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Pasteurella multocida

A Study on the Isolation, Identification and
Characterization of New Zealand Strains.

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ABSTRACT

Pasteurella multocida is a small Gram negative bacillus which causes disease in many animal species. Its pathogenicity is attributed to two mechanisms: invasiveness and toxin production. The proportion of strains which produce toxin is low but such strains cause severe atrophic rhinitis in swine. Invasiveness is the predominant mechanism of pathogenicity and is largely dependant on the possession of a capsule.

P. multocida is divided into five serotypes (A, B, D, E and F) based on the antigenic structure of the capsule and a correlation exists between serotype, disease and the animal species affected. However, many isolates are non-typable due to the possession of a non-antigenic (hyaluronic acid) capsule. The presence of an antigenic capsule and a hyaluronic acid capsule are independant. ie one, both or neither may be present.

To facilitate the isolation of *P. multocida* we assessed the selective medium of Smith and Baskerville(1983) and found that many New Zealand isolates of *P. multocida* grew poorly in the absence of blood (not present in Smith and Baskerville's medium). Furthermore, many isolates were inhibited by Polymixin and the high alkalinity (pH 8.6) of the medium.

On the basis of these observations we formulated a modified selective medium which omitted Polymixin and was composed of a blood agar base at neutral pH. However, it contained three antibiotics (Gentamycin, Bacitracin and Mycostatin) present in the original medium of Smith and Baskerville. This modified medium propagated all our test isolates of *P. multocida* which were derived from seven

animal species (fowl, sheep, goat, cattle, rabbit, dog, cat). It also suppressed the background flora present in swabs taken from the nasal cavity of rabbits.

Both the traditional isolation technique of plating specimens on blood agar and the modified selective medium were used to obtain isolates of *P. multocida* from several species of domestic animal. These were: pigs (15 isolates), rabbits (25), cats (10), dogs (21) and deer (1). Isolates were examined in some detail.

Isolates were serotyped using the indirect haemagglutination assay (IHA). They were found to be Type A (7%), B (1%), D (9%) or untypable (83%). This is similar to overseas findings.

The IHA assay is a laborious technique so two possible alternatives were examined. viz sodium dodecyl sulphate polyacrylamide-gel analysis (SDS-PAGE) of proteins and restriction endonuclease analysis (REA) of DNA. Both techniques failed to distinguish between *P. multocida* serotypes in the sense that isolates were extremely heterogeneous and most gave a unique pattern both with SDS-PAGE and REA.

This heterogeneity allowed the identification of individual strains of *P. multocida* and consequently SDS-PAGE was used in an epidemiological investigation which traced the origin of an outbreak of respiratory disease in rabbits and provided evidence that the outbreak was not (as was earlier believed) due to the introduction of rabbits from overseas into a New Zealand colony. Furthermore, using this approach we were able to show that strains which cause severe pneumonia were not confined to the lower respiratory tract but could also be carried in the nasal cavity. This is consistent with the accepted hypothesis that *P. multocida* is an opportunistic pathogen.

Severe atrophic rhinitis is a disease of pigs which is caused by toxin-producing strains of *P. multocida*. The disease is responsible for much economic loss overseas but has not been reported in New Zealand. We obtained a toxigenic strain of *P. multocida* and used it to validate an *in vitro* (mammalian cell culture) method for detecting toxin-producing strains. This method was then used to examine New Zealand isolates of *P. multocida*. None of these were shown to be toxigenic. This suggests that toxigenic strains, if present in New Zealand, are not common.

Diseases due to *P. multocida* are commonly treated with antimicrobial agents such as the Penicillins, Aminoglycosides and Sulphonamides. Strains which are resistant to these agents are present in overseas countries and resistance is associated with the presence of plasmids.

We determined the minimum inhibitory concentrations (MIC), of four antimicrobials (Penicillin G, Streptomycin, Tetracycline and Sulphadiazine) for New Zealand isolates. Considerable variation was found between isolates but no isolate was of sufficiently high resistance to be considered "resistant". This was despite the fact that 12 of 73 (16%) of the field isolates possessed plasmids of a similar size (1Mdal to 5Mdal) to plasmids known to carry antibiotic resistance markers.

Despite its importance as a pathogen of swine, cattle, sheep, fowl and rabbits, *P. multocida* has been little studied in New Zealand. This thesis represents a preliminary stage to a fuller understanding of the importance of *P. multocida* in this country and the best means of control. The possibility of outbreaks of disease due to *P. multocida*, eg atrophic rhinitis, may well stimulate further work on this organism.

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INTRODUCTION

Pasteurella multocida is associated with many species of animals usually as a commensal of the upper respiratory tract but it is also a potential pathogen. As a pathogen it causes a range of diseases. These include pneumonia of several animal species, shipping fever of cattle and sheep, fowl cholera of domestic hens and atrophic rhinitis of swine.

These diseases have resulted in considerable economic loss and therefore it is not surprising that *P. multocida* has been intensively studied overseas. The studies however were for many years less productive than they could have been for three main reasons. The wide host range of *P. multocida* was not recognized which led to a multiplicity of names for what we now recognize as one bacterial species. The organism was not adequately distinguished from either *Yersinia* spp or *P. haemolytica* and the antigenic complexity of *P. multocida* was continually underestimated. Even today our understanding of *P. multocida* leaves much to be established but the fact that it continues to be an important pathogen of animals ensures that it will continue to be intensively studied.

Two general mechanisms, invasiveness and toxin production, are responsible for the pathogenicity of *P. multocida*. The former is probably the more important and depends to a large extent on the possession of a capsule. There are three common capsular types, viz A, B and D. These determine the serotype and a correlation exists between the serotype, the disease caused and the host species affected.

Toxin producing strains of *P. multocida* have been recognized only recently (De Jong, Oei and Tetenburg, 1980), are apparently rare and so far have been limited to pigs where they cause a severe form of

atrophic rhinitis. This disease is common in Europe and, if media reports are reliable ("The Dominion" December 22, 1987) also affects pigs in Australia. As yet severe atrophic rhinitis has not been reported in New Zealand although a mild form of the disease, of undetermined aetiology, has been reported (Hodges, 1981) in pigs in the Waikato.

Treatment of disease due to *P. multocida* usually involves one or a combination of the following antimicrobial agents, Penicillin, Tetracycline, Streptomycin and Sulphonamides. The continual use of these drugs has led to the development of resistant strains of *P. multocida* and this resistance is associated with plasmids.

A major practical difficulty in studying the prevalence of *P. multocida* in animals is that since it is carried in the nasal tract in relatively low numbers it may frequently go undetected when nasal swabs are examined by the standard technique of plating on blood agar.

A further difficulty in studying the organism is that the only available technique to determine the capsular type is the indirect haemagglutination assay (IHA). This is laborious, is often interfered with by the presence of non-antigenic hyaluronic acid on the bacterial surface and frequently gives negative results because many isolates of *P. multocida* do not possess a capsule. These difficulties have to some extent inhibited epidemiological studies on the different antigenic types.

The above considerations resulted in the formulation of the following aims for this research project:

1. To develop a selective medium which would facilitate the isolation of *P. multocida* from nasal swabs.

2. To obtain and examine isolates of *P. multocida* from several species of animal in New Zealand.
3. To establish the IHA assay and use it to determine the serotypes of New Zealand isolates of *P. multocida*.
4. To examine the possibility of using alternative approaches for differentiating between serotypes of *P. multocida*. This involved the examination of proteins (by SDS-PAGE) and DNA (by REA) to determine if any correlation between serotype and proteins (or DNA) could be established which might act as an alternative method for determining the serotype.
5. To establish an *in vitro* mammalian cell culture method for the detection of exotoxin-producing strains of *P. multocida* and to use this method to determine if New Zealand isolates produce exotoxin.
6. To screen New Zealand isolates of *P. multocida* for resistance to antibiotics (Penicillin, Streptomycin, Tetracycline and Sulphonamide). To examine these isolates for the presence of plasmids and to determine if a correlation exists between antibiotic resistance and the presence of plasmids.

CHAPTER 1

Historical Review.

1.1 Classification and Nomenclature

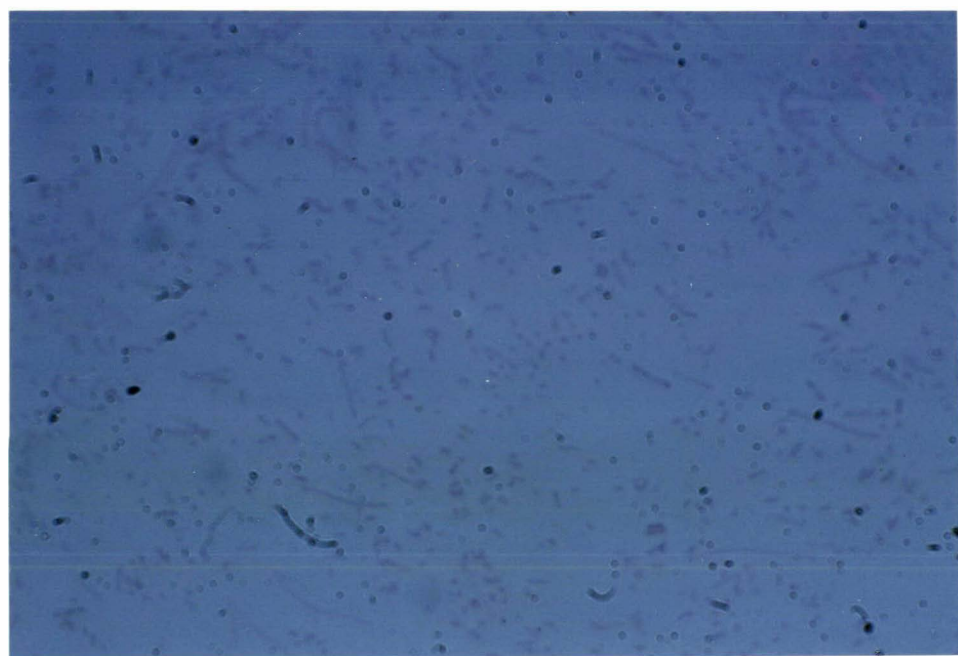
The *Pasteurellaceae* are a family of Gram negative, facultatively anaerobic coccobacilli which are nonmotile, nonsporeforming and exhibit pleomorphism (Figure 1.1). They are distinguishable from the *Enterobacteriaceae* and *Vibrionaceae* because they are oxidase and catalase positive, do not produce appreciable amounts of hydrogen sulphide and in the case of most species do not produce gas from the fermentation of carbohydrate. The family is divided into three genera: *Pasteurella*, 6 species; *Haemophilus*, 19 species and *Actinobacillus*, 5 species (Mannheim, 1984). Using biochemical tests *Pasteurella* can be differentiated from *Haemophilus* and *Actinobacillus*. Furthermore *Haemophilus* spp require certain growth factors whereas *Pasteurella* spp are non-fastidious organisms which grow on simple media (Mannheim, 1984). Members of the genus *Pasteurella*, in particular *P. haemolytica*, are related to the actinobacilli (Smith, 1974) and it can be difficult to distinguish between the two. However the actinobacilli possess a unique sticky colonial texture which is useful in differentiating between the two genera (Mannheim, 1984).

The first report of the isolation of a bacterium fitting the description of the organism now known as *P. multocida* was made by Bollinger in 1878 (see Carter, 1976). At this time Louis Pasteur demonstrated that this organism causes fowl cholera. He then produced the world's first laboratory made vaccine to combat the disease (see Mannheim, 1984). In 1883 Burril (see Namioka, 1978) isolated *P. multocida* from the blood of a domestic fowl suffering from fowl cholera. He described the organism and called it *Micrococcus gallicidus*. This name did not achieve general acceptance

Figure 1.1 Gram Stain of *P. multocida*.

The cells are small, Gram negative, pleomorphic rods.

1000x Magnification.



and it was still known as 'the bacillus of fowl cholera' when isolated and described by Peroncito in 1887 (see Smith and Wilson, 1985). It was in that year that Trevisan coined the generic name *Pasteurella* as a tribute to Pasteur for his earlier work on fowl cholera (see Carter, 1984; Smith and Wilson, 1985).

Despite the above reports Lehmann and Neumann[1889] (see Namioka, 1978) are given credit for the first attempt at comprehensively describing *P. multocida* which they designated *Bacterium multocidum*. However in 1893 Sternberg named the organism

Bacterium septicaemiae haemorrhagicae after isolating it from a case of haemorrhagic septicaemia, a disease of cattle reared in hot countries (Namioka, 1978). This proliferation of names is not surprising because at the end of the 19th century it was considered that isolates derived from different host species represented different species of the same genus. This view was derived from the common observation that during naturally occurring outbreaks of disease, transmission of *P. multocida* rarely occurred between different host species (Smith, 1974). This phenomenon will be discussed later. Following this inaccurate line of reasoning Lignieres[1900] (see Smith and Wilson, 1983; Carter, 1967) added a specific name for each organism based upon the animal which it was isolated. For example, *Pasteurella* spp causing fowl cholera was designated *P. aviseptica*. That causing septicaemia and haemorrhage in cattle was designated *P. bovisseptica* whereas if it was isolated from sheep Lignieres called it *P. oviseptica* (Smith and Wilson, 1983; Carter, 1967).

In preparing Topley and Wilsons first edition of 'Principals of Bacteriology, Virology and Immunity' [1929] (see Smith and Wilson, 1983) the authors recognized that on morphological and biochemical grounds the strains of Lignieres could be grouped as a single species. To encompass this new grouping Topley and Wilson (see Smith and Wilson, 1983) coined the name *Pasteurella septica* in 1929. This name gained wide but not universal acceptance and has been used

until quite recently, especially in the British Commonwealth (Carter, 1967). Notwithstanding the conclusion that *P. septica* could infect several species of animal, isolates of the organism tended to be more pathogenic for the species of animal from which they were isolated than for other animal species. Topley and Wilson[1929] (see Smith, 1974) therefore concluded that *P. septica* could be subdivided into 'biotypes'. However the nature of the differences between 'biotypes', except for differences in host range, was not specified (Smith, 1974).

Despite the prestige associated with Topley and Wilson and their first edition of 'Principals of Bacteriology, Virology and Immunity' the name *P. septica* did not achieve universal approval because it did not comply with the rules of nomenclature (see Smith and Wilson, 1983). Consequently in 1939 Rosenbusch and Merchant (see Smith and Wilson, 1983) proposed the alternative name *Pasteurella multocida*. This name was accepted by the authors of Bergey's Manual in 1948 (see Smith and Wilson, 1983; Namioka, 1978). However the picture was complicated by the temptation to classify strains causing haemorrhagic septicaemia of cattle and buffaloes under the separate species name *Pasteurella bovis*. This temptation was finally resisted since positive differentiation from other members of *P. multocida* could be made only by immunological or serological means. ie. It represents a different serotype rather than a different biotype of the same organism (Carter, 1967).

At this point the reader may deduce, if only from the title of this thesis, that the name *P. multocida* has generally been accepted following its acceptance by Bergey's Manual in 1948. However for many years after the first edition of the manual was published strains of *P. multocida* have been referred to as 'the haemorrhagic septicaemia pasteurellae'. This loose designation was used by some authors to include both *P. multocida* and *P. haemolytica* (Carter and Bain, 1960). This term is unsatisfactory even if limited to *P. multocida* because in practice many strains do not cause

haemorrhagic septicaemia and nowadays the term haemorrhagic septicaemia is reserved to describe acute systemic pasteurellosis in cattle (Carter and Bain, 1960; Carter, 1967).

Talbot and Sneath(1960) examined many strains of *Pasteurella* using classical morphological and biochemical tests. They concluded that *P. multocida* was only distantly related to

Pasteurella pseudotuberculosis and the plague bacillus

Pasteurella pestis. However these latter two organisms were found to be closely related to each other. This implied that the genus *Pasteurella* should be split (Talbot and Sneath, 1960).

Investigations by Smith and Thal[1965] (see Carter, 1967) support Talbot and Sneaths findings. Furthermore Smith and Thal (see Carter, 1967) demonstrated that members of the genus *Pasteurella* fell into two groupings. One group contained the oxidase positive species *P. multocida*, *P. pneumotropica*, *P. ureae*, and *P. haemolytica* [*P. aerogenes* and *P. gallinarum* have since been added to this grouping]. The second group contained the oxidase negative organisms *P. pestis* and *P. pseudotuberculosis*. With this information in mind this second grouping was removed from the genus *Pasteurella* and is now classified in the genus *Yersinia* as *Y. pestis* and *Y. pseudotuberculosis* (Carter, 1967).

The species name 'multocida' is derived from the Latin adjectives 'multus' [many] and 'cidus' [killing]. Hence the name means 'many killing' which is appropriate because *P. multocida* causes mortality in a wide range of hosts. Pedantically speaking it should be spelt multicida, however the Greek 'multocida' has become the accepted version. The logical pronunciation is therefore mul/to/ci/da and not mul/toc/i/da as is commonly used.

Note: The above section illustrates the main historical landmarks in the nomenclature of *P. multocida*. Between 1878 and 1929 a vast range of names were generated for the many biotypes of this organism. These names and their references have been reported by Hussaini[1966] (see Smith, 1974).

1.2 Serological Characterisation of *P. multocida*

1.2.1 Early Attempts at Grouping

The elucidation of *P. multocida* serology has been a slow process. This has been due in part to a continual underestimation of the organism's complexity. Lignieres[1901] (see Carter, 1967) named strains after the animal species from which they were isolated. By doing this he may have suspected that serological differences existed between isolates from different hosts. However, most early investigations seem to have assumed that the species was serologically homogeneous. This did not apparently inhibit them from suggesting a multitude of different names for the organism.

Using serological techniques the existence of differences between isolates of *P. multocida* was shown independently by several workers. Cornelius[1929], Yusef[1935] and Ochi[1933] (see Carter, 1967) using 'agglutinin absorption', precipitation and agglutination assays respectively were able to distinguish four serological types. Ochi[1933] (see Namioka and Murata, 1961²) named his four serological groups A, B, C and D. He observed that domestic fowls were not attacked by *P. multocida* strains of bovine origin. Reciprocally, cattle and buffalo were not attacked by fowl isolates. Ochi's type A consisted of cultures isolated from cases of fowl cholera and were found to possess pathogenicity for fowl and small laboratory animals only. Type B strains were isolated from cases of haemorrhagic septicaemia of cattle and from similar diseases of sheep and swine. These strains did not produce fowl cholera in

domestic birds. This represents the first clear indication that the host range of *P. multocida* is related to the serotype. Ochi's Type C possessed no pathogenicity for fowls and failed to produce either haemorrhagic septicaemia in cattle or a similar condition in sheep and swine. Ochi's type D was dissimilar to types A, B and C. However we will not consider it further because it has since been shown to equate with the current species *P. haemolytica* (Carter, 1967).

Note: Strictly speaking systemic pasteurellosis is a bacteraemia and not a septicaemia since *P. multocida* does not multiply in the bloodstream. However this thesis follows the general practice and uses the designation septicaemia when this is the accepted terminology for a particular disease. The usage of the term pasteurellosis is discussed in Section 1.5.1.

Until the late 1930's whole organisms were used as both immunising antigens and as reacting antigen in serological tests. However in 1938 Pirotsky (see Carter, 1967), using an agar-gel immunodiffusion procedure (AGID), studied polysaccharide extracts from smooth cultures of *P. multocida*. He found that antiserum prepared using whole organisms reacted with the polysaccharide extracts of some isolates only. That is, he showed that serological specificity was at least in part determined by the polysaccharide capsule (see Carter, 1967). This led him to suggest that *P. multocida* isolates could possibly be divided into serological groups based on the antigenicity of the polysaccharide (Carter, 1967). However he found that some strains did not react with any antisera, including antiserum against the strain under test. This is a problem which persisted even when other typing methods were developed. As a result many isolates are untypable by the various typing methods employed and this remains true up to the present day. The problem of untypable strains is returned to later.

Following Pirotsky's suggestion that *P. multocida* could be classified serologically on the basis of its capsular polysaccharide Rosenbusch and Merchant[1939] (see Carter, 1967; Roberts, 1947) used tube agglutination reactions augmented with biochemical tests to divide *P. multocida* into three groups. The biochemical tests were based on differences in the fermentation of xylose, arabinose and dulcitol, however this approach has not apparently been continued by other workers and its usefulness is uncertain. Also the serological tests were not definitive because they detected many cross reactions between *P. multocida* isolates. These cross reactions were probably due at least in part to the use of whole organisms rather than extracts. Nevertheless Little and Lyon[1943] (see Carter, 1967) identified three serological groups of *P. multocida* using slide agglutination tests. They found that more isolates were typable by this method than by using the tube agglutination procedure of Rosenbusch and Merchant (see Carter, 1967). However the slide agglutination method gave even more cross reactions than the tube agglutination method of Rosenbusch and Merchant (see Carter, 1967).

In hindsight the cross reactions were probably due to the antiserum causing agglutination of organisms on the basis of their capsular polysaccharide and/or their somatic antigens. These two antigen systems are known to exist and vary more or less independantly. It is therefore not surprising that complicated cross reactions occur when reacting complex antigen preparations with equally complex antiserum. Somatic antigens are discussed later.

A different approach to the serological classification of *P. multocida* was taken by Roberts(1947). He passively immunized mice with antisera raised to *P. multocida* strains from different animals and from wide geographical sources. When challenged the immunized animals were found to be protected against some strains but not others. These 'mouse protection' tests suggested that some isolates were closely related serologically whilst others were readily distinguishable because of a lack of cross protection. From his

results Roberts speculated that *P. multocida* was composed of four different serological groups. These he named with the Roman numerals I, II, III and IV. He observed that typable strains of *P. multocida* isolated from cases of haemorrhagic septicaemia in cattle fell into group I (Roberts, 1947). These bovine group I strains probably equate with Ochi type B grouping (see Namioka and Murata, 1961²). Interestingly Roberts also isolated many 'untypable' strains. That is, antiserum to any of the four groups failed to protect passively immunized mice against some field isolates.

1.2.2 Discovery of Capsular Types

I. A Serological Approach

Carter(1952) attempted to group strains of *P. multocida* using two methods. These were the tube agglutination typing method of Ochi[1933] (see Carter, 1967) and Little & Lyon's[1943] (see Carter, 1967) slide agglutination test. He was unsuccessful in the sense that he could not reproduce the earlier published work. Carter(1952) then attempted to group strains using an AGID test employing a saline extract as antigen. This approach is somewhat similar to that of Pirotsky (see above), however Pirotsky used what was then called "Boivin" antigen which in practice probably contains both capsular polysaccharide and heat stable somatic antigens. In contrast Carter's saline extracts probably consisted mostly of capsular polysaccharide. Using this method Carter was successful in demonstrating three serological groups (Carter, 1952). However it was unsatisfactory for screening large numbers of isolates since it involved both the consumption of large amounts of reagents and suffered from the problem of cross reactions when field isolates, as opposed to 'prototype strains' were examined.

The failure of traditional tests to clearly define serological differences in isolates led Carter(1955) to use what was then a relatively new typing procedure. This was the Indirect

Haemagglutination Assay (IHA). This method involved several steps. Bacterial capsule was first removed from cells by saline and heat extraction. This extract was then mixed with human type O erythrocytes. The extracted capsular material sticks to the erythrocytes and remains even after the cells have been washed. The 'sensitized' (capsular polysaccharide coated) erythrocytes were then mixed with serial dilutions of *Pasteurella* antiserum and incubated for several hours. By this time the coated erythrocytes may or may not be agglutinated. Agglutination indicates that the *P. multocida* strain being tested is of the same serological group as the strain used to produce the antiserum and vice versa. This method had a further benefit in that it could be used to assay the titre of antibody to the capsular polysaccharide.

The IHA assay is superior to previous methods, especially agglutination tests, for several reasons.

- i. It requires only small quantities of antigen.
- ii. Fewer strains are found to be untypable.
- iii. The results showed greater reproducibility.
- iv. Fewer isolates showed cross reactions.

Most of these advantages can be attributed to the greater sensitivity of the IHA assay. However the method has the disadvantage of being very laborious.

Carter(1955) found that human Type O erythrocytes were the most suitable for his IHA assay because they did not agglutinate in the presence of normal rabbit or bovine serum. Sheep and chicken erythrocytes were unsatisfactory because they contain antigens which cross-react with antigens of *P. multocida* (Carter(1955)).

Using the IHA assay Carter(1955) identified four serological groups. These he designated Types A, B, C, and D. They equated with Roberts's mouse protection groups II, I, III and IV respectively (Carter, 1955).

A further capsular type was identified in 1961 (Carter, 1961). It was isolated from several cases of bovine haemorrhagic septicaemia in central Africa. This new group was designated Type E. It is serologically related to Type B but sufficiently dissimilar to warrant the separate serological classification (Carter, 1961).

Recently a new capsular type, designated Type F, was isolated from turkeys (Rimler and Rhoades, 1987). It showed some similarity to Type D by the acriflavin test. To date little information is available on this Type.

The above information suggests that there are six types of *P. multocida*. However Carter(1963) suggested that Type C should be dropped from the classification as "it does not represent either an important type or group". He consistently found that antibody titres to Type C were low and considered that it was "not a capsular type in the same sense that the others were" (Carter, 1967). Namioka(1978) reexamined the existence of Type C *P. multocida*. He concluded that the Type C reference strain had become antigenically rough. This implies that a Type C grouping may exist but, if so, no standard fully encapsulated prototype is available.

Several important modifications of the IHA assay have been made since its introduction by Carter(1955). Carter and Rappay(1961) modified the assay by using formalinised human type O erythrocytes. This procedure extended the shelf life of the sensitized erythrocytes and also resulted in antibody reactions of higher titre which further increased the sensitivity of the test (Carter and Rappay, 1961). Another modification was the pretreatment of mucoid, hyaluronic-acid-containing strains with hyaluronidase. This resulted in an increase in the numbers of strains which were typable (Carter, 1972). This is discussed later.

In further developing the IHA assay Sawada, Rimler and Rhoades(1982) substituted glutaraldehyde fixed sheep erythrocytes for human erythrocytes. This in itself is an advantage because sheep blood is readily available in Veterinary laboratories. Furthermore fixation with glutaraldehyde overcomes the problem of heterophilic antibodies and further extends the shelf life of the cells. IHA assays performed with Human type O erythrocytes or fixed sheep erythrocytes essentially give the same results (Sawada, Rimler and Rhoades, 1982) .

II. A Non Serological Approach

Preceding the serological identification of capsular types by IHA assay, various attempts were made at grouping strains of *P. multocida* by other criteria (Carter, 1967). This approach was based on colonial morphology and the host species from which different colonial types were isolated. The colonial types fell into three groups: mucoid, smooth and rough (Carter, 1967). Braun[1953] (see Carter, 1957¹) found that the acriflavin test (see below), first introduced by Allesandrini and Sabatucci[1933] (see Carter, 1957) was a more reliable indicator of variant type than was simple observation of colonial morphology (Carter, 1957¹). Mucoid colonies contain large amounts of capsule which reacts with acriflavin to give a slimy precipitate. Rough colonies possess little, if any, capsule. They flocculate in acriflavin whereas the intermediate 'smooth' variety reacts to give a homogeneous suspension. Rough strains tend not to be pathogenic (Carter, 1957¹; Smith and Wilson, 1983). This method of grouping strains was of considerable value initially, however it was largely superseded by the superior discriminating power of the IHA assay. Nevertheless rough variants are untypable by the IHA method due to their lack of capsule so the acriflavin test is still a useful tool for screening isolates to establish if they are rough (Carter, 1957¹) .

1.2.3 Discovery of Somatic Antigenic Groups

Many of the earlier serological studies on *P. multocida* resulted in the publication of results which were not confirmed by other workers. This was in part due to a lack of appreciation of the antigenic complexity of the organism (Namioka and Murata, 1961²). Carter (1952) reported that *P. multocida* possessed a somatic antigen common to all members of the species. This view changed in 1956 when Yaw and coworkers (see Namioka and Bruner, 1963) stated that the wide differences observed in host range could not be explained by variation in capsular type alone. Consequently they suggested that organisms with different somatic antigens might exist to account for this phenomenon. Carter supported this concept (Carter, 1961; Carter, 1963). He had typed many strains by their capsular antigen and suggested that other (surface) antigens probably existed.

It might be assumed that *P. multocida* somatic antigens could be investigated using one of the techniques previously used for studying the capsular antigen. However difficulties were experienced when conventional agglutination procedures were used to study the somatic antigen. For example, some isolates were not agglutinated by homologous antisera while others showed multiple cross reactions (Carter, 1967). This was probably due to the test interacting with the capsular polysaccharide

A breakthrough occurred with the work of Namioka and Murata (1961²). They found that agglutination tests yielded more consistent results if cultures, whether used for antigen or antiserum preparation, were first treated with Molar HCl which removed the capsular polysaccharide. Cross reactions if they still occurred could be minimised by absorbing the rabbit sera with cross reacting strains thus resulting in what they called 'specific factor' sera specific for somatic antigens. This approach of increasing the specificity of serological reactions by absorption is similar to that used for enteric bacteria such as the salmonellae. For this reason the

somatic antigen of *P. multocida* is often referred to as the 'O' antigen (Carter, 1967).

Namioka(1961², 1961³), used cross absorbtion methods to divide the somatic antigens of *P. multocida* into two groups: 'common' and 'specific' (see Figure 1.2). He further demonstrated the existence of six specific somatic antigens three of which were associated with strains possessing Carter's Type A capsule and three with Type D. By combining capsular type nomenclature with the somatic antigens Arabic number the strains were assigned to a 'Serotype' (Namioka, 1961³).

Later Namioka(1963) identified additional somatic antigens and demonstrated the relative independence of somatic and capsular antigens. ie He had earlier shown that a strain of *P. multocida* with a specific capsular type could possess one of several somatic antigens. He now showed that a strain possessing a specific somatic group could possess one of several capsular antigens. For example (Table 1.1), a strain of *P. multocida* possessing a Group 3 somatic antigen could possess either a Type A or a Type D capsule. Other somatic antigens were found only in association with one specific type of capsular polysaccharide. For example, Group 9 somatic antigen has only been found associated with a Type A capsule. To date twelve somatic groups have been identified and their relationship with the capsular type has been determined (Table 1.1).

Table 1.1 *P. multocida* Somatic Groups and their Relation to Capsular Type.*

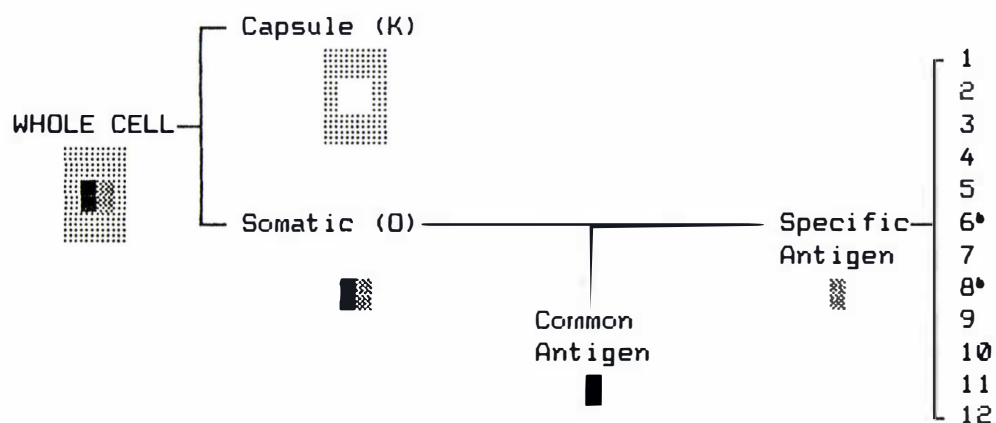
Capsular Type	Somatic Group	Serotype
A	1	1A
	3	3A
	5	5A
	7	6A
	8	8A
	9	9A
B	6	6B
	11	11B
D	1	1D
	2	2D
	3	3D
	4	4D
	10	10D
E	6	6E
F**	1	1F
	3	3F
	7	7F
	12	12F

* From Carter(1967)

** Based on Rimler and Rhoades(1987)

The existence of somatic subgroups was shown by Namioka(1961³) and Namioka and Murata(1963).

Figure 1.2 Antigenic Characteristics of *P. multocida*.^a



* Based on Namioka(1978)

* Composed of several subgroups

1.2.4 Agreement between Typing Methods

Shigidi and Mustafa(1980) found that there was good agreement between the tube agglutination assay of Namioka and the AGID method in identifying the somatic antigens. This implies that both methods involve the same antigens. However serotypes determined by one typing method do not always correlate with results using a different method. Brogden and Packer(1979) compared six different typing methods. These were:

- i. The slide agglutination test of Little and Lyon [see Section 1.2.1].
- ii. The Indirect Haemagglutination Assay of Carter [see Section 1.2.2].
- iii. The AGID test of Heddleston [see Section 1.2.3].
- iv. The slide and tube agglutination assays of Namioka [see Section 1.2.1].
- v. The mouse protection test of Roberts [see Section 1.2.1].
- vi. The acriflavin/hyaluronidase decapsulation test of Carter [see Section 1.2.2].

Brogden and Packer(1979) concluded that a correlation between typing methods could not be shown. Often isolates belonging to one serotype would fall into several serotypes when a different typing method was used. This phenomenon is probably due to the complex nature of the *P. multocida* capsule. Isolates with one or two serotyping antigens in common may differ in other antigens which another typing method recognises (Brogden and Packer, 1979).

Differences between results of IHA and AGID typing can possibly be explained by the involvement of different antigens. Originally it was thought that the IHA antigen was lipopolysaccharide (Carter and Rappay, 1962). However it has since been shown that lipopolysaccharide is the major antigen responsible for typing based on the AGID test whereas the capsular polysaccharide determines the results obtained by IHA (Manning, 1984).

1.2.5 Bacteriophage Typing

The existence of *P. multocida*-specific bacteriophages has been known for many years. No success has been achieved in using bacteriophage as a typing tool (Carter, 1967).

1.3 Antigenic Structure of *P. multocida*

The occurrence of antigenic polysaccharides in *P. multocida* was first reported over fifty years ago by Hoffenreich[1928] and Dingle[1934] (see Carter and Bain, 1960). Since then the antigenic structure of the organism has been shown to be complex.

Carter(1952) used tube agglutination and capsular swelling techniques to show that *P. multocida* possessed a capsular antigen. He presumed this to be composed of polysaccharide and produced evidence which suggested that strains also had a common (polysaccharide containing) somatic antigen. Carter and Annau(1953) later analysed capsular extracts from several *P. multocida* strains and confirmed that the capsular antigen was indeed composed of polysaccharide. Interestingly a non-immunogenic mucoid polysaccharide was also recovered and using a hyaluronidase-depolymerisation test it was identified as hyaluronic acid (Carter and Annau,1953).

Many freshly isolated strains of *P. multocida* do not agglutinate when mixed with homologous antiserum (Carter, 1972). This is surprising because the possession of a capsule can be demonstrated in many of these strains by using simple capsule-staining methods. Furthermore the binding of Type-specific antibody to this capsule has been shown by capsular swelling techniques (The capsule enlarges when antibody binds to it) (Carter, 1952). With the knowledge that many encapsulated strains of *P. multocida* do indeed bind Type-specific antibody it is now thought that the non-immunogenic

hyaluronic acid on the surface forms a lattice framework throughout the capsule, rather than a discrete layer. This lattice interferes with agglutination reactions by sterically hindering the binding of one antibody molecule to more than one bacterium. Binding of the antibody to more than one site on a single host is unimpaired (Carter, 1967; Carter and Bain, 1960).

The problems associated with the inhibition of agglutination due to hyaluronic acid extends to the indirect haemagglutination assay (IHA). This led Carter (1972) to modify the IHA assay. This was discussed in Section 1.2.2.

The production of hyaluronic acid is most prolific in Carter Type A strains. This endows the colonies with a mucoid appearance (see Figure 1.3) and thus they can be distinguished from Type D strains (see Figure 1.4) which produce little hyaluronic acid and form smooth colonies. It is not present in Type B strains and attempted decapsulation of these strains by hyaluronidase leaves them unaffected (Carter, 1967; Carter and Bain, 1960).

From the work done by Carter and Annau (see above) it may have been deduced that capsule extracts of *P. multocida* contain only one antigenic species ie polysaccharide. However Bain (1955) used a saline/potassium thiocyanate extraction method followed by a polar-organic-solvent separation method to isolate both a protein fraction and a polysaccharide fraction from the capsule of Roberts Type I strains. This type of *P. multocida* is equivalent to Carter Type B strains (see Section 1.2.2). He then demonstrated that the protein complex was a protective antigen because rabbit antiserum when absorbed with the protein complex had a diminished protective power as shown by the mouse protection test. This clearly showed that the polysaccharide was not the only protective antigen in capsule extracts. Bain (1955) suggested that this protein component could be used in future *P. multocida* vaccines.

Figure 1.3 Type A Colonies of *P. multocida*.

The colonies are approximately 2.0mm in diameter after 24hrs incubation at 37°C on blood agar.

Note that the colonies are mucoid. This is in contrast to Type D colonies (see Figure 1.4) which are smooth.

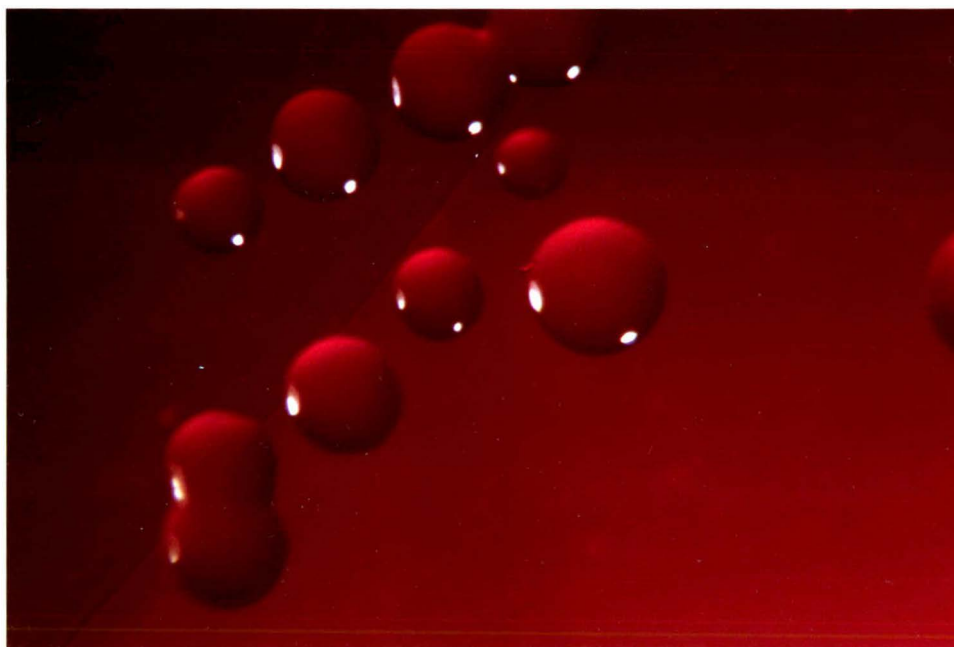
10x Magnification

Figure 1.4 Type D Colonies of *P. multocida*.

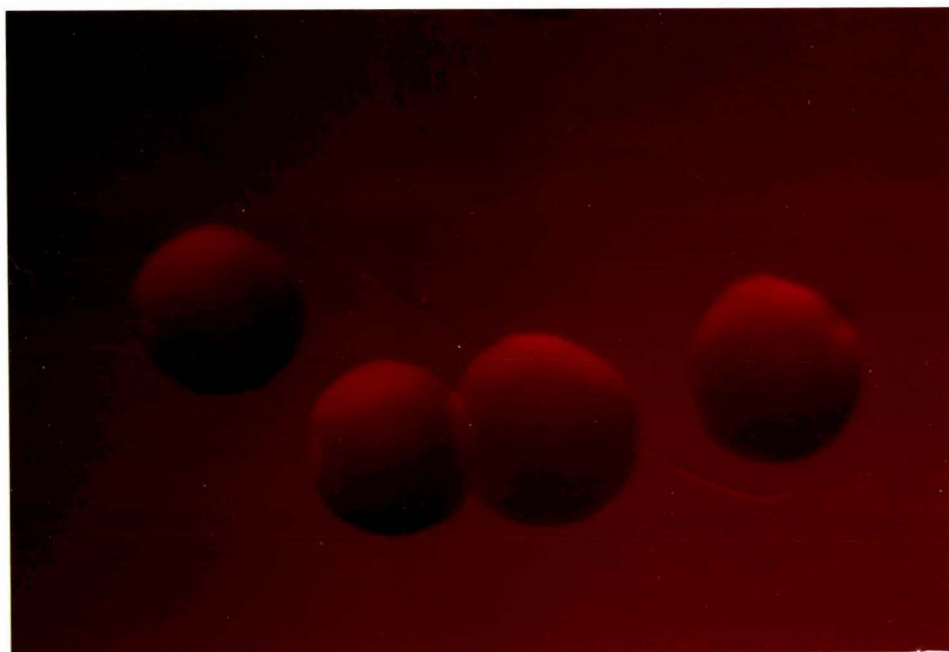
The colonies are approximately 2.0mm in diameter after 24hrs incubation at 37°C on blood agar.

Note the smooth appearance of colonies as illustrated by the demarcation of adjacent colonies. This is in contrast to Type A colonies (see Figure 1.3) which are mucoid.

10x Magnification



1-3



1-4

Bain [1955] (see Carter, 1967; Carter and Bain, 1960) continued his antigenic analysis of *P. multocida* and using AGID procedures revealed twelve antigens in saline extracts. Prince and Smith(1966¹) used AGID and immunoelectrophoretic [IEP] techniques to identify eighteen soluble antigens of *P. multocida*. Sixteen of these antigens were shown to be common to Type A and B strains. Furthermore several of these sixteen antigens were found to be shared by some other Gram negative organisms. The remaining two antigens, denoted α and β , were found to be serotype specific. That is, found only in Type A and Type B strains of *P. multocida* respectively (Prince and Smith, 1966¹). It seems reasonable to suppose that these two antigens (α and β) represent the Type-specific antigens. Clearly the type specific antigen exists against a complex background of shared or partially shared antigens.

Since the work of Prince and Smith(1966¹) the number of known surface antigens has been further increased. Bhasin and Lapointe-Shaw(1980) used crossed immunoelectrophoresis to analyse a strain of *P. multocida* belonging to Carter's Serotype 5:A. They described nineteen cell associated antigens.

Penn and Nagy(1974) used AGID and immunoelectrophoretic techniques to study extracted cellular material from Carter Type B and E strains of *P. multocida*. They reported several lines of precipitation by AGID. However, they observed that two lines represented the most common surface antigens. Using sodium deoxycholate depolymerisation analysis they demonstrated that one of these lines was a non-proteinaceous Type-specific capsular antigen (Penn and Nagy, 1974). By the same method they showed that the other line contained protein. Interestingly Type B proteinaceous antigens were precipitated by antisera prepared by using whole Type E cells as immunizing antigen and vice versa. This indicated that the proteinaceous antigens extracted from the capsule of Type B and E strains of *P. multocida* were similar (Penn and Nagy, 1974). Furthermore Type B and E possess other common antigens because

Namioka and Murata(1964) found that they both belonged to the same somatic grouping [viz group 6], with the exception of a single isolate of a Serotype 11:B strain (Namioka and Murata[1964] see Carter, 1967).

It could therefore be concluded that Type B and E strains of *P. multocida* represent similar organisms differing only in their capsule. This similarity is reinforced by the knowledge that Type B and E strains cause similar diseases. This will be returned to later.

The work of Penn and Nagy(1974) (see above) showed that saline extraction of *P. multocida* yielded a complex mixture of antigens. Clearly if one wished to study capsular polysaccharide alone a much more specific extraction and purification technique had to be developed. A suitable method was established two years later by Penn and Nagy(1976). Starting with saline extracts of *P. multocida* Types B and E they used a polar-organic-solvent based fractional precipitation method to separate capsular polysaccharide antigen from the endotoxin. They then used biological tests to show that the capsular polysaccharide (non-pyrogenic) had been completely freed from the endotoxin (pyrogenic) fraction. The purified capsular antigen was shown to be a high molecular weight acidic polysaccharide which was poorly immunogenic in rabbits. However a dose dependent antibody response was achieved in cattle as shown by the mouse protection test (Penn and Nagy, 1976). This clearly demonstrates that the capsular polysaccharide is an important protective antigen. Multicomponent whole-cell vaccines are often toxic because of the presence of endotoxin so it is advantageous to use a purified capsular antigen so these workers exploited the finding of a dose dependent response in cattle. Using purified capsule from both Type B and Type E *P. multocida* they produced a commercial haemorrhagic septicaemia vaccine (Walker and Foster, 1981).

The above discussion illustrates that the antigenic makeup of *P. multocida* is complex and that the capsular antigen and somatic antigen vary more or less independantly from strain to strain (see Section 1.2.3). However the two antigens are not easily separable in the laboratory.

It is now generally accepted that the "capsule", that is, the fraction removed from intact cells by saline extraction, is made up of four basic components (Carter, 1967):

- i. Polysaccharide
- ii. Hyaluronic Acid
- iii. Lipopolysaccharides
- iv. Proteins

The polysaccharide is responsible for Type specific immunity.

Hyaluronic acid is non-antigenic and probably augments the organisms virulence.

Lipopolysaccharide is a "contaminant" of the capsular extract. That is, it represents the somatic antigen, of which there are twelve types known (see Section 1.2.3). Somatic antigens are normally found associated with the intact cell, however a small proportion of these antigens are found in saline extracted capsule preparations. The somatic antigens represent protective antigens although they are of lesser importance than the capsular polysaccharide [see below].

Protein complexes extracted from capsular material have not been widely studied. However, those associated with Type B and E strains of *P. multocida* are similar, if not identical [see above]. Although protein components derived from the capsule have been shown to be protective (Bain, 1955) a single homogeneous protein which protects has yet to be isolated. However partially purified protein fractions have been successfully used to immunize mice, cattle (Carter, 1967)

and rabbits (Bain, 1955). Recently Kajikawa and Matsumoto(1984) demonstrated that a protein component was also protective in birds. They prepared a saline extract of *P. multocida* Serotype 5:A and using gel filtration isolated a (polysaccharide free) capsule-associated protein component which was protective for turkeys (Kajikawa and Matsumoto, 1984).

1.4 Toxigenic Strains of *P. multocida**

The above discussion implies that *P. multocida* is a pathogen solely because of its invasiveness. However within the last decade the importance of a second virulence factor, toxin production, has been established.

Smith[1957] (see Carter and Bain, 1960) demonstrated the presence of a heat labile toxin in the supernatant of several *P. multocida* strains after cell lysis. He found that sterile filtrates of week-old broth cultures contained 20 to 160 mouse lethal doses. This toxin could be inactivated by formalin but (surprisingly) was resistant to proteolytic enzymes (see Carter and Bain, 1960).

The significance of Smiths' [1957] work was not appreciated until 1975 when Il'ina and Zasukhin (see De Jong, Oei and Tetenburg, 1980) showed that strains of *P. multocida* which contained a heat labile toxin, as determined by the mouse lethality test of Smith[1957], could be isolated from the nasal cavity of swine suffering from progressive atrophic rhinitis [AR] (a description of this disease is given in Section 1.5.6). They demonstrated that toxigenic filtrates from these strains would produce AR-like lesions in the nasal tract of specific pathogen free [SPF] piglets. This strongly suggested that toxigenic strains of *P. multocida* were the cause of AR. It is tempting to speculate that the toxigenic strains isolated by Smith over a decade earlier [see above] could also produce AR-like lesions in SPF piglets. However this must remain speculation because the

origins of Smiths's strains are not clear and furthermore they are not now available.

The use of SPF piglets in surveys to demonstrate AR-lesion-producing strains is expensive and cumbersome. This problem was partially overcome when De Jong, Oei and Tetenburg(1980) demonstrated that toxin-containing extracts of *P. multocida*, as determined by the SPF piglet method, caused dermonecrotic lesions in guinea pigs when injected intradermally. This discovery led to the term Dermonecrotic Toxin (DNT) being used as a name for this toxin.

The use of guinea pigs to test for *P. multocida* toxin was more convenient than intranasal inoculation of SPF piglets. However *in vivo* tests, including the guinea pig skin test, are both expensive and cumbersome when used to screen large numbers of isolates. This problem was surmounted when Rutter and Luther(1983) showed that a cytopathic effect is produced in embryonic bovine lung (EBL) cell cultures when they were exposed to sterile filtrates of DNT-producing strains of *P. multocida*. Pennings and Storm(1984) showed that a similar cytopathic effect could be produced in presensitized [see below] Vero cells. These observations were important because many isolates could now be conveniently screened for toxin production.

Many *P. multocida* isolates have been examined and workers have looked for a correlation between capsule type and toxin production. Toxigenic strains of *P. multocida* have been found to be predominantly Type D (Nakai, Sawata and Kume, 1985; Sawata et al, 1984). The remainder are Type A (Pijoan et al, 1984). An equally important question is the distribution of toxin producing strains among host species. This has apparently not recieved much attention and at present all known toxigenic strains have been isolated from swine.

The Nature of *P. multocida* Dermonecrotic Toxin

P. multocida dermonecrotic toxin is a heat labile immunogenic 160 000 Mr protein which contains seventeen different amino acids (Nakai et al, 1984¹; Nakai et al, 1984²; Rutter and Luther, 1983; Rutter et al, 1984; De Jong, Oei and Tetenburg, 1980). The toxin is synthesized in the cytoplasmic space during the logarithmic growth phase of *P. multocida* *in vitro*. Release of toxin into the surrounding medium occurs during the stationary phase of the growth curve (Sawata and Kume, 1985; Rutter and Mackenzie, 1984).

The mode of action of *P. multocida* toxin is unknown. Toxic effects are similar to those produced by a dermonecrotic toxin of *Bordetella bronchiseptica* however the two molecules are serologically distinct (Nakai et al, 1984¹). Toxin-containing filtrates have no significant haemolytic or phospholipase activity (Rutter and Luther, 1984).

Pennings and Storm(1980) found that an enhanced toxic effect in Vero cells could be produced by pretreatment of the cells with a phosphodiesterase inhibitor. Phosphodiesterases catalyse the conversion of cAMP to 5'AMP. This suggests that the cytopathic effect may be caused by intracellular accumulation of cAMP. This action is similar to the enterotoxins produced by some members of *Enterobacteriaceae* and *Vibrionaceae* (Speirs, Stavric and Konowalchuk, 1977; Konowalchuk, Speirs and Stavrik, 1977; Guinee and Jansen, 1979).

*Note: also see Sect 1.5.6

1.5 Disease Associations and Epidemiology of *P. multocida*

1.5.1 General

The principal diseases caused by *P. multocida* are fowl which affects domestic and wild birds, haemorrhagic septicaemia of cattle and buffaloes, shipping fever of several host species especially sheep and cattle, primary or secondary pneumonia and abscesses in a wide range of animals. These diseases often involve other agents in concert with *P. multocida*. The most frequent coinfecting agents are *P. haemolytica* and Parainfluenza type 3 virus [PI3].

The following table, which is not exhaustive, illustrates the wide range of animals from which *P. multocida* has been isolated, either as a disease agent or as a commensal. Animals for which the organism is a major pathogen are indicated, as are animals of commercial importance to New Zealand.

Table 1.2 Host range of *P. multocida*.*

Cow**	Turkey**	Mink
Deer**	Buffalo	Mole
Dog**	Caribou	Mouse
Duck**	Cat	Monkey
Goat**	Chipmunk	Muskrat
Horse**	Dove	Peacock
Domestic Hen**	Gerbil	Pelican
Rabbit**	Guinea Pig	Rat
Swine**	Kangaroo	Vole
Sheep**	Man	Vulture

*: From Carter and Bain(1960) and Carter(1967).

** : *P. multocida* is a major disease-causing agent.

Bold Print: Animal of commercial importance to New Zealand.

As the above table illustrates *P. multocida* causes many diseases of many animals. However, the organism is normally associated with animals as a commensal of the upper respiratory tract (URT) but it cannot usually be isolated from healthy lungs (Carter, 1967; Carter

and Bain, 1960; Hamdy, Pounder and Ferguson, 1959; Ngatia et al, 1985; Magwood Barnum and Thompson, 1970; Smith, 1955).

Commensal strains of *P. multocida* are important opportunistic pathogens and physiological "stresses" are usually given the credit [if that is the right word!] for initiating most outbreaks of pasteurellosis (Carter, 1967). Such predisposing factors as "stress" cause the commensal to become disseminated. This was demonstrated by Cavellero and Sala[1951] (see Carter and Bain, 1960) who injected cortisone (which is released in large amounts by the body during periods of stress) into rats which had an inapparent nasal *P. multocida* infection. They observed that after treatment the inapparent infection rapidly progressed to an acute systemic pasteurellosis and many of the animals died from the bacteraemia.

The pathogenic mechanisms by which *P. multocida* cause disease are not known, however it is thought that bacterial endotoxin is responsible for the clinical effects and death in acute bacteraemias. The numbers of organisms present in animals dying of acute pasteurellosis is enormous (Carter, 1967) so a high concentration of endotoxin would be present in the bloodstream.

It is generally accepted that natural infections of *P. multocida* are acquired by inhalation or ingestion of the organisms. However, Daubney and Hudson(1934) suggested that arthropods may be vectors of haemorrhagic septicaemia. This view was supported by MacAdam(1962) who demonstrated the transmission of acute pasteurellosis from cattle to rabbits using ticks as a vector. This is interesting but disease due to *P. multocida* in rabbits is not usually associated with the same types of strains as those which cause acute pasteurellosis in cattle so it is probably not important in field conditions.

1.5.2 Cattle

The most important diseases of cattle associated with *P. multocida* are shipping fever, haemorrhagic septicaemia and pneumonia. Shipping fever is the most important of these and hence has been the most studied. This is an acute systemic pasteurellosis which typically occurs after an animal has spent long periods in a stressed state. eg in transit. It is found world-wide and is one of the most economically important diseases of feedlot cattle in the United States (Carter and Bain, 1960). However, despite the wide-spread occurrence of the disease it has not been reported in New Zealand.

Shipping fever is usually due to *P. multocida* in association with other bacteria and viruses. It is thought to be initiated by a lung infection which progresses rapidly to a bacteraemia resulting in a high grade fever and often death. This view is supported by the work of Panciera and Corstvet (1984¹). They inoculated both *P. multocida* and *P. haemolytica* into the lungs of calves. This induced pneumonic lesions. All of the animals developed acute pneumonic pasteurellosis and some animals subsequently died of a bacteraemia which can be regarded as experimental shipping fever.

The conclusion that two or more organisms combine to cause shipping fever does not at all imply that they play equal roles throughout the disease. Clearly the one which becomes most widely disseminated throughout the body is ultimately the one which kills the animal. If so, then the most critical organism is *P. multocida* because Collier (1968) said that this organism is the principal cause of the advanced clinical stages of shipping fever.

Haemorrhagic septicaemia is a clinically similar disease to shipping fever but it contrasts in that it is an acute systemic pasteurellosis caused by some strains of *P. multocida* only (see Section 1.6) and is found predominantly in tropical countries.

Pneumonia in cattle can be caused by *P. multocida* either alone or in association with other organisms. This was shown by Carter and Rousell(1958) who isolated both *P. multocida* and *P. haemolytica* from the pneumonic lesions of lungs obtained from calves which had died from pneumonia. It is now generally accepted that *P. multocida* and other agents, especially *P. haemolytica* and Parainfluenza Type 3 (PI3) virus, combine with physiological stress to precipitate pneumonia.

The contribution to the initiation of pneumonia in cattle, due to *P. multocida*, by non-*P. multocida* organisms has raised the question of whether the presence of the disease in a herd can be correlated with the type of nasal flora carried by the animal. Magwood, Barrum and Thompson(1970) surveyed the bacterial flora of the nasal cavity of clinically normal calves from both healthy and pneumonia-prone herds. They found that *P. multocida* was one of the most widely distributed organisms in both populations. However, the pneumonia prone herd was not associated with a nasal population differing markedly from the norm. But it was observed that *P. multocida* dominated the nasal flora of the pneumonia prone herd, in a state of active colonization for several days at a time and that bacteria were shed in large numbers during this process. This might suggest that pneumonia prone animals are physiologically more susceptible to *P. multocida* dominating their nasal flora. However in their report, Magwood, Barrum and Thompson(1970) did not say if the types of *P. multocida* strains which were isolated from the two herds differed from each other so it can be speculated that the pneumonia prone herd may have been associated with a strain of *P. multocida* commensal which was more virulent (that is, it had a higher propensity for being an opportunistic pathogen) than the strains found in the normal herd. Furthermore, the dominance of *P. multocida* in the nasal flora is a cyclic event so the lack of large numbers of *P. multocida* in the nasal passages of pneumonic animals may be due to the time lag between high numbers of the organism and the onset of clinical symptoms. At the clinical stage the numbers of the

organism in the nasal cavity would have returned to a low part of the cycle.

Cases of *P. multocida* associated mastitis have been reported in cattle both overseas (Carter and Bain, 1960; Carter, 1976) and in New Zealand (Carter[1972] (see Woodgyer, 1976). However, unlike sheep [see Section 1.5.3] mastitis of this aetiology is not a major problem.

In general, despite the overseas situation pasteurellosis is not a major problem in the New Zealand cattle industry. This might be due (Curtis, 1972) to the extensive pastoral rearing methods used in this country. It has been speculated that would change if more intensified methods of beef production are embarked upon (Curtis, 1972).

1.5.3 Sheep

The main diseases of sheep which are caused by *P. multocida* are shipping fever, pneumonia and mastitis (Carter, 1967). The remarks concerning shipping fever of cattle also apply to sheep so will not be discussed further except to say that deaths due to systemic pasteurellosis (a category which includes shipping fever) which are not complicated by pneumonia are rare. This implies that the major route of infection is via the lungs. Unlike shipping fever of cattle, *P. multocida* and *P. haemolytica* play roles of equal importance in causing systemic pasteurellosis of sheep (Biberstein and Kennedy, 1959).

Pasteurella pneumonia of sheep is more important in temperate climates than in the tropics. However, *P. haemolytica* is considered to be a more common cause of this disease than *P. multocida*. This comment especially applies to lambs where *Pasteurella pneumonia* occurs principally within the first month of life and is the cause of considerable loss of animals (Marsh, 1953; Carter, 1976).

Nevertheless *P. multocida* does play a not-insignificant role in causing pneumonia in lambs. This was demonstrated by Stevenson(1969) who used *P. multocida* in combination with mycoplasmas to produce pneumonia in stressed lambs. However, he could not produce the disease in stressed lambs using *P. multocida* alone nor in normal lambs using either combination (Stevenson, 1969). This suggests that other organisms and stress are important in predisposing the animal to disease caused by *P. multocida*.

The above discussion does not imply that *P. multocida* and *P. haemolytica* are not isolated together from sheep pneumonias. *P. multocida* is often isolated from sheep pulmonary lesions in conjunction with *P. haemolytica*, however in line with the general rule with sheep pneumonia *P. haemolytica* usually predominates (Hamdy, Pounder and Ferguson, 1959).

Belschner[1951] (see Marsh, 1953) isolated *P. multocida* from the lungs of sheep which had died from pleuropneumonia. He concluded (incorrectly) that the organism was the aetiological agent of this disease. Since this work it has been established that *Mycoplasma mycoides* is the agent of pleuropneumonia. However, Belschner's conclusions do illustrate the importance of *P. multocida* as a secondary disease causing agent. This is enhanced by St George(1972) who demonstrated the presence of *P. multocida* in pneumonic lungs of Australian sheep which had died of 'Summer Pneumonia'. It is now generally accepted that *P. haemolytica* is the agent of this disease. Notwithstanding this, Tadayan, Cheema and Muhammed(1980) isolated *P. multocida* from lung abscesses in Iranian sheep. This suggests that *P. multocida* nonetheless can cause primary lung disease in these animals.

From the above discussion it can be appreciated that *P. multocida* is associated with sheep diseases in many countries. However the role of *P. multocida* in New Zealand flocks, although suspected, is poorly understood. Alley(1975) surveyed the respiratory tract flora of both

normal and pneumonic sheep. Despite demonstrating the presence of a heterogeneous bacterial population he did not report the presence of *P. multocida*. Salisbury(1957) and Downey(1957) isolated a *P. multocida*-like organism from sheep suffering from enzootic pneumonia (ie chronic non-progressive pneumonia [CNP]). However it is more probable that their isolates were *P. haemolytica* since this organism is almost ubiquitous in CNP lesions (Prince, 1985).

Note: A problem in interpreting early literature on *P. multocida* is that in many accounts the identification of the species is not sufficiently clear to differentiate the organism from other *Pasteurella* species, in particular *P. haemolytica*. For this reason many of the earlier reports of *P. multocida* should be treated with a degree of scepticism.

1.5.4 Goats

The main diseases of goats caused by *P. multocida* are pneumonias and systemic pasteurellosis. Infections occur world-wide although it would seem from the literature that a higher prevalence occurs in tropical countries. Pande(1941) isolated an organism which fitted the description of *P. multocida* from the lungs of many goats which had died from pleuropneumonia in India. He cited earlier outbreaks which had decimated goat herds in the area and suggested that *P. multocida* was the causal agent. However since 1941 *Mycoplasma mycoides* has been accepted as the cause of pleuropneumonia. It is therefore likely that the organisms described by Pande(1941) represent secondary invaders associated with the primary mycoplasmal disease.

Borgman and Wilson(1955) showed that *P. multocida* could be isolated from a large proportion of lungs from goats which had died from pneumonia. The disease picture is similar to that which affects sheep (Borgman and Wilson, 1955). Usually a few animals die suddenly

due to a bacteraemia. This is followed by clinical evidence a lung infection within other members of the herd. The main signs are lethargy, anorexia, a mucopurulent discharge from the eyes and nose, cough, dyspnoea and an elevated temperature. The disease takes one to two weeks to run its course. Deaths occur from the onset of clinical signs with mortality rates averaging approximately 10% of the infected herd (Borgman and Wilson, 1955).

The finding that *P. multocida* can be isolated from a large proportion of goats which die from pneumonia or systemic pasteurellosis suggests that, like cattle and sheep, *P. multocida* is a commensal of the upper respiratory tract and is also an opportunistic pathogen. This would imply that a large proportion of normal animals would carry the organism in their nasal cavity. This is supported by Ngatia et al (1985) who surveyed the nasal flora of clinically normal goats in the United States. They isolated *P. multocida* from approximately half of the animals studied. Furthermore, they showed that *P. multocida* is one of the most predominant bacteria found in the normal nasal flora in the sense that it can be recovered from a high proportion of animals.

The above discussion suggests that the types of diseases associated with *P. multocida* in goats are similar to those found in sheep. However, much of the literature has been taken at face value and as with sheep many earlier reports of *P. multocida* as a cause of disease have confused the role of this organism (usually a secondary invader) with that of the primary pathogen. eg pleuropneumonia is caused by *Mycoplasma mycoides*. Furthermore, early identification procedures did not take into account the the large numbers of *Pasteurella* and related species which are now recognised. Consequently some reports of a *P. multocida*-like organism may have actually referred to *P. haemolytica* or a *Yersinia* spp. Nonetheless *P. multocida* is clearly an important pathogen of goats especially in tropical countries. The organism has not been reported as a problem

in New Zealand but with the growing emphasis on farming goats here this could change.

1.5.5 Rabbits

Rabbits are extremely susceptible to *P. multocida* infections and the organism is responsible for economic loss in production units and complications of laboratory research projects (Percy, Bhasin and Rosendale, 1986). A number of clinical manifestations occur including 'snuffles', pneumonia, otitis media, conjunctivitis, pyometra, orchitis, subcutaneous abscesses and septicaemia (Flatt, 1974; Carter, 1976; DiGiacomo et al, 1987; Hagen, Gorham and Flatt, 1976).

The most common form of pasteurellosis in rabbits is snuffles. This disease is characterised by a serous or mucopurulent nasal exudate. Rhinitis develops after two weeks from the initial nasal infection. It is a common but surprising finding that preweanlings are not affected, even if the doe is infected (Giacommo, Garlinghouse and Van Hoosier, 1983). This apparent resistance of preweanlings seems to be unique to rabbits.

P. multocida exists as an upper respiratory tract commensal in a high proportion of clinically normal rabbits. If resistance is lowered, for example due to stress related experimental use, infection can spread to other parts of the body resulting in clinical manifestations (Flatt, 1974).

From the upper respiratory tract *P. multocida* infection can spread to the lungs and cause acute pneumonia. This often leads to death due to septicaemia which is responsible for between five and fifty percent of deaths in overseas rabbit colonies (Flatt, 1974; Manning et al, 1986).

Spread of *P. multocida* to the middle ear leads to otitis media. This can be complicated by further spread to the inner ear [otitis interna]. This infection is characterised by head tilt with an associated loss of balance and is called torticollis or colloquially as wry-neck). An animal in this condition is difficult to treat with antibiotics due to the inaccessibility of the site of infection (Flatt, 1974). Untreated cases often lead to death due to starvation because the animal is unable to orientate itself to eat.

1.5.6 Swine

P. multocida causes a variety of diseases in swine. The more important of these is atrophic rhinitis [AR], secondary pneumonia and swine plague. Next to the mycoplasmas, *P. multocida* is the most common organism isolated from pneumonic pig lungs (Gois, Kukea and Sieak, 1980). In New Zealand *P. multocida* has been isolated from cases of swine abortion Carter[1972] (see Woodgyer, 1975).

AR in swine is characterised by degeneration of the nasal turbinates resulting in snout deformity. It is of considerable economic importance overseas, especially in Europe (Pederson and Nielsen, 1983). A mild form of AR has been detected (Hodges, 1981) in New Zealand, however there is no report of the isolation of *P. multocida* from pigs with this condition.

Overseas research has shown that the presence of AR in a herd correlates with the presence of *Bordetella bronchiseptica* and/or toxigenic strains of *P. multocida* [the toxin of *P. multocida* was discussed in Section 1.4] (De Jong and Akkermans, 1986; Rhodes et al, 1987; Sawata et al, 1984; Lugtenburg, Van Boxtel and De Jong, 1984). *B. bronchiseptica* alone, or in concert with non-toxigenic *P. multocida*, causes a mild, transient turbinate atrophy known as 'regressive AR'. Toxigenic *P. multocida* causes a more severe progressive atrophy of the turbinates. This suggests that the toxin is responsible for the more severe lesions. This is supported by

Pederson and Barfod(1982) who instilled purified *P. multocida* toxin into the nasal cavities of pigs and produced lesions which were similar to those of progressive AR. The severity of the lesions produced was proportional to the amount of toxin given (De Jong and Akkermans, 1986; Pederson and Barfod, 1982; Pederson and Barfod, 1981; De Jong, Oei and Tetenburg, 1980).

Chemical irritation of the nasal mucosa of pigs prior to the introduction of toxigenic *P. multocida* results in a more rapid onset of clinical AR. This raises the possibility that *B. bronchiseptica* may cause a mild irritation which progresses to progressive clinical AR after infection by toxigenic *P. multocida*. While the importance of coinfecting agents remains to be clarified in detail, the mode of action of *P. multocida* toxin is to stimulate bone reabsorption and suppress osteoid synthesis (Gois, John-Barnes and Ross, 1983; Pederson and Elling, 1984). This explains the changes in gross snout morphology observed in this disease.

1.5.7 Deer

Little is known about pasteurellosis in deer but a haemorrhagic-septicaemia-like disease due to *P. multocida* has been described in reindeer (Nordkvist and Karlsson[1962], see Carter, 1967). The strains involved were not typed but from the nature of the disease it would seem that deer may be susceptible to the same *P. multocida* serotypes as cattle and these strains may cause similar diseases in both species.

1.5.8 Cats and Dogs

P. multocida disease in cats and dogs is not as common as it is in the animals mentioned earlier in this section. Nevertheless it has been isolated as a commensal of the mouth and throat of a large proportion of cats and dogs overseas (Carter and Bain, 1960; Carter, 1967). This would appear to be also the case in New Zealand cats

because Woodgyer(1975) has shown that 42% of a sample of New Zealand cats carry *P. multocida* in their mouths.

As a disease agent of cats and dogs *P. multocida* has been found associated with abcesses of both species, abortion in dogs and pneumonia in cats (Carter and Bain, 1960; Namioka and Bruner, 1962; Carter, 1967). Little work has been done in New Zealand to investigate disease caused by *P. multocida* in these animals. However, Carter[1972] (see Woodgyer, 1976) isolated the organism from a cat with pneumonia. This is a solitary isolation but it is nonetheless consistent with overseas findings.

1.5.9 Man

Although not common, human infections due to *P. multocida* are becoming more widely recognised. Infection does occassionally result from dog and cat bites so it is not surprising that biochemically and morphologically human strains tend to be very similar to feline strains (Talbot and Sneath, 1960) however the respiratory tract can become infected via other sources. The strains involved appear to be opportunistic pathogens producing local lesions only. While infection as a result of animal bites clearly represent a zoonosis patients suffering from respiratory tract infections are predominantly from farming backgrounds. This also suggests a zoonotic relationship (Carter, 1967; Rutter and Luther, 1984).

P. multocida has been isolated from one human sputum sample in New Zealand. Interestingly the patient had symptoms similar to those of tuberculosis (Miller, 1963). .

1.6 The Relationship of Type of *P. multocida* to Hosts and Diseases

This section considers the capsular Type (ie A, B, D, E, F), which alludes only to the capsular polysaccharide. It is to be

distinguished from the Serotype, which refers to the combination of capsular type and the somatic antigen (eg 1:A, 3:A, 3:F, 6:B, 6:E).

I. Relationship of Capsular Type to Disease.

Diseases due to *P. multocida* can often be correlated with the host animal affected and the strain Type (ie A, B, D, E or F) associated with the disease (see Table 1.3).

Table 1.3 Relationship of *P. multocida* Capsular Type to Disease.*

Type	Disease Association
A	: Fowl cholera. Primary and secondary infections in a wide range of species.**
B	: Haemorrhagic septicaemia of cattle and buffaloes. Swine plague.
D	: Primary and secondary infections in a wide range of animal species.**
E	: Haemorrhagic septicaemia of cattle in Central Africa.
F	: Primary disease of turkeys.

* Based on Carter(1976).

** See Table 1.2 for a list of species involved.

As illustrated above *P. multocida* is an important disease-causing agent. Nevertheless, the organism exists primarily as part of the normal flora and can be recovered from the nasal cavity of healthy animals. For example Types A, B and D strains are recoverable from the nasal cavities of healthy cattle. However the distribution of serotypes in the nose is not exactly paralleled by the relative prevalence of these types as disease causing agents. Thus in cattle

Type A is by far the most common type isolated from pneumonic lungs. This disease is predominantly found in temperate climates. On the other hand haemorrhagic septicaemia is a disease of cattle which were reared in tropical climates and is associated with strains of *P. multocida* Type B. The reason for this is unclear but *P. multocida* is an opportunistic pathogen and like many such pathogens it tends to cause disease following subjection of the host to "stress". It might plausibly be suggested that the stress associated with cold, wet weather predisposes the animal to the Type A related disease, which is pneumonia. Hot, dry weather on the other hand, might predispose the animal to the Type B related disease, haemorrhagic septicaemia.

II. Relationship of Serotype to Disease.

"Serotype" is defined as a the combination of capsular type with somatic antigen.

P. multocida strains which cause fowl cholera are all the same capsular type. ie Type A. However Roberts(1947), using the mouse protection test, established the existence of three immunological "types" associated with fowl cholera. The idea that several "types" of *P. multocida* could cause fowl cholera was reinforced by Heddleston[1962] (see Carter, 1967) who studied isolates using both an immunization-challenge test in chickens and a complement fixation test. His results demonstrated the existence of two "types" of *P. multocida* from cases of fowl cholera. These two "types" also differed biochemically in their abilities to ferment xylose and dulcitol.

Thus Roberts[1947] found that three serological "types" of *P. multocida* were responsible for fowl cholera, whereas Heddleston[1962] had proposed that only two "types" were involved. However all isolates were Type A strains.

Namioka (see Section 1.2.3) discovered different somatic antigens of *P. multocida* and gave them a number to distinguish them from (capsular) Types. It is reasonable to suggest that the differences in the avian isolates of Roberts[1947] and Heddleston[1962] lay in their somatic antigens. This was supported when Namioka and Bruner(1963) found that Serotypes 5:A and 8:A were the most prevalent isolates from fowl cholera. They further showed that these two serotypes equated to the two immunogenic types isolated by Heddleston[1962] (see Carter, 1967). Namioka and Murata(1964) reiterated the role of Serotype 5:A as a causal agent of fowl cholera and in addition refined Serotype 8:A to Subserotype 8a:A (8a being a subgroup of somatic group 8). They also added an additional Serotype [9:A] to compile a final list of three Serotypes causing fowl cholera. This conclusion is consistent with the earlier findings of Roberts[1947] (see above) who said that three different strains were involved.

It should be clear from the above discussion that fowl cholera is caused by certain Serotypes of *P. multocida* only. Interestingly correlations have also been seen between particular *P. multocida* Serotypes and the age and species of bird (domestic hen, turkey, duck etc) affected. For example Heddleston[1962] (see Carter, 1967) observed that his type 1 strain [equivalent to Carter Serotype 5:A] was pathogenic for 16-week-old and 45-week-old chickens. Type 3 [equivalent to Carter Serotype 8:A] was pathogenic for 16-week-old turkeys and 45-week-old chickens but was of low pathogenicity for 16-week-old chickens.

Since the work (see above) which demonstrated the involvement of certain Serotypes of *P. multocida* in causing fowl cholera, other workers have shown that such a relationship occurs with diseases in some other animal species (see Table 1.4).

Table 1.4 Relationship of *P. multocida* Serotype to Host and Disease.

Animal Host	Disease	Serotype	Reference
Fowl	Fowl Cholera	5:A 8a:A 9:A	Carter(1967), Namioka and Murata(1964)
Cattle	Haemorrhagic -Septicaemia	6:B 6:E	Shigidi and Mustafa(1979)
Swine	Pneumonia	1:A* 3:A 1:D 2:D 4:D	Carter(1967), Carter and Bain(1960), Namioka and Bruner(1963)
Rabbits	Respiratory Tract	12:A. 3:A 12:D 3:D	Giacommo, Garlinghouse and Van Hoosier(1983), Manning <i>et al</i> (1986)

* Predominant disease-causing Serotype.

In summary the identification of "Serotypes" on the basis of both the capsule and somatic antigens has allowed a more refined analysis of the relationship between this and disease produced in a variety of hosts. The above discussion indicates that some correlation exists between the Serotype of *P. multocida*, the species of host infected and the disease caused.

1.7 Immunity and Vaccines

Immunity to *P. multocida* is primarily humoral (Carter, 1967) and the antibodies produced are bactericidal (Walker and Foster, 1981).

The first *P. multocida* vaccine was accidentally produced by Louis Pasteur in 1878. This was in the form of a broth culture of (almost certainly Type A) organisms which had been left to stand at room temperature for such a length of time as to render the organisms non-infectious. This was discovered by Pasteur after he unsuccessfully tried to produce fowl cholera in chickens using the old culture. However, the significance of the discovery was not appreciated until he reinoculated the same fowl with a fresh virulent strain. The birds did not contract the disease and Pasteur correctly concluded that they had somehow been 'protected' by the old culture.

Active immunization of fowl with avirulent vaccines was continued for several decades after Pasteur's discoveries. However their popularity diminished because vaccines prepared, at least ostensibly, by Pasteur's method were not consistently effective (Carter and Bain, 1960).

Early attempts were made at passively immunizing animals against several *P. multocida* diseases especially shipping fever which requires only short term protection (Carter and Bain, 1960). However, this was discontinued due to a general lack of efficacy. In hindsight this is not surprising because only one type of *P. multocida* was used to produce the antiserum whereas the disease can involve another type of *P. multocida* or even be due to other organisms, eg *P. haemolytica*. Therefore the immunizing sera, which was serotype specific, was not protecting against diseases caused by other organisms.

Bain[1952] (see Carter, 1967) prepared a vaccine for haemorrhagic septicaemia (a disease of cattle in tropical countries) by blending organisms with mineral oil and lanolin. Good protection was obtained with a minimum of tissue damage. Bain stated that an oil adjuvant combined with a high number of organisms were both important factors in preparing vaccines to *P. multocida* (see Carter, 1967).

The demonstration by Bain[1952] (see above) of the importance of using an adjuvant in *P. multocida* vaccines led Heddleston and Hall[1958] (see Carter, 1967) to study several adjuvants for use with fowl cholera vaccines. They found that antigen emulsified in an oily adjuvant elicited greater protection than aqueous suspensions. This finding was similar to that of Bain's. Later Heddleston in association with Reisinger[1959] (see Carter, 1967) used mineral oil and arlacel as an adjuvant for a fowl cholera vaccine containing a single (Serotype 5:A) strain of *P. multocida*. This vaccine was found to be effective against challenge from the same strain of organism. However, when used in the field it was found to be ineffective due to the occurrence of "vaccine breaks". In hindsight, these "vaccine breaks" were not surprising because later Namioka and coworkers[1962-1964] (see Section 1.4) showed that fowl cholera was caused by three Serotypes of *P. multocida*. Although this information was not known to Heddleston and Reisinger they did nonetheless suspect the involvement of more than one strain in the aetiology of the disease. In 1960 (see Carter, 1967) they modified their vaccine by adding an extra strain of *P. multocida* (Serotype 8a:A). This vaccine was found to be effective in the field. They further modified this vaccine by substituting the oil-arlacel adjuvant with aluminium hydroxide. They found that this elicited protection for the same length of time as oil-arlacel (approximately one year) but did cause as much tissue damage (Carter, 1967). Consequently aluminium hydroxide has since become the adjuvant of choice for most *P. multocida* vaccines whether for fowl cholera or other diseases (Walker and Foster, 1981).

Heddleston and Reisinger's two-serotype-containing vaccine afforded protection in the field although it was later shown that three types of *P. multocida* are responsible for fowl cholera. That it was effective could suggest that the third type (Serotype 9:A) was not present in the areas where flocks were immunized. However Serotype 9:A was the last fowl cholera strain to be described (Namioka and Murata, 1964) which suggests that it is not as important as Serotypes 5:A or 8a:A in causing fowl cholera.

Since 1960 *P. multocida* vaccines have predominantly been 'bacterin' based. That is, made from killed cultures or components of killed cultures (Carter, 1967). The diseases protected against include fowl cholera, shipping fever and swine pneumonia. There are many reports of success or failure in immunizing animals against *P. multocida* using bacterins. However, as a general rule it can be said that bacterins are not usually effective unless used with an adjuvant. If successful, protection is usually only short term.

Until the last decade whole-cell *P. multocida* vaccines were relatively undefined. That is, they consisted of an inactivated cell suspension standardized on the basis of cell numbers and blended with an adjuvant. Such vaccines act by stimulating bactericidal antibodies which are directed against cell surface antigens and as such tend to be sero-specific.

If the number of serotypes of a pathogen is limited the formulation of a vaccine is relatively simple. However, when several serotypes are involved as is the case with *P. multocida* infections of some host species, multicomponent vaccines must be used. These vaccines may not be practical. For example if a high number of cells for each of several serotypes are added to a vaccine, toxic levels of endotoxin may be present or the required inoculum may be excessively large. It would therefore be advantageous to purify immunizing antigens and such a vaccine has been prepared for haemorrhagic septicaemia. Purified capsular polysaccharide is not immunogenic in

all animals (Penn and Nagy, 1976) but when purified from strains of *P. multocida* which cause haemorrhagic septicaemia, ie Serotypes 6B and 6E (the bovine-haemorrhagic-septicaemia strains), it has been shown to be immunogenic in cattle (Penn and Nagy, 1976). This has been exploited and a multi-strain vaccine containing the purified antigens (B and E) is now available commercially (Walker and Foster, 1981).

The haemorrhagic septicaemia vaccine discussed above is exceptional because With some other animal diseases, eg pneumonia in sheep (Cameron and Bester, 1984), an effective whole-cell multicomponent vaccine has been effective.

It has been shown that a combined infection of *Bordetella bronchiseptica* and dermonecrotic-toxin-producing *P. multocida* Type D will induce progressive atrophic rhinitis (AR) in pigs (see Section 1.5). Pedersen and Barfod(1982) vaccinated pregnant gilts with a whole-cell bacterin made from a DNT-producing *P. multocida* Type D. They demonstrated that both the gilt and its offspring were protected against AR. However, it has since been found that Type A strains of *P. multocida* can also produce DNT (Pi Joan et al, 1984) which suggests that an effective AR vaccine will need to be composed of more than one strain of *P. multocida*.

The desirability of producing an effective non-toxic vaccine for *P. multocida* led to at least one highly unorthodox approach (Eisenstein[1978] see Phillips and Rimler, 1984). This approach involved the extraction of ribosomes from the bacteria and using them as an immunizing antigen. Contrary to what might reasonably be expected, this was highly successful. However, in a more critical study of this approach, Phillips and Rimler(1984) reinvestigated the use of ribosomes and showed that when highly purified, their immunizing capacity was diminished or lost. However this immunizing capacity was restored not only to *P. multocida* ribosomes but also *Aspergillus* spp ribosomes and avian ribosomes provided that these

were exposed to trace amounts of *P. multocida* lipopolysaccharide (LPS).

With hindsight it is clear that the ribosomes act as a potent adjuvant for some surface antigens of *P. multocida*. While the original report that *P. multocida* ribosomes were an effective antigen to protect against *P. multocida* is at least (in one sense) nonsense, nevertheless it is correct in that they act as potent vaccines because they have a strong adjuvant effect on trace amounts of *P. multocida* LPS antigens. This approach therefore seems to be of potential use because ribosomes themselves are not toxic and the trace amounts of LPS needed to be immunogenic are not toxic at these levels.

More recently, ie within the last year, the possibility of a live *P. multocida* vaccine has been reexamined. The reader may recall that live (or at any rate not intentionally killed) *P. multocida* vaccines were used by Pasteur.

Shimizu et al(1987) used ultra violet and chemical mutagenesis to treat a strain of DNT-producing *P. multocida* Type D. They thus obtained a temperature sensitive (ts) mutant. Wild type strains grow between 30°C and 41°C but the mutant did not grow above 34°C. ie it would not grow at physiological temperature. This implies that the ts strain could be used as a live vaccine because it cannot grow in the host animal and consequently cannot cause disease. They then used this ts strain to immunize mice and found that the mice were protected against the wild type strain. This work was done only recently and as yet the approach has not been extended to the immunization of animals of greater commercial importance than mice. It is however a promising avenue.

Digiacoio et al(1987) used selective media to isolate a streptomycin dependent (Sm^d) strain of *P. multocida* Serotype 12:A. They then grew this strain in broth culture and administered it intranasally into

rabbits. Thirty days later they challenged half of the treated rabbits with the equivalent wild type. The remainder of the treated rabbits were challenged with streptomycin sensitive cells of a different Serotype (3:A). The rabbits treated with the wild type 12:A all developed a mild nasal infection which resolved after two weeks. Those challenged with the 3:A strain developed a chronic nasal infection which did not progress to the severe condition. Unvaccinated controls all developed severe pasteurellosis and some died.

These results suggest that the Sm^d strain may be effective at protecting rabbits against severe disease due to Type A serotypes of *P. multocida*. However to give complete protection the results would imply that a mixture of serotypes are necessary.

1.8 Antibiotic Resistance and the Presence of Plasmids

The amount of literature concerning the antibiotic sensitivities of *P. multocida* is vast (Carter and Bain, 1960) but the relationship of this to plasmid carriage has not been extensively examined. Chang and Carter (1976) demonstrated that multiple drug resistance is commonly found in strains of *P. multocida* isolated from cattle and swine. They showed that over 80% of isolates possessed resistance to one or more of the drugs Streptomycin, Penicillin, Tetracycline and Chloramphenicol. Resistance to Streptomycin was found to be the most frequent. Berman and Hirsh (1978) isolated a strain of *P. multocida* from fowl cholera of turkeys and showed it to be resistant to Tetracycline, Streptomycin and sulphonamides. They further demonstrated that it carried two resistance-carrying plasmids [R-plasmids]. One of these was shown to encode resistance to Streptomycin and sulphonamides. The other coded resistance to all three of the antibiotics. Both plasmids were shown to be non transmissible (Berman and Hirsh, 1978).

Hirsh, Martin and Rhoades(1985) surveyed many turkey strains of *P. multocida* for plasmids. Approximately 60% contained plasmids and 10% of these encoded resistance to Tetracycline, Streptomycin and sulphonamides. These R-plasmids were nontransmissible. These findings are similar to both those of Berman and Hirsh(1978) [see above] who also worked with *P. multocida* isolated from turkeys and Silver et al(1979) who demonstrated R-plasmids in strains of *P. multocida* isolated from cattle with pneumonia.

The above discussion shows that a significant proportion of *P. multocida* strains carry one or more plasmids. Furthermore a significant and growing proportion of these strains carry plasmids which encode resistance (R-plasmids) to Tetracycline, Streptomycin and Sulphonamides. These are the predominant antibiotics used for therapy and prophylactic treatment of diseases caused by *P. multocida*. This suggests that the evolution of resistant strains is continuing and in future they may become a major problem as the use of these antibiotics is continued. For example Sulphonamides have been added to chicken feed to prevent fowl cholera from becoming established in flocks. This has probably assisted the selection of strains of *P. multocida* which carry Sulphonamide resistance markers.

Woodgyer(1976) demonstrated that strains of *P. multocida* isolated from cats in New Zealand were not resistant to Streptomycin, Sulphonamide, Tetracycline, Chloramphenicol or Penicillin. This would appear to be the only report of antibiotic sensitivities being determined for *P. multocida* strains which have been isolated from New Zealand animals so it is not surprising that the presence of R-plasmids in New Zealand strains also has not been reported.

1.9 Isolation and Identification of *P. multocida*

Many strains of *P. multocida* are highly pathogenic for mice and rabbits. Pure cultures of the organism can be recovered from these animals after intraperitoneal inoculation (Pedersen and Nielsen, 1983). However this method is not as effective as using solid media for reasons which are discussed later (see Section 2.1).

The pasteurellae grow well on blood agar so this is the medium of choice for the isolation of *P. multocida* from clinical specimens. *P. multocida* is difficult to isolate if the medium used does not contain blood (Carter, 1984). However selective media have been described for the isolation of *P. multocida* from heavily contaminated materials (See Chapter 2).

P. multocida can, with experience, be identified, in mixed culture on solid medium. Typical colonies, grown on 5% horse blood agar, are 1-5mm in diameter after 48hrs growth at 37°C (see Figures 1.3 and 1.4). They are raised, greyish in colour, non haemolytic and usually possess a smooth to mucoid appearance, depending on the amount of capsule produced.

The Pasteurellaceae are facultatively anaerobic Gram negative coccobacilli. They are distinguishable from the other two families of this group [Enterobacteriaceae and Vibrionaceae] because they are oxidase positive, nonmotile and most species ferment glucose to acid but without gas production. The pasteurellas are distinguished from *Haemophilus* because they do not require the growth factors NAD and Haemin (Carter, 1984). The colonial morphology of the actinobacilli is similar to *P. multocida* and it can be difficult to distinguish between the two. However they can be differentiated because the actinobacilli possess a unique 'sticky' colonial texture (Smith, 1974).

The Genus *Pasteurella* is composed of six species (Carter, 1984). *P. multocida* is distinguishable from the other five species biochemically and the main distinguishing reactions are presented in Table 1.5. Colonies of *P. multocida* on blood agar have a distinctive odour useful in its identification.

Table 1.5 Differential Characteristics of *Pasteurella* Species.^a

Characteristics	species					
	M	P	H	U	A	G
β Haemolysis	-	-	+	-	-	-
Growth on McConkey Agar	-	-	+	-	+	-
Indole Production	+	+	-	-	-	-
Urease Activity	-	+	-	+	+	-
Acid from Lactose	-	v ^b	v ^b	-	-	-

^a based on Carter (1984)

^b 'variable reaction'

LEGEND

M: *P. multocida*

P: *P. pneumotropica*

H: *P. haemolytica*

U: *P. ureae*

A: *P. aerogenes*

G: *P. gallinarum*

CHAPTER 2

Isolation of *P. multocida* from the Nasal Cavity: Evaluation and Modification of a Selective Medium.

2.1 Introduction

P. multocida causes many diseases in different animal species (Carter and Bain, 1960) and it is possible to isolate the organism from several organs. Depending on the site of infection, it may or may not be associated with an excess of other bacteria. eg it may be present in pure (or nearly pure) culture and in high numbers in subcutaneous abscesses.

In contrast nasal swabs usually contain many species of bacteria in low numbers (Ngatia et al, 1985; Carter and Bain, 1960). These may include bacteria which are present in greater numbers or replicate more rapidly than *P. multocida*. Others (eg some motile species of *Enterobacteriaceae* and *Vibrionaceae*) can spread on the surface of an agar plate. The presence of such organisms in the nasal flora hampers the detection of *P. multocida* because they overgrow the medium (Morris, 1958; Smith and Baskerville, 1983; Knight, Paine and Speller, 1983). This problem can be solved by selecting for *P. multocida* *in vivo* or *in vitro*.

Encapsulated strains of *P. multocida* are pathogenic for mice so a pure culture may be obtained by intraperitoneal inoculation of nasal material. This method has several disadvantages. For example, if more than one strain of *P. multocida* is present it selects the strain which is most virulent for mice (Pedersen and Nielsen, 1983) and the large numbers of mice required for routine screening and isolation work makes this method cumbersome and expensive (Pedersen and Nielsen, 1983).

Various *in vitro* selective media for the isolation of *Pasteurella* species have been reported. These use a basal medium incorporating the selective properties of a high pH and antimicrobial chemicals including antibiotics (Carter, 1967; Morris, 1958; Smith and Baskerville, 1983; Knight, Paine and Speller, 1983; De Jong and Borst, 1985). A selective medium (8HPG) designed specifically for the isolation of *P. multocida* from the nasal tract of pigs has been described by Smith and Baskerville(1983). It is based on alkaline nutrient agar [pH 8.6] which also contains four antibiotics viz Mycostatin, 50Uml⁻¹; Bacitracin, 5Uml⁻¹; Polymixin, 0.2ugml⁻¹ and Gentamycin, 0.03ugml⁻¹.

For a selective medium to be effective it must have two main attributes: it must efficiently propagate the target organism but must suppress the background flora.

P. multocida colonizes many of the animal species which are found in New Zealand therefore an ideal selective medium should allow the isolation of the organism from all of these animals. To evaluate such a medium it is necessary to obtain isolates of *P. multocida* from a variety of animals, to test the selective medium's ability to efficiently propagate the isolates and to evaluate the ability of the selective medium to inhibit the growth of contaminating organisms, especially those present in the upper respiratory tract.

This chapter evaluates the selective medium of Smith and Baskerville(1983) and describes the development and testing of a modified medium for isolating *P. multocida* from New Zealand animals.

2.2 Materials and Methods

2.2.1 Preparation of Media

BA (Blood Agar)

See appendix.

BA + Individual Antibiotics

BA plates containing different concentrations of one of four antibiotics were prepared as follows:

Medium	Antibiotic Concentrations					
BA plus Mycostatin: [BA+M]	Uml ⁻¹	2.5	5	10	25	50
BA plus Bacitracin: [BA+B]	Uml ⁻¹	0.25	0.5	1	2.5	5
BA plus Polymixin : [BA+P]	ugml ⁻¹	0.01	0.02	0.04	0.1	0.2
BA plus Gentamycin: [BA+G]	ugml ⁻¹	0.0015	0.003	0.006	0.015	0.03

BA + 4 Antibiotics (BA+4)

Antibiotic Solution (see 8HPG Medium)

Liquefied BA

The 8HPG antibiotic solution was used to prepare 100% 'BA + 4 Antibiotic' plates. Dilutions of this antibiotic solution were used to prepare BA containing 5, 10, 15, 20, 25, 50, 75 percent strength media.

BHI Broth (Brain Heart Infusion)

See appendix.

8HPG Medium (selective medium as per Smith and Baskerville, 1983)

Mycostatin (Squibb)	:500 000U
Bacitracin (Sigma)	: 50 000U
Polymixin B Sulphate (Sigma)	: 2000ug
Gentamycin Sulphate (Sigma)	: 300ug
Distilled Water	: 100ml
Nutrient Agar (pH 8.6)	

- i. The antibiotics were dissolved in the distilled water and sterilized by filtration using a 0.2um filter.
- ii. This antibiotic solution was stored at +4°C in a dark bottle.
- iii. For use 1ml of the antibiotic solution was mixed with 99ml liquefied (50°C) nutrient agar. This was dispensed equally between four standard petri dishes.

Modified Selective Medium (SM)

A mixed antibiotic solution was prepared as follows:

Mycostatin (Squibb)	:500 000U
Bacitracin (Sigma)	: 50 000U
Gentamycin Sulphate (Sigma)	: 300ug
Distilled Water	: 100ml

1ml of this solution was mixed with 99ml liquefied (50°C) BA and dispensed between four standard petri dishes.

Nutrient Agar [pH 8.6]

Nutrient Agar [pH 7.4]

The medium [NA] (Difco) was prepared as per manufacturer's instructions. The pH was adjusted using 1M NaOH and the medium was dispensed into 100ml bottles and autoclaved. For use the medium was melted and 25ml aliquots were dispensed between four standard petri dishes as required.

N.B. Plates were stored at +4°C for not more than one week.

2.2.2 Preparation and Standardization of Cultures

Ten cultures [see below] of *P. multocida* from a range of hosts listed below were grown, assayed and stored using the following protocol:

Cultures

- | | |
|-----------|-----------|
| 1. Fowl | 6. Sheep |
| 2. Fowl | 7. Goat |
| 3. Bovine | 8. Cat |
| 4. Swine | 9. Rabbit |
| 5. Dog | 10. Swine |

- i. Cultures were removed from -80°C storage and propagated on BA for 12hrs at 37°C.
- ii. Colonies were transferred to 5ml BHI broth using a bacteriological loop. This was incubated at 37°C on an orbital shaker (at approximately 120rpm) until the culture had reached mid-logarithmic growth phase (ie O.D ≈ 0.2 at 500nm).

- iii. Sterile glycerol was added to give a final concentration of 20%. The mixture was mixed thoroughly and 1ml aliquots were stored at -80°C .
- iv. A 1x1ml aliquot from each of the ten cultures was used to establish the viable count on BA.

2.2.3 Evaluation of 8HPG Medium

- i. One culture from each of ten different stock cultures of *P. multocida* [see 2.2.2] was removed from -80°C storage and thawed.
- ii. The cultures were diluted in sterile BHI broth so that 50-100 viable organisms were contained in a known volume within the range of 20-100ul.
- iii. A volume [see ii.] from each of the ten cultures sufficient to produce 50 to 100 colonies on BA was inoculated on to the surface of ten of each of the following agar plates: 8HPG, BA, NA[pH 8.6], NA[pH 7.4].
- iv. Using a glass spreader the inocula were spread over the surface of the plates and left to dry.
- v. The plates were then inverted and incubated at 37°C for 48hrs and the colony counts recorded.

2.2.4 The Effect of Antibiotics on the Growth of *P. multocida* and the Suppression of Other Organisms

- i. The nasal cavity of ten New Zealand White rabbits (from a *P. multocida*-free colony) were individually swabbed with

cotton buds soaked in BHI broth. Each swab was placed in 5ml BHI broth and transferred to the laboratory.

- ii. The broths containing the swabs were agitated using a vortex mixer.
- iii. One culture from each of ten different stock cultures of *P. multocida* [see 2.2.2] was removed from -80°C storage and thawed.
- iv. The cultures were diluted in
 - a. sterile BHI broth and
 - b. rabbit nasal material containing broth (see ii.)so that 50-100 viable organisms were contained in a known volume of diluent within the range of 20-100ul, for each dilution of the ten cultures.
- v. A volume of each of the diluted cultures was then inoculated on to the surface of some or all of the following media: BA, BA+4, BA+M, BA+B, BA+P, BA+G.
- vi. The inocula were distributed over the surface using a glass spreader and the plates were left to dry.
- vii. The plates were then inverted and incubated 48hrs at 37°C and the colonies of *P. multocida* were counted.

2.2.5 Inoculation of Modified Selective Medium (SM)

- i. The nasal cavity of ten New Zealand White rabbits (from a *P. multocida*-free colony) were individually swabbed with cotton buds which had been soaked in BHI broth. Each swab was placed in 5ml BHI broth and transferred to the laboratory.

- ii. The broths containing the swabs were agitated using a vortex mixer.
- iii. One culture from each of ten different stock cultures of *P. multocida* [see 2.2.2] was removed from -80°C storage and thawed.
- iv. The cultures were diluted in
 - a. sterile BHI broth and
 - b. rabbit nasal material containing broth (see ii.)so that 50-100 viable organisms were contained in a known volume of diluent within the range of 20-100ul, for each dilution of the ten cultures.
- v. A volume of each of the diluted cultures was then inoculated respectively on to the surface of each of a BA plate and an SM plate.
- vi. Using a glass spreader the inocula were distributed over the surface of the plates and left to dry.
- vii. The plates were then inverted, incubated 48hrs at 37°C and colonies counted.

2.3 Results

2.3.1 Evaluation of 8HPG Medium

An initial experiment evaluated the efficiency of 8HPG medium for propagating a range of *P. multocida* isolates derived from different species. An identical inoculum of ten *P. multocida* isolates from eight different host species was plated on four different media (*viz* alkaline nutrient agar plus four antibiotics (8HPG), alkaline

nutrient agar (NA-A), neutral pH nutrient agar with neutral pH (NA), blood agar (BA)). The number of resultant colonies was expressed as a percentage of those growing on blood agar. The results are shown on Table 2.1. Note that only 1 of 10 isolates grew on 8HPG medium. More isolates (7 out of 10) grew on (NA-A) which represents 8HPG medium without antibiotics and more (9 out of 10) grew if a neutral pH was used in addition to no antibiotics (NA).

Table 2.1 The Ability of 8HPG Medium to Propagate *P. multocida**

Media	Isolate No.									
	1	2	3	4	5	6	7	8	9	10
8HPG	0	0	0	0	0	0	0	0	0	80
NA-A	0	0	15	55	10	50	0	35	20	75
NA	40	35	20	35	30	10	15	35	0	105
BA	100	100	100	100	100	100	100	100	100	100

*Results expressed as % growth [N° colonies rounded to the nearest 5%] compared to the number of colonies on BA.

Legend

Media

8HPG : Selective medium of Smith and Baskerville(1983)
(see Section 2.2.1)
NA-A : Nutrient agar [pH 8.6]
NA : Nutrient agar [pH 7.4]
BA : Blood agar

Origin of *P. multocida* Cultures

1. Fowl 6. Sheep
2. Fowl 7. Goat
3. Bovine 8. Cat
4. Swine 9. Rabbit
5. Dog 10. Swine

In a subsequent experiment the medium of Smith and Baskerville (1983) was modified by substituting blood agar at neutral pH for alkaline nutrient agar. This modified medium was tested using a series of dilutions of the mixed antibiotics for its ability to (a) propagate strains of *P. multocida* and (b) suppress other organisms. The results are shown on Table 2.2 and 2.3. In Table 2.2 it can be seen that an antibiotic concentration of 25% or less will not suppress the background flora whereas a concentration of 50% will suppress background flora but is inhibitory for 3 out of 10 isolates (note that [Table 2.3, isolate 6] even a concentration of 10% inhibited the growth of one sheep strain). We conclude that no dilution of the antibiotic mixture fulfilled the two necessary criteria (to propagate the target bacteria but suppress other organisms) for an effective selective medium. This led us to test the inhibitory effect of the individual antibiotics on *P. multocida* isolates.

2.3.2 The Effect of Antibiotics on the Growth of *P. multocida* on BA

An experiment was designed where the inhibitory effects of the four antibiotics were tested individually using the four most sensitive strains of *P. multocida* as test organisms. The results (Table 2.4) show that three antibiotics (Mycostatin, Gentamycin and Bacitracin) are not significantly inhibitory even at concentrations used in 8HPG medium. In contrast, no growth occurred in the presence of the fourth antibiotic (Polymixin) when this was present at the concentration ($0.2\mu\text{gml}^{-1}$) used in 8HPG medium. This antibiotic was inhibitory to one ovine strain of *P. multocida* even at $0.02\mu\text{gml}^{-1}$ (one tenth of the concentration used in 8HPG).

Table 2.2 The Effects of Different Levels of a Combination of Antibiotics on *P. multocida**

Isolate	-----Antibiotic Level-----									
	0%		25%		50%		75%		100%	
	P	N	P	N	P	N	P	N	P	N
1	100	og	75	og	85	70	50	55	0	0
2	100	og	105	og	100	90	105	90	85	85
3	100	og	65	og	0	0	0	0	0	0
4	100	og	105	og	0	0	0	0	0	0
5	100	og	65	og	75	50	70	65	50	45
6	100	og	0	og	0	0	0	0	0	0
7	100	og	90	og	15	10	0	0	0	0
8	100	og	90	og	85	75	90	75	0	5
9	100	og	20	og	20	15	0	0	0	0
10	100	og	110	og	80	75	80	80	90	85

*Results expressed as % growth [N° colonies rounded to the nearest 5%] compared to the number of colonies on BA.

Legend

Media

BA with % 8HPG antibiotic component as indicated

Scoring

og: Plate overgrown by background flora

Inocula

P: Pure broth culture of *P. multocida* containing 50-100cfu per inoculum
 N: *P. multocida* free rabbit nasal swab material seeded with 50-100 cfu *P. multocida* per inoculum

Origin of *P. multocida* Cultures

1. Fowl	6. Sheep
2. Fowl	7. Goat
3. Bovine	8. Cat
4. Swine	9. Rabbit
5. Dog	10. Swine

Table 2.3 The Effects of Low Levels of a Combination of Antibiotics on *P. multocida**

Note: These strains were the four most antibiotic sensitive as determined from Table 2.2.

Isolate	----- Antibiotic Level -----											
	0%		5%		10%		15%		20%		25%	
	P	N	P	N	P	N	P	N	P	N	P	N
3	100	og	95	og	100	og	90	og	85	og	60	og
4	100	og	100	og	100	og	100	og	95	og	95	og
6	100	og	70	og	0	og	0	og	0	og	0	og
9	100	og	95	og	100	og	90	og	90	og	20	og

Results expressed as % growth [N colonies rounded to the nearest 5%] compared to the number of colonies on BA.

Legend

Media

BA with % BHPG antibiotic component as indicated

Scoring

og: Plate overgrown by background flora

Inocula

P: Pure broth culture of *P. multocida* containing 50-100cfu per inoculum

N: *P. multocida* free rabbit nasal swab material seeded with 50-100 cfu *P. multocida* per inoculum

Origin of *P. multocida* Cultures

3. Bovine 4. Swine
6. Sheep 9. Rabbit

2.3.3 Evaluation of Modified Selective Medium (SM)

From the above results it was concluded that an effective selective medium might be composed of blood agar at a neutral pH containing three antibiotics (viz Mycostatin, Bacitracin and Gentamycin) at concentrations equal to that of 8HPG medium. ie with no Polymixin addition. This modified selective medium (SM) was evaluated by plating standard inocula of ten *P. multocida* test strains with and without nasal flora. The results are shown in Table 2.5 and Figure 2.1. It can be seen (Table 2.5) that for any test strain, at least 65% of the organisms plated on BA can be recovered on SM. That is, SM propagated *P. multocida* with an efficiency which we considered to be similar to that of BA. In a parallel experiment the same number of organisms (mixed with nasal swab material) was plated on BA and SM. It was found (Table 2.5) that no *P. multocida* colonies were identified on BA. The selective medium produced identifiable *P. multocida* colonies which were counted (Table 2.5). On the selective medium a number of the colonies represented the background growth of organisms which could not be readily quantitated because they were too small (see Figure 2.1). It should however be noted that in the selective medium which was heavily contaminated with nasal swab material at least 60% of the total number of *P. multocida* colonies present on blood agar were observed.

Figure 2.1 The Suppression of Nasal Bacterial Flora by a Selective Medium (SM) .

A nasal swab was taken from a rabbit and broken off into broth. A low concentration (enough to give approximately 50 cfu) of *P. multocida* was added and the preparation was plated on Blood Agar (left) and Selective Medium (right) and incubated for 48hrs at 37°C.

Note that the selective medium suppressed the background flora but permitted the growth of *P. multocida* colonies.



Table 2.4 The Effects of Individual Antibiotics at Different Concentrations on the growth of *P. multocida**

Isolate N°	Mycostatin Uml ⁻¹						Bacitracin Uml ⁻¹						Polymixin ugml ⁻¹						Gentamycin ugml ⁻¹ (x10 ⁻⁶)					
	BA	25	50	10	25	50	25	50	10	25	50	01	02	04	10	20	15	30	60	15	30			
3: Cattle	100	85	95	80	80	80	95	85	95	80	75	80	95	80	0	0	95	80	80	80	80			
4: Swine	100	80	90	90	80	80	80	85	95	95	95	75	95	95	0	0	95	80	80	80	80			
6: Sheep	100	50	95	90	95	75	95	75	75	95	95	75	0	0	0	0	95	75	95	90	75			
9: Rabbit	100	95	80	95	80	80	95	95	90	95	95	95	95	90	20	0	95	95	95	90	95			

* Results expressed as % growth [N° colonies rounded to the nearest 5%] compared to the number of colonies on BA.

Table 2.5. The Ability of SM Medium to Recover *P. multocida**.

Media	Isolate No.									
	1	2	3	4	5	6	7	8	9	10
BA	100	100	100	100	100	100	100	100	100	100
SM	95	90	90	100	90	65	100	90	65	65
BA*	cg	cg	cg	cg	cg	cg	cg	cg	cg	cg
SM*	60	75	85	95	75	60	85	85	60	65

*Results expressed as % growth [N° colonies rounded to the nearest 5%] compared to the number of colonies on BA.

Legend

<u>Media</u>	<u>Origin of <i>P. multocida</i> Cultures</u>
BA : Blood agar inoculated with pure culture	1. Fowl 6. Sheep
BA* : Blood agar inoculated with nasal material	2. Fowl 7. Goat
SM : Modified selective medium inoculated with pure culture	3. Cattle 8. Cat
SM* : Modified selective medium inoculated with nasal material	4. Swine 9. Rabbit
	5. Dog 10. Swine

Note: The numbers of colonies recorded for SM* plates did not include species of background flora which were not suppressed by the antibiotics. However it can be seen from Figure 2.1 that in spite of other organisms *P. multocida* could be distinguished.

2.4 Discussion

The detection of low numbers of *P. multocida* in the nasal cavity is difficult. To increase the efficiency of detection a selective medium can be used because it has two important properties: it

efficiently propagates the required bacteria and suppresses other organisms. Smith and Baskerville(1983) used a selective medium (8HPG). This had an nutrient agar base, was modified by adjustment to alkaline pH and contained four antibiotics. It was developed for use with pigs.

Our initial experiments investigated the relative ability of 8HPG and non-selective media to propagate (mainly New Zealand) isolates of *P. multocida* from a wide variety of animal species. The results (Table 2.1) indicated that 8HPG medium failed to grow 9 of 10 isolates tested but it should be noted that the only strain which grew was isolated from pigs. The other porcine strain did not grow. This data demonstrates that 8HPG medium is not satisfactory for the isolation of *P. multocida* at least from species other than pigs.

The results (Table 2.1) suggested that for the efficient isolation of *P. multocida* it was necessary to modify the medium by the addition of blood and by the use of a neutral pH. This combined change gave an increased efficiency of propagation of *P. multocida* from a standard inoculum (Table 2.1). These changes are clearly advantageous for propagating pure cultures of *P. multocida* but the medium is not inhibitory for contaminating organisms present in clinical material such as nasal swabs. However, when the four antibiotics present in 8HPG medium were added to this modified medium it suppressed not only the background flora but also most of the standard test strains of *P. multocida* (Table 2.2). Furthermore, when the mixture of antibiotics were diluted to 50%, 25% or even a lower concentration, they remained inhibitory for some strains of *P. multocida* but below a concentration of 50% failed to suppress the background flora. Thus, there was no concentration of the mixed antibiotic which was non-inhibitory for *P. multocida* and suppressed the background flora.

This led us to assess the effect of the antibiotics singly. In our hands three of the antibiotics (*viz* Mycostatin, Bacitracin and

Gentamycin) at the full concentration described for 8HPG medium did not significantly suppress the growth of any test strain of *P. multocida*. In contrast to this the fourth antibiotic (Polymixin) was highly inhibitory (see Table 2.4). These results led us to formulate a modified medium based on 8HPG. However this modified medium differs from 8HPG medium in three respects: It is composed of a blood agar base with a neutral pH and does not contain Polymixin. It resembles 8HPG medium in three respects: It contains Mycostatin, Bacitracin and Gentamycin at the concentrations specified by Smith and Baskerville(1983). This modified medium had only a marginal inhibitory effect on any of the test strains but suppressed the background flora present in nasal swabs taken from rabbits (see Table 2.5 and Figure 2.1).

CHAPTER 3

Isolation of *P. multocida*, Serotyping of Isolates and Comparison of These by SDS-PAGE and Restriction Endonuclease Analysis (REA).

3.1 Introduction

The range of host species colonized by *P. multocida* is unusually wide and the organism almost certainly causes significant disease in animals in New Zealand. Nevertheless the importance of *P. multocida* in this country has been little studied and isolates from different host species have not been compared. As a contribution to the assessment of *P. multocida* strains present in New Zealand we have, in this section, described the isolation of *P. multocida* from several species and compared the isolates with reference to the following properties:

- A. Serotypes.
- B. Their protein as demonstrated by SDS-PAGE.
- C. The cleavage of their DNA by Restriction Endonuclease Analysis.

3.2 Materials and Methods.

3.2.1 Isolation and Identification of *P. multocida*

3.2.11 Materials

Blood Agar (BA)

Brain Heart Infusion Broth (BHI)

Materials for Identification Tests

See appendix

Modified Selective Medium (SM)

See section 2.2.1

3.2.12 Methods

I. Isolation and Identification of *P. multocida* from Pig and Rabbit Lungs.

- i. Pneumonic lungs were obtained from pigs at slaughter and from angora rabbits which had died from pneumonia.
- ii. A small piece of pneumonic lung tissue was removed and rubbed over one quadrant of a BA plate. A bacteriological loop was then used to streak the inoculated material to obtain single colonies. The culture was incubated for 24hours at 37°C.
- iii. *P. multocida* was provisionally identified by colonial morphology, Gram and oxidase reactions and identification was completed as per Section 1.9).
- iv. *P. multocida* isolates were cultured in 5ml BHI broth for 24hrs at 37°C on an orbital shaker (set at approximately 120 cpm). Glycerol was added to a final concentration of 20% and the culture was stored at -80°C in 2ml aliquots.

II. Isolation and Identification from Rabbit Nasal Cavities.

- i. The nasal tract of angora rabbits was swabbed with cotton buds soaked in BHI broth.
- ii. The broths containing the swabs were agitated using a vortex mixer.

- iii. 1ml aliquots were inoculated on to one SM plate each. The inoculum was spread and left to dry.
- iv. The plates were incubated at 37°C for 48hrs and examined.
- v. *P. multocida*-like colonies were identified as described above.

III. Isolation and Identification from Cats and Dogs.

- i. Isolates already identified as *P. multocida* were supplied by the Ministry of Agriculture and Fisheries (MAF), Palmerston North as blood agar cultures streaked for single colonies.
- ii. Several colonies were restreaked on to BA and propagated for 24hrs at 37°C. The identification of the cultures as *P. multocida* was then confirmed as described above.

3.2.2 Procedures for the IHA Assay.

3.2.21 Materials

Alsevers Solution (pH 6.1)

Glucose	:20.5gl ⁻¹
Trisodium Citrate:	8.0gl ⁻¹
NaCl	: 4.2gl ⁻¹
Citric Acid	: 0.55gl ⁻¹
Distilled Water	

The pH was adjusted using 0.1M NaOH and the solution was autoclaved and stored at +4°C.

Formalin-PBS

Formalin was mixed with PBS to a final concentration of 0.4% and stored at +4°C in a dark container.

1.0% Glutaraldehyde-PBS

- i. 2ml of 50% Glutaraldehyde Solution (Sigma) was mixed with 98ml PBS [see appendix].
- ii. The solution was autoclaved and stored at +4°C.

Microtitre plates

Linbro Titretrek 12x8x0.25ml V bottomed plates. .

0.85% NaCl Solution

See appendix

PBS (Phosphate Buffered Saline)

See appendix

PBS-A Solution

Bovine Serum Albumin (BSA) (Sigma):0.25g
Sodium Azide (NaN_3) (Sigma):1g
PBS to 1000ml

The components were dissolved in the PBS, sterilized by filtration and stored at +4°C.

PBS-B Solution

This was prepared as per PBS-A Solution (see above) with the omission of BSA.

3.2.22 Methods

I. Production of *P. multocida* Antiserum in Hens.

Step 1: Preparation of Antigen (Bacterin).

- i. A culture was removed from -80°C storage and propagated 12hrs at 37°C on blood agar (BA).
- ii. Five dextrose starch agar plates were inoculated with colonies using a bacteriological loop. The inoculum was distributed over the surface using a glass spreader.
- iii. The plates were dried and incubated for 18hrs at 37°C .
- iv. Colonies were scraped from the plates using a sterile flat edged spatula, suspended in approximately three volumes of Formalin-PBS and incubated for 48hrs at 37°C with occasional mixing.
- v. A loopfull of the suspension was streaked on to a BA plate and incubated for 24hrs at 37°C to confirm that the culture had been sterilised.
- vi. The suspension was diluted with sterile PBS to a turbidity equal to M^CFarland's Standard #4.

- vii. The diluted suspension [referred to as a bacterin] was stored in 3ml aliquots at -20°C .

Step 2: Immunization Procedure.

- i. 1-2ml blood was collected from the wing vein of each of two domestic hens.
- ii. Each serum was separated, preserved (by adding NaN_3 (Sigma) to a final concentration of 0.1%) and stored at -20°C .
- iii. Each hen was inoculated both intravenously with 1ml bacterin and intramuscularly (0.5ml into each leg of each hen) with 1ml of antigen in FCA prepared as follows:

Freunds Complete Adjuvant	:1.5ml
Whole Cell Bacterin	:0.5ml
Purified Capsule* $[100\text{mgml}^{-1}]$:0.5ml

* Note: See section IV for preparation.

- iv. Intravenous injections of 1.0ml of bacterin were given twice weekly for up to three months or until an acceptable serum antibody titre was reached. An acceptable titre was defined as at least a 16 fold increase above the pretitre (see Section VI).

Step 3: Collection of Antiserum.

Hens were exsanguinated and the serum was separated and preserved by adding NaN_3 (Sigma) to a final concentration of 0.1%. It was then dispensed in 5ml aliquots and stored at -20°C .

II. Standardization of Antisera.

Serum antibody titres to Types A, B, and D *P. multocida* were measured by the following modification of the technique described in Section VI:

- i. Using PBS-A serial two-fold dilutions of a serum were made in triplicate in a microtitre plate. The dilution range used was from 2 to 2048 fold.
- ii. 25ul aliquots of Type A, B and D sensitized (ie antigen coated) GRBCs [see Section V] were added to one row of wells each.
- iii. The plate was rocked gently and the cells were left to settle [approximately two hours].
- iv. The titre of antiserum was defined as the greatest serum dilution which caused agglutination.
- v. Using PBS-B solution, an aliquot of each stock antiserum was diluted so that it had a titre of 8 units. ie caused agglutination up to a dilution of 1:8.
- vi. If any antiserum cross-reacted with a heterologous antigen it was absorbed with the heterologous antigen (before diluting) as follows:

Absorption of Serum by Heterologous *P. multocida* Strains.

The antiserum was mixed with a 1/10th volume saline-extracted capsule [see Section III] of the heterologous *P. multocida* antigen and was retested for specificity. If the cross reaction was not eliminated larger volumes of capsule were added until no cross reaction was observed.

III. Preparation of Saline Extracted Capsule.

- i. A culture was removed from -80°C storage and propagated 12hrs at 37°C on blood agar (BA).
- ii. Ten dextrose starch agar plates were inoculated with colonies of propagated culture using a bacteriological loop. The inoculum was distributed over the surface using a glass spreader.
- iii. The plates were dried and incubated for 18hrs at 37°C .
- iv. Colonies were scraped from the plates using a sterile flat edged spatula and suspended in approximately one volume of PBS.
- v. Hyaluronidase [Sigma] was added to give a final concentration of 200Uml^{-1} and the preparation was incubated at 37°C for 2hrs with occasional mixing.
- vi. The digest was heated at 100°C for 1hr, left to cool and centrifuged at $10\,000\times g$ for 20mins.
- vii. The supernatant was collected, preserved by adding NaN_3 (Sigma) to a final concentration of 0.1% and stored at $+4^{\circ}\text{C}$.

IV. Preparation of Purified Capsule.

- i. A culture was removed from -80°C storage and propagated on blood agar (BA) for 12hrs at 37°C .
- ii. 5ml BHI broth was inoculated with colonies using a bacteriological loop and incubated at 37°C on an orbital shaker (at approximately 120rpm) until the culture reached mid-logarithmic growth phase. That is, it had an optical density (OD) of approximately 0.2 at 500nm using a Spectronic 20.
- iii. 1l dextrose starch broth was inoculated with 4ml of the broth culture and incubated at 37°C for 18hrs on an orbital shaker (at approximately 120rpm).
- iv. NaCl was added to give a final concentration of 2%. Formalin was also added to give a final concentration of 0.4%.
- v. This preparation was incubated at 37°C for a further 24hrs and centrifuged at $10\,000\times g$ for 20mins.
- vi. The supernatant was retained and the pellet discarded.
- vii. Hyaluronidase [Sigma] was added to the supernatant to give a final concentration of 200Uml^{-1} . It was then incubated for 2hrs at 37°C in a water bath.
- viii. The digest was passed through filters of successively smaller pore diameter down to $0.45\mu\text{m}$.
- ix. The filtrate was concentrated twenty-fold using an Amicon filter (UM10). This was performed at $+4^{\circ}\text{C}$ using pressurized nitrogen (N_2).

- x. The concentrate was dialysed against several changes of distilled water, at +4°C for 5days.
- xi. Sodium acetate was added to give a final concentration of 1% (this aids the precipitation of antigens).
- xii. 3 volumes of methanol were added. The suspension was mixed and allowed to stand for 5mins at room temperature.
- xiii. The precipitate was removed by filtration using Whatman N^o1 paper and the filtrate retained. The precipitate (which contained most of the endotoxin) was discarded.
- xiv. 1 volume of acetone was added to the filtrate. The suspension was allowed to stand at 4°C for 12hrs. During this time a precipitate (capsular polysaccharide) formed, settled out and adhered to the surface of the tube.
- xv. The tube and contents were centrifuged at 10 000xg for 30mins.
- xvi. The supernatant was decanted and discarded. The centrifuge tube, which contained the precipitate (purified capsule) was then left at 37°C for 12hrs to dry.
- xvii. The precipitate was removed from the tubes by scraping out using a spatula, weighed and reconstituted in distilled water to give a 100mgml⁻¹ solution. This was preserved by adding NaN₃ (Sigma) to a final concentration of 0.1% and stored at +4°C.

V. Preparation of Sensitized Blood Cells.

Step 1: Preparation of GRBCs.

- i. 20ml of sheep blood was collected in a heparinized 'vacutainer'.
- ii. The blood was mixed with 100ml of cold Alsevers solution and centrifuged at 650xg at 8°C for 20mins.
- iii. The cells were washed six times by repeatedly suspending in 5 volumes of cold 0.85% NaCl solution and centrifuging as above.
- iv. The cells were then suspended in approximately nine volumes of sterile cold PBS to give an approximate 10% suspension.
- v. An equal volume of a 1.0% glutaraldehyde-in-PBS solution was added to the washed blood cell suspension. This effectively gave a 5% blood cell suspension.
- vi. This was incubated at +4°C for two hours with slow mixing.
- vii. The cells were centrifuged at 25°C for 10mins and then washed three times with cold sterile PBS.
- viii. After the final wash they were suspended in approximately nine volumes of PBS-B solution (to give an approximate 10% suspension) and stored at +4°C until required.

Step 2: Sensitization of GRBCs.

For each isolate

- i. 0.2ml of a 10% suspension of GARBCs [from Step 1] were mixed with an equal volume of a 25% suspension of saline extracted capsule [from Section III] in PBS.
- ii. The mixture was incubated at 37°C for one hour with slow mixing.
- iii. The cells were then washed three times in cold sterile PBS, centrifuging at 650xg for 10mins each time.
- iv. They were then suspended in 4ml PBS-A solution to give an approximate 0.5% suspension of sensitized cells and stored at +4°C until required.

VI. Procedure for the Indirect Haemagglutination Assay (IHA).

for each of Type A, B and D sera:

- i. Using a microtitre plate, 25ul of standardized antiserum (containing 8 haemagglutinating units) was diluted in two-fold steps using PBS-A solution to give a 1 in 8 dilution.
- ii. 25ul of sensitized GRBCs [see Section V] was added to each well. The plate was rocked and left to settle [approximately two hours].
- iii. After this time the capsular polysaccharide *P. multocida* isolate [see results] was determined from the pattern of agglutination.

3.2.3 Procedures for SDS-PAGE Analysis.

3.2.31 Materials

10% Acetic Acid Solution

A stock solution (101) was prepared and stored at room temperature. Used solution was recycled by passing through decolourizing charcoal.

Running Acrylamide

Acrylamide : 300.0g
Methylene-bis-Acrylamide : 5.0g
Distilled Water to 1000ml

- i. The acrylamide was added to approximately 70% of the Distilled Water and stirred until the solution returned to room temperature.
- ii. The Methylene-bis-Acrylamide was added and the preparation was made up to volume with Distilled Water, passed through one layer of Whatman N^o1 paper and stored at +4°C.

Stacking Acrylamide

Acrylamide : 300.0g l⁻¹
Methylene-bis-Acrylamide : 16.0g l⁻¹
Distilled Water

This mixture was prepared as per Running Gel (see below).

Ammonium Persulphate Solution

A 10% solution was prepared and kept for no longer than one hour.

Brain Heart Infusion Broth (BHI)

See appendix

Coomassie-Blue Reagent

Coomassie-Blue G-250 : 0.1g
95% Ethanol : 50.0ml
85% wv⁻¹ Phosphoric Acid : 100.0ml
Distilled Water to 1000ml

- i. The ethanol and phosphoric acid were mixed and then the dye was completely dissolved in the solution.
- ii. The mixture was made up to volume, passed through two layers of Whatman N^o1 paper and stored at room temperature.

Isopropanol Stain

Isopropyl Alcohol (Propan-2-ol) : 250ml
Acetic Acid (Glacial) : 100ml
Coomassie Blue R-250 (Brilliant Blue) : 0.4g
Distilled Water to 1000ml

- i. The Isopropyl Alcohol and Acetic Acid were mixed together.
- ii. The Coomassie Blue dye was dissolved completely in the mixture before making up to volume with Distilled Water and storing at room temperature.

SDS Sample Buffer

β -Mercaptoethanol :10.0ml
Upper Tris Buffer :25.0ml
SDS : 6.0g
Distilled Water to 100ml

The components were mixed and stored at room temperature.

10% SDS Solution

A 10% wv^{-1} solution of Sodium Dodecyl Sulphate (Lauryl Sulphate) (Sigma) was prepared in Distilled Water and stored at room temperature.

TEMED

N,N,N^1,N^1 -Tetramethylethylenediamine (Sigma)

Tracking Dye

Bromophenol Blue :0.10g
Glycerol :8.0ml
Distilled Water to 10ml

The components were mixed and stored at room temperature.

Tris-Glycine Reservoir Buffer (pH 8.3)

Trizma Base : 6.07g
Glycine : 28.8g
SDS : 2.0g
Distilled Water to 2000ml

The components were mixed and stored at room temperature.

Lower Tris Buffer (pH 8.8)

Trizma Base : 18.17g
10% SDS Solution : 4.0ml
Distilled Water to 100ml

This mixture was prepared as per Upper Tris Buffer (see below).

Upper Tris Buffer (pH 6.8)

Trizma Base : 6.06g
10% SDS Solution : 4.0ml
Distilled Water to 100ml

- i. The Trizma Base was added to approximately 90ml Distilled Water and stirred until the mixture returned to room temperature.
- ii. The pH was adjusted with 12N HCl and the mixture was brought up to volume and stored at +4°C.

3.2.32 Methods

I. Preparation of Samples for Protein Assay.

for each sample

- i. A 100ml BHI broth culture of *P. multocida* (which was in the mid-logarithmic phase of growth) was centrifuged at 10 000xg for 20mins, washed twice with cold PBS, and the pellet suspended in approximately one volume of cold PBS.
- ii. The suspension was placed in an ice bath and the cells were disrupted by sonic vibration at 70cps for 3mins.
- iii. The lysed cell suspension was stored at -20°C.

II. Protein Assay.

- i. The lysed cell material was removed from -20°C storage and a 0.1ml aliquot was diluted ten-fold, thirty-fold and one hundred-fold in 0.2M NaOH.
- ii. 0.1ml aliquots of the above dilutions were heated for 3 mins in a boiling water bath and left to cool.
- iii. 5ml of Coomassie-Blue Reagent was added to each of the 0.1ml samples and mixed.
- iv. The absorbance of each sample at 595nm was measured and their protein content calculated from a standard curve which was constructed from the absorbances of a range of Bovine Serum Albumen (BSA) (0-100ug/0.1ml) which had been assayed using the method described above.

III. Preparation of Gels. (170mmx130mmx15mm)

A) Mould Preparation

Gel moulds were prepared as per Ionas(1983). These consisted of two suitable sheets of glass held together by 15mm-thick perspex spacers coated with petroleum jelly.

B) Preparation and Pouring of Gel Components

Running Gel: A 10% acrylamide running gel was prepared as follows.

Lower Tris Buffer (pH 8.8)	: 5.0ml
Running Acrylamide	: 6.7ml
Distilled Water	: 8.3ml
Ammonium Persulphate Soln	: 0.1ml
TEMED	: 0.01ml

- i. The buffer, acrylamide and distilled water were mixed together by swirling so as not to create bubbles.
- ii. The ammonium persulphate and TEMED were mixed in to the mixture and an 11cm long gel was immediately poured by decanting sufficient into the prepared mould.
- iii. The mould was rocked to create an even surface on the top of the gel. A small volume ($\approx 1.0\text{ml}$) of distilled water was then distributed over the surface of the mixture and the gel was left to polymerize.

Stacking Gel: A 30% acrylamide/ 1.6% Methylene-bis-acrylamide stacking gel was prepared as follows.

Upper Tris Buffer (pH 6.8)	: 2.5ml
Stacking Acrylamide	: 1.5ml
Distilled Water	: 6.0ml
Ammonium Persulphate Soln	: 0.05ml
TEMED	: 0.01ml

- i. The buffer, acrylamide and distilled water were mixed together by swirling so as not to create bubbles.
- ii. The ammonium persulphate and TEMED were mixed in to the mixture and the surface of the (polymerized) running gel was washed with approximately 0.5ml of the mixture after the water overlay had been decanted. The mould was then filled with stacking-gel mixture to 5.0mm from its top, a 10 tooth comb was inserted to a depth of 10-15mm and left until the gel had polymerized.
- iii. The comb was carefully removed, the wells were then washed out with 10ml of Tris-Glycine Buffer and the bottom perspex spacer was removed
- iv. The assembly was attached to a discontinuous-buffer vertical electrophoresis tank (see Ionas, 1983) and the tanks were filled with Tris-Glycine buffer.

IV. Preparation of Samples for Electrophoresis.

- i. To a 100ul aliquot of lysed cell material was added 125ul of SDS Sample Buffer and 25ul Tracking Dye. The mixture was agitated using a vortex mixer and heated at 100°C for 3mins (to solubilize the protein).

- ii. The preparation was then cooled for 5mins at room temperature and centrifuged at 2000xg for 10mins at room temperature.
- iii. Using a micropipette, volumes of supernatant which contained 80ug* protein were added to the acrylamide wells (Section III).

* 80ug had previously been determined (results not shown) as the optimum loading of *P. multocida* protein for a 10% polyacrylamide gel.

V. Gel Electrophoresis and Staining.

- i. The loaded buffer tank was connected to a suitable power supply (anode to top tank).
- ii. The gel was electrophoresed at a constant current of 15mA until the tracking-dye front reached the stacking-gel-running-gel interface. The current was then reduced to 10mA until the dye front was 1cm from the bottom of the gel. That is, the dye front had electrophoresed across 10cm of running gel.
- iii. The power source was switched off and the gel was removed from the mould, immersed in Isopropanol Stain and left overnight on a shaker.
- iv. The gel was destained in 10% Acetic Acid solution and photographed using Kodak Techpan film.

3.2.4 Procedures for Restriction Endonuclease Analysis (REA).

3.2.41 Materials

0.2M EDTA

EDTA (di-sodium salt) :22.33g

Glacial Acetic Acid

Distilled Water to 300ml

- i. The EDTA was mixed with approximately 280ml Distilled Water, adjusted to pH 7.2 with Glacial Acetic Acid and brought to volume with Distilled Water.
- ii. The solution was sterilized by autoclaving and stored at +4°C.

Electrophoresis (E) Buffer 10x Concentrated

Trizma Base (Sigma) :96.88g

EDTA : 7.44g

Sodium Acetate : 8.20g

Glacial Acetic Acid

Distilled Water to 2000ml

- i. The Trizma Base, EDTA and Sodium Acetate were dissolved in approximately 1800ml Distilled Water and adjusted to pH 7.8 using Glacial Acetic Acid.
- ii. The mixture was made up to volume with Distilled Water and stored at +4°C. For use it was diluted 10X in distilled water.

Pronase 25mgml⁻¹ Solution (Sigma)

A solution was prepared in distilled water, incubated for 2hrs (to remove DNase activity and stored at -20°C.

Restriction Endonuclease (RE) Buffer

As prescribed by the manufacturers of the restriction endonuclease used.

Running Buffer

1M Tris-HCl	:0.10ml
0.2M EDTA	:0.05ml
Glycerol	:2.00ml
10% SDS Solution	:0.05ml
Distilled Water	to 10.0ml

The components were mixed and stored at room temperature.

10% SDS Solution

A 10% wv⁻¹ Sodium Dodecyl Sulphate (Lauryl Sulphate) (Sigma) solution was prepared with distilled water and stored at room temperature.

Saline-Tris-EDTA (STE) Buffer 10x Concentrated

5M NaCl Solution :20.0ml
1M Tris-HCl Buffer :50.0ml
0.2M EDTA : 5.0ml
Distilled Water to 100ml

The components were mixed, sterilized by autoclaving and stored at room temperature. For use it was diluted 10x*.

*Note: This dilution is taken into account with most of the buffer formulae described in this section.

Tris-EDTA (TE) Buffer

1M Tris-HCl Buffer :40.0ml
0.2M EDTA :20.0ml
Distilled Water to 4000ml

The components were mixed freshly for use.

1M Tris-HCl Buffer

Trizma HCl :47.28g
5M NaOH
Distilled Water to 300ml

- i. The Trizma HCl was mixed with approximately 280ml Distilled Water, adjusted to pH 7.5 with Glacial Acetic Acid and brought to volume with Distilled Water.
- ii. The solution was sterilized by autoclaving and stored at +4°C.

3.2.42 Methods

I. Extraction of DNA.

- i. A 50ml BHI broth culture of *P. multocida* which was in the late-logarithmic phase of growth (that is, had an optical density (OD) of approximately 0.6 at 500nm) was centrifuged at 13 500xg for 20mins, washed twice with 20ml cold PBS, and the pellet suspended in Saline-Tris-EDTA (STE) buffer to approximately 5ml .
- ii. The suspension was placed in a 15ml capped centrifuge tube, 100ul of a 25mgml⁻¹ solution of Pronase (Sigma), 200ul of a 10% SDS solution and 5 units of DNase-free Ribonuclease was added and the mixture was incubated overnight at 50°C in a water-bath.
- iii. 0.3ml of a 3.4M solution of sodium perchlorate was added to the tubes and reincubated for 1hr. During this incubation period a 25:24:1 phenol-chloroform-isoamyl-alcohol solution (Extraction Mixture) was prepared and equilibrated by adding 1/10th volume of STE buffer and standing for 30mins.
- iv. 5ml of Extraction Mixture was added to the treated cell suspension, the tubes were capped and then rocked until a milky emulsion was formed.
- v. The tubes were left to stand (vertically) for 10mins and centrifuged at 10 000xg at +4°C for 20mins.
- vi. Using serological pipettes the upper layer (aqueous) was removed, re-extracted until clear and dialysed against TE buffer at +4°C for two days.

- vii. The dialysed fraction was decanted into sterile 4ml bottles, incubated at 65°C for 10mins (to neutralize DNase) and stored at +4°C.

II. Assay of DNA.

- i. 1ml of a 1/20 solution of dialysed DNA in TE buffer was placed in a cuvette and its absorbance* at the following wavelengths was measured using TE buffer as a blank:
- 258nm. Absorbance of DNA.
270nm. Absorbance of Phenol.
300nm. Absorbance of Protein.

* Using a Cecil CE272 Spectrophotometer.

- ii. Suitably extracted DNA preparations were deemed to have 258/270 and 258/300 values of >1.0.
- iii. The DNA content of the preparation was calculated using the following formula*:

$$\frac{[\text{DNA mg ml}^{-1}] = \text{OD}_{258} - \text{OD}_{300}}{20} \quad \times \text{dilution factor.}$$

where OD = optical density at defined wavelengths.

20 = DNA absorbance constant (1mgml⁻¹ DNA has an OD of 20 absorbance units at 258nm).

*Brenner and Falkow(1971)

In the above case the dilution factor is 20 so the formula condenses to:

$$[\text{DNA mg ml}^{-1}] = \text{OD}_{258} - \text{OD}_{300}$$

III. Digestion of DNA with Restriction Endonuclease.

- i. A volume of DNA solution containing 5 μ g DNA was placed in a 2ml Eppendorf Microfuge tube with RE Buffer and mixed.
- ii. 5 U Restriction Enzyme (ie 1 U per 1 μ g DNA) was added, mixed by tapping the tube, pulsed in a Microfuge and incubated at 37°C for 45mins.
- iii. The tube was then incubated at 65°C for 10mins (to inactivate the enzyme) and cooled rapidly at -20°C (to prevent reannealing of the cut DNA).
- iv. A 1/20 volume of 5M NaCl was added and mixed by tapping the tube. 2 volumes of cold (-20°C) ethanol was added, mixed by inversion and left at -20°C for at least 2hrs for the DNA to precipitate.
- v. The tube was centrifuged for 10mins (in a Microfuge), the supernatant was decanted and discarded, the pellet washed once with cold ethanol and left for 30mins at 37°C to dry.
- vi. The DNA was solubilized by adding 45 μ l of Running Buffer and mixing by tapping the tube. It was then centrifuged for 10mins in a Microfuge and a 40 μ l aliquot of supernatant was removed, placed into a well in an agarose gel and electrophoresed.

IV. Preparation of 0.7% Agarose Gels.

- i. 0.7g Ultrapure Agarose (Bio-Rad) was mixed with 100ml E Buffer in a round-bottomed 250ml flask, boiled under reflux for 5mins on a heating mantle and left to cool to approximately 50°C.

- ii. Using Cellotape a gel mould was prepared by sticking a length of tape around the perimeter of a 20cmx16cm glass plate and sealing one face (ie the bottom of the plate).
- iii. The mould was levelled and the cooled gel mixture decanted in to it. Bubbles were removed using a hot wire and a 10 tooth (2mmx10mm) comb was pressed into on end of the gel and left to set.
- iv. The comb and Cellotape was removed. The plate and gel were placed in a continuous-buffer horizontal electrophoresis tank and E Buffer was added until the gel was submerged. DNA samples were added to the wells and the system was connected to a suitable power source (wells at anode end) for electrophoresis as follows.

V. Electrophoresis and Photographing Gels.

- i. A constant voltage of 80v was applied across the gel until the buffer front^{*} had travelled 15cm. The power source was disconnected and the gel visualized by sliding off the glass plate on to an ultra violet light box.
- ii. A permanent record was made by exposing with Tri-X film for 15secs at an aperture of 4.5 and developing as per manufacturers instructions.

^{*} This is taken as being RNA which has been placed in a spare well and visualized using long-wavelength UV light.

3.3 Results

3.3.1 Isolation of *P. multocida* and Some Epidemiological Aspects

Some strains of *P. multocida* were obtained from overseas culture collections. These prototype strains had been isolated from a range of animals (fowl, buffalo, pig, goat, dog and sheep). Three of these strains (fowl, buffalo and pig) were serotypes A, B and D respectively and were used to produce standard antiserum for the IHA assay (see Section 3.2.2).

To obtain field isolates from New Zealand animals we attempted to isolate *P. multocida* from a range of domestic animals and obtained a total of 72 isolates from five species. These were (with number of isolates in brackets): Dog (21), Cat (10), Deer (1), Rabbit (25) and Swine (15).

Canine strains were isolated from pre-mating vaginal swabs. Nine feline strains were isolated from wounds. The remaining feline isolate was from a pneumonic lung. The cervine strain was isolated from a lung. These dog, cat and deer strains were supplied to us by The Ministry of Agriculture and Fisheries (MAF) at Palmerston North. However most* rabbit and swine strains were isolated in the course of the present study.

* One rabbit and one swine isolate were supplied by MAF.

Isolates of *P. multocida* from Rabbits.

Isolates were obtained from three rabbit colonies located in different areas (Patea, Waitotara Valley and Upper Hutt). Healthy animals and animals which had clinical signs of respiratory disease were chosen at random and their nasal cavities swabbed. The swab material was plated on selective medium (see Chapter Two) for the isolation of *P. multocida*. If the animal had died from pneumonia a piece of pneumonic (ie consolidated) lung tissue was plated on blood agar.

P. multocida was isolated from the nasal tract of rabbits in all three colonies. The results (Table 3.1) show that the proportion of diseased rabbits which showed carriage of *P. multocida* was higher than that found in healthy animals. This difference was significant ($p < 0.01$) when the combined results of the three colonies were analysed. The difference was also significant for the Patea ($p < 0.01$) and Upper Hutt ($p < 0.025$) data when treated separately. The remaining data (Waitotara Valley) was not a large enough sample size to apply statistical analysis. Furthermore (results not shown), several of the Patea rabbits were suffering from torticollis, a symptom of inner ear infection which is associated with *P. multocida* (see Section 1.5.5). The organism was isolated from the nasal cavity of all of these animals.

Table 3.1 *P. multocida* Strains Isolated from Rabbits.

Origin of Strain	Date of Isolation	Condition of Animal	Animals Tested	PM* Positive
Patea	June 1987	Healthy	20	1
		Diseased	33	17
		Total	53	18
Waitotara Valley	June 1987	Healthy	3	0
		Diseased	1	1
		Total	4	1
Upper Hutt	August 1987	Healthy	43	1
		Diseased	13	4
		Total	56	5

* PM = *P. multocida***Porcine Isolates**

Swine strains were isolated from pneumonic lung tissue, obtained at slaughter (Kiwi Bacon Company), from pigs which came from at least five different farms in the Manawatu. Pneumonic lungs were sampled once in winter and once in summer. The results are shown on Table 3.2.

Table 3.2 The Proportions of Pneumonic Lesions from which *P. multocida* was Isolated in Winter and Summer.

Date Examined	Total N° of Pigs	Lungs with Lesions		Recovery of <i>P. multocida</i>	
		N°	%	N°	%
June 1986	300	20	7	3	15
December 1986	100	30	30	14	47

The proportion of lungs with lesions in December (30 out of 100) was significantly different ($p < 0.01$) than the proportion found in June (20 out of 300). The proportion of pneumonic lungs which contained *P. multocida* in December (14 out of 30) was higher than that found in June (3 out of 20) but this was not significant at the 95% level ($p < 0.1$).

3.3.2 Serotyping: Validation of IHA Assay and its Application to the Typing of Field Strains

Using the IHA assay (see Section 3.2.22) *P. multocida* isolates were serotyped. ie They were assigned to Type A, B, D or were regarded as Non-typable (NT). The results of serotyping are shown in the following two sections:

3.3.21 Validating the IHA Assay

We initially attempted to produce type-specific antiserum in rabbits. This was unsatisfactory (see discussion) but we were successful in producing typing serum in hens by the method described in Section 3.2.

Prototype strains (see Section 3.3.1) were examined by the standard IHA assay used to establish the serotype of isolates. The results are shown in Table 3.3.

Table 3.3 Serotyping Prototype Strains of *P. multocida* by IHA Assay.

Animal of Isolation	N° of Strains	Serotype	Result of IHA Serotyping
Bovine	1	B*	B
Fowl	2	A*, nk	A, A
Swine	2	D*, D	D, D
Sheep	1	nk	A
Goat	1	nk	D
Dog	1	nk	NT

Legend NT: Non-typable (see Section 3.3.2)
 nk: Serotype not known by supplier
 *: Strains used to produce standard antiserum for IHA assay

The antiserum prepared against the prototype strains were Type-specific (ie Anti-A serum reacted with Type A but not Type B or D antigen etc). We conclude that in our hands the IHA assay can distinguish A, B and D strains. The remaining four prototype strains (viz Fowl, Swine, Sheep and Goat) were found to be of the most probable serotype considering the species from which they were isolated. Thus, sheep and goats are known to be associated with A and D strains which is consistent with our results and furthermore, dog strains tend to be rough and therefore untypable. We conclude that although the number of strains of known serotype is small the results validate the IHA assay.

The IHA assay was then used to serotype field isolates of *P. multocida* (see Section 3.3.1). The results are shown in Table 3.4.

3.3.22 Serotyping of Field Isolates

Table 3.4 Serotypes of Field Isolates as Determined by the IHA Assay.

Host	N° of Isolates of Each Serotype				Total
	A	B	D	NT*	
Dog	2	0	0	19	21
Cat	3	1	1	5	10
Pig	0	0	4	11	15
Deer	0	0	1	0	1
Rabbit	0	0	0	25	25

* NT = Non-typable

These results are discussed later (see Section 3.4) but the finding that all (25) rabbit isolates are untypable was unexpected because the literature indicates that most rabbit *P. multocida* isolates are typable. However, our results might be explained if the isolates represented one or a few strains which had become disseminated. To investigate this possibility we examined and compared the proteins of rabbit isolates by SDS-PAGE.

3.3.3 SDS-PAGE Analysis of Strains of *P. multocida*

The protein patterns of a variety of microorganisms have been compared using SDS-PAGE but no work has apparently been done with *P. multocida* apart a limited study of membrane proteins of exotoxigenic strains from swine (Lugtenburg, 1984) and the characterization of lipopolysaccharides in some rabbit strains (Manning et al, 1986). If *P. multocida* was a homogeneous species with respect to protein patterns then the technique could be used to identify the organism. If isolates from a variety of host species were heterogenous between species but homogeneous within a species it could be used to identify the species of origin. If (as we initially and correctly assumed in the present work) isolates from a single species of host animal are heterogeneous it could be used to identify strains.

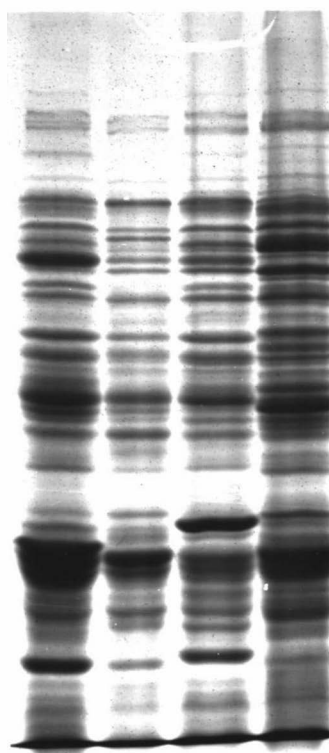
This section examined the 25 rabbit isolates by SDS-PAGE to establish if they represented few or many strains of the organism. However, to obtain an overall picture of the heterogeneity of *P. multocida* initial experiments examined strains from different host species.

One representative strain from each of four hosts was compared. The results are shown in Figure 3.1. It can be seen that some bands are common to all four strains. However the overall patterns show that *P. multocida* is heterogeneous when isolated from different species.

Figure 3.1 SDS-PAGE Protein Analysis of *P. multocida* Isolated from Several Animal Species.

The isolates (left to right) were obtained from a rabbit, pig, cat and dog.

Note that the isolates can be distinguished despite the presence of some common bands.



In further experiments (results not shown) some common bands were found between *P. multocida* and *P. haemolytica* so SDS-PAGE is not an appropriate method to use to identify the bacterial species.

Eight representative strains from each of three hosts (swine, dog and cat) were then examined. The results (Figures 3.2, 3.3, 3.4) show that strains isolated from within each of the host species are heterogeneous in that although many bands were common, each of the eight isolates had a unique overall pattern of bands.

Three strains of *P. multocida* which had been isolated from rabbits of three different colonies in New Zealand were examined. The results are shown in Figure 3.5 where it can be seen that the strains are different with respect to banding patterns. We therefore conclude that strains of *P. multocida* isolated from rabbits (like canine, feline and porcine strains) are heterogeneous. This led us to examine strains from within individual rabbit colonies.

Four representative strains of *P. multocida* from each of two rabbit colonies (Patea and Upper Hutt) were compared by SDS-PAGE. The results are shown in Figures 3.6 where it can be seen that strains isolated from the same rabbit colony were homogeneous. This finding has implications which are considered in the discussion (Section 3.4).

3.3.4 Epidemiological Tracing of *P. multocida* by SDS-PAGE

So far SDS-PAGE examination of *P. multocida* strains from rabbits was limited to nasal isolates. They were homogeneous within rabbit colonies but heterogeneous between colonies. It is generally assumed that during the course of disease *P. multocida* spreads from the nasal cavity to the lungs (particularly with rabbits in a stressed condition). This implies that the strains carried in the nasal cavity should be identical to those which colonize the lungs. This

Figure 3.2 SDS-PAGE Analysis of *P. multocida* Isolates from Swine.

Note that the isolates can be distinguished despite the presence of some common bands.

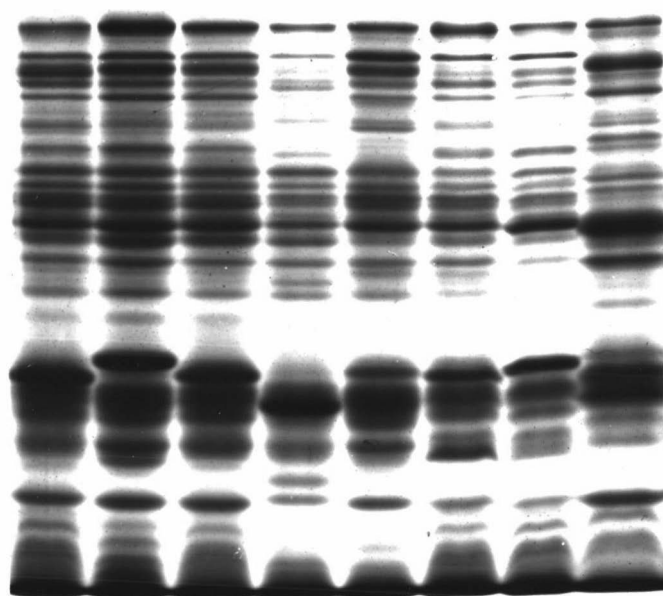


Figure 3.3 SDS-PAGE Analysis of *P. multocida* Isolates from Dogs.

Note that the isolates can be distinguished despite the presence of some common bands.

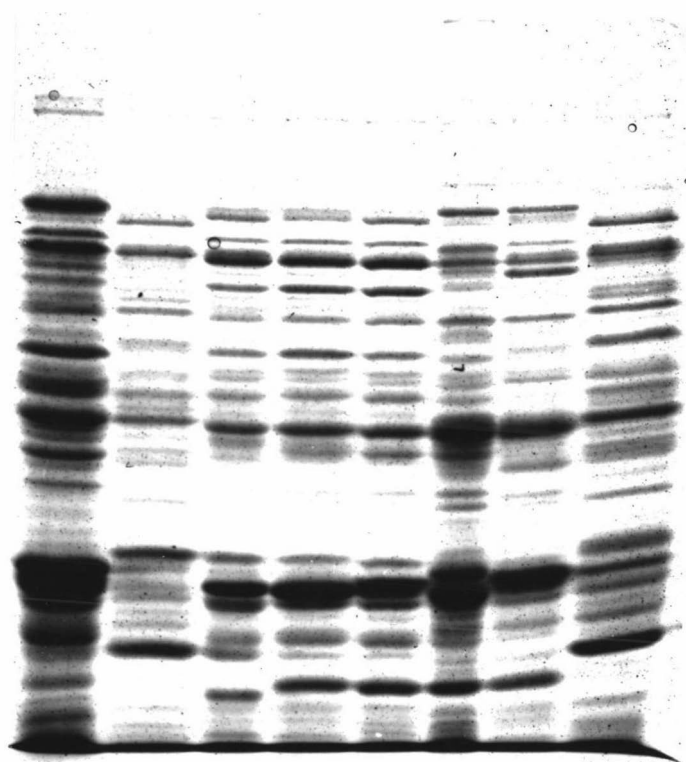


Figure 3.4 SDS-PAGE Analysis of *P. multocida* Isolates from Cats.

The isolates can be distinguished despite the presence of some common bands.

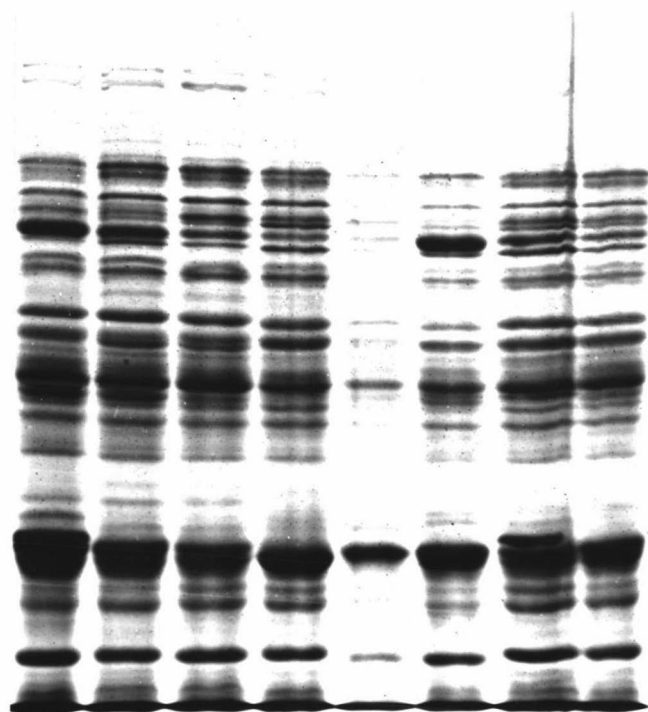


Figure 3.5 SDS-PAGE Analysis of *P. multocida* Isolates from Rabbits.

The isolates were derived from three colonies of rabbits from (left to right) Patea, Waitotara Valley and Upper Hutt.

Note that the isolates can be distinguished despite the presence of some common bands.

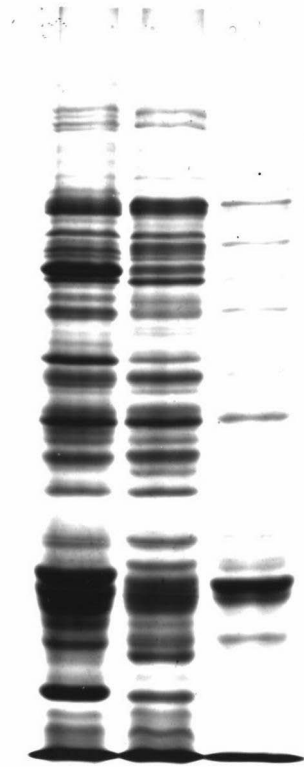
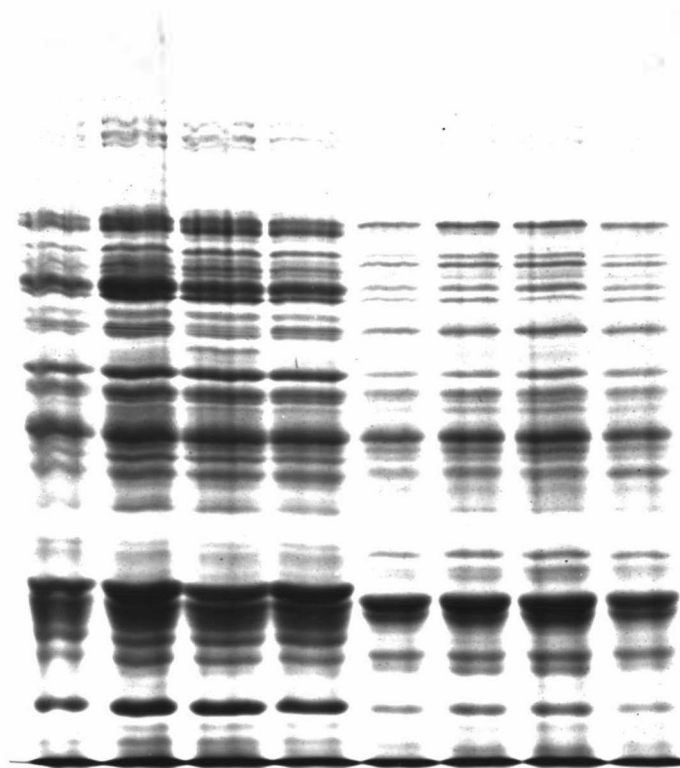


Figure 3.6 SDS-PAGE Analysis of *P. multocida* from Rabbits.

Several Isolates Derived from Rabbits in Each of Two Colonies are Compared.

Four isolates (left) were from a Patea rabbit colony and four (right) from an Upper Hutt colony.

Note that the isolates from any one rabbit colony cannot be distinguished but isolates from the two colonies are different.



was tested by comparing nasal and lung isolates derived from the same rabbit colonies.

We compared two nasal strains of *P. multocida* with two strains which had been isolated from the lungs of rabbits with pneumonia. The results (Figure 3.7) indicate that the nasal and lung strains are homogeneous with respect to their protein patterns so we conclude that they represent the same strain.

3.3.5 The Source of a *P. multocida* Isolate Associated with an Epidemic of Snuffles^{*}

The isolation of *P. multocida* from Patea rabbit nasal swabs coincided with an outbreak of snuffles. This outbreak was explained by the importation of a new batch of rabbits from Germany in March 1987. Since *P. multocida* is a heterogeneous species and varies between colonies in New Zealand it can be reasonably assumed that the pathogenic strain of *P. multocida* imported from Germany would be different to local strains. The MAF laboratories had previously isolated (September 1986) a strain of *P. multocida* from this colony (Patea). If the outbreak of snuffles was caused by the introduction of a new strain of *P. multocida* derived from the infected rabbits then it would be reasonable to assume that the MAF isolate obtained before importation would differ from the new strain derived from the imported rabbits.

Using SDS-PAGE we compared the protein pattern of the rabbit isolate with the patterns of three recent (March 1987) nasal isolates of *P. multocida* from the same rabbit colony (Patea). The results are shown in Figure 3.8. It can be seen that, contrary to expectations, the strains were homogeneous. This is discussed later (Section 3.4).

^{*} See Section 1.5.5

Figure 3.7 SDS-PAGE Analysis of *P. multocida* from Rabbits.

A comparison of lung and nasal tract isolates.

Four isolates, from one rabbit colony, are compared. Two isolates (left) were obtained from the lungs of rabbits which had died of pneumonia. The remaining isolates (right) originated from nasal swabs taken from rabbits with respiratory disease.

The lung and nasal tract isolates are indistinguishable.

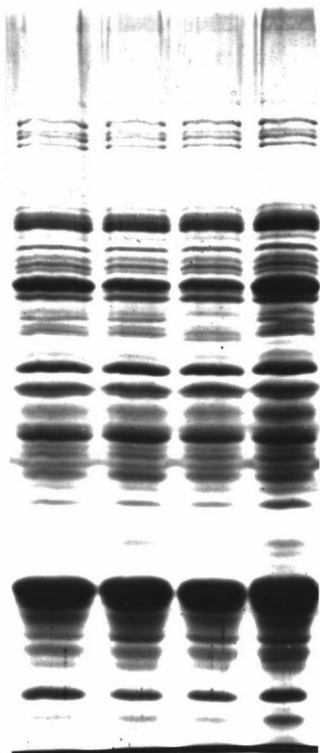
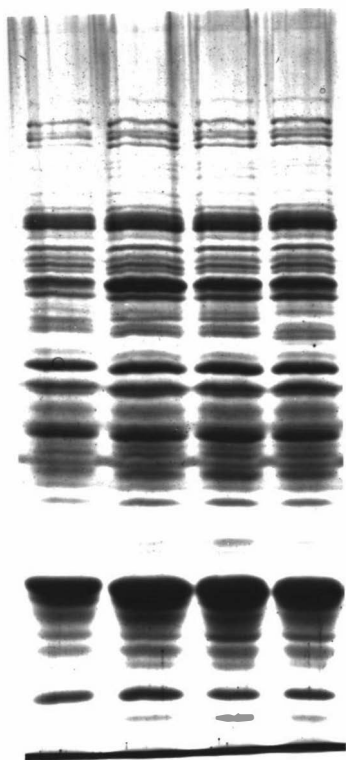


Figure 3.8 SDS-PAGE Analysis of *P. multocida* Isolates from Rabbits.

A comparison of isolates obtained before and after the introduction of animals, from overseas, to a rabbit colony.

Four isolates, from one rabbit colony, are compared. One isolate (left) was obtained from the lung of a rabbit, which had died of pneumonia, before the introduction of imported rabbits into the colony. The remaining isolates (right) originated from nasal swabs taken from imported rabbits which had contracted respiratory disease after introduction to the colony.

Note that all four isolates are indistinguishable.



3.3.6 Comparison of *P. multocida* Serotypes with SDS-PAGE and REA

The detection of the serotype of an isolate of *P. multocida* is a labour-intensive process. Isolates of *P. multocida* can be distinguished By SDS-PAGE and REA can be used to distinguish other organisms. With this in mind we examined serotypes of *P. multocida* to see if they could be correlated with either SDS-PAGE or REA patterns.

Using SDS-PAGE we examined representative strains of *P. multocida* Types A, B and D. The results are shown in Figure 3.9. It can be seen that some bands are common to all serotypes, however no bands were unique to any one serotype so we conclude that "serotyping" *P. multocida* by SDS-PAGE of proteins is not possible.

It is possible (even if not particularly likely!) that REA digest patterns could be correlated with *P. multocida* serotypes so we examined representative strains of *P. multocida* Types A and D by REA (see Section 3.2.4) using EcoR1. The result (Figures 3.10 and 3.11) show that although certain bands are common to all test strains of *P. multocida*, no band is unique to one serotype. This situation was also found using a different six-base cutting enzyme (Xho-1) (see Figures 3.12 and 3.13).

Since neither the SDS-PAGE or the REA pattern produced correlations in any obvious way with the serotype of *P. multocida* we conclude that *P. multocida* cannot be "serotyped" by either SDS-PAGE of proteins or by REA of DNA.

Figure 3.9 SDS-PAGE Analysis of *P. multocida* Serotypes A, B and D.

Note that the protein banding patterns cannot be related to their serotypes. It follows that the serotype of a new isolate cannot be established by examining the proteins by SDS-PAGE.

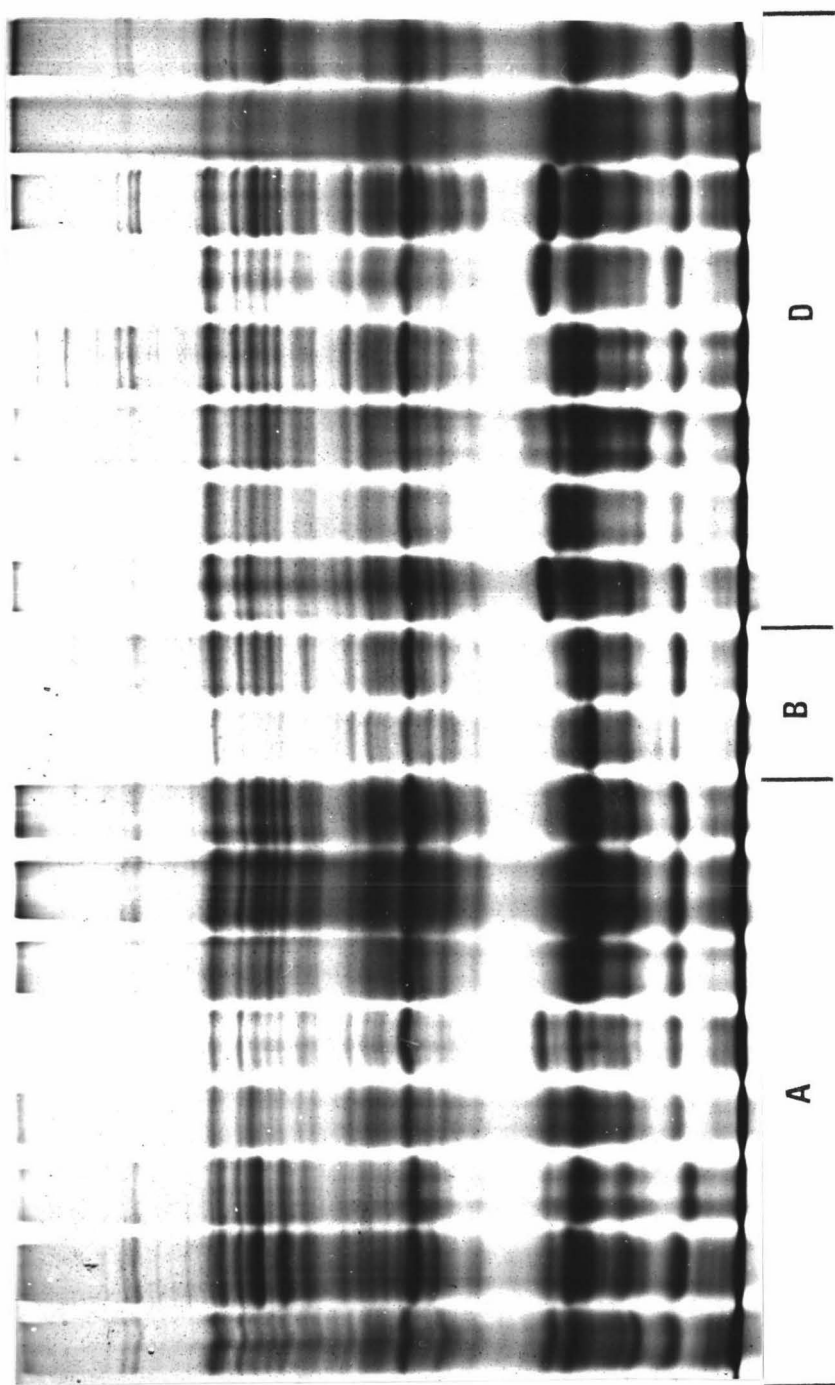
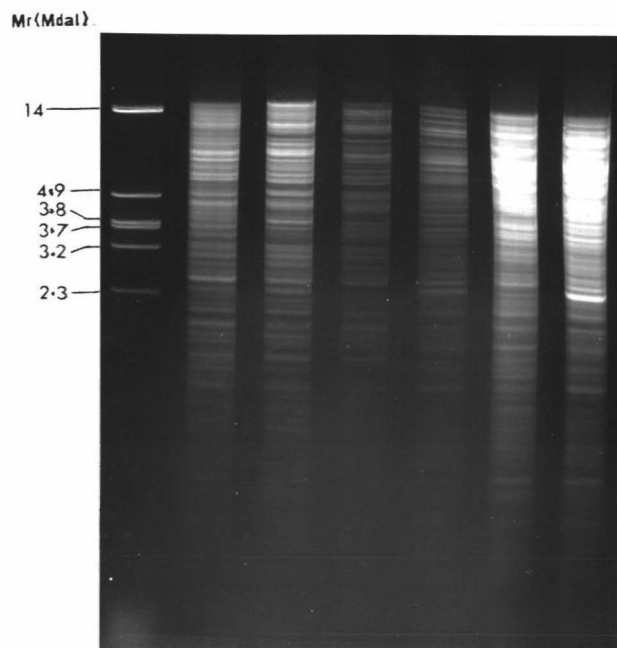


Figure 3.10 Restriction Endonuclease Analysis of *P. multocida*.

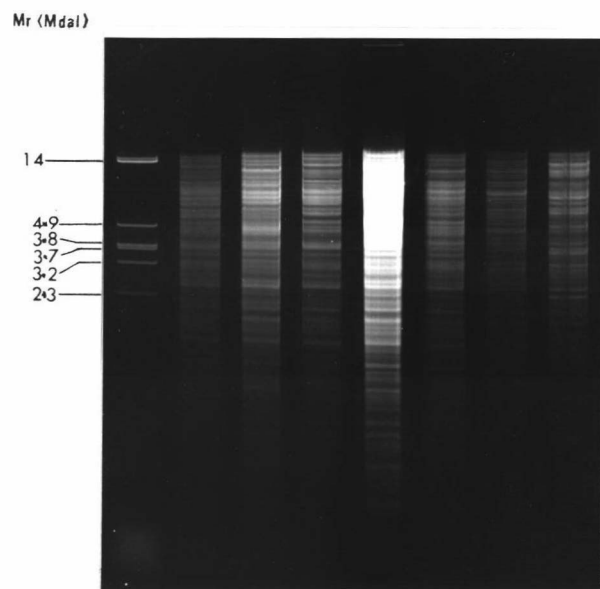
- a. A comparison of serotype A isolates using Eco R1.
- b. A comparison of serotype D isolates using Eco R1.
- c. A comparison of serotype A isolates using Xho 1.
- d. A comparison of serotype D isolates using Xho 1.

The left hand track in each photograph (digested DNA from Phage Lambda) represents molecular weight markers.

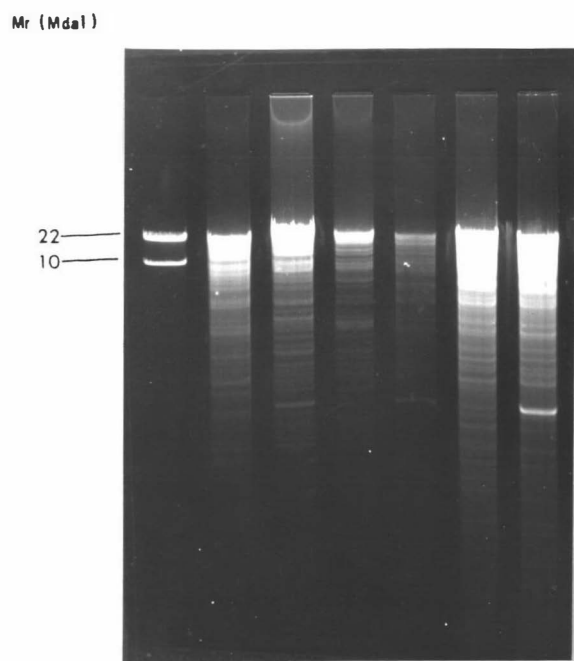
Heterogeneous patterns were obtained for all isolates. ie no two isolates were identical. This extreme heterogeneity indicates that the REA patterns cannot be correlated with the serotype. ie REA of the DNA gives no information about the serotype.



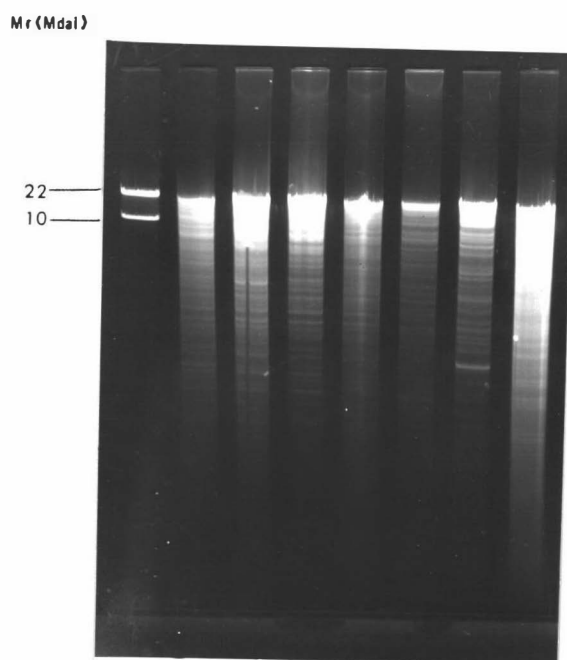
a



b



c



d

3.4 Discussion

P. multocida is an organism which causes disease in many species of animals. We isolated *P. multocida* from the species investigated so clearly *P. multocida* is ubiquitous in New Zealand animals. In our limited study the distribution of serotypes in New Zealand was similar to that found overseas and in rabbits at least, the strains which colonized the lungs were identical to nasal isolates.

The major problem in our hands was the production of type-specific antisera. Repeated attempts (data not shown) were made at producing antiserum in rabbits but with little success. This problem had also been encountered by Prince (1985) when she attempted to produce type-specific antiserum to *P. haemolytica*. She found that satisfactory antiserum could be produced in domestic hens although cross reactions occurred. Following the work of Prince (see above) we used domestic hens to produce satisfactory type-specific antisera to *P. multocida*. However we also obtained cross reacting serum but this was removed by absorption with heterologous antigens.

The hope that the SDS-PAGE and REA patterns could be correlated with the serotypes was not realised so at present there is no alternative to serotyping by the classical method.

The SDS pattern of individual isolates were so heterogeneous that individual strains could be differentiated. This allowed some epidemiological aspects of disease in rabbits to be studied. This is considered in the general discussion.

CHAPTER 4

The Detection of Toxigenic Strains of *P. multocida* Derived from New Zealand Animals.

4.1 Introduction

Atrophic Rhinitis (see Section 1.5.6) is a major cause of economic loss in European swine herds and a mild form has been recognised in New Zealand. The cause of this disease has been attributed to strains of *P. multocida* which produce a heat labile exotoxin. This exotoxin can be demonstrated *in vitro* because it produces a cytopathic effect (CPE) in Embryonic Bovine Lung (EBL) cells (see Section 1.4).

Exotoxigenic strains of *P. multocida* have not been isolated from New Zealand swine herds nor have they been isolated from other species in the world. It is not clear however if exotoxigenic strains are confined to pigs or whether the absence of reports of toxigenic strains in other species reflects the apparent absence of a systematic search for toxin producing isolates from other species.

This section establishes an *in vitro* assay for the exotoxin of *P. multocida* using a standard of exotoxigenic *P. multocida* and based on the method described by Rutter and Luther(1983). It has been further modified by using the method described by Sawata and Kume(1985) for preparing cell-free filtrates of the test organism. Using this assay strains which had been isolated from pigs in New Zealand, overseas strains and New Zealand strains other than swine were examined.

4.2 Materials and Methods

4.2.1 Preparation of Reagents and Media

Acidic Gentian Violet Solution

A 0.2% wv^{-1} solution of gentian violet in 2% acetic acid was prepared and stored at room temperature.

Bicarbonate Solution (4.4%)

Sodium Hydrogen Carbonate : 4.4g
Distilled Water to 100ml

The components were mixed and dispensed in 20ml aliquots. The lids were firmly tightened, the preparations were autoclaved and then stored at +4°C.

Blood Agar (BA)

See appendix.

BHI Broth

See appendix.

Bovine Serum

This was clarified, sterilized by passing through a 0.2 μ m filter and stored in 50ml volumes at -20°C.

Cell Culture Microtitre Plates

Falcon 96-well flat bottom disposable.

Eagles Base

This was prepared as per manufacturers (Gibco) directions from dri-form material (Gibco), sterilized by passing through a 0.2um filter and stored in 380ml aliquots at +4°C until required.

Minimal Eagles Medium (MEM)

4.4% Bicarbonate Solution :	20.0ml
TPB	: 40.0ml
PSK	: 1.0ml
Eagles Base	: 380.0ml
Amphotericin B (5mgml ⁻¹) :	0.2ml

Bicarbonate Solution, TPB, PSK, Eagles Base and were prepared separately (see individual headings).

All components were added to the Eagles Base.

N.B. The colour of the medium should be tangelo orange when used. If not then it is required to restore the correct pH by bubbling sterile CO₂ through the medium. Warm medium to 37°C before using.

Complete MEM Medium

This was prepared as per MEM with the addition of 40ml Bovine Serum (to give a final concentration of 10%).

Fixing Solution

A 12% solution of formalin in PBS was prepared and stored at room temperature.

Freezing Mixture

Complete MEM Medium : 33.5ml
Bovine Serum : 6.5ml
Dimethylsulphoxide (DMSO) : 10.0ml

The components were dissolved in the distilled water. The preparation was then sterilized by passing through a 0.2um filter and stored in 5ml volumes at -20°C.

Phosphate Buffered Saline (PBS)

See appendix

PSK (Pen/Str/Kan)

Penicillin : 1×10^7 IU
Streptomycin : 10g
Kanamycin : 10g
PBS to 1000ml

The components were dissolved in the PBS. The preparation was then sterilized by passing through a 0.2um filter and stored in 2ml volumes at -20°C.

Trypsin Versene Solution (TV)

NaCl : 0.8g
KCl : 0.04g
Glucose : 0.1g
NaHCO₃ : 0.058g
Phenol Red : 0.02ml
Trypsin : 0.05g
EDTA : 0.02g
Distilled Water to 100ml

The components were dissolved in the distilled water. The preparation was then sterilized by passing through a 0.2um filter and stored in 2ml volumes at -20°C.

Tryptose Phosphate Broth (TPB)

Tryptose (Difco) : 20.0g
Glucose : 2.0g
NaCl : 5.0g
Na₂HPO₄ : 2.5g
Distilled Water to 1000ml

The components were mixed, dispensed in 40ml aliquots, autoclaved and then stored at +4°C.

4.2.2 MethodsI. Cultivation of Embryonic Bovine Lung (EBL) Cells.

Note: An Embryonic Bovine Lung was kindly donated by Gibco (NZ) Ltd. This lung was stored for twelve hours (on ice) before use.

- i. A small portion of tissue (approx 5cmx5cmx1cm) was removed from a lobe of a foetal calf lung (the foetus was aged between one and two months from term).
- ii. The tissue was washed with MEM.
- iii. Small pieces (approx 5mm³) were removed, washed in fresh Trypsin Versene Solution (TV) and transferred to a 250ml conical flask which contained a small magnet ("flea").
- iv. The pieces were washed twice in TV. Fresh TV was added and stirred at 37°C using the 'flea' which was spun eccentrically (to aid disrupting the tissue) until the medium became turbid due to individual cells.
- v. The medium was then transferred, using a pipette, to a sterile centrifuge tube in an ice bath. The removed (turbid) medium was replaced with fresh TV and the process was repeated five times.
- vi. The pooled suspended cells were centrifuged for 10 minutes and the supernatant discarded.
- vii. The pellet was resuspended in approximately 30 volumes of Complete MEM Medium.
- viii. The resuspended cells were pooled. 5ml aliquots were dispensed into 50ml tissue culture flasks and incubated in 5% CO₂ atmosphere at 37°C.
- ix. Flasks were examined daily for the adherence and growth of the cells into monolayers.
- x. Monolayers not required for immediate use were passaged to obtain a stock of cells which were then stored (see next section) in liquid N₂.

II. Storage of Cultured Cells.

- i. The monolayer was trypsinized and the cells were suspended in 5ml Complete MEM Medium.
- ii. The cells were diluted to give a final concentration of $1 \times 10^7 \text{ ml}^{-1}$.
- iii. 0.5ml aliquots of Freezing Mixture were dispensed into sterile glass ampoules. These were placed on ice and 0.5ml of cell suspension was added to each.
- iv. The ampoules were sealed, placed in an insulated container which was placed for 12hrs at -80°C .
- v. The ampoules were then transferred to LN_2 .

III. Source of *P. multocida* Isolates.

Strains of *P. multocida* were obtained from a range of animals as described in Section 3.3.1. A total of 72 New Zealand isolates were obtained from five species. These were (with number of isolates in brackets): Dog (21), Cat (10), Deer (1), Rabbit (25) and Swine (15). In addition eight prototype strains were imported. These strains had been isolated from a range of animals (fowl, buffalo, pig, goat, dog and sheep). One porcine strain, known to produce exotoxin, was used as a positive control.

IV. Preparation of Cell-Free Filtrates of *P. multocida*.

- i. A culture was removed from -80°C storage and propagated on blood agar (BA) for 12hrs at 37°C .

- ii. Colonies were inoculated into 10ml of BHI broth and this was then incubated at 37°C on an orbital shaker (at approximately 120rpm) for 48hrs.
- iii. The cells were disrupted by sonic vibration for 10mins at 70cps and the preparation was centrifuged for 60mins at 10 000xg at 4°C.
- iv. The supernatant was collected, passed through a 0.2um filter and stored at +4°C for up to 48hrs.

V. Procedure for the *in vitro* Assay of *P. multocida* in Embryonic Bovine Lung Cells (EBL assay).

- i. A suspension of EBL cells containing between 1.5×10^5 and 2.0×10^5 cells.ml⁻¹ in Complete MEM was prepared.
- ii. *P. multocida* cell-free filtrates (see above) were diluted 10, 30, 100, 300 and 1000 fold using the diluted cell suspension as diluent. Toxin-free controls were included.
- iii. A 0.15ml aliquot of each dilution was dispensed into the wells of a cell culture microtitre plate and incubated at 37°C in a humidified-5% CO₂ atmosphere until monolayers were formed in the wells which represented negative controls.
- iv. The cells were then fixed, stained (see below) and examined microscopically for the presence or absence of a cytopathic effect.

Fixing and Staining EBL Cell Cultures

- i. Medium was removed from the plates.

ii. 2-3 drops of a solution containing equal volumes of Fixing Solution and Acidic Gentian Violet Solution were dispensed into each well and the plate was incubated for 2hrs at 37°C in a humidified container.

iii. The wells were washed with water and dried at 37°C.

4.3 Results

Validation of the EBL Assay and its Application to the Screening of Field Strains of *P. multocida* for Exotoxin Production.

Initially we attempted to produce a CPE in "sensitized" Vero cells using a known toxigenic strain of *P. multocida* (data not shown). This was unsuccessful but we were able to produce a positive reaction in cultured EBL cells using the procedure described in Section 4.2.

Using the EBL assay (see Section 4.2.2) *P. multocida* isolates were screened for exotoxin production. The results are shown in the following two sections.

4.3.1 Validation of the EBL Assay

Prototype strains (see Section 4.2, Part III) were examined by the EBL assay. The results are shown in Table 4.1.

Both porcine prototype strains caused a CPE in EBL cells (see Figures 4.1 and 4.2) although (results not shown) the positive control produced a higher titre. The isolates obtained from other species did not produce a CPE. It was concluded that the assay conditions used were adequate to detect *P. multocida* exotoxin.

Figure 4.1 Cell Culture Monolayer of Embryonic Bovine Lung Cells.

A cell-free extract of a non-toxigenic strain of *P. multocida* was added to the culture.

Note that the monolayer is intact after four days incubation. The cells are large with pale staining nuclei (compare with Figure 4.2).

40x Magnification.

Stained with Gentian Violet.

Figure 4.2 Cell Culture Monolayer of Embryonic Bovine Lung Cells.

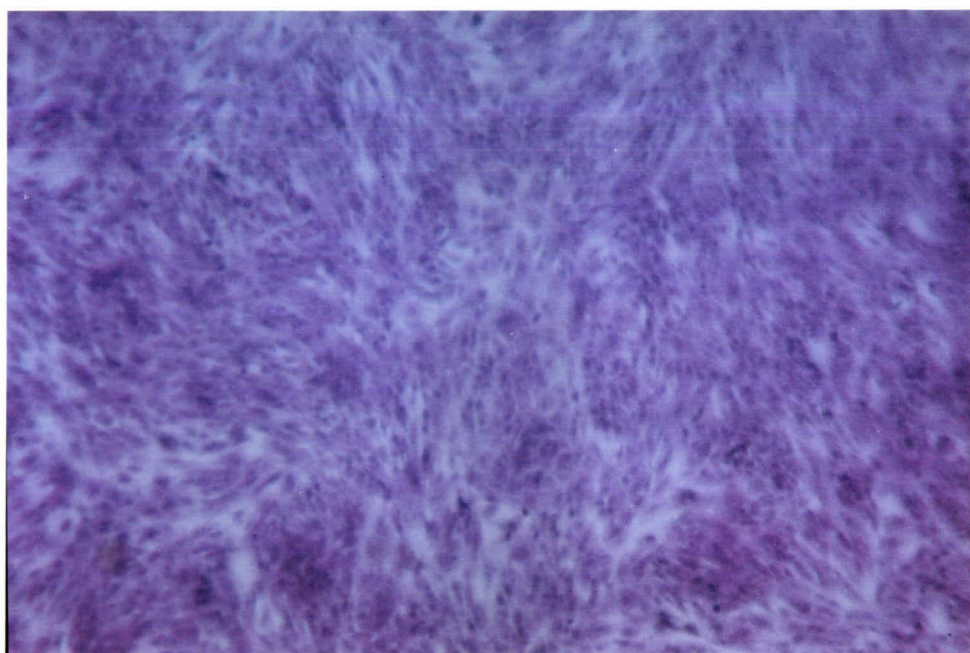
The cytopathic effect of *P. multocida* exotoxin.

A cell-free extract of a toxin-producing strain of *P. multocida* was added to the culture.

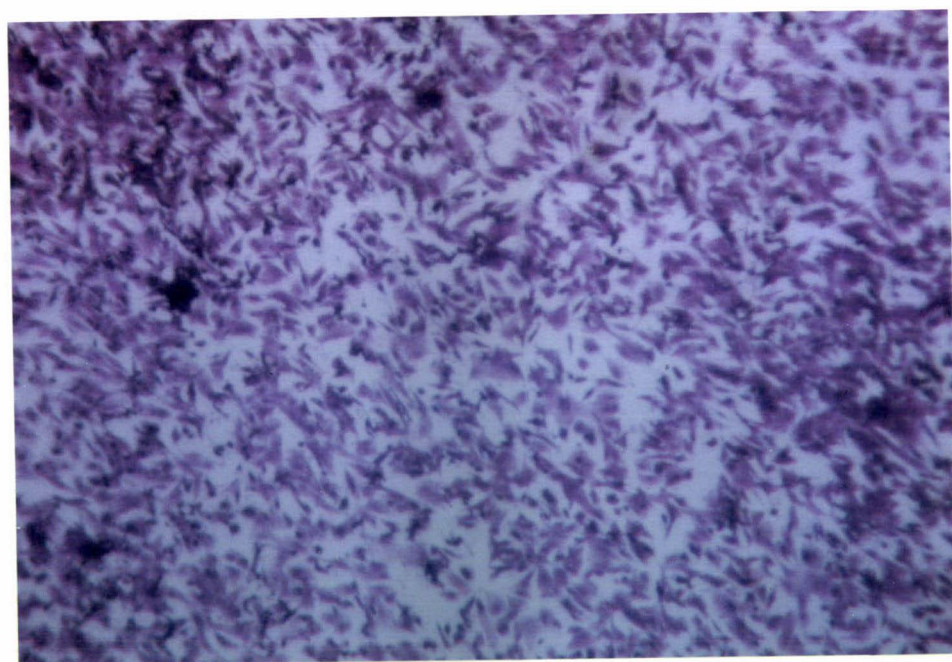
Note that the monolayer is incomplete after four days incubation. The cells are small and spindle shaped with darkly staining nuclei compared with normal cells (see Figure 4.1).

40x Magnification.

Stained with Gentian Violet.



4.1



4.2

Table 4.1 Assay of Prototype Strains of *P. multocida* for Exotoxin Production

Animal of Isolation	N° of Strains	CPE Production
Bovine	1	-
Fowl	2	-, -
Swine	2	+*, +
Sheep	1	-
Goat	1	-
Dog	1	-

Legend +: CPE production
 -: No CPE production
 *: Known toxigenic strain (Positive control)

4.3.2 Assay of Field Isolates

The EBL assay was then used to screen field isolates of *P. multocida* (see Section 4.2, Part III). The results are shown in Table 4.2 where it can be seen that no New Zealand isolates produced exotoxin. This is discussed in Section 4.4.

Table 4.2 Assay of Field Isolates of *P. multocida* for Exotoxin Production

Animal Host	Exotoxin Production		Total
	+	-	
Dog	0	21	21
Cat	0	10	10
Pig	0	15	15
Deer	0	1	1
Rabbit	0	25	25

Legend +: Exotoxin production
 -: No exotoxin production

4.4 Discussion

No New Zealand field isolates were shown to produce exotoxin. This suggests that either toxigenic strains of *P. multocida* are not present in New Zealand or are present at a frequency which is too low to be detected with the sample tested.

It must be appreciated however that during the course of this study we were not able to identify any herd of pigs which had Atrophic Rhinitis. We conclude that a reassessment of the presence of toxigenic strains in pigs should be undertaken in the event that an outbreak of Atrophic Rhinitis occurs.

CHAPTER 5

Comparison of New Zealand Isolates of *P. multocida* With Particular Reference to Plasmids.

5.1 Introduction

Prevention and treatment of diseases due to *P. multocida* may be effected using the antibiotics Penicillin, Tetracycline, Streptomycin and Sulphonamides. The routine use of these agents overseas has led to an increase in the prevalence of resistant strains of *P. multocida* and resistance has been associated with the possession of plasmids (Hirsch, Martin and Rhoades, 1985) which are between 1 Megadalton (Mdal) and 5 Mdals in size.

Plasmids can carry information for the synthesis of proteins. This section examines strains of *P. multocida* isolated in New Zealand for the presence of plasmids (between 1 Mdal and 5 Mdals in size) and attempts to establish if a relationship exists between the carriage of plasmids, antibiotic resistance and the presence of unique proteins as evidenced by SDS-PAGE analysis.

5.2 Materials and Methods

Source of *P. multocida* Isolates.

Strains of *P. multocida* were obtained from a range of animals as described in Section 3.3.1. A total of 70 New Zealand isolates were obtained from five species. These were (with number of isolates in brackets): Canine (19), Feline (10), Cervine (1), Laprine (25) and Porcine (15). In addition three prototype (1 canine, 2 swine) strains were tested.

5.2.1 Examination of *P. multocida* for Plasmid Carriage.

5.2.11 Materials

See Section 3.2.4

5.2.12 Methods

- i. DNA was extracted and assayed as described in Section 3.2.4, Parts I. and II. DNA was also extracted from a culture of *E. coli* V517 which was used as a marker strain because it contains eight plasmids of known molecular weights.
- ii. A volume which contained 5ug DNA was placed into sterile Eppendorf tubes, a 1/20 volume of 5M NaCl was added and the preparations were mixed by tapping the tubes. 2 volumes of cold (-20°C) ethanol were added, mixed by inversion and left at -20°C for at least 2hrs for the DNA to precipitate.
- iii. The tubes were centrifuged for 10mins (in a Microfuge), the supernatants were decanted and discarded, the pellets washed once with cold ethanol and left for 30mins at 37°C to dry.
- iv. The DNA was solubilized by adding 45ul of Running Buffer and mixing by tapping the tubes. They were then centrifuged for 10mins in a Microfuge and a 40ul aliquot of supernatant were removed, placed in wells in a 0.7% agarose gel which had been prepared as described in Section 3.2.4, Part IV.
- v. The samples were then electrophoresed, stained and photographed as described in Section 3.2.4, Part V.

5.2.2 *P. multocida*: Determination of the Minimum Inhibitory Concentrations* of Antibiotics.

* Based on the Agar Dilution Test of Washington and Barry as described by Lennette, Spaulding and Truant (1974).

5.2.21 Materials

Antibiotic Sensitivity Plates

MHA plates were prepared which contained one of three antibiotics (Tetracycline, Streptomycin and Sulphadiazine) at concentrations ranging from $0.25\mu\text{gml}^{-1}$ to $2048\mu\text{gml}^{-1}$. Further plates were prepared which contained Penicillin at concentrations ranging from 0.25 IUml^{-1} to 2048 IUml^{-1} .

Blood Agar (BA)

See appendix

Brain Heart Infusion Broth (BHI)

See appendix

Mueller-Hinton Agar (MHA)

Mueller-Hinton Broth (Difco) was prepared as per manufacturer's instructions with the addition of 17g l^{-1} agar. The medium was dispensed into 100ml bottles, autoclaved and 25ml aliquots were dispensed between four standard petri dishes as required.

NaCl Solution (0.85%)

See appendix

5.2.22 Methods

- i. A culture of *P. multocida* was removed from -80°C storage and propagated on blood agar (BA) for 12hrs at 37°C.
- ii. 10ml BHI broth was inoculated with colonies and incubated at 37°C on an orbital shaker (at approximately 120rpm) until the culture reached mid-logarithmic growth phase. That is, it had an optical density (OD) of approximately 0.2 at 500nm using a Spectronic 20. A culture of *E. coli* (ATCC 25922) was also grown and used as a control because the MIC of several antibiotics to this strain were known.
- iii. The turbidity of the culture was adjusted to a M^CFarlands Standard #1 using 0.85% NaCl Solution* and diluted by adding approximately one volume of diluent.

* Note. Antibiotic plates were inoculated with test strains within 30mins from this time.
- iv. 0.5ml volumes of each test strain were delivered into the wells of a "Steers Replicator" and Antibiotic Sensitivity Plates were then stamped.
- vi. The plates were dried, incubated for 72hrs at 37°C and examined for growth.
- v. The Minimum Inhibitory Concentration of the antibiotics was designated as the lowest concentration of antibiotic which prevented growth of the test strain.

5.2.3 Procedures for SDS-PAGE Analysis

See Section 3.2.3

5.3 Results

Seventy three (73) *P. multocida* isolates from several different species of animals (see Section 5.2.2) were screened for the presence of small (ie between 1 Mdal and 5 Mdal) plasmids. The results (Table 5.1) show that plasmids were present in isolates of *P. multocida* from cats and dogs but were not detected in isolates from rabbits, pigs or deer (see Table 5.1). In the case of those isolates which contained plasmids (see Table 5.2) two plasmid species were invariably present.

Table 5.1 Assay of Strains of *P. multocida* for Plasmids.

Animal of Isolation	N° of Strains	N° with Plasmids
Dog	20	5
Cat	10	7
Rabbit	25	0
Pig	17	0
Deer	1	0

The plasmids within an isolate were distinguished by their mobility but the mobility of plasmids from different isolates were similar if not identical (see Figures 5.1, 5.2 and Table 5.2) with two exceptions (C5 and D5).

Figure 5.1 Agarose-Gel Electrophoresis of DNA from Plasmid-Containing Feline Isolates of *P. multocida*.

The mobility of plasmids contained in seven feline isolates were compared.

The left hand track (plasmids recovered from *E. coli* V517) represents molecular weight markers.

Note that any one isolate possessed two species of plasmid which are similar, if not identical, to the plasmids found in the other six feline isolates. Similar plasmids were present in some canine isolates (see Figure 5.2).

Mr (Mdal)

chromosome

4.8

3.7

3.4

2.6

2.0

1.8

1.4

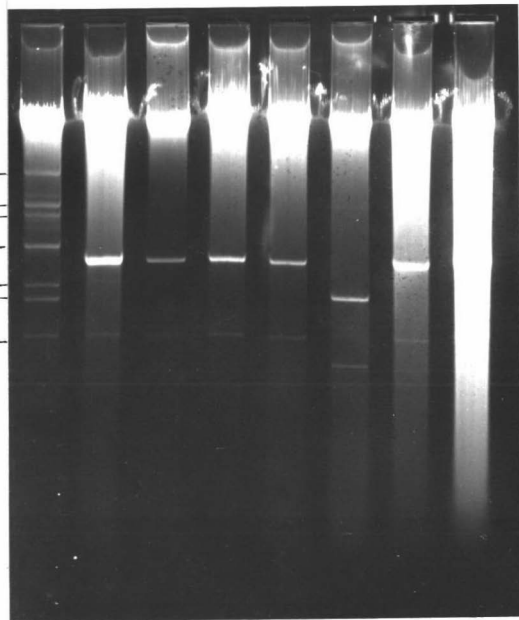


Figure 5.2 Agarose-Gel Electrophoresis of DNA from Plasmid-Containing Canine Isolates of *P. multocida*.

The mobility of plasmids contained in five canine isolates were compared.

The left hand track (plasmids recovered from *E. coli* V517) represents molecular weight markers.

Note that any one isolate possessed two species of plasmid which are similar, if not identical, to the plasmids found in the other four canine isolates. Similar plasmids were present in some feline isolates (see Figure 5.1).

Mr (Mdal)

chromosome

4.8
3.7
3.4
2.6
2.0
1.8
1.4

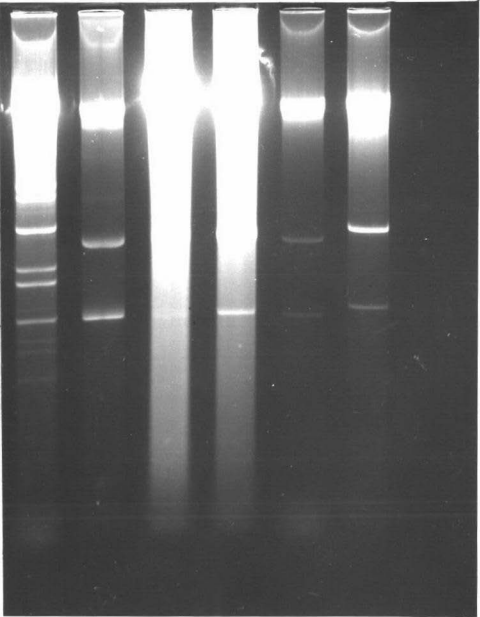


Table 5.2 An Evaluation of Plasmids Carried by Strains of *P. multocida*.

Animal of Isolation	Isolate Identity	Plasmid Description		
		N°	Mr1	Mr2
Cat	C1	2	2.4	1.4
	C2	2	2.4	1.4
	C3	2	2.4	1.4
	C4	2	2.4	1.4
	C5	2	1.8	(1.4
	C6	2	2.4	1.4
	C7	2	2.4	1.4
Dog	D1	2	2.4	1.4
	D2	2	2.4	1.4
	D3	2	2.4	1.4
	D4	2	2.4	1.4
	D5	2	2.5	1.5

Legend Mr1: Approximate molecular weight (Mdal) of largest plasmid
 Mr2: Approximate molecular weight (Mdal) of smallest plasmid

5.3.1 The Antibiotic Sensitivity of Strains of *P. multocida*

The antibiotic sensitivity (MIC) of isolates were determined and the results obtained for strains with and without plasmids were compared (see Table 5.3). The strains varied in their sensitivities but none were "resistant"* to any of the antibiotics in the sense normally used in a diagnostic laboratory to define resistance. Furthermore, there were no consistant differences in antibiotic sensitivity between strains which carried plasmids and those which did not carry plasmids.

* A strain of *P. multocida* is considered to be resistant if it grows in the presence of: $\geq 32 \text{ IU ml}^{-1}$ Penicillin, $\geq 32 \text{ ug ml}^{-1}$ Tetracycline, $\geq 32 \text{ ug ml}^{-1}$ Streptomycin or $\geq 1024 \text{ ug ml}^{-1}$ Sulphonamide.

Table 5.3 Minimum Inhibitory Concentrations of Antibiotics For
P. multocida.

Animal of Isolation	Isolate Identity	Minimum Inhibitory Concentration			
		Pe IUml ⁻¹	Te ugml ⁻¹	Sm ugml ⁻¹	Su ugml ⁻¹
Cat	C1	1	16	2	64
	C2	≤0.25	8	1	64
	C3	≤0.25	2	2	16
	C4	≤0.25	8	2	8
	C5	0.5	16	2	256
	C6	8	16	0.5	256
	C7	≤0.25	8	1	256
	C8	1	16	2	64
	C9	0.5	16	2	256
	C10	≤0.25	16	1	128
Dog	D1	≤0.25	2	2	256
	D2	1	16	2	128
	D3	8	16	0.5	256
	D4	0.5	16	2	128
	D5	1	16	2	128
	D6	1	≤0.25	4	16
	D7	1	16	2	64
	D8	0.5	32	32	128
	D9	0.5	8	2	256
	D10	4	16	2	32
	D11	≤0.25	2	2	512
	D12	1	128	4	256
	D13	8	16	0.5	64
	D14	≤0.25	8	0.5	128
	D15	≤0.25	8	2	256
	D16	1	16	2	128
	D17	1	16	2	256
	D18	≤0.25	2	2	32
	D19	1	16	2	256
	D20	≤0.25	8	1	128
Pig	P1	1	≤0.25	2	32
	P2	1	≤0.25	2	64
	P3	1	≤0.25	2	64
	P4	0.5	32	4	64
	P5	0.5	8	2	128
	P6	1	16	2	128
	P7	0.5	16	2	128
	P8	1	16	2	64
	P9	1	16	2	64
	P10	1	2	2	128
	P11	1	16	2	256

Table 5.3 Cont^d

Rabbit •	P12	≤0.25	16	2	256
	P13	≤0.25	8	1	256
	P14	≤0.25	8	1	128
	P15	0.5	8	1	128
	P16	≤0.25	8	1	128
	P17	0.5	8	2	128
	R1	0.5	8	1	256
	R2	≤0.25	4	1	256
	R3	≤0.25	4	1	256
	R4	≤0.25	2	1	256
	R5	0.5	2	1	256
	R6	≤0.25	2	1	128
	R7	0.5	2	1	256
	R8	0.5	2	1	256
	R9	≤0.25	2	1	64
	R10	≤0.25	4	1	256
	R11	0.5	4	1	256
	R12	≤0.25	2	1	256
	R13	≤0.25	2	1	256
	R14	0.5	4	1	256
	R15	≤0.25	2	1	256
	R16	0.5	4	1	256
	R17	≤0.25	4	1	256
	R18	0.5	2	1	256
	R19	≤0.25	2	1	256
	R20	≤0.25	2	1	256
	R21	0.5	4	2	128
	R22	0.5	2	2	128
	R23	≤0.25	4	2	128
	R24	0.5	2	2	128
	R25	0.5	2	2	128
Deer	X1	0.5	8	1	256

Legend: Antibiotics**• Notes**

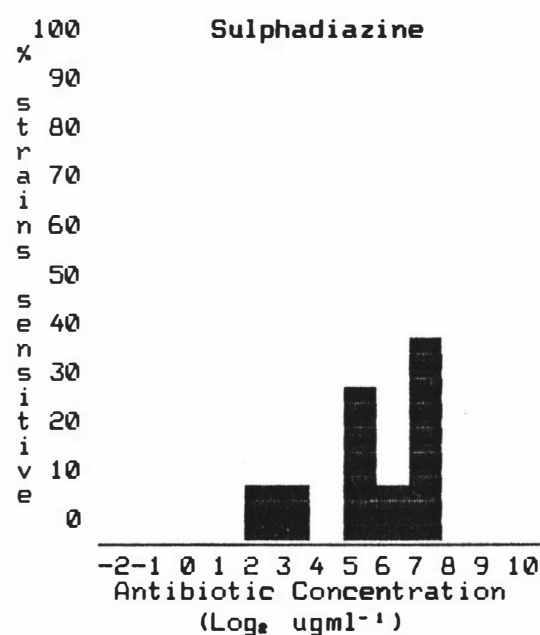
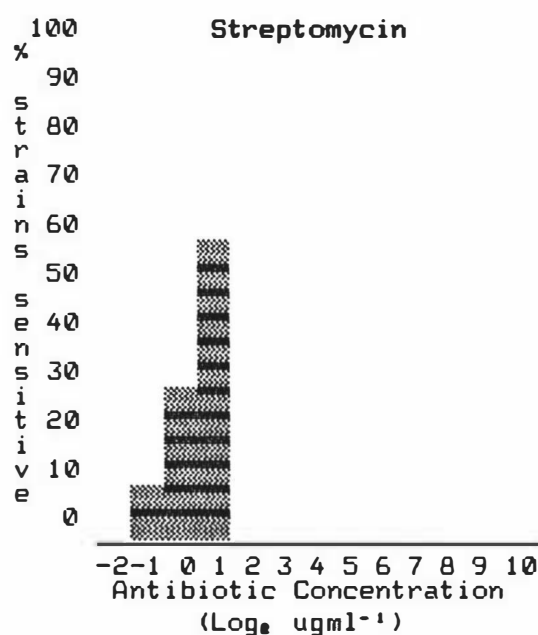
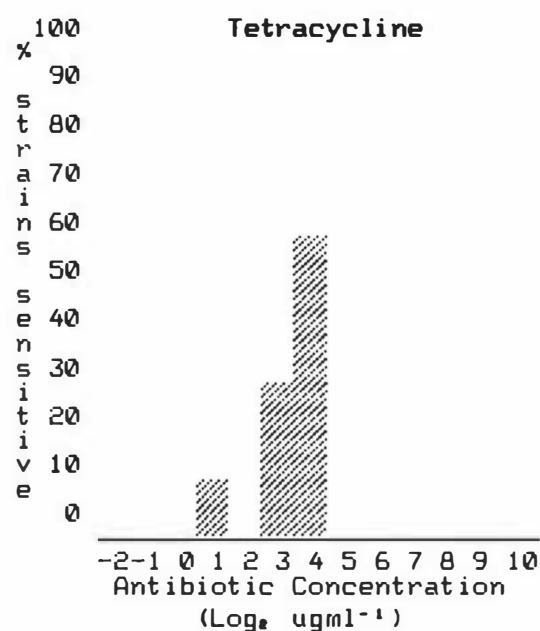
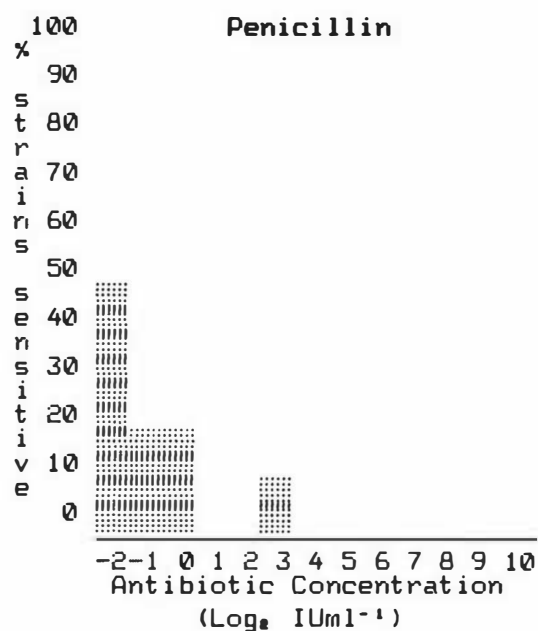
Penicillin G (Pe)
Tetracycline HCl (Te)
Streptomycin SO₄ (Sm)
Sulphadiazine (Su)

R1-R19 from Patea colony
R20 from Waitotara Valley colony
R21-R25 from Upper Hutt colony

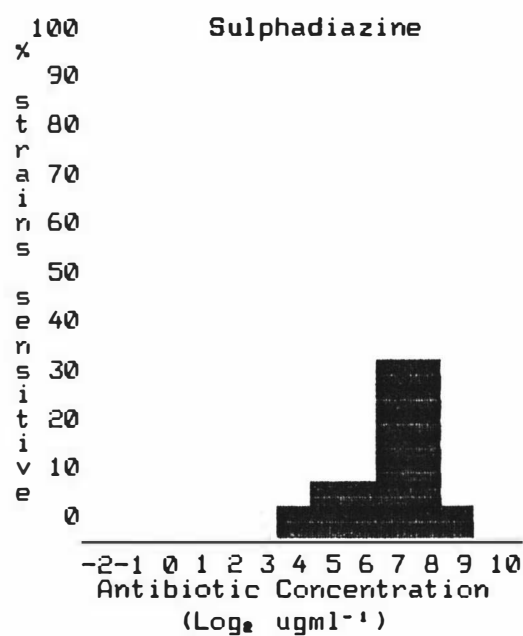
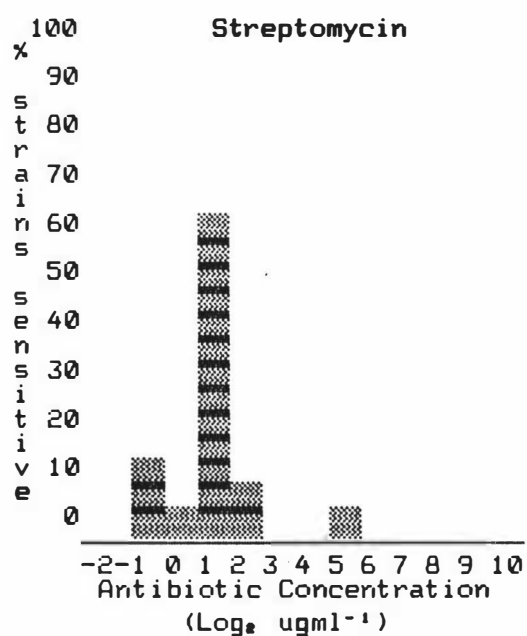
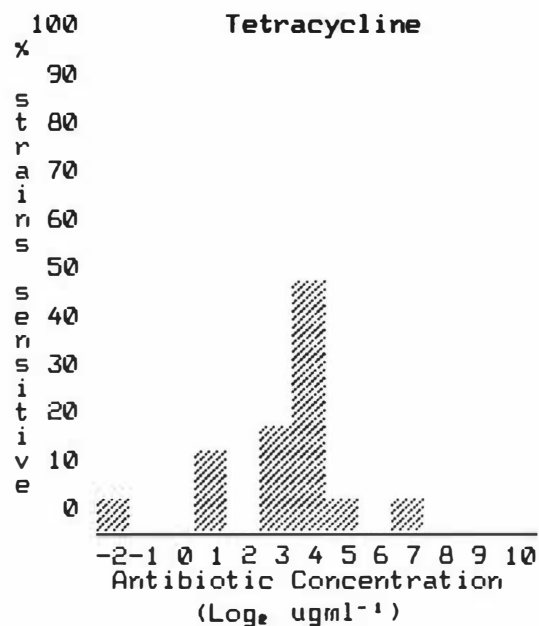
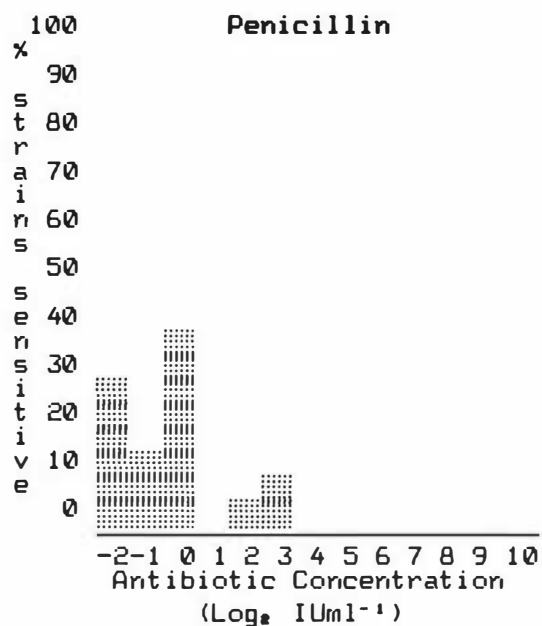
Canine, feline and porcine strains (see Table 5.3) of *P. multocida* showed more variation in their sensitivities to antibiotics than rabbit isolates. This can be seen in Figure 5.3 and is discussed in Section 5.4.

Figure 5.3 Variation of MICs * Between Strains of *P. multocida*.

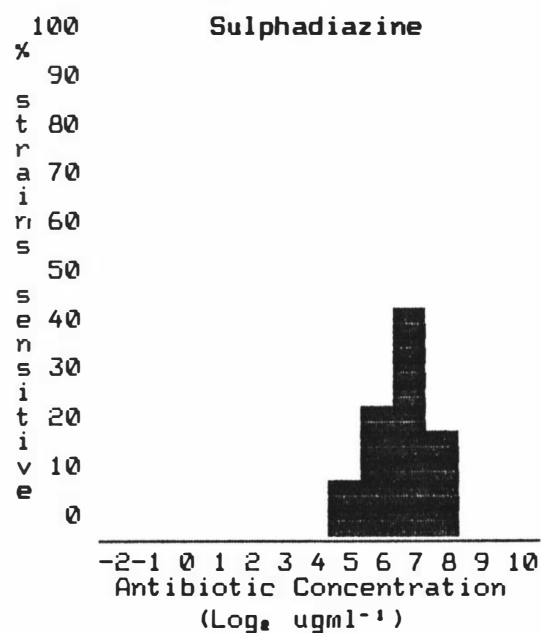
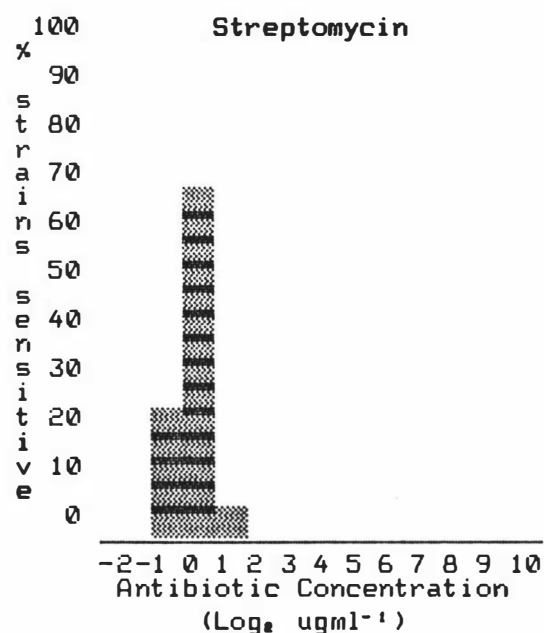
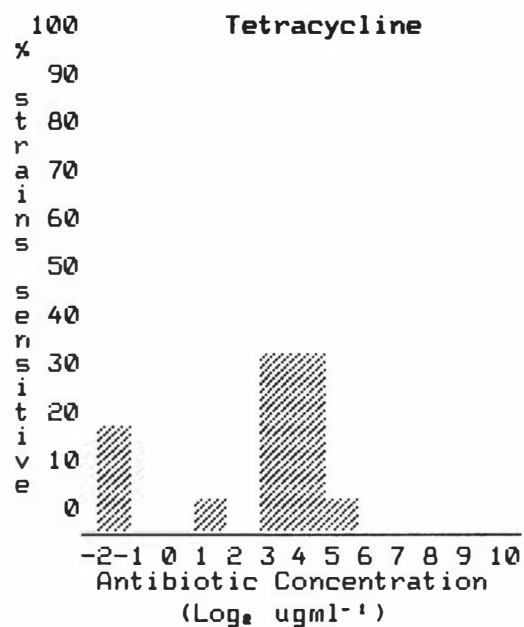
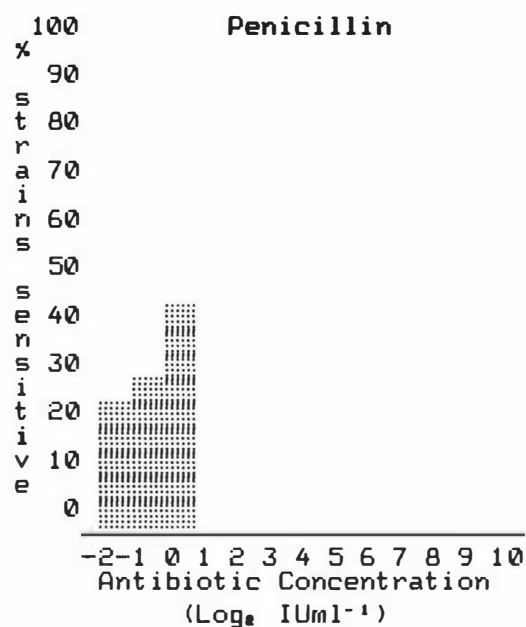
* See Legend

I. Feline Strains

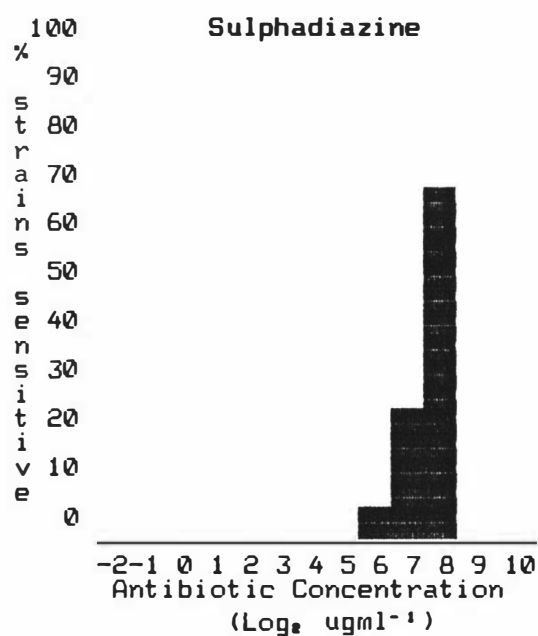
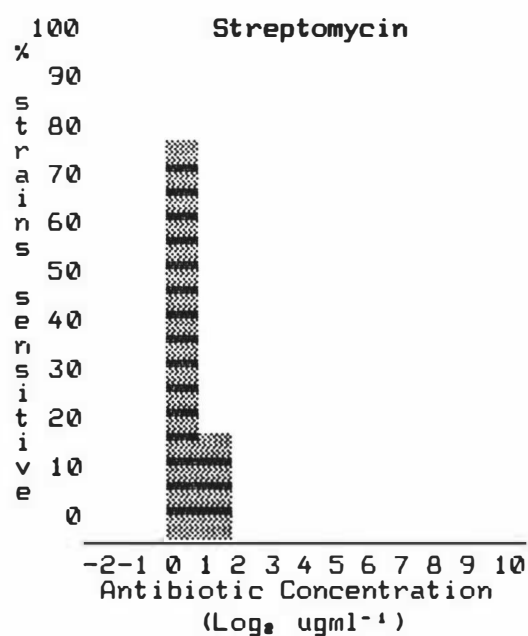
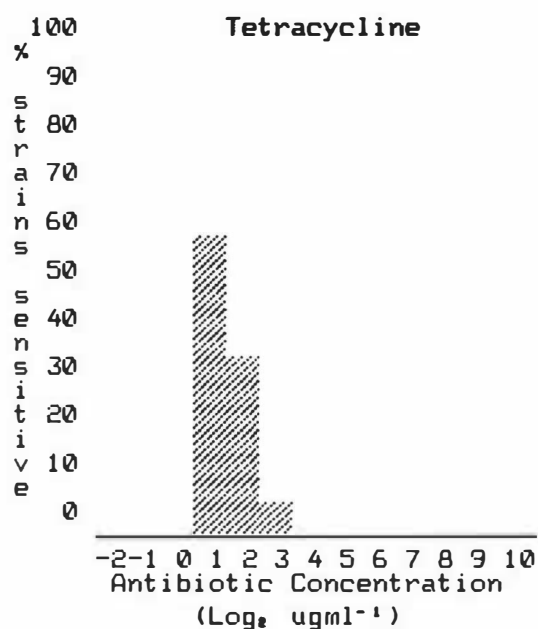
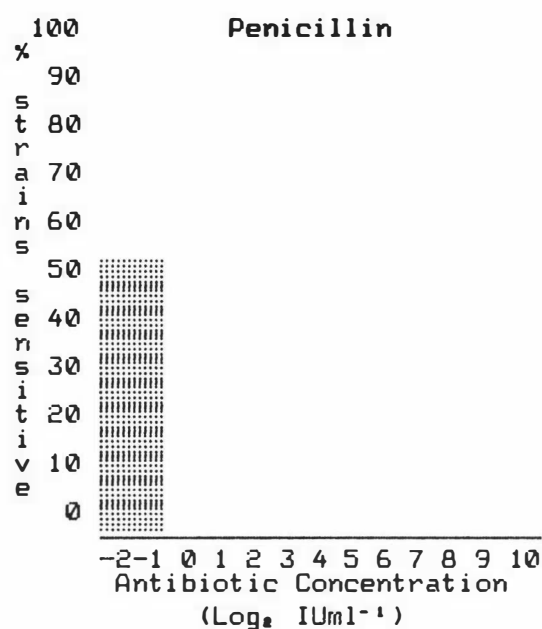
II. Canine Strains



III. Porcine Strains



IV. Rabbit Strains



Legend: Antibiotics:

Penicillin G	(Pe)
Tetracycline HCl	(Te)
Streptomycin SO ₄	(Sm)
Sulphadiazine	(Su)

5.3.2 Comparison of Proteins of *P. multocida*. Plasmid Carrying Strains Versus Non-Plasmid Carrying Strains

The proteins of 16 strains of *P. multocida* (8 canine, 8 feline) were compared by SDS-PAGE. Half of the 16 strains tested (4 canine, 4 feline) carried plasmids. The results are shown in Figures 5.4 and 5.5. The SDS-PAGE pattern of strains with and without plasmids did not allow us to distinguish between the two groups. We conclude that if the plasmids code for proteins then these are not readily detectable by SDS-PAGE.

5.4 Discussion

Isolates of *P. multocida* from New Zealand animals were screened for plasmids. We found that some canine and feline strains were the only isolates to carry plasmids (Table 5.1). It was interesting that all of these plasmid-carrying isolates had two plasmids and that plasmids from both the dog and cat strains were, with two slight exceptions, the same size (ie 2.4 Mdal and 1.4 Mdal, see Table 5.2). This suggests that the plasmids of different isolates might be related or are identical.

Antibiotic resistance can be associated with plasmid carriage in *P. multocida* (Silver et al, 1978). We examined strains of *P. multocida* for their sensitivities (MICs) to the four main therapeutic antibiotics (Penicillin, Tetracycline, Streptomycin and Sulphonamides) and found that no isolate was resistant. Furthermore, the plasmid-carrying isolates showed similar sensitivities to those isolates which did not carry plasmids. We conclude that the plasmids did not carry antibiotic resistance markers at least to the antibiotics tested (Penicillin, Tetracycline, Streptomycin and Sulphonamides).

Figure 5.4 SDS-PAGE Analysis of *P. multocida* Isolates from Dogs.

A comparison of plasmid-containing isolates with isolates which do not contain plasmids.

Four plasmid-containing isolates (left) and four isolates (right) which did not possess plasmids were compared.

The patterns were examined for the presence of an extra band (or bands) common to the plasmid-containing isolates. No such bands were detected.

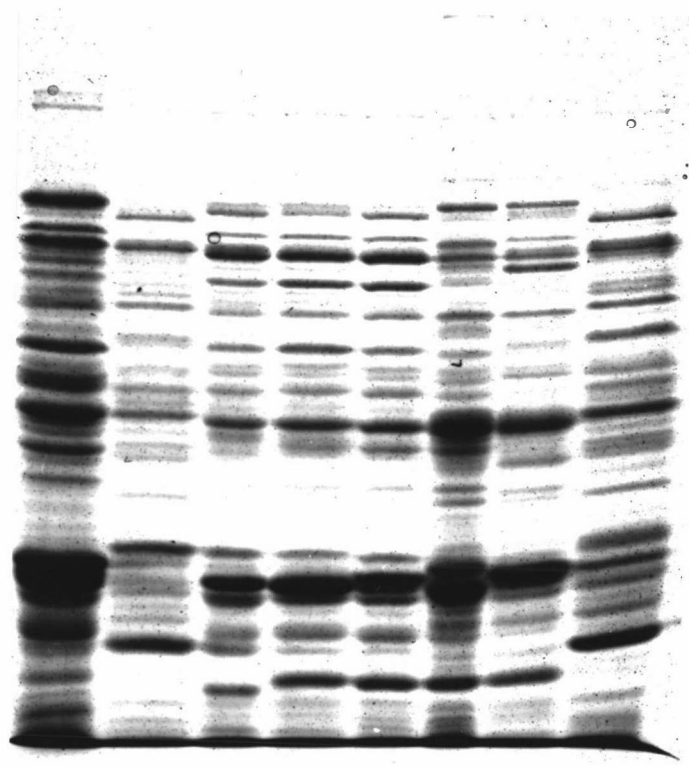
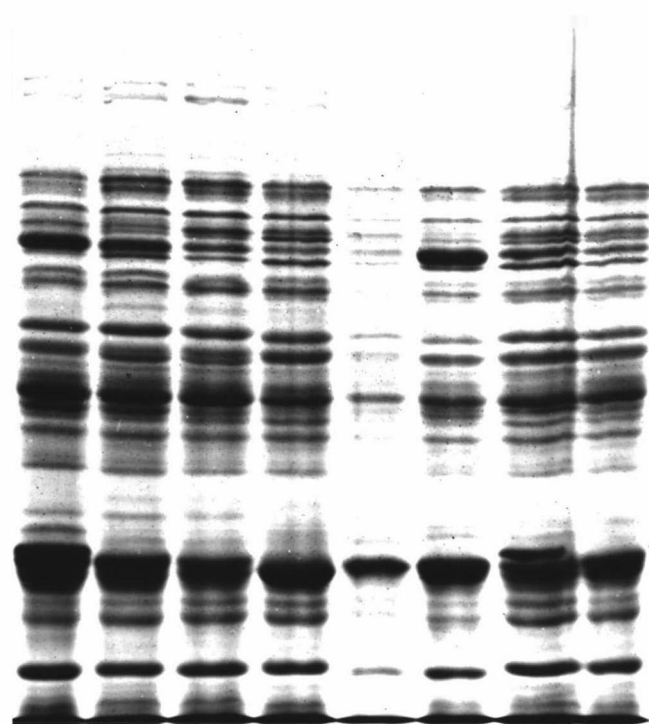


Figure 5.5 SDS-PAGE Analysis of *P. multocida* Isolates from Cats.

A comparison of plasmid-containing isolates with isolates which do not contain plasmids.

Four plasmid-containing isolates (left) and four isolates (right) which did not possess plasmids were compared.

The patterns were examined for the presence of an extra band (or bands) common to the plasmid-containing isolates. No such bands were detected.



It was observed (Table 5.3) that strains of *P. multocida* isolated from rabbits exhibited very similar levels of sensitivity to all of the test antibiotics. This was in contrast to feline, canine and porcine strains which showed considerably more variation. Earlier work (see Section 3.4) had shown that isolates obtained from individual rabbit colonies were homogeneous with respect to the proteins (as demonstrated by SDS-PAGE) and therefore probably represent multiple isolates of one strain. The similarity in the antibiotic sensitivities of rabbit isolates supports this conclusion.

CHAPTER 6

General Discussion.

Pasteurella multocida, as its name implies, causes disease in many different animals. It is therefore not surprising that for decades the organism has been designated by different names because of its wide host range and the many diseases which it causes. The discovery (within the last thirty years) that *P. multocida* is composed of different serotypes led to the recognition that isolates from various host species and diseases belonged to a single species and underlines the pathological significance of this bacterial species.

Because of its importance as a pathogen *P. multocida* has been much studied overseas but, perhaps surprisingly, has been little studied in New Zealand despite the economic importance of animals in this country. This thesis should be regarded as a contribution to the understanding of the distribution of and the variations between *P. multocida* strains recovered from animals in New Zealand. Thus we described the isolation of *P. multocida* from different species of animal in New Zealand and compared these isolates using several techniques.

Typically *P. multocida* is found in animals in association with other bacteria which are usually present in large excess, so efficient isolation of *P. multocida* requires a selective medium. Consequently we assessed the value of selective medium 8HPG which has been described by Smith and Baskerville (1983) who used it to isolate *P. multocida* from the nasal tract of pigs. In our hands it was unsatisfactory because it inhibited the growth of many New Zealand isolates of *P. multocida* derived from a range of host species (see Table 2.1). We therefore attempted to formulate a more suitable medium.

6.1 The Development of a Selective Medium for the Isolation of *P. multocida* in New Zealand

The components of 8HPG medium were examined individually (see Section 2.3) and it was found that the basal medium (Nutrient Agar) failed to propagate several of our test strains and others were inhibited by the high alkalinity of 8HPG medium. In addition to this, 8HPG medium contained polymixin which inhibited a significant proportion of the New Zealand isolates of *P. multocida*.

Following assessment of the inhibitory effects of individual antibiotics on a range of New Zealand strains of *P. multocida* we formulated a modified selective medium (SM) which had a blood agar base at neutral pH (Section 2.2.1) and contained Bacitracin, Gentamycin and Mycostatin as described for the original medium (8HPG). SM (see Table 2.5) was found to propagate all of the New Zealand test strains of *P. multocida* and suppressed most bacteria (see Figure 2.1) which were present in nasal swabs derived from rabbits. It was therefore used to isolate *P. multocida* from the nasal cavity of rabbits including healthy animals and animals with respiratory disease. We believe that this medium has considerable potential for use in routine veterinary investigations. It could for example be used to screen the nasal flora of imported pigs for the presence of *P. multocida*. Isolates obtained could then be examined for the production of exotoxin (using the cell culture method described in Chapter 4) and thus prevent the introduction of severe atrophic rhinitis into the country.

6.2 Isolation and Serotyping of *P. multocida* from New Zealand Animals

Using traditional isolation methods (plating on blood agar) augmented with our selective medium, *P. multocida* was isolated from several species of animal in New Zealand (ie Cat, Dog, Rabbit, Pig and Deer).

It was found (see Table 3.1) that a higher proportion of rabbits with clinical respiratory tract infection carried *P. multocida* in their nasal tract than did healthy controls. This is consistent with the generally accepted concept that *P. multocida* carried in the upper respiratory tract can (especially in the presence of stress) spread to the lungs and cause pneumonia. Further experiments which examined isolates by SDS-PAGE analysis (see Section 6.4) confirmed that the strains isolated from the lungs of diseased rabbits were indistinguishable from the strains isolated from the nasal cavity.

The indirect haemagglutination assay (IHA) is the accepted method for typing strains of *P. multocida*. This technique, which has not previously been used in New Zealand, had to be validated in our hands before field isolates could be typed. A major problem was to produce type-specific antiserum. As the literature will verify (Brogden and Packer, 1979), this process is difficult because, on its own, the type-specific antigen (capsular polysaccharide) is poorly antigenic or non antigenic.

Using whole cells and purified capsular antigen of *P. multocida* with Freund's Complete Adjuvant as an immunizing antigen we failed to produce type-specific antiserum in rabbits but later produced typing antisera of adequate specificity by immunizing domestic hens. While much of the literature suggests that rabbits are satisfactory for producing *P. multocida* typing serum the fact that some other workers used hens, which are not common experimental animals, suggests that others have experienced difficulty in immunizing rabbits with this antigen.

Using the IHA assay and avian antisera we were able to distinguish between serotypes A, B and D (see Table 3.3).

Isolates of *P. multocida* from dogs, cats and pigs in New Zealand were predominantly Types A and D so we concluded, unsurprisingly,

that the serotypes carried by these hosts in New Zealand are the same as those carried by the same species of animal overseas. A single isolate from a deer proved to be Type D. We are aware of only one deer isolate being typed overseas. It also was Type D.

Two findings were unexpected (see Table 3.4): A feline isolate of *P. multocida* was Type B. These are normally associated with cattle and buffaloes. All the rabbit isolates (from three rabbit colonies) were untypable which is in contrast to overseas findings where approximately 50% of rabbit strains are untypable and the remainder are Types A and D.

Notwithstanding the latter result, which is discussed below, we conclude that the New Zealand results mirror overseas findings.

6.3 SDS-PAGE Analysis of Field Isolates of *P. multocida*

The effort involved in serotyping *P. multocida* by IHA is considerable and an alternative method is not available. It seemed possible that there might be a correlation between proteins of *P. multocida* (as examined by SDS-PAGE analysis) and the serotype of an isolate. We examined this possibility but found that *P. multocida* isolates were heterogeneous not only when isolated from different host species (Figure 3.1) but also when multiple isolates were obtained from a single host species (Figures 3.2, 3.3 and 3.4). Therefore, our attempt to correlate proteins with serotypes was not successful which perhaps accounts for the absence of data concerning SDS-PAGE profiles of *P. multocida* in the literature.

The extreme heterogeneity of SDS-PAGE patterns of *P. multocida* allowed us to use SDS-PAGE analysis to compare nasal and lung isolates from rabbits (see Figure 3.7) and we were able to show that the same strains were involved (see Section 3.3) which supports the concept that pneumonia is caused by the dissemination of nasal strains.

Examination of *P. multocida* proteins by SDS-PAGE also enabled us to cast light on the source of *P. multocida* which caused an outbreak of respiratory disease in rabbits. Disease had occurred after the introduction of imported rabbits into a colony so it was concluded that a newly introduced strain of *P. multocida* had caused the outbreak. However SDS-PAGE analysis of isolates obtained from diseased rabbits before and after the importation of new rabbits (Figure 3.8) showed that the strains were indistinguishable. Since isolates from different flocks are heterogeneous we conclude that the pathogenic strain of *P. multocida* was present in New Zealand before the importation of the new rabbits and that the disease outbreak was probably unconnected with the introduction of a new isolate of *P. multocida*.

As alluded to above it was found that all rabbit isolates were untypable when examined by the IHA assay. This was unusual (see Chapter 3) so we speculated that these isolates represented multiple isolations of one, or at at any rate, a small number of strains. This was supported because when examined by SDS-PAGE (Figure 3.6) isolates from individual rabbit colonies were homogeneous. Thus, since only three rabbit colonies were examined our rabbit isolates probably represent only three strains.

In conclusion, SDS-PAGE was found to be a useful tool to study epidemiological aspects of disease (at least in rabbits) caused by *P. multocida* but could not be used to distinguish between serotypes.

6.4 Comparison of *P. multocida* Serotypes by Restriction Endonuclease Analysis (REA)

As discussed above the IHA assay is a laborious technique so an alternative method of serotyping *P. multocida* would be advantageous. SDS-PAGE analysis did not correlate with serotype so we considered REA as a possible alternative because it had been successfully used

by Marshall, Wilton and Robertson (1981) to differentiate between serovars of *Leptospira*. However, the electrophoresis patterns of digested DNA from *P. multocida* (see figures 3.10, 3.11, 3.12, 3.13) did not correlate with serotypes. The heterogeneous patterns of different isolates would, like SDS-PAGE, allow epidemiological studies of individual strains but we did not pursue this approach with respect to REA.

6.5 Assay for Exotoxigenic Strains of *P. multocida*

Progressive atrophic rhinitis (AR) is an important disease of pigs in overseas countries and a mild form has been described in New Zealand. The disease is caused by exotoxin producing strains of *P. multocida*. These can be detected *in vitro* by producing a cytopathic effect (CPE) in cultured tissue cells by exposing them to cell-free extracts of toxin-producing strains of *P. multocida*.

We sought to establish if toxigenic strains of *P. multocida* are present in this country using cell cultures as a screening method. This method had to be validated in our hands and has not been previously used in New Zealand.

Embryonic Bovine Lung (EBL) cells and "sensitized" Vero cells have been used to detect exotoxigenic strains of *P. multocida* (see Section 1.4). In our hands a CPE was not visible in "sensitized" Vero cells (data not shown) but was produced in EBL cells (see Figures 4.1 and 4.2). Consequently the EBL cell culture assay was used to screen field isolates for exotoxin production.

None of the New Zealand strains of *P. multocida* which were tested (see Table 4.2) produced a CPE in EBL cells. ie none produced exotoxin. This was not unexpected because none of our New Zealand isolates had been recovered from pigs with AR and we were unable to identify any herd with the disease during the course of this study. We conclude that the presence of toxigenic strains of *P. multocida*

must remain an open question until either an outbreak of AR occurs and isolates from the nasal tract of these pigs can be examined or in the absence of the disease. This requires a project which systematically screens animals, especially pigs, in New Zealand for the carriage of *P. multocida* and examines isolates for the production of endotoxin.

6.6 Antibiotic Resistance and its Relationship to Plasmids in New Zealand Strains of *P. multocida*

The occurrence of antibiotic resistance in *P. multocida* is a problem in some countries and has been attributed to the (largely indiscriminant) use of antibiotics. Penicillin, Tetracycline, Streptomycin and Sulphonamides, are often used to treat disease due to *P. multocida* in domestic animals in New Zealand so it therefore seemed possible that "resistant" strains of *P. multocida*, ie strains with a sensitivity below a defined level (see Section 5.3.1) could be isolated from animals in this country.

We determined the sensitivity (MIC) of New Zealand strains of *P. multocida* but found that none had a resistance high enough to be designated "resistant". Cat, dog and pig isolates possessed variable sensitivities but rabbit isolates were homogeneous. This latter result was not surprising because it had previously been concluded (see Section 3.4) that our rabbit isolates represented multiple isolations of a total of three strains only.

The presence of antibiotic resistance in *P. multocida* is often associated with plasmids between 1 Mdal and 5 Mdals in size (Hirsch, Martin and Rhoades, 1985). Methods for extracting plasmids from bacteria normally involve a technique (eg Ekhardt method) which is gentle enough to extract, in an intact form, relatively large (over 100Mdals) plasmids. However, we used a harsher method (see Section 5.2.12), which extracted total DNA for use in both the plasmid assay and REA. This method did not recover large plasmids but we

demonstrated (see Figures 5.1 and 5.2) that plasmids up to approximately 5 Mdals in size were present. Therefore this method was suitable for detecting resistance plasmids in *P. multocida*.

We found that several cat and dog strains of *P. multocida* possessed plasmids which had molecular weights of approximately 1.4 Mdals and 2.4 Mdals. However, since these strains did not possess "resistance" to the test antibiotics we conclude that these plasmids, unlike similar plasmids in *P. multocida* isolated overseas, do not carry resistance markers.

6.7 Outlook

Despite its importance as a pathogen of swine, cattle, sheep, fowl and rabbits, *P. multocida* has been little studied in New Zealand. This thesis represents a preliminary step to a fuller understanding of the importance of *P. multocida* in this country and to the best means of control. The possibility of outbreaks of atrophic rhinitis due to the introduction of toxigenic strains of *P. multocida* in "carrier pigs" may well stimulate further work of this nature.

APPENDIX

Blood Agar 5%

Defibrinated Horse Blood : 50ml l^{-1}

Blood Agar Base

- i. The Blood Agar Base was melted and cooled to 50°C .
- ii. Defibrinated Horse Blood was added to give a final concentration of 5% and 25ml volumes of this preparation were dispensed in standard petri dishes.

Blood Agar Base (Difco)

The medium was prepared as per manufacturers instructions, autoclaved in 100ml bottles and stored at room temperature.

Brain-Heart Infusion Broth (Difco)

The medium was prepared as per manufacturers instructions and autoclaved in appropriate containers.

Dextrose Starch Agar (Difco)

The medium was prepared as per manufacturers instructions, autoclaved in 100ml bottles and stored at room temperature.

Dextrose Starch Broth (pH 7.3)

Proteose Peptone N ^O 3 (Difco)	:15.0gl ⁻¹
Bacto Dextrose (Difco)	: 2.0gl ⁻¹
Soluble Starch	:10.0gl ⁻¹
NaCl	: 5.0gl ⁻¹
Disodium Phosphate	: 3.0gl ⁻¹
Distilled Water	

The components were mixed and the pH adjusted using NaOH. The preparation was then autoclaved in appropriate containers and stored at +4°C.

Kovacs Reagent*

Amyl, Isoamyl, or Isobutyl Alcohol	: 15.0ml
para-Dimethylaminobenzaldehyde**	: 1.0g
Concentrated Hydrochloric Acid	: 5.0ml

The aldehyde was dissolved completely in the alcohol. The acid was then added (slowly) and the preparation was stored at +4°C in a dark container.

* Based on Blair et al(1970).

** The raw aldehyde should be light of colour.

M^CConkey Agar (Difco)

The medium was prepared as per manufacturers instructions, autoclaved in 100ml bottles and stored at room temperature. As required 100ml of medium was melted and dispensed between four standard petri dishes.

Motility Medium*

Peptone : 10g l⁻¹
NaCl : 5g l⁻¹
Davis Agar: 3g l⁻¹
Distilled Water

The components were dissolved in the Distilled Water, adjusted to pH 7.5 using NaOH, dispensed in tubes, autoclaved and stored at +4°C.

* Based on Blair et al(1970)

NaCl Solution (0.85%)

NaCl was dissolved in Distilled Water to give a final concentration of 0.85%. The solution was then autoclaved and stored at +4°C.

Oxidase Reagent*

- i. A fresh 1% solution of Di- or Tetra-methyl-p-phenylenediamine dichloride was prepared in distilled water.
- ii. Ascorbic acid (a stabilizer) was added to give a final concentration of 0.11%.
- iii. 5mmx50mm strips of Whatman N^o1 filter paper were soaked in the preparation, air dried at 37°C and stored at 4°C in a sealed container.

* Based on Blair et al(1970).

PBS (Phosphate Buffered Saline)

NaCl : 8.5gl⁻¹
 Na₂HPO₄.12H₂O : 13.5gl⁻¹
 NaH₂PO₄.2H₂O : 0.4gl⁻¹
 Distilled Water

The components were mixed, autoclaved and stored at +4°C.

Peptone Water (pH 7.2)

Peptone : 10gl⁻¹
 NaCl : 5gl⁻¹
 Distilled Water

The components were mixed and the pH was adjusted using NaOH. The preparation was then autoclaved and stored at +4°C.

Peptone Water Sugars

Bromocresol Purple : 25ml l^{-1}

Peptone Water (pH 7.2-7.3)

- i. The ingredients were mixed and 5ml volumes were dispensed in 1/2 oz bottles.
- ii. A durham tube was added to each bottle and the preparations were autoclaved.
- iii. The preparations were stored at $+4^{\circ}\text{C}$. At the time of use, 0.25ml of sterile 10% sugar solution was added to each bottle.

Triple Sugar Iron Agar (TSI)

The medium was prepared as per manufacturers instructions.

Tryptso-Soy Agar (Difco)

The medium was prepared as per manufacturers instructions, autoclaved in 100ml bottles and stored at room temperature. As required 100ml of medium was melted and dispensed between four standard petri dishes.

Tryptso-Soy Broth (Difco)

The medium was prepared as per manufacturers instructions.

Urea Agar (Difco)

The medium was prepared as per manufacturers instructions.

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