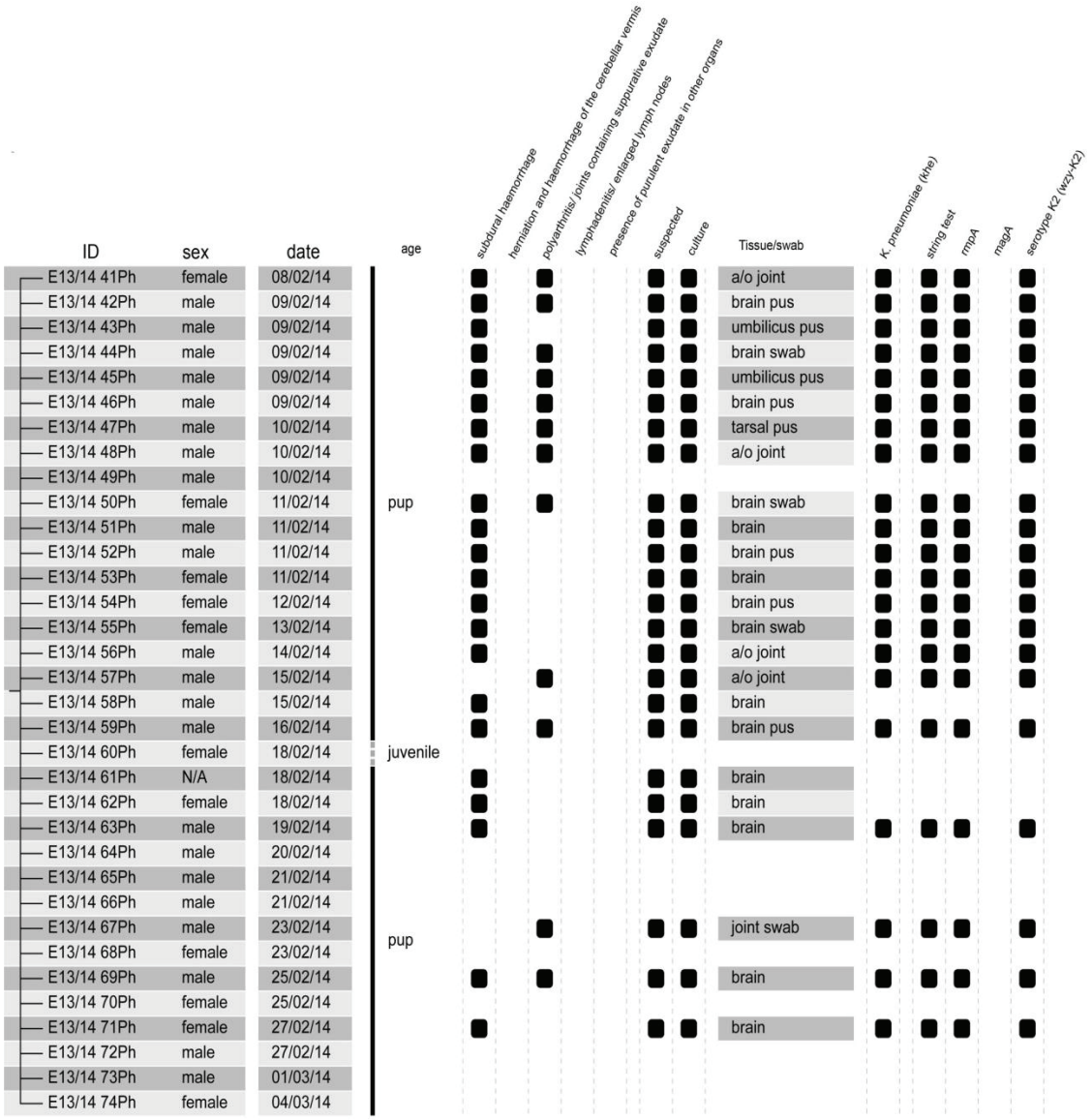


A3.3 Case selection and culture of *K. pneumoniae* from archived samples 2013/14 breeding season (1)

A total of 71 Enderby Island NZSL pups from 2013/2014 were necropsied. A total of 51 cases were further investigated with bacterial culture. *K. pneumoniae* were isolated from tissues of 47 of these NSZL pups.



A3.3 Case selection and culture of *K. pneumoniae* from archived samples 2013/14 breeding season (2)



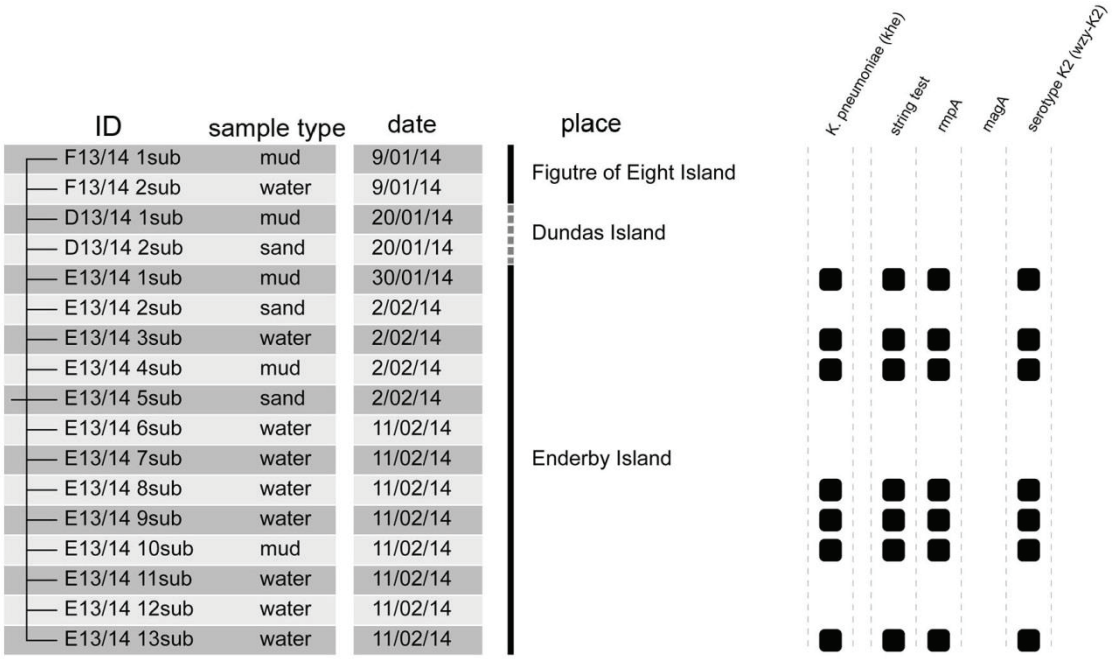
A3.4 **Zone diameter Breakpoints for antimicrobial sensitivity test (CLSI, 2015)**

Antimicrobial agent	Disk content	Interpretive categories and zone diameter breakpoint (mm)		
		S	I	R
Ampicillin	10µg	≥17	14–16	≤13
Amoxicillin-clavulanate	20/10µg	≥18	14–17	≤13
ceftriaxone	30 µg	≥23	20–22	≤19
ciprofloxacin	5 µg	≥ 31	21–30	≤ 20
cefuroxime sodium	30µg	≥18	15–17	≤ 14
gentamicin	10 µg	≥15	13–14	≤12
sulfamethoxazole/trimethoprim	23.75/1.25µg	≥16	11–15	≤10

S=Susceptable, I=Intermediate, R=Resistant

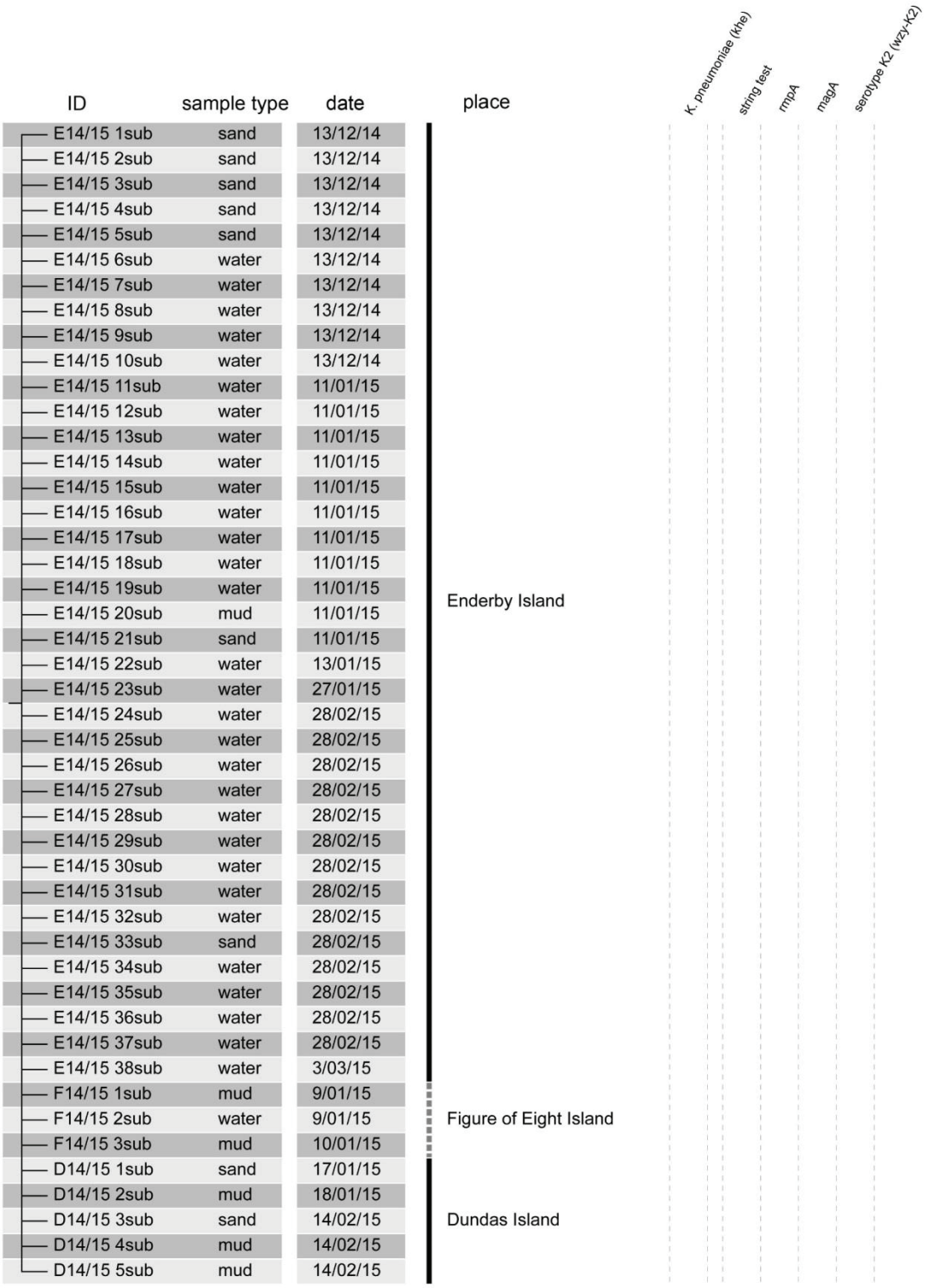
A4.1 Substrate samples culture results: Auckland Islands 2013/2014 breeding season

A total of 13 samples were collected, 7 samples were positive *K. pneumoniae*. All the positive samples were serotype K2, string test positive, *rmpA* positive and *magA* negative.



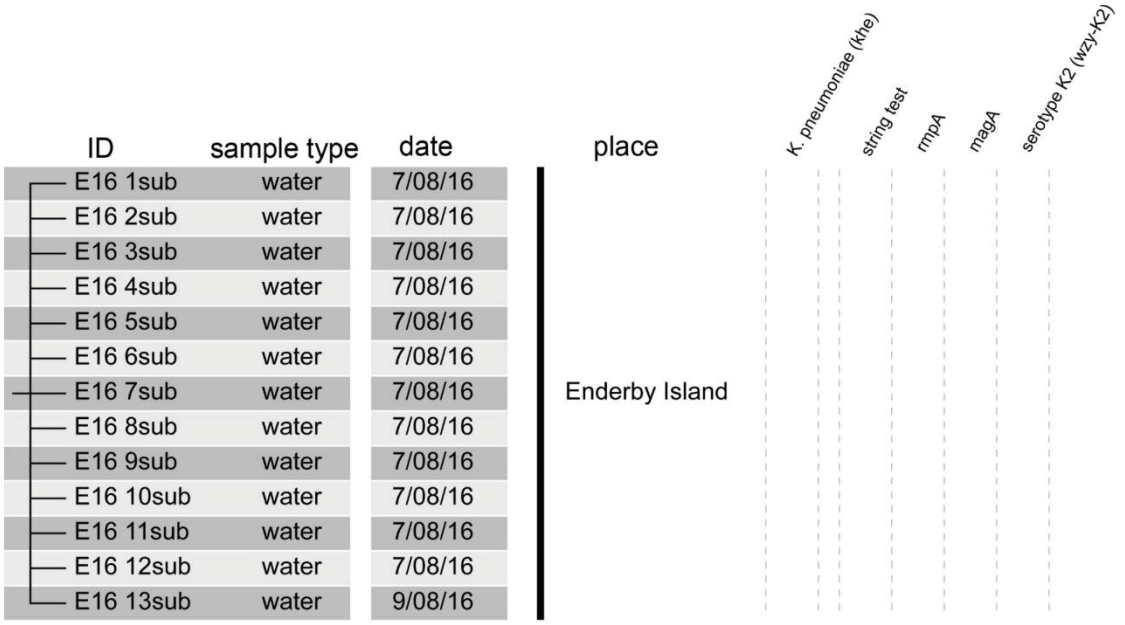
A4.1 Substrate samples culture results: Auckland Islands 2014/2015 breeding season

A total of 46 samples were collected during 2014/2015 breeding season were negative for *K. pneumoniae.*



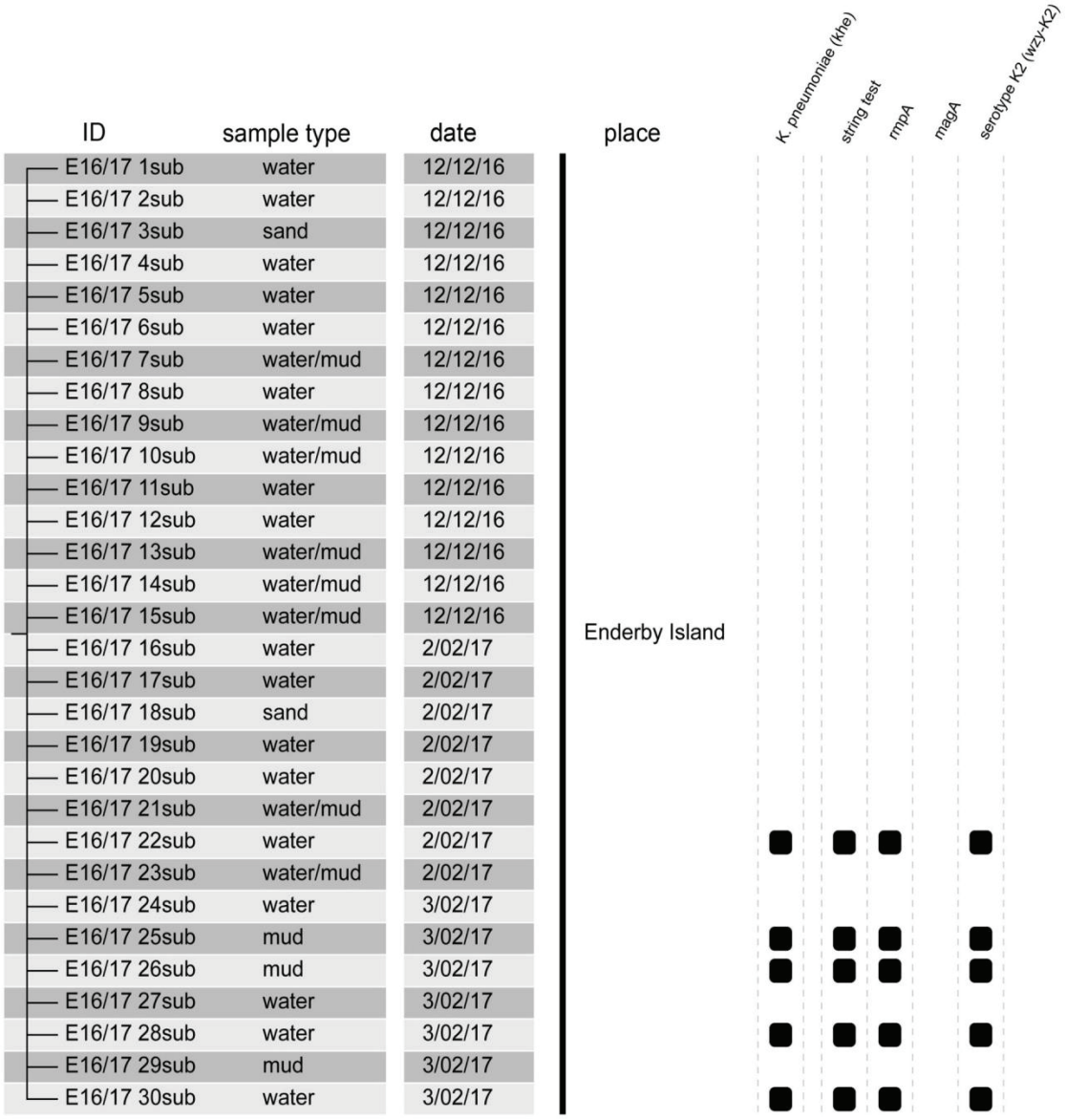
A4.1 Substrate samples culture results: Auckland Islands 2016 winter season

A total of 13 samples were collected during August were negative for *K. pneumoniae.*



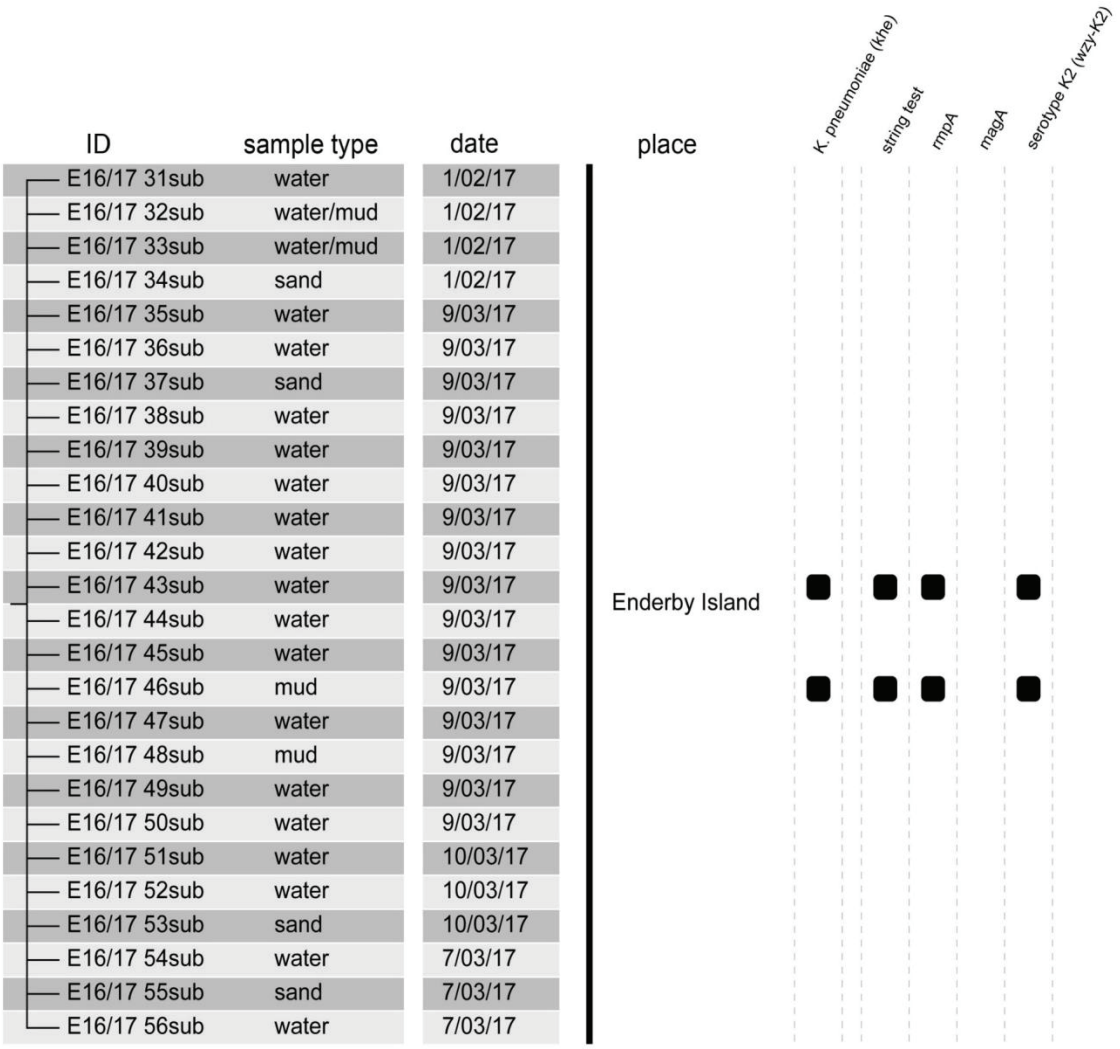
A4.1 Substrate samples culture results: Auckland Islands 2016/17 breeding season (1)

A total of 56 samples were collected, 7 samples were positive *K. pneumoniae*. All the positive samples were serotype K2, string test positive, *rmpA* positive and *magA* negative.



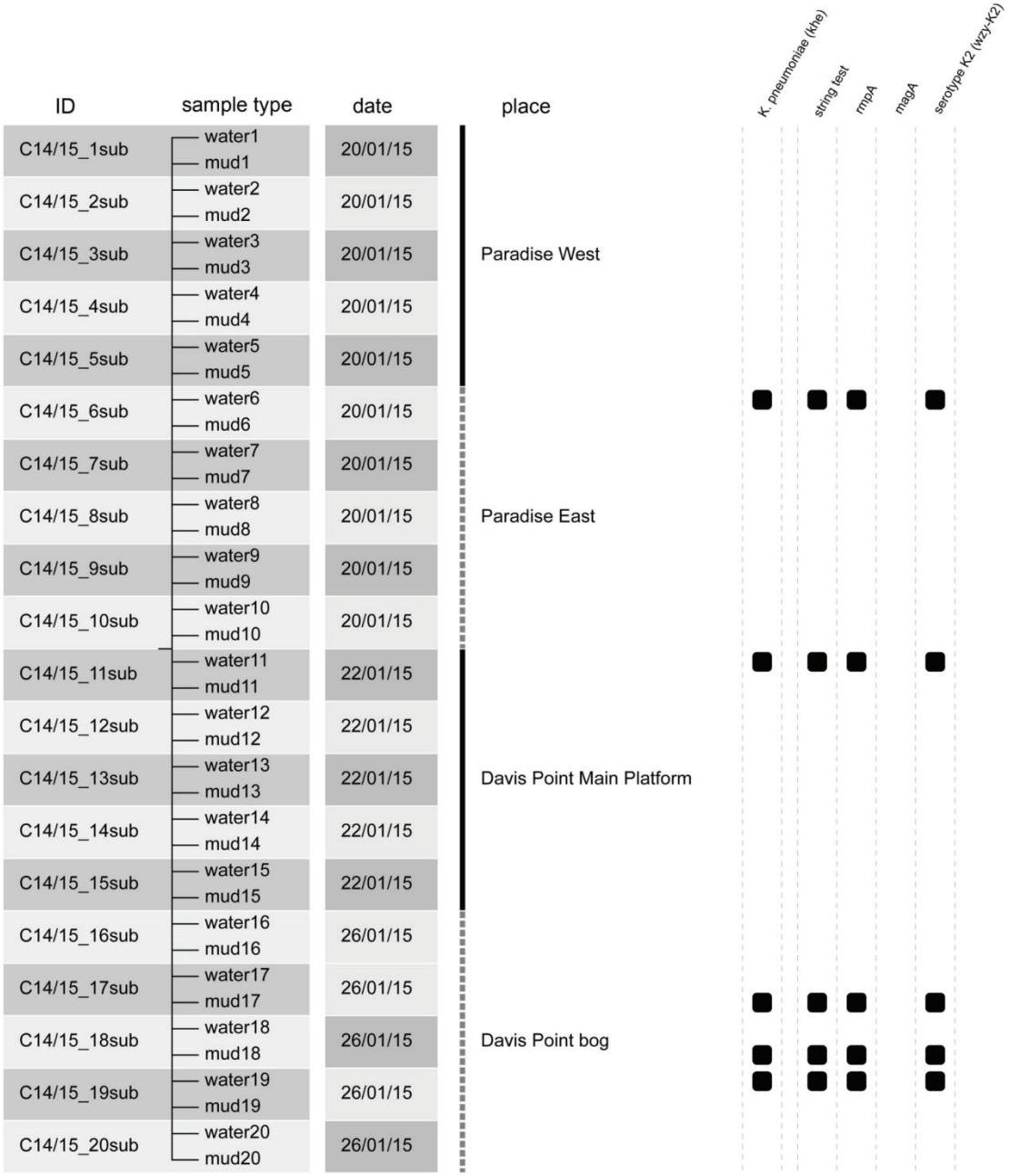
ID	sample type	date	place
E16/17 1sub	water	12/12/16	
E16/17 2sub	water	12/12/16	
E16/17 3sub	sand	12/12/16	
E16/17 4sub	water	12/12/16	
E16/17 5sub	water	12/12/16	
E16/17 6sub	water	12/12/16	
E16/17 7sub	water/mud	12/12/16	
E16/17 8sub	water	12/12/16	
E16/17 9sub	water/mud	12/12/16	
E16/17 10sub	water/mud	12/12/16	
E16/17 11sub	water	12/12/16	
E16/17 12sub	water	12/12/16	
E16/17 13sub	water/mud	12/12/16	
E16/17 14sub	water/mud	12/12/16	
E16/17 15sub	water/mud	12/12/16	
E16/17 16sub	water	2/02/17	Enderby Island
E16/17 17sub	water	2/02/17	
E16/17 18sub	sand	2/02/17	
E16/17 19sub	water	2/02/17	
E16/17 20sub	water	2/02/17	
E16/17 21sub	water/mud	2/02/17	
E16/17 22sub	water	2/02/17	
E16/17 23sub	water/mud	2/02/17	
E16/17 24sub	water	3/02/17	
E16/17 25sub	mud	3/02/17	
E16/17 26sub	mud	3/02/17	
E16/17 27sub	water	3/02/17	
E16/17 28sub	water	3/02/17	
E16/17 29sub	mud	3/02/17	
E16/17 30sub	water	3/02/17	

A4.1 Substrate samples culture results: Auckland Islands 2016/17 breeding season (2)



A4.2 Substrate samples culture results: Campbell Island 2014/15 breeding season

A total of 40 samples were collected, 5 samples were positive *K. pneumoniae*. All the positive samples were serotype K2, string test positive, *rmpA* positive and *magA* negative.



A5.1 Reference genome data retrieved from publicly available sources used in Chapter 5.

Isolate	Reference	Country	year	infection	K type
CAS683	(Stahlhut et al., 2009)	US	2004	Liver abscess	K1
CAS685	(Stahlhut et al., 2009)	US	2005	Liver abscess	K1
CAS686	(Stahlhut et al., 2009)	US	2005	Liver abscess	K1
CAS687	(Stahlhut et al., 2009)	Canada	2005	Liver abscess	K1
CAS688	(Stahlhut et al., 2009)	US	2005	Liver abscess	K2
CAS689	(Stahlhut et al., 2009)	Canada	2005	Liver abscess	K2
CAS690	(Stahlhut et al., 2009)	Canada	2005	Liver abscess	K1
CAS691	(Stahlhut et al., 2009)	Canada	2005	Liver abscess	K1
CAS692	(Stahlhut et al., 2009)	Canada	2006	Liver abscess	K1
CAS694	(Stahlhut et al., 2009)	Canada	2006	Liver abscess	K1
CAS695	(Stahlhut et al., 2009)	Canada	2006	Liver abscess	K1
CAS698	(Struve et al., 2015)	US	2006	Liver abscess	K1
CAS699	(Struve et al., 2015)	US	2007	Liver abscess	K1
CAS701	(Struve et al., 2015)	US	2007	Liver abscess	K1
CAS726	(Sobirk et al., 2010)	Sweden	2008	Liver abscess	K1
CAS727	(Holmås et al., 2014)	Norway	2008	Liver abscess	K1
CAS813	(Gundestrup et al., 2014)	Denmark	2010	Liver abscess	K1
CAS905	(Holmås et al., 2014)	Norway	2010	Liver abscess	K1
CAS906	(Holmås et al., 2014)	Norway	2010	Liver abscess	K1
CAS951	(Struve et al., 2015)	Norway	2012	Liver abscess	K1
CAS983	(Yu et al., 2007a)	Taiwan	1996	Liver abscess	K1
CAS986	(Yu et al., 2007a)	Taiwan	1996	Liver abscess	K1
CAS987	(Yu et al., 2007a)	Taiwan	1996	Liver abscess	K1
CAS988	(Yu et al., 2007a)	Taiwan	1997	Liver abscess	K1
CAS989	(Yu et al., 2007a)	South Africa	1996	Pneumonia	K1

CAS990	(Yu et al., 2007a)	South Africa	1996	Pneumonia	K1
CAS991	(Yu et al., 2007a)	South Africa	1997	Pneumonia	K1
CAS992	(Yu et al., 2007a)	South Africa	1997	Pneumonia	K1
TG01970	(Struve et al., 2015)	US	2006	Respiratory tract	Non-K1/2
TG21587	ATCC	US	1990-1991	-	K2
TG21950	(Struve et al., 2015)	US	2010	Urinary tract	K2
TG21974	(Struve et al., 2015)	US	2011	Bacteraemia	K2
California1	(Holt et al., 2015)	US	1997-2008	-	K2
California2	(Holt et al., 2015)	US	1997-2008	-	K2

A5.2 Virulence genes

Gene	Hypermucoviscous phenotype	Organism
rmpA	Regulator of the mucoid phenotype A	*K. pneumoniae* CG43
rmpA2	Regulator of the mucoid phenotype A2	*K. pneumoniae* CG43
magA	Mucoviscosity associated gene A	*K. pneumoniae* NTUH_K2044
rsc A	Regulator of capsule synthesis A	*K. pneumoniae* NTUH_K2044
rsc B	Regulator of capsule synthesis A	*K. pneumoniae* NTUH_K2044
	LPS synthesis	
wabG	glucuronic acid transferase	*K. pneumoniae* NTUH_K2044
uge	Uridine diphosphate galacturonate 4-epimerase	*K. pneumoniae* NTUH_K2044
	Iron up take system	
iroN	Catecholate siderophore receptor	*K. pneumoniae* NTUH_K2044
irp2	Yersiniabactin biosynthesis	*Yersinia pestis* CO92
iucD	Aerobactin siderophore synthesis	*K. pneumoniae* CG43
iutA	Ferric siderophore receptor	*K. pneumoniae* NTUH_K2044
ybtS	Salicylate synthase Irp9	*K. pneumoniae* NTUH_K2044
kfu	Iron acquisition system	*K. pneumoniae* NTUH_K2044
	Fimbriae	
mrkD	Type 3 adhesin	*K. pneumoniae* NTUH_K2044
	Other	
allS	DNA-binding transcriptional activator allS	*K. pneumoniae* NTUH_K2044

A5.3 Reference plasmids used in Chapter 5.

NCBI reference sequence	Size (bp)	Organism
NC005249	219385	*K. pneumoniae* CG43
NC006625	224152	*K. pneumoniae* NTUH_K2044
JN420336	267242	*K. pneumoniae* NDM-MAR
BX664015	274762	Serratia marcescens R478
CP000966	91096	*K. variicola* strain 342
KF954760	139941	*K. pneumoniae* strain BK30683
DQ298019	9294	*K. pneumoniae* IGMS32
AY046276	50969	*Salmonella enterica* subsp. *enterica* serovar Typhimurium R46
CP023920	4619	*K. pneumoniae* strain FDAARGOS_440
CP000670	137010	*Yersinia pestis* Pestoides F
AP001918	99159	*Escherichia coli* K-12
DQ449578	98264	*K. pneumoniae* strain NK245
CP003223	122799	*K. pneumoniae* HS11286
CP000648	175879	*K. pneumoniae* MGH 78578
JN233704	208191	*K. pneumoniae* strain ST258

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