STUDIES ON *PASTEURELLA HAEMOLYTICA*: COMPARISON OF SEROTYPING TECHNIQUES AND SURVEYS OF THE PREVALENCE OF SEROTYPES IN SHEEP AND GOATS IN NEW ZEALAND

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY AT MASSEY UNIVERSITY, NEW ZEALAND.

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ABSTRACT

P.haemolytica is the aetiological agent of pneumonic pasteurellosis in sheep and goats, and, as a secondry invader it also exacerbates lesions of chronic non-progressive pneumonia (CNP). These diseases cause considerable economic loss to the New Zealand farming industry.

P.haemolytica exists as 15 serotypes and immunity is serotype specific. Vaccines against *P.haemolytica* are produced, but it is not known if the serotypes contained in the vaccine are the same as those causing disease in New Zealand as there is a lack of information on the prevalence and distribution of the serotypes of *P.haemolytica* in this country. This is largely due to the technical difficulties involved in typing isolates because the standard method, the indirect haemagglutination assay, (IHA), is laborious and may give anomalous results due to cross-reactions.

The present investigation was undertaken with two major aims: to replace IHA with a more convenient typing system, *viz.* agar gel immunodiffusion, (AGID), and to use AGID to survey the serotypes of *P.haemolytica* present in CNP lesions of sheep, pneumonic pasteurellosis of sheep, pneumonic pasteurellosis of goats and the nasal cavities of goats.

Difficulties were encountered in the preparation of rabbit antisera to some of the 15 prototype strains. These difficulties were overcome by using domestic hens when necessary. Using these sera it was possible to distinguish the 15 prototype strains by IHA, and following absorption of sera, by AGID. The results obtained by IHA and AGID were in agreement, at least when prototype strains were examined. It was necasary to show that AGID is able to correctly establish the serotype of field isolates of

P.haemolytica. Hence 25 caprine isolates of *P.haemolytica* from field cases of pneumonic pasteurellosis were serotyped by both IHA and AGID. In 24 cases the results from the two tests agreed. In the remaining case IHA indicated that the isolate was serotype A2 or A11. We were able to show that this isolate gave a line of identity with antigen prepared from the prototype strain of A11, but showed no line of identity in the AGID with any other antigen preparation. Taking this as the critical criterion we concluded that this isolate was serotype A11, although IHA showed a 2-fold preference for A2 over A11. Since AGID was shown to be a reliable test we used it alone for future serotyping, for two reasons: it is more convenient, and any cross-reactions that do occur may be resolved by looking for a line of identity between antigens of the isolate and a prototype antigen. In the case of two serotypes involved in many cross-reactions, namely A1 and A7, the capsular polysaccharide was purified by organic solvent precipitation. This purified polysaccharide was used to test for a line of identity with reacting isolates. This eliminates the possibility that the line of identity seen was due to a non-serotype-specific antigen.

Four surveys (two in sheep, two in goats) of the serotypes of *P.haemolytica* present in New Zealand were undertaken. The first involved 139 isolates derived from ovine lesions of CNP collected from 4 areas of New Zealand. A total of 9 serotypes were found. Serotypes A1 (31.7%), A2 (47.8%) and A7 (10%) made up 89.5% of the total.

A smaller survey of 18 isolates from pneumonic pasteurellosis of sheep revealed 6 serotypes, including 1 isolate of T10, a serotype and biotype not previously found in New Zealand. A1 (11.1%) and A2 (61.1%)

were the predominant serotypes present and represented 72.2% of the total.

The 25 isolates of *P.haemolytica* from caprine pneumonic pasteurellosis contained only 4 serotypes. A2 represented 80% of the total.

14 isolates of *P.haemolytica* were obtained from the nasal cavities of 109 goats. Only 2 serotypes were isolated. 13 isolates were A2 and the remaining isolate was A11.

The implications of these results for vaccine manafucture were discussed and it was suggested that a vaccine containing A2, A1 and A7 (in order of importance) should control CNP in sheep and pneumonic pasteurellosis in both sheep and goats.

Field isolates of *P.haemolytica* were compared with prototype strains for capsule production (using Laurell Rocket test), and antibiotic sensitivities. The total proteins of caprine and ovine strains were also compared, using SDS-PAGE. Laurell Rocket tests showed that the prototype strains produced more capsular polysaccharide than did any of our field isolates. All isolates of *P.haemolytica* showed some resistance to streptomycin while none were resistant to more than 4µg/ml chloramphenicol or penicillin so these are the drugs of choice. No difference was found within a serotype between the total proteins of caprine and ovine isolates by SDS-PAGE.

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INTRODUCTION

Pasteurella haemolytica is a small Gram-negative rod found as a commensal in the nasopharynx of sheep and goats. In both species it causes pasteurellosis and it is also involved in chronic non-progressive pneumonia (CNP) of sheep. Pasteurellosis occurs as two distinct disease entities: septicaemic pasteurellosis and pneumonic pasteurellosis. Septicaemic pasteurellosis is found in New Zealand, but is rare (Hartley *et al.*, 1955). The pneumonic form also occurs in New Zealand where it is a common and widespread disease (Salisbury, 1957). It is caused by *P.haemolytica* often in conjuction with parainfluenza virus type 3 (Harbourne, 1979). It is a rapidly fatal disease of economic significance and is found in both sheep and goats.

The aetiology of CNP is not universally agreed. However most workers concur that *M.ovipneumoniae* and *P.haemolytica* are involved. *M.ovipneumoniae* initiates lesions which are exacerbated by *P.haemolytica* (Jones *et al.*, 1978). The disease is widespread and prevalent in New Zealand sheep aged between 6 and 9 months. CNP was estimated to have caused a $26 million loss in 1975 (Dysart, 1976). Treatment of pasteurellosis or CNP is seldom a practical proposition, for economic reasons. Furthermore acute pasteurellosis is often diagnosed only at post mortem as it is often a rapidly fatal disease. However an effective *P.haemolytica* vaccine could prevent losses due to pneumonic pasteurellosis and CNP.

Immunity to *P.haemolytica* depends on a immune response to the capsular polysaccharide (Biberstein *et al.*, 1965b). However there are 15 serotypes of *P.haemolytica* based upon the capsular polysaccharide (Fraser

et al., 1982). Obviously any *P.haemolytica* vaccine should contain the serotype or serotypes which most frequently cause disease in New Zealand. This requires information on the prevalence of the various serotypes in New Zealand and their association with disease in sheep and goats. This in turn requires a practical method by which isolates can be serotyped.

The method of serotyping *P.haemolytica* is, by convention, the indirect haemagglutination assay, (IHA) (Biberstein *et al.*, 1960). This method is laborious and the results may be difficult to interpret due to cross-reactions between serotypes. The agar gel immunodiffusion (AGID) test is a more convenient method for serotyping, but it has been used only by Murasachi *et al.*, (1965), who found that it was less than satisfactory due to the number of cross-reactions that occured.

This led us to undertake the following investigation.

1/ Comparison of IHA and AGID as methods of serotyping *P.haemolytica* with the aim of developing AGID as a standard serotyping method.

2/ The use of AGID to survey the prevalence of serotypes of *P.haemolytica* found in:

a) The lungs of sheep with CNP

b) The lungs of sheep with pneumonic pasteurellosis

c) The lungs of goats with pneumonic pasteurellosis

d) The nasal cavities of apparently healthy goats.

3/ To examine field isolates of *P.haemolytica* as follows:

a) To compare the production of capsular polysaccharide by prototype and field isolates, using the Laurell Rocket Test.

b) To compare the proteins of caprine and ovine isolates by SDS-PAGE (only isolates from the same serotype were

compared).

c) To establish the antibiotic sensitivities of isolates of *P.haemolytica* from pneumonic pasteurellosis of sheep and goats.

CHAPTER 1

Historical review

1.10 Classification of P.haemolytica

The genus *Pasteurella* includes 4 main species (*P.multocida*, *P.haemolytica*, *P.pneumotropica* and *P.ureae*) plus up to 3 more of uncertain affiliation.

The *Pasteurella* are small, Gram-negative, non-motile rods. They are aerobes or facultative anaerobes with a fermentative metabolism. They may be differentiated from the *Enterobacteriaceae* as they are oxidase positive and produce acid but no gas and no H_2S in triple-sugar-iron slants. They may be distinguished from fermentative bacteria (other than the *Enterobacteriaceae*) on the basis of tests for oxidase, urease and indole activity.

P.haemolytica can be distinguished from other *Pasteurella* as it produces a zone of haemolysis on blood agar and does not produce urease.

1.11 Biotypes

The heterogeneity *P.haemolytica* was first recognised by Biberstein *et al.*, (1958), who described two types of the organism which differed in colonial characteristics, crystal violet uptake and serology but did not define them further. Smith, (1959), agreed with Biberstein's division of *P.haemolytica* and designated the two forms of *P.haemolytica*, the «T strains» and the «A strains». Perhaps more importantly, Smith, (1961), found that the two strains were associated with different diseases. A

strains were found in the lungs of pneumonic lambs and sheep but were also associated with septicaemia of lambs aged less than three months whereas T strains were associated only with septicaemia of older lambs.

Smith, (1961), showed that the strains, now referred to as biotypes, could be readily distinguished on primary isolation on the basis of colony morphology. T biotype colonies were larger than that of A biotypes and possessed dark centres. A biotype colonies were an even grayish colour sometimes with a central thickening. When dispersed in water T biotypes formed a lace-like pattern whereas A biotypes dispersed evenly. However the biotypes are similar in the degree of haemolysis produced on blood agar (Smith, 1961).

The biotypes can also be distinguished by fermentation reactions: the T strains all ferment trehalose and fail to ferment arabinose within 10 days. In contrast, A strains fail to ferment trehalose but ferment arabinose within 10 days (Smith, 1959). It was this characteristic fermentation pattern which led to the two biotypes being called T (for trehalose) and A (for arabinose), respectively. The biotypes may also be differentiated on the basis of other fermentation reactions. The A biotypes ferment starch (Smith, 1961) and xylose (Shreeve *et al.*, 1970) whereas the T biotypes ferment salicin and mannose (Shreeve *et al.*, 1970).

Growth curves of A and T strains in broth are initially similar with both reaching similar maximum viable counts. However after this, the viable counts of T strains decline slowly while those of A strains fall sharply. As a consequence of this, after 24 hours incubation the viable count of T biotypes may be 40 times that of the A biotypes (Smith, 1959)

Smith, (1959), found that the A biotype were markedly more sensitive to penicillin than the T biotype. The A biotype is also significantly more

sensitive to tetracycline, ampicillin, cephalothin, chloramphenicol, erythromycin and furadantin (Biberstein *et al.*, 1979). A biotypes are inhibited by much lower concentrations of basic fuchsin, methylene blue and brilliant green than the T biotypes (Olmos *et al.*, 1979).

The differences between the A and T biotypes extend to their capsules. By electron-microscopy Gilmour *et al.*, (1985), showed that while the capsules of A biotypes are large and well defined, those of T biotypes are thinner and less defined. Adlam *et al.*, (1984) and (1985), chemically analysed the capsular polysaccharides of *P.haemolytica* serotypes A1 and T4 and found that while the capsular polysaccharide of A1 was a conventional Gram-negative polymer, that of T4 was a teichoic acid, a molecule not usually associated with Gram-negative organisms.

The differences between the A and T biotypes of *P.haemolytica* are now known to be more fundamental than variations in growth patterns, capsules and antibiotic susceptibilities. Thus DNA-RNA hybridizations between the A and T biotypes (Biberstein *et al.*, 1968), suggest that the differences between the two types are equivalent to those between different species.

These differences between A and T biotypes led Gilmour *et al.*, (1985), to suggest that the A and T biotypes should be recognised as separate species.

1.12 **Antigens of P.haemolytica.**

Biberstein *et al.*, (1960), heated broth cultures at 56°C and used the supernatant «soluble antigens» as the basis of a serotyping system for *P.haemolytica*. They used autoclaved cells to study the somatic antigens by agglutination tests. Six somatic groups were detected. Somatic group A (not

to be confused with biotype A) contains serotypes 1, 5, 6, 7, 8 and 9, while somatic group B contains serotype 2 strains.

Immunity to *P.haemolytica* depends largely on the serotype (Knight *et al.*, 1969). Hence from a vaccine point of view, the association of serotype (rather than somatic type) with disease is of overriding importance and has received most attention.

Cameron, (1966), stated that the main antigen detected in the serotyping of *P.haemolytica* by IHA was a polysaccharide, but that protein antigens were also capable of producing agglutination in the IHA. Himmel *et al.*, (1982), extracted a highly immunogenic protein from a number of *P.haemolytica* serotypes. This protein was toxic to bovine alveolar macrophages. It was neutralized by species-specific antisera, with homologous antisera giving better neutralization than heterologous (Shewen *et al.*, 1983).

Tabatabai *et al.*, (1981), isolated from A1 a soluble, extracellular antigen that had neuriminidase activity. This activity was inhibited by antisera to *P.haemolytica* serotype A1 but not by normal serum.

Donachie *et al.*, (1984)., made sodium salicylate extracts of *P.haemolytica* serotypes A1 and A6. They determined the chemical composition of these extracts and suggested that sodium salicylate extracted a complex of lipid, polysaccharide and protein from the outer membrane. A number of antigens were detected. For both serotype A1 and A6 a dominant serotype-specific antigen was detected by counter immunoelectrophoresis (CIE).

Adlam *et al.*, (1984) and (1985), purified the serotype-specific capsular polysaccharide of *P.haemolytica* serotypes A1 and T4 and characterized them by chemical analysis and NMR spectroscopy. The two polymers were chemically dissimilar. The T4 capsule was composed of

teichoic acid. The partially purified polymers were antigenic in sheep (although completly purified polymers were not). The purified polysaccharide reacted with A1 antisera only, in the agar gel immunodiffusion test. Antisera raised against the partially purified polysaccharide reacted with sodium salicylate extracts of homologous cells, producing a line of identity with the purified polysaccharide. This led Adlam *et al.*, (1984), to suggest that the sodium salicylate extracts and purified polysaccharide are similar or identical immunologically and that this also implies that when present in the cell wall, the capsular polysaccharide is attached to lipid components.

1.13 **Number of Serotypes.**

Early work on the serology of *P.haemolytica* established the antigenic separation of *P.haemolytica* and *P.multocida* (Newsome *et al.*, 1932). In subsequent studies Tweed *et al.*, (1930), postulated only 1 serotype whereas Montgomerie *et al.*, (1938), suggested that there were 3 serotypes of *P.haemolytica*. Nearly 20 years later, Carter, (1956), reported the species to be antigenically homogeneous.

The definitive work on the serotyping of *P.haemolytica* was done by Biberstein *et al.*, (1960). This group typed 98 isolates of *P.haemolytica* by the indirect haemagglutination assay, (IHA), and found that they could be divided into 10 serotypes (numbered 1-10) or were untypable.

Subsequently 5 additional serotypes have been identified (Biberstein and Gills, 1962; Biberstein and Thompson, 1966; Pegram *et al.*, 1979; Fraser *et al.*, 1982). It should be noted that the most recent addition to the *P.haemolytica* serotypes, T15, is serologically related to serotype T3 and

it is uncertain if the two are antigenically distinct (Fraser *et al.*, 1982).

1.14 **Untypable Strains**

A number of isolates of *P.haemolytica* do not react in the IHA with antisera to any of the 15 existing serotypes. Antisera raised against these isolates do not give interpretable IHA results even when tested against the homologous strains (Aarsleff *et al.*, 1970; Frank, 1980). Many of these untypable strains are also difficult to assign to a biotype as their fermentation patterns are often anomalous (Aarsleff *et al.*, 1970). Frank, (1970), tested a number of untypable strains by rapid plate agglutination and suggested that some strains may be untypable due to their lack of serotype-specific antigen. Untypable strains are usually isolated from the nasal cavity but are rarely, if ever, recovered from pathogenic lesions. From a veterinary point of view they are probably unimportant.

1.15 **Identification of Serotypes**

The most widely used method of serotyping *P.haemolytica* is the indirect haemagglutination assay (IHA), first described by Biberstein *et al.*, 1960). Modifications to this method include the use of glutaraldehyde-fixed sheep RBC (Sawada *et al.*, 1982), and the use of microtitre plates (Fraser *et al.*, 1983).

The IHA involves the adsorption of capsular material from heat-treated culture supernatants of the strain to be tested to glutaraldehyde-fixed sheep RBC. These antigen-coated RBC are added to serial dilutions of antisera to all 15 serotypes. This test is laborious as it involves 15

titrations for each isolate. It may also be difficult to interpret because cross-reactions may occur (Biberstein, 1965a).

An agglutination test for the serotyping of *P.haemolytica* was described by Frank and Wessman, (1978). It gave essentially the same results as the IHA but more cross-reactions were found.

Muraschi *et al.*, (1965), extracted «somatic antigens» from *P.haemolytica* and used the extracts in an agar gel immunodiffusion (AGID) test to compare serotypes. The gel precipitation lines were to a large extent serotype specific. This is not as surprising as may be first thought because the method of extraction used to prepare the «somatic antigens» would also extract the serotype specific capsular polysaccharide.

It was suggested by Biberstein, (1965a) that minor antigens were responsible for cross-reactions observed in IHA. However Gonzalez-Rayos *et al.*, (1986), inserted two recombinant plasmids made from the genome of *P.haemolytica* serotype A1 and coding for a soluble surface antigen into *E.coli*. *E.coli* cells carrying the plasmids were agglutinated strongly by antiserum to *P.haemolytica* A1 and weakly by antisera to A2 and A7. They were found to have patches of extra-cellular material on their surfaces. Southern blot hybridization using the DNA from recombinant plasmids as a probe showed that the probe hybridized strongly to DNA from serotype A1 and weakly to DNA from serotypes A2 and A7. This suggests that the cross-reactions observed are due to shared determinants in the capsular material of different serotypes rather than shared protein or other minor antigens.

1.16 **Relationships Between Serotype And Biotype**

Biotype and serotype are not independent. Biberstein *et al.*, (1962), tested 37 strains, which represented all known serotypes of *P.haemolytica*

for fermentation reactions and antibiotic sensitivity patterns and assigned them to either biotype A or T. Serotypes 1, 2, 5, 6, 7, 8, 9 and 11 were all biotype A, while serotypes 3, 4 and 10 were biotype T. Serotypes 12, 13 and 14 were isolated subsequently and were found to be biotype A while the latest serotype, 15, belongs to the T biotype, as would be expected because it is closely related to serotype 3 (Fraser *et al.*, 1982).

In contrast to the above, workers in Kenya (Mwangota *et al.*, 1978) indicated that nasal isolates, from sheep and goats, of serotypes 3, 4, 6, 10 and 12 were found to contain both A and T biotypes within a single serotype. However this work is not convincing and has not been confirmed.

1.20 **Commensal Association of P.haemolytica with Sheep and Goats**

P.haemolytica is a commensal which may be isolated from the nasal cavity and tonsils of apparently healthy sheep and goats. The two biotypes appear to localise in different regions. In a survey by Gilmour *et al.*, (1974), *P.haemolytica* was isolated from 95% of the ovine tonsils examined and from 64% of the ovine nasopharyngeal swabs. Of these isolates, 65% of those from tonsils but only 6% of those from nasopharynxes were T biotype while 60% of *P.haemolytica* isolates from the nasopharynx and 20% of those from the tonsils were A biotype. The remaining isolates gave anomalous fermentation patterns and thus could not be biotyped.

P.haemolytica has been isolated from the nasal cavities of normal goats with a prevalence of from 5% (Ojo, 1970) to 64% (Ngatia *et al.*, 1985). The biotypes of the isolates are unknown. *P.haemolytica* has also been isolated from the tonsils of goats, (Mwangota *et al.*, 1972), although the biotypes of these isolates are uncertain.

1.30 **Diseases Associated with P.haemolytica in Sheep.**

The most important of the *P.haemolytica* infections are the pneumonias: chronic non-progressive pneumonia (CNP) and acute pneumonic pasteurellosis. These are considered below in more detail.

P.haemolytica is also involved in a septicaemic disease of sheep caused in young lambs by either A or T biotype and in older lambs by biotype T.

Arthritis has been observed asted lungs and numerous blood splashes on the heart. Lesions may be found in the alimentary tract. These lesions appear to spread from the tons this association may be found in the field, although *P.haemolytica* has not been isolated from the diseased joints.

Ovine mastitis is frequently caused by *P.haemolytica*. Systemic infections following mastitis are not uncommon and sheep may die or lose a mammary gland due to gangrene (Biberstein, 1981).

P.haemolytica has been associated with ulcerative infections of the oesophagus and pharynx, though it is not confirmed that the organism causes the lesions. Also *P.haemolytica* has been isolated from parasitic gastro-enteritis infections, and from encephalitis and meningitis (Harbourne, 1979).

1.31 **Pneumonic Pasteurellosis**

Synonyms include enzootic pneumonia (Note: although this term is used as a synonym for pneumonic pasteurellosis in New Zealand, elsewhere it refers to CNP), acute necrotising pneumonia, acute exudative pneumonia and acute pneumonia. The disease may occur in any age of sheep at all times of the year, although outbreaks are more common in spring or early autumn (Harbourne, 1979).

Outbreaks are often sudden. The proportion of animals affected ranges from single sporadic cases to 10% of the flock (Harbourne, 1979).

Symptoms include respiratory distress, often accompanied by coughing, discharges from the nose and eyes, high fever and occasionally diarrhoea. Affected animals usually die within 24-36 hours of the onset of the disease (Harbourne, 1979).

The pathology of pneumonic pasteurellosis is characterised by a severe fibrinous pleurisy with pleural adhesions. The apical and cardiac lobes are often consolidated with the remainder of the lung showing acute congestion (Salisbury, 1957).

Histopathological examination shows a cellular exudate containing neutrophils,macrophages and detached alveolar epithelial cells, (Alley, 1975a), with «oat cells» lying at the edge of the lesion (Harbourne, 1979).

P.haemolytica biotype A may be isolated in profusion from the lesions and pericardial fluid of pneumonic lungs. Ocasionally a T biotype may be isolated from a pneumonic lesion (Gilmour, 1978).

It has been suggested that the pneumonia is associated with a viral infection such as parainfluenza virus type 3 (PI-3) or adenovirus (Harbourne, 1979).

The factors governing the prevalence of pneumonic pasteurellosis are not well known. It is not known why some flocks are affected and others not (Harbourne, 1979).

Although spring and autumn, rather than winter show peaks in prevalence, cold weather may be a pre-disposing factor, especially in cases associated with a viral infection (Harbourne, 1979). As well, mustering, over-heating and transportation, humidity and changes in diet have all been

implicated, as have dipping, castrating and dosing (Gilmour, 1978). All these factors involve stress.

1.32 **Septicaemic Pasteurellosis**

Septicaemic pasteurellosis of sheep is caused by both the A and T biotypes of *P.haemolytica*. In lambs under 2 months, infection with *P.haemolytica* biotype A usually causes septicaemia rather than pneumonia (Gilmour, 1978). In older lambs and sheep, septicaemic pasteurellosis is associated with *P.haemolytica* biotype T.

Septicaemic pasteurellosis occurs in all ages of sheep throughout the year, but appears to be more prevalent in 1-2 year old sheep during autumn, coinciding with the movement of sheep and changes in feeding (Gilmour, 1978). Bad weather or disease also seem to be predisposing factors (Dyson *et al.*, 1981).

Affected sheep are commonly found dead. Symptoms include dullness with an unwillingness to move, laboured breathing, frothy discharge from the mouth and high temperature (Gilmour, 1978).

Post mortem examination shows enlarged, haemorrhagic lymph nodes, swollen and congested lungs and numerous blood splashes on the heart. Lesions may found in the alimentary tract. These lesions appear to have spread from the tonsils (Dyson *et al.*,1981).

Fatal septicaemic disease may also be produced in lambs by inoculation of heat-killed bacteria (Smith, 1960) and from the pathology of the disease it seems unlikely that it is a true septicaemia (if this is defined as requiring multiplication of bacteria in the blood), but is a toxaemia because the organisms multiply in the tissues. Death is due to liberated endotoxin (Dyson *et al.*,1981).

It has been postulated that the T biotype organisms commonly found in the tonsils, may multiply and invade surrounding tissues. From there they spread to the liver, spleen and kidney (Dyson *et al.*, 1981).

1.33 **Chronic Non-Progressive Pneumonia**

Synonyms for chronic non-progressive pneumonia or CNP include enzootic pneumonia (Note: although this term is used as a synonym for pneumonic pasteurellosis in New Zealand, elsewhere it refers to CNP), sub-acute pneumonia, summer pneumonia (in Australia), acute exudative pneumonia, hogget pneumonia, atypical pneumonia (Stamp *et al.*, 1963), proliferative interstitial pneumonia (Sullivan *et al.*, 1973)

CNP is prevalent in New Zealand sheep between the ages of 3 and 10 months (hence the name hogget pneumonia), unlike acute pneumonic pasteurellosis which may be found in all ages of sheep.

The disease is infrequently fatal and as the name suggests, is chronic, which distinguishs it from acute pneumonic pasteurellosis, and non-progressive, which distinguishs it from the progressive pneumonia produced by maedi virus and related agents.

CNP elicits few clinical signs. In experimentally induced pneumonia (Jones *et al.*, 1982) animals showed depression, with slight fever and coughing. Sullivan *et al.*, (1973), reports that an affected flock showed poor growth and exercise intolerance.

The macroscopic appearance of the lesions shows differing degrees of consolidation over the antereo-ventral parts of both lungs. Lesions vary in colour between red and gray with red lines of collapse present in some cases. In severe cases the affected lobes are noticeably firm and thicker than normal. Occasionally fibrinous adhesions are found between the

affected lobes and the pleura. Abcesses may be found associated with lesions of CNP in older lambs.

The predominant feature of CNP lesions by light microscopy is alveolar collapse. This is associated with neutrophil infiltration and cellular exudate composed primarily of macrophages. As well, severe hyperplasia of both the bronchiolar and alveolar epithelium may be found (Alley, 1975a).

Damage by CNP tends to be localised and less than that caused by pneumonic pasteurellosis (Alley, 1975a). The aetiology of CNP is complex and has not yet been unequivocally established, although most workers do agree on the organisms involved.

St. George, (1972), found that the bacteria most frequently isolated from pneumonic sheep lungs were *P.haemolytica*, *Corynebacterium pyogenes* and *Neisseria* species. *Mycoplasma* species were also isolated, as were a low number of viruses.

P.haemolytica can be isolated from about 60% of pneumonic lung lesions but from only about 6% of normal lungs (Alley, 1975b). The organism can also be found in about 75% of nasal cavities (Alley, 1975b). *P.haemolytica* produces pneumonia when inoculated intrabronchially in large (10^{10} organisms) doses (Smith, 1964). However this disease (pneumonic pasteurellosis) is not CNP and obviously the relationship of *P.haemolytica* to CNP is not a straight-forward one of cause and effect.

M.ovipneumoniae was recovered from the nasal cavities of 70% of pneumonic sheep and from 100% of pneumonic lungs. Titres of 10^6-10^7 organisms per gram of lung were present. *M.ovipneumoniae* was isolated from only 20% of normal lungs and then with a titre of less than 10^3 (Alley *et al.*, 1975). *M.ovipneumoniae* is capable of producing macroscopic lesions when inoculated intratracheally (Sullivan *et al.*, 1973).

Alley and Clarke, (1977), found that the number of *M.ovipneumoniae* and bacteria found in the lungs correlated with the histopathological lesions such as high numbers of neutrophils and severe epithilial hyperplasia.

Jones *et al.*, (1978), produced lung lesions identical to the field cases by the intratracheal inoculation of pneumonic lung suspensions, and by inoculations of a mixed culture of *M.ovipneumoniae*, *M.arginini* and *P.haemolytica*. This implies that more than one organism may be necessary to establish the disease.

1.40 **Diseases Associated with P.haemolytica in Goats.**

The majority of reports of *P.haemolytica* infections in goats refer to pneumonic diseases and these appear to be the most important. However Gourlay and Barber, (1960), report the isolation of *P.haemolytica* from cases of septicaemia in young kids and this disease is similar to that found in lambs.

Bhagwan and Singh, (1972), examined pneumonia in goats and concluded that there were 6 different pathological conditions. They gave no indication of the micro-organisms involved.

In a review on caprine pneumonia Ojo, (1977), described 2 diseases, contagious caprine pleuropneumonia, associated with *Mycoplasma* species and a pneumonic pasteurellosis infection involving either *P.haemolytica* or *P.multocida*. Pande, (1942) and Borgman and Wilson, (1955), described outbreaks of disease, in India and the USA respectively, but it is not clear whether the organisms involved are *P.haemolytica* or *P.multocida* or both and whether or not any other micro-organisms were involved. Misra *et al.*, (1970), reported pneumonic pasteurellosis in kids due to *P.haemolytica*. This disease appears similar in sheep and goats. Symptoms

include nasal discharge and cough often associated with sudden death (Misra *et al.*,1970). The pathology of the disease is also similar to that of sheep, with consolidation of the lobes of the lungs, exudate in the thoracic cavity and inflammation of the pleural cavity (Ojo, 1976). Predisposing factors appear to be the same; thus Ojo, (1976), reported that the incidence of caprine pneumonia increased with increase in relative humidity and rainfall.

It appears that the acute pneumonic pasteurellosis pneumonia in goats and sheep are alike, in aetiology, symptoms and predisposing factors, as well as the pathology of the lesions produced.

1.50 **Importance of Pasteurellosis in Sheep and Goats**

Pneumonic pasteurellosis was first described by Dungal, (1931), in Iceland, where it caused considerable losses. The disease has subsequently been described in the USA (Newsome *et al.*, 1932), the UK (Montgomerie *et al.*,1938), New Zealand (Salisbury, 1957), South Africa (Cameron *et al.*, 1970) and Kenya (Mwangota *et al.*,1978). In New Zealand the disease may cause heavy losses in some years with up to 15% mortality (Salisbury, 1957). Between 9% (Davis, 1974) and 12% (Pyke, 1974) of all sheep deaths on the farm have been attributed to pneumonia. In New Zealand, Davies, (1986), estimated that pleurisy caused by pneumonic pasteurellosis alone costs between $7 and $12 million every year in rejected carcases.

Septicaemic pasteurellosis has been reported from the UK (Stamp *et al.*, 1955), from New Zealand (Hartley *et al.*, 1955) and from the USA (Biberstein *et al.*, 1959) and would appear to be as geographically widespread but not as prevalent as pneumonic pasteurellosis. In New Zealand septicaemic pasteurellosis does not appear to be as important as overseas,

although up to 7% of neonatal lamb mortalities may be attributed to it (Hartley *et al.*, 1955).

It is difficult to assess the importance of pneumonic pasteurellosis in goats as little work has been done in temperate countries. However if the disease in goats follows the same pattern as that in sheep, which appears likely, pneumonic pasteurellosis may be an important disease.

1.60 **Importance of Chronic Non-Progressive Pneumonia**

Chronic non-progressive pneumonia is highly prevalent in New Zealand with 70% to 80% of some flocks being affected (Alley, 1975a). Although it is rarely fatal it is economically important for two reasons: CNP may cause pleural adhesions in some lambs, (Alley, 1975a) leading to downgrading or rejection of carcasses and CNP diminishes weight gain in lambs (Jones *et al.*, 1982). It has been estimated that 3% of lambs killed in New Zealand have pleural adhesions (Dysart, 1976) and this alone has been estimated to have cost $26 million in lost revenue in the 1982/1983 season. The economic loss due to poor weight gain is harder to assess. It was found (Jones *et al.*, 1982), that lambs with experimentally induced CNP needed 25% more food and 9 additional weeks to reach the same live-weight as uninfected controls. This has important economic consequences.

1.70 **Transmission of Disease**

One of the major problems in the use of sheep as experimental animals for the investigation of immunity to *P.haemolytica* is the difficulty in inducing the disease. Smith, (1964), produced an acute pneumonia in conventionally raised sheep with large ($6\text{-}18\times10^{10}$) intrabronchial inoculations of *P.haemolytica*. However, inoculations of 1×10^{8} or less were

non-fatal. Gilmour *et al.*, (1975), produced an experimental pneumonia, identical to field cases of pneumonic pasteurellosis in specific pathogen free (SPF) lambs, by exposure to $10^{4ø8}$ bacteria in an aerosol. However this method was not succesful in producing pneumonia in all lambs. Sharp *et al.*, (1978), consistently produced a severe pasteurellosis pneumonia (identical to the field cases) in SPF lambs by inoculation with parainfluenza virus type 3 followed by an aerosol of *P.haemolytica* serotype A1. The problem with this method is that SPF lambs are expensive to obtain and maintain and are only available seasonally. Gilmour *et al.*, (1982a) produced a pneumonic pasteurellosis, similar though not identical to the naturally occuring disease, in conventionally raised lambs by the intravenous inoculation of 5% sterile agar, followed by an aerosol of *P.haemolytica*.

The above experiments refer to pneumonic pasteurellosis. CNP however is consistently produced using an inoculum of pneumonic lung homogenate. Jones *et al.*, (1978), produced «atypical pneumonia» by endobronchial inoculations of pneumonic lung homogenate. This disease could not be distinguished from field cases of chronic-non-progressive pneumonia. Alley *et al.*, (1979), transmitted CNP by aerosols of pneumonic lung homogenate.

1.80 **Antibiotic Treatment**

Treatment of acute pneumonic or septicaemic pasteurellosis even if it were economic may be difficult for two reasons.

1/ The disease, especially the septicaemic form, has a rapid course. Affected animals may die within 24-36 hours of onset of symptoms, (Harbourne, 1979).

2/ It is difficult to assess in the time available which antibiotics should be used.

Veterinary handbooks are not specific in their recomendations for treatment of *P.haemolytica* infections. Whitten, (1971), suggests that treatment with «antibiotics or sulphonamides» is useful. However no particular antibiotic is recommended and no information on dosage is given. The International Encyclopedia of Veterinary Medicine, (1966), advises that early administration of streptomycin or penicillin may be beneficial. Marsh, (1965), suggests that the regime followed in the cattle disease - tetracycline administered intravenously, followed by penicillin and streptomycin intramuscularly or intraperitonially should be used. Salisbury, (1957), reported the successful treatment of pneumonic pasteurellosis with penicillin, while Carter, (1967), suggested that the sulphonamides are effective against *P.haemolytica* infections, at least in cattle. Gilmour *et al.*, (1982b), recommend the use of oxytetracycline for the treatment of pneumonic pasteurellosis.

Reports on the minimun inhibitory concentration (MIC) of different antibiotics for *P.haemolytica* vary. It should be remembered that the A and T biotypes have different sensitivity patterns. Smith, (1959), found the A biotype to be markedly more sensitive to penicillin than the T biotype. As well the A biotype is significantly more sensitive to tetracycline, ampicillin, cephalothin, chloramphenicol, erythromycin and furadantin (Biberstein *et al.*, 1979).

Unless specifically mentioned all subsequent MIC data refers to the A biotypes.

ANTIBIOTIC	**MIC** (µg/ml)	**References**
Penicillin	0.008 - 12.5	Biberstein *et al.*,1979, Maysson *et al.*,1959.
Ampicillin	0.0125	Biberstein *et al.*,1979.
Erythromycin	0.2 - 0.8	Biberstein *et al.*,1979, Maysson *et al.*,1959.
Streptomycin	12.5	Maysson *et al.*,1959.

Chang *et al.*, (1967), found that 90% of their isolates from bovine and porcine disease were resistant to 10µg/ml streptomycin, while over 50% were resistant to 30µg/ml tetracycline and/or 10units/ml penicillin. However only 1.4% were resistant to 30µg/ml chloramphenicol.

Martin *et al.*, (1983), regarded as resistant any strain that was not killed in growth conditions by 10 units penicillin, 5µg tetracycline or 30 µg chloramphenicol.

In 1980, Zimmerman *et al.* reported a strain of *P.haemolytica*, biotype T, which had a MIC of 128µg/ml penicillin, 128µg/ml ampicillin, 32µg/ml streptomycin and 64µg/ml tetracycline. This strain was isolated from the lung of a feedlot calf. The tetracycline and steptomycin resistance genes were carried on separate small, non-transmissable plasmids. In the laboratory this resistance could be transferred by transformation to *E.coli*.

Amstutz *et al.*, (1982), found that 90% of *P.haemolytica* strains isolated from nasal swabs from feedlot cattle were resistant to one or more

antibiotics. This included 1 strain resistant to >200µg/ml penicillin, 100µg/ml oxytetracycline and >200µg/ml streptomycin. This multiple resistance pattern is typical of plasmid mediated resistance. These reports are from America where the heavy use of antibiotics in feedlot cattle is widespread.

In Britain, Wray *et al.*, (1983), isolated a multipley resistant isolate of *P.haemolytica*, serotype A1, from pneumonic lesions in a calf. Resistance could not be transferred to *E.coli*, unlike the isolate made by Zimmerman *et al.*, (1980).

Allan *et al.* (1985), isolated three different serotypes of *P.haemolytica* from cases of bovine pneumonic pasteurellosis, A1, (the predominant isolate) A2 and A6. The majority of the A1 strains were resistant to lincomycin and streptomycin and some were also resistant to ampicillin, penicillin and oxytetracycline. None were resistant to chloramphenicol. The A2 and A6 strains were resistant to lincomycin and streptomycin but not to any other antibiotics.

With the presence of plasmid-mediated resistance to certain antibiotics and their widespread use in animal husbandry, the number of resistant strains of *P.haemolytica* might be expected to increase. However a report from America (Gilson *et al.*, 1982), suggests otherwise. A comparison of microbial susceptibility tests found that between 1974 and 1978, the proportion of isolates of *P.haemolytica* (from bovine, ovine, porcine and caprine sources) resistant to certain antibiotics actually dropped. No reason is suggested for this.

1.90 **Immunity to P.haemolytica**

Biberstein *et al.*, (1965b), inoculated mice with serotype A1, A2, T4 or T10, then challenged them with the homologous organism, or with strains differing in either capsular or somatic antigens, or strains differing in both these antigens. They found that the greatest degree of protection was obtained when the immunizing and challenging strains were identical. A lesser degree of protection was found when only the capsular antigens were common, and still less when only the somatic antigens were the same.

Thus, while the capsular antigens (upon which the serological typing is based) are the most important, they are not the only antigens involved in protective immunity.

Cameron, (1966), showed that a phenol-water extract of polysaccharide had immunizing properties in mice, as did a protein fraction of the cells.

Knight *et al.*, (1969), immunized mice with serotypes A1, A2 or A5. Their results again indicated that immunity to challenge was influenced by both capsular antigens, and to a lesser degree, somatic antigens. However excellent protection was afforded by vaccination with an A1 strain, against an A2 strain challenge, although the two serotypes had neither capsular nor somatic antigens in common. This suggests that factors other than the capsular or somatic antigens may be involved. However the results of Knight *et al.*, (1969) do not agree with those of other workers (Gilmour *et al.*, 1979; Evans *et al.*, 1979a).

Evans *et al.*, (1979a) inoculated mice with a trivalent vaccine which contained capsular extracts of A1 and A6 and heat-killed whole cells of A2. This vaccine protected against challenge with serotypes A1 or A6, but not against A2 or A9. It was concluded that immunity to *P.haemolytica* is type specific, but that it is difficult to protect against A2.

Mice inoculated with a polyvalent vaccine including *P.haemolytica* serotypes A2, T3 and T10 showed a protective response to challege with serotype A1 or *E.coli* (Evans *et al.*, 1979b), but only if the vaccine was given 12-24 hours before challenge. This rapid response and cross-protection with *E.coli* suggest that this immunity was non-specific and induced by the endotoxin rather than the capsular antigens.

Tayadon *et al.*, (1981), prepared a number of different fractions of a *P.haemolytica* strain and used these to immunize mice and hamsters. Their results showed that capsular antigen gave the best protection. A KSCN extract provided more protection than an NaCl extract. However the degree of purification of these extracts did not allow unequivocal determination of the role that the capsular antigens play in protective immunity, especially as an ethanol-acetone purified polysaccharide produced less immunity than the other two.

In summary it appears that while the serotype specific capsular antigens are the most important in the production of protective immunity, other antigens are also involved. This tends to suggest that antibody rather than CMI confers resistance to *P.haemolytica*.

However as Wells *et al.*, (1979), found lambs with passively acquired antibody to *P.haemolytica* were not protected against challenge with homologous organisms, cell-mediated immunity may be important in resistance to disease. However, Macdonald *et al.*, (1983), found that *P.haemolytica* was sensitive to an antibody and a complement-mediated system in bovine serum. More work is required to study the relative importance of antibody and CMI in immunity to *P.haemolytica*.

1.91 **Vaccines**

Vaccines against *P.haemolytica* are currently available commercially, although their efficacy has been questioned (Cameron *et al.*, 1970; Gilmour, 1978). Gilmour *et al.*, (1979), vaccinated SPF lambs with capsular extracts of A1 or A6 in Freund's Complete, or Freund's Incomplete Adjuvant, and demonstrated that this gave protection against challenge with homologous organisms. Gilmour *et al.*, (1979), also used whole cells of serotype A2 with adjuvant as an immunizing antigen This did not protect against challenge with the homologous serotype as well as did the other two serotypes.

Gilmour *et al.*, (1982c), vaccinated conventionally reared lambs and calves with a sodium salicyate extract of A1 in adjuvant and then challenged them with an intravenous inoculation of agar plus an aerosol of A1. This vaccine afforded protection in both species.

Wells *et al.*, (1984), assessed the efficacy of a multivalent commercially available vaccine produced by Hoechst. Heptavac-P contains capsular antigens from A1, T3, T4, A6, A7, A9 and T10, plus whole cells of serotype A2 as well as a number of clostridial antigens. Experiments used conventionally raised lambs and SPF lambs and compared the response of vaccinated and unvaccinated animals to challenge by intranasal and intrabronchial inoculations of PI-3, followed by an aerosol of *P.haemolytica*. The vaccine gave a significant level of protection against experimentally induced pneumonic pasteurellosis, although it did not entirely prevent deaths in the vaccinated groups. When the multivalent vaccine was compared to a monovalent A6 preparation, the multivalent vaccine was just as effective against challenge by A6. The least protection was given against challenge with *P.haemolytica* serotype A2. The difficulty

in producing immunity to serotype A2 was confirmed in other experiments (Gilmour *et al.*, 1979; Evans *et al.*, 1979a). Unfortunately serotype A2 is the predominant serotype involved in disease, both in Britain and New Zealand (Rodger, 1982; Prince, 1985). Jones *et al.*, (1986), found that the multivalent Hoechst vaccine that provided significant protection in a PI-3 virus-*P haemolytica* challenge situation (Gilmour *et al.*, 1979), did not protect against challenge with *M.ovipneumoniae-P.haemolytica*. This suggests that while vaccines may protect against acute pneumonic pasteurellosis more work is required to produce a vaccine to protect sheep against CNP, the lesions of which are at least partially produced by *P.haemolytica*. This difficulty is complicated if serotype A2 is involved in CNP.

In summary, although the serotype-specific capsular polysaccharide is the predominant antigen involved in immunity to *P.haemolytica*, other antigens *viz.* endotoxin and protein do contribute. It is difficult to produce an immunity to serotype A2, even using homologous antigens. The importance of serotypes in immunity makes it necessary to determine the serotypes of *P.haemolytica* present in New Zealand before any attempt is made to produce a vaccine. This is the major thrust of the present work.

CHAPTER 2

Comparison of the Indirect Haemagglutination Assay and the Agar Gel Immunodiffusion Test using Prototype Strains of P.haemolytica and Field Isolates

2.1 Introduction

The indirect haemagglutination assay, (IHA), as described by Biberstein *et al.*, (1960) and later modified by Sawada *et al.*, (1982) and Fraser *et al.*, (1983), is the standard technique for serotyping *P.haemolytica*. Capsular material from the isolate to be typed is adsorbed to glutaraldehyde-fixed sheep RBC. These are added to serial 2-fold dilutions of antisera to all 15 serotypes. This test is extremely laborious and also prone to cross-reactions (Biberstein, 1965a).

The agar gel immunodiffusion test, (AGID) as described by Muraschi *et al.*, (1965) and Prince, (1985), shows a certain amount of promise as a possible replacement for IHA. However both workers found that cross-reactions between serotypes occured. This gave rise to ambiguous results when attempting to establish the serotypes of some isolates of *P.haemolytica* by AGID.

In this chapter we compare the two serotyping methods using antigens prepared from the prototype strains of *P.haemolytica* and antigens derived from field isolates of the organism.

2.2 Materials and Methods

2.21 Preparation of Antigen for Immunising Rabbits

Prototype strains of serotypes A1, T4, A5, A8, A9, A14 and T15 were inoculated into 10 ml of BHI broth and incubated at 37°C overnight. The cultures were centrifuged at 2,000g for 10 minutes and the pellet was resuspended in 10 ml of PBS. The suspended cells were heat-killed at 56°C for 30 minutes and stored at 4°C.

Inoculation Schedule for Rabbits

1.5 ml of cell suspension and 1.5 ml of Freund's Complete Adjuvant were mixed and emulsified using a vortex mixer to produce a water-in-oil emulsion. 1.5 ml of the emulsion was inoculated intramuscularly into each hind leg. After 7 days a further 1 ml of cell suspension was inoculated intramuscularly and this was repeated at 3-4 day intervals for one month.

The rabbits were bled from the ear vein 1 week after the last inoculation and the sera were titrated using AGID. If the titre was >8 the rabbit was exsanguinated by cardiac puncture. If the titre was <8 the rabbit was given a further two inoculations and bled a week later.

Rabbits produced high titre antisera to the serotypes A1, T4, A5, A8, A9 A14 and T15 but in our hands, did not produce adequate antisera to the remaining 8 serotypes. For the latter domestic hens were used to raise antisera.

Antigen Preparation for Immunising Hens

Domestic hens were used to produce antisera to *P. haemolytica* serotypes A2, T3, A6, A7, T10, A11, A12 and A13.

The following method of antisera production is adapted from that of Newman *et al*, (1982). 0.1 ml of a broth culture of *P.haemolytica* was placed on a blood agar plate and streaked to give single colonies. The plates were incubated overnight. This method gives heavier growth than the spread plate technique. The cells were suspended in 3 ml of PBS and heat-killed at 56°C for 30 minutes. This suspension was adjusted to a turbidity equal to McFarland Standard 3 and stored at 4°C.

Inoculation Schedule for Hens

1 ml of suspension and 1 ml of Freund's Complete Adjuvant were mixed to form a water-in-oil emulsion. This was inoculated intramuscularly. At the same time 1 ml of suspension was inoculated intravenously. These inoculations were followed by 1 ml intravenously at weekly intervals for 1 month.

Hens were bled 1 week after the last inoculation and the sera were titrated using AGID. If the serum had a titre >8 the hen was exsanguinated by decapitation. If the serum had a titre <8 the hen was given a further 2 inoculations and exsanguinated 1 week after the last inoculation.

2.22 **Indirect Haemagglutination Assay (IHA)**

Production of Glutaraldehyde-Fixed RBC. Sheep blood from the jugular vein was collected in heparinised vacutainers. The RBC were washed 3 times in PBS by centrifuging for 5 minutes at 3,000g. The final deposit was resuspended in PBS to give a 20% suspension. An equal volume of 0.2% glutaraldehyde in PBS was added to the cell suspension and incubated at 37°C for 30 minutes. The cells were then washed 5 times in PBS and resuspended in PBS containing 0.1% sodium azide, to give a 10% supension. This suspension was stored at 4°C for not longer than 3 months.

Preparation of Antigen. For each *P.haemolytica* serotype a 10 ml aliquot of BHI broth was inoculated and shaken at 37°C overnight. This culture was heat-killed at 56°C for 30 minutes.

Coating of RBC with Antigen. The stored glutaraldehyde-fixed RBC were re-washed and suspended in PBS to give a 5% suspension. An aliquot was added to 9 times its volume of the antigen to give a final concentration of 0.5% RBC. The RBC-antigen mixture was incubated at 37°C for 30 minutes and the RBC were then washed 3 times with PBS and suspended in PBS to give a 0.5% suspension.

IHA Test. 2-fold dilutions of antisera of each of the fifteen *P.haemolytica* serotypes were prepared in microtitre plates, using 50 µl aliquots. 50 µl of the antigen-coated RBC were added to each cavity. Controls without antibody were included.

The titre of each antiserum was taken as the highest dilution which showed total agglutination of the RBC.

2.23 Agar Gel Immunodiffusion Test

Agar Gel

Sodium Chloride	20g
Noble Agar	2.5g
Sodium Azide	0.25g
Distilled Water to	250 ml

This was boiled to dissolve the agar and 25 ml aliquots were dispensed into petri plates. Wells were cut using a template.

Preparation of Antigen for AGID. Isolates were inoculated into 10 ml aliquots of BHI broth and incubated at 37°C, with shaking overnight, after which the cultures were heat-killed at 56°C for 30 minutes.

When an isolate did not react with antisera to any of the 15 serotypes, a concentrated antigen preparation was produced. The isolate was streaked on blood agar and incubated overnight. Cells were removed with 3 ml of PBS and heat-killed at 56°C for 30 minutes.

Antigen from each isolate was tested against all 15 antisera.

Adsorption of Antisera. Antisera was adsorbed by mixing 1.0 ml of antisera with a 0.5 ml pellet of the appropriate heterologous cells. The mixture was left at room temperature for 1 hour and centrifuged to remove the cells.

2.24 Purification of Capsular Polysaccharide

See Appendix.

2.3 Results

2.31 **IHA**. Table I records the IHA titres of antisera to each of the 15 serotypes of *P.haemolytica* titrated against RBC sensitised with antigen from each of the 15 prototype strains.

Titre of Antibody Raised Against the 15 P.haemolytica Serotypes Tested by Indirect Haemagglutination Assay Against All 15 Serotypes.

Antisera

Antigen	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15
A1	**64**	16	2	<2	<2	2	16	2	<2	2	4	2	8	<2	<2
A2	8	**128**	<2	2	<2	2	8	2	<2	2	4	2	8	<2	<2
T3	2	8	**16**	2	<2	<2	4	<2	<2	2	<2	2	2	<2	16
T4	2	8	8	**32**	2	2	16	<2	<2	8	8	2	32	2	2
A5	<2	2	<2	<2	**8**	<2	<2	4	<2	<2	<2	<2	2	<2	<2
A6	4	4	2	<2	<2	**32**	8	<2	2	2	2	<2	8	<2	<2
A7	2	16	2	2	2	<2	**64**	<2	<2	8	8	4	8	<2	<2
A8	<2	2	2	2	2	32	16	**32**	<2	4	4	<2	4	<2	<2
A9	4	8	2	2	2	<2	4	<2	**16**	2	2	4	4	<2	<2
T10	<2	16	2	2	2	2	4	2	<2	**64**	4	8	16	<2	<2
A11	2	16	<2	<2	<2	<2	8	<2	<2	2	**512**	<2	8	<2	<2
A12	2	4	2	<2	<2	<2	8	<2	2	2	8	**8**	16	<2	<2
A13	<2	16	2	<2	<2	<2	8	<2	<2	8	16	<2	**32**	<2	<2
A14	<2	8	<2	<2	<2	<2	16	<2	<2	<2	8	<2	2	**32**	<2
T15	2	8	32	2	<2	<2	8	<2	2	2	2	2	2	<2	**32**

Table I

Defining a 4-fold or higher difference in titres in favour of the homologous organism as «specific» the following antisera were specific; A1, A2, T4, A5, A7, A8, A9, T10, A11, A14.

Antisera to serotypes T3, A6, A12, A13, T15 showed cross-reactions with heterologous antigens. See discussion.

2.32 **AGID**. Antisera raised against the 15 serotypes of *P.haemolytica* tested by AGID with antigens prepared from all serotypes.

Reactions of Antibody to All 15 P.haemolytica Serotypes Tested by AGID.

Antisera

Antigen	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15
A1	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A2	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-
T3	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
T4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
A5	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
A6	+	-	-	-	-	++	-	-	-	-	-	-	-	-	-
A7	+	-	-	-	-	-	++b	-	-	-	-	-	-	-	-
A8	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
A9	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
T10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
A11	+	-	-	-	-	-	-	-	-	-	++	-	-	-	-
A12	+	-	-	-	-	-	-	-	-	-	-	++	+	-	-
A13	+	-	-	-	-	-	+	-	-	-	-	+	++	-	-
A14	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
T15	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+

Table II

+ and ++ refer to the intensity of the AGID lines. ++ indicates the more readily visible ie. stronger line.

(a) = two gel precipitation lines

(b) = three gel precipitation lines

+ and ++ refer to the intensity of the AGID lines seen.

* = antiserum adsorbed with cells of a heterologous serotype as follows:

A1 was adsorbed with A7 cells.

A7 was adsorbed with A13 cells.

A12 was adsorbed with A13 cells.

A13 was adsorbed with A12 cells.

Note: T3 and T15 were not adsorbed as they are regarded as antigenically similar or identical. Apart from this serotype no further cross-reactions were observed.

2.33 **AGID Reactions of Adsorbed Antisera.** Antisera were raised against all 15 *P.haemolytica* serotypes. Those that cross-reacted with heterologous serotypes in the AGID test (see Table II) were adsorbed with heterologous cells. The adsorbed antisera were retested by AGID. The results are recorded in Table III.

Reactions of Adsorbed Antibody to all P.haemolytica Serotypes Tested by AGID

	Adsorbed Antisera														
	*						*					*	*		
Antigen	**A1**	**A2**	**T3**	**T4**	**A5**	**A6**	**A7**	**A8**	**A9**	**T10**	**A11**	**A12**	**A13**	**A14**	**T15**
A1	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A2	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
T3	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
T4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
A5	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
A6	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-
A7	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
A8	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
A9	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
T10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
A11	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-
A12	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
A13	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
A14	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
T15	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+

Table III

Figure 1

IHA of a field isolate of *P.haemolytica*, serotype A2. Rows A-H contain dilutions of antisera to *P.haemolytica* serotypes A1-A8.

Note: Although the isolate is clearly serotype A2, there are cross-reactions with antisera to serotypes A1 (row A), A5, (row E) and A7 (Row G). These cross-reactions may, on occasions make IHA results difficult to interpret.

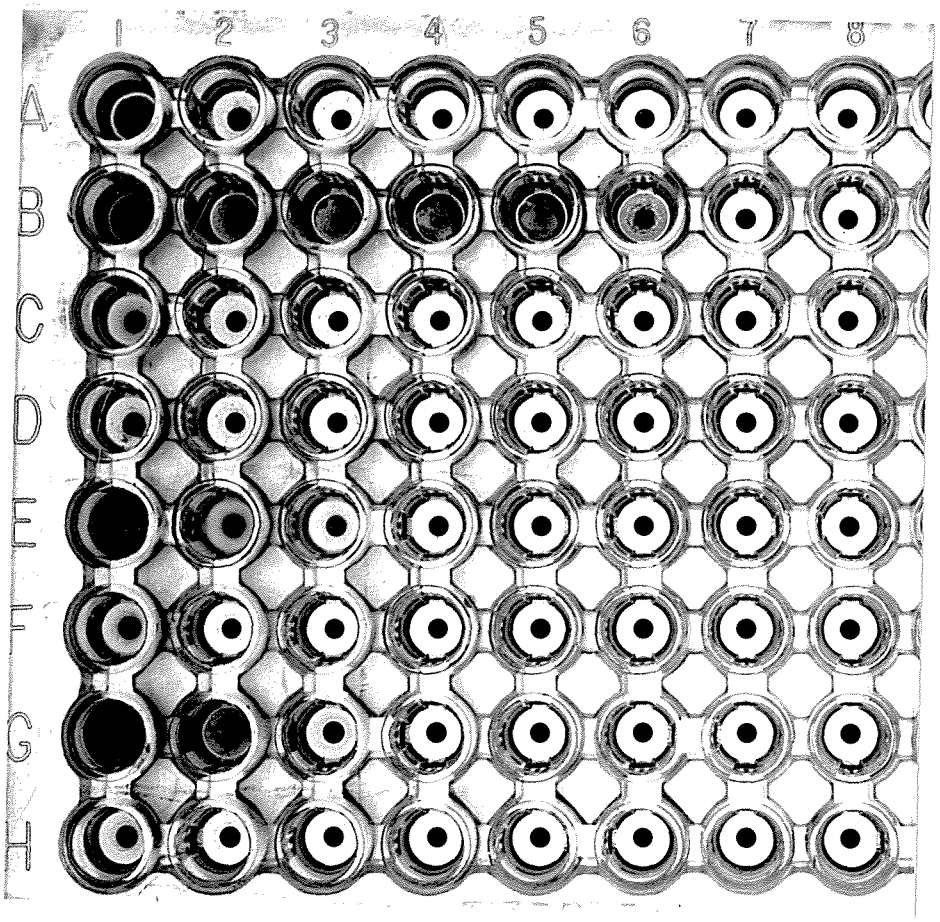

1
2
3
4
5
6
7
8
A
B
C
D
E
F
G
H

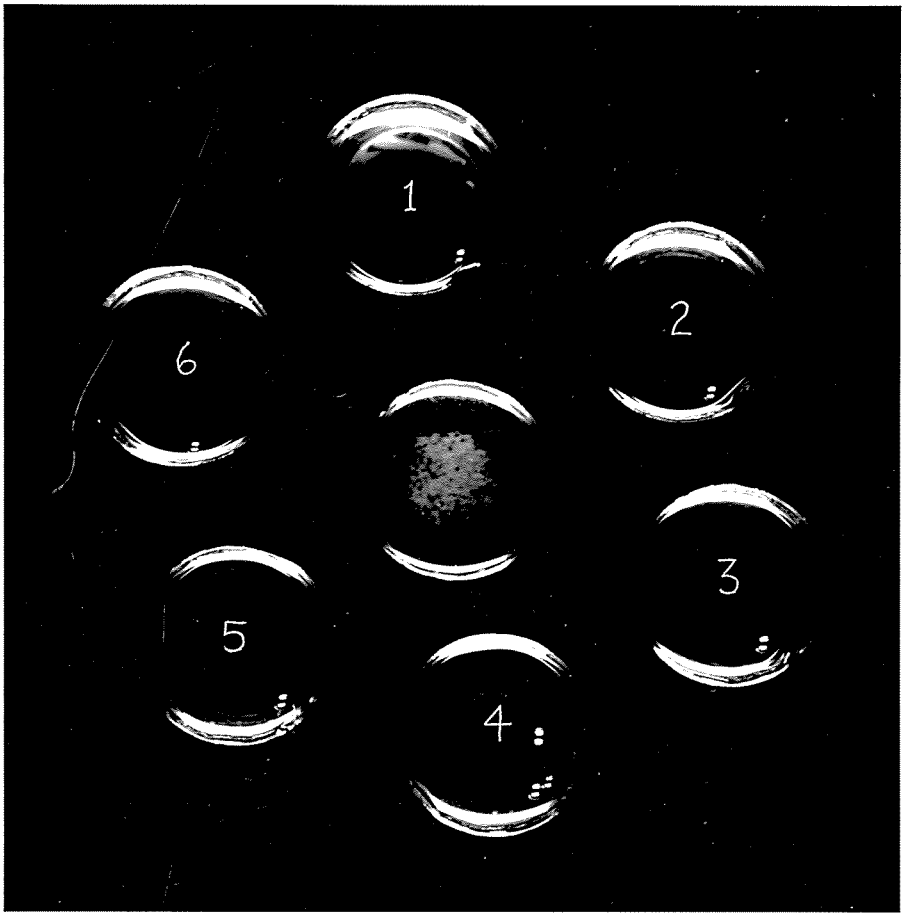
Figure 2

AGID of two field isolates of *P.haemolytica*, serotype A2 against antisera to A2 (central well).

Prototype A2 (wells 2 and 5)

Field isolates (wells 3 and 4, wells 1 and 6)

Note: A line of identity can be seen between the prototype antigen and that of the field isolate. This is the definitive test of the serotype of an isolate.


1
2
3
4
5
6

2.34 **Serotyping of Field Isolates.** The serotypes of isolates derived from cases of caprine pneumonic pasteurellosis were investigated by both IHA and AGID. Table IV records the AGID results and the serotypes inferred from them.

Investigation of the Serotypes of 25 Isolates of P.haemolytica Recovered from Goats with Acute Pneumonia, Using Adsorbed Antisera in the AGID Test.

	Antisera															
Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result
4600	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
3981	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	A7
4206	-	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
12445	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
5850	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
2348	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
2557	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
2351	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
655	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
631	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
3198	-	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
113/20	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
112/014	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2

Table IV

Table IV continued

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result
3372	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
3952	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
2883	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
3068	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
3452	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
3056	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
2695	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
17585	-	-	-	-	-	-	++a	-	-	-	-	-	-	-	-	A7
966	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	A6
1847	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	A2/A6
13287	-	+a	-	-	-	-	-	-	-	-	+	-	-	-	-	A2/A11
12251	-	-	-	-	-	-	+	-	-	-	++b	-	-	-	-	A7/A11

+ and ++ refer to the AGID lines seen. ++ indicates a more readily visible line.

(a) = two gel precipitation lines.

(b) = three gel precipitation lines.

2.35 **Serotyping of Field Isolates.** The serotypes of isolates derived from cases of caprine pneumonic pasteurellosis were investigated by both IHA and AGID. Table V records the results of the IHA and the serotypes inferred from them.

Investigation Using IHA of the Serotypes of 25 Isolates of P.haemolytica Recovered from Goats with Acute Pneumonia.

Antisera.

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result
4600	32	512	8	4	8	4	32	2	4	8	32	4	16	2	4	A2
3981	<2	2	2	2	<2	<2	32	<2	<2	<2	4	4	4	<2	<2	A7
4206	<2	32	<2	2	<2	<2	2	<2	<2	2	8	<2	8	<2	<2	A2
12445	<2	64	<2	<2	<2	<2	2	<2	<2	<2	8	<2	8	<2	<2	A2
5850	<2	64	<2	2	2	2	4	2	<2	2	8	<2	<2	<2	<2	A2
2348	<2	32	<2	2	2	<2	2	2	2	<2	8	2	8	2	<2	A2
2557	2	64	2	2	2	2	2	2	2	2	4	8	8	<2	<2	A2
2351	<2	32	<2	<2	<2	<2	2	2	2	<2	8	2	8	<2	<2	A2
655	2	64	2	2	2	<2	2	2	2	4	4	8	8	<2	<2	A2
631	2	64	2	2	2	<2	2	2	2	2	4	8	8	<2	<2	A2
3198	64	512	8	8	8	4	32	2	2	32	64	4	16	64	4	A2
113/20	16	512	4	4	2	4	16	2	<2	4	2	2	4	2	2	A2
112/014	64	512	8	4	2	4	32	4	<2	8	<2	4	8	4	4	A2

Table V

Table V continued

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result
3372	16	256	4	2	4	4	16	<2	2	8	32	8	16	4	2	A2
3952	32	512	4	4	8	4	16	2	2	8	<2	4	16	16	2	A2
2883	32	1024	4	32	32	8	128	8	4	8	32	4	16	2	4	A2
3068	16	512	4	4	8	4	16	4	4	16	<2	2	64	16	8	A2
3452	<2	32	<2	<2	<2	<2	2	2	<2	<2	16	2	8	<2	<2	A2
3056	<2	32	<2	2	2	<2	2	2	2	<2	16	2	8	<2	<2	A2/A11
2695	<2	32	<2	2	<2	<2	2	2	2	<2	16	2	8	2	2	A2/A11
17585	2	8	4	4	2	<2	16	2	4	2	4	4	4	2	2	A7/A2
966	4	2	2	2	2	16	8	<2	<2	2	4	8	8	<2	<2	A6/A7/A11
1847	4	2	2	<2	<2	16	4	<2	<2	2	4	8	4	<2	<2	A6
13287	2	64	<2	2	2	<2	2	2	<2	2	16	<2	<2	<2	<2	A2
12251	<2	32	<2	2	<2	<2	2	2	2	<2	16	2	8	2	2	A2/A11

2.35 **Serotyping of Field Isolates.** The serotypes of isolates derived from cases of caprine pneumonic pasteurellosis were investigated by both IHA and AGID. Table VI compares the results of the two tests and draws conclusions as to the serotypes of the isolates.

Serotypes of 25 Caprine Isolates of P.haemolytica: Comparison of AGID Results with IHA Results.

Isolate	IHA		AGID		Comments
	Type	Preference	Type	Cross-Reaction	
4600	A2	16x	A2	-	Both tests are
3981	A7	8x	A7	-	satisfactory and the
4206	A2	4x	A2	-	results agree.
12445	A2	8x	A2	-	
5850	A2	8x	A2	-	
2348	A2	4x	A2	-	
2557	A2	8x	A2	-	
2351	A2	4x	A2	-	
655	A2	8x	A2	-	
631	A2	8x	A2	-	
3198	A2	8x	A2	-	
113/20	A2	32x	A2	-	
112/014	A2	8x	A2	-	

Table VI

Table VI continued

3372	A2	8x	A2	-	
3952	A2	16x	A2	-	
2883	A2	8x	A2	-	
3068	A2	32x	A2	-	
3452	A2	2x	A2	-	The results agree but
3056	A2	2x	A2	-	IHA on it's own is
2695	A2	2x	A2	-	equivocal as there is
17585	A7	2x	A7	-	only a 2-fold preference
966	A6	2x	A6	-	for any one serotype.
1847	A6	2x	A6	A2	AGID showed cross-reaction
13287	A2	2x	A2	A1	IHA showed only a 2-fold
12251	A2	2x	A11	A7	preference for one serotype and the results do not agree

Note: In the cases of isolates 1847, 13287, and 12251, the antigen prepared from the isolate was retested alongside prototype or (in the case of A7), purified polysaccharide. Isolate 1847 showed a line of identity with the A6 prototype antigen, but not with the A2 prototype antigen. Isolate 13287 showed a line of identity with the A2 prototype antigen, but not with the A11 antigen. Isolate 12251 produced no gel precipitation lines with A2 antisera in the AGID. It showed a line of identity with A11 prototype antigen and no line of identity with the A7 purified polysaccharide.

Figure 3

AGID of adsorbed antisera to serotype A1 (central well) against:

1/ Prototype A1 antigens (well 1)

2/ A1 purified capsular polysaccharide (well 2)

Note: There is a clear line of identity between the two antigens. The precipitation line produced by the purified polysaccharide is sharper and more distinct than that of the prototype antigen. This is probably due to its antigenic uniformity.

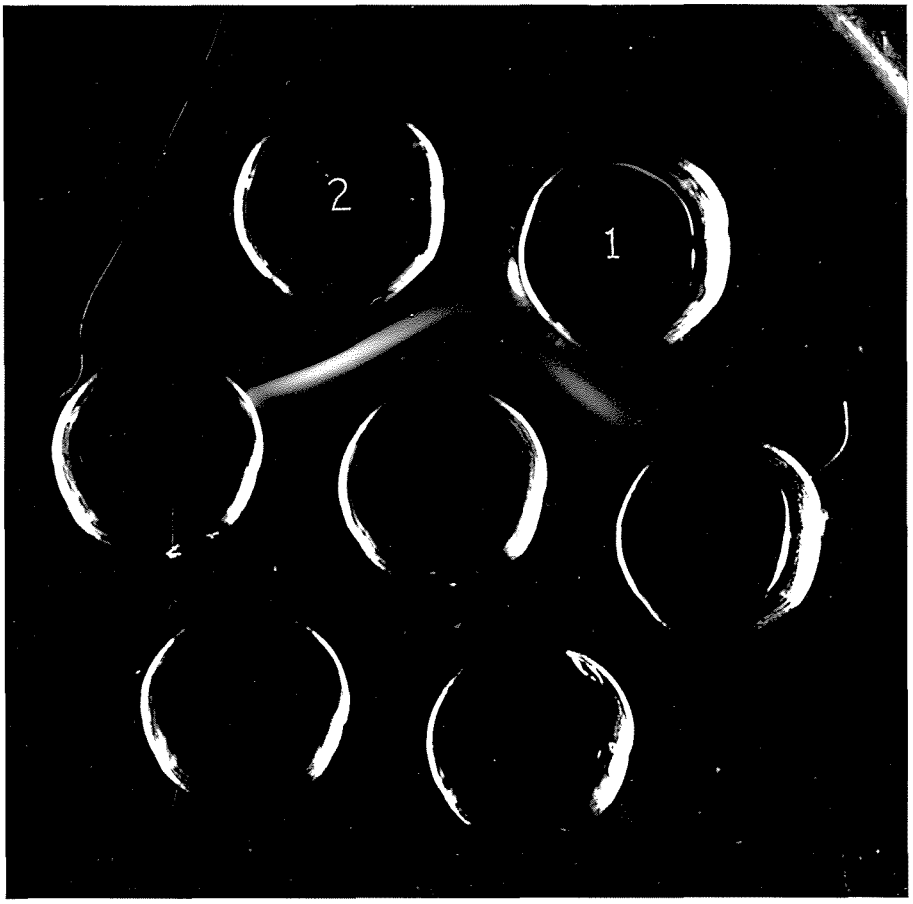

2
1

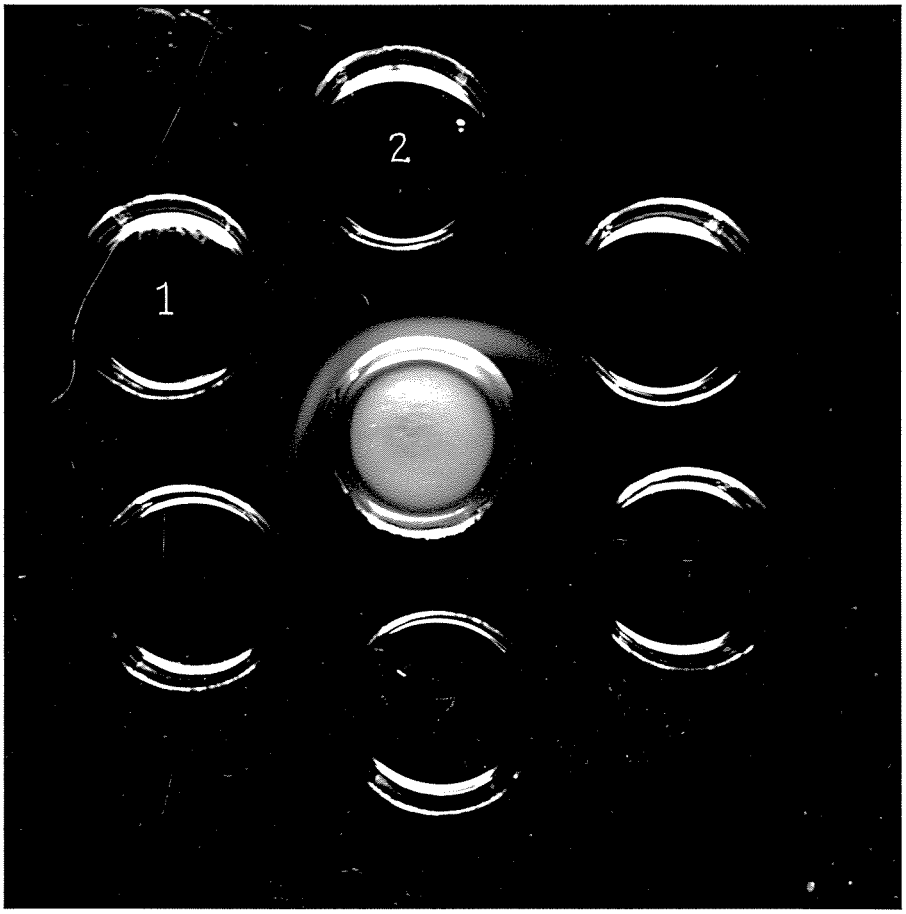
Figure 4

AGID of unadsorbed antisera to serotype A7 (central well) against:

1/ Prototype A7 antigens (well 1)

2/ A7 purified capsular polysaccharide (well 2)

Note: There is a clear line of identity between the capsular polysaccharide and one, but only one, of the precipitation lines produced by the prototype A7 antigens. We conclude that the innermost precipitation line produced by the A7 prototype antigens is due to the capsular polysaccharide. The other two lines are probably due to protein or endotoxin antigens.


2
1

2.4 **Discussion**

IHA is the standard method of serotyping *P.haemolytica*. It depends on:

1/ The ability of RBC to bind the capsular polysaccharide strongly and the absence of binding of other cellular antigens to RBC.

2/ The availability of antisera to the capsular polysaccharide.

Antiserum production and specificity

In our hands rabbits did not produce adequate antisera to 8 of the 15 serotypes of *P.haemolytica* (A2, T3, A6, A7, T10, A11, A12 and A13). For this reason antisera to the above types were produced in domestic hens.

It was somewhat unexpected that rabbits failed to produce reasonable titres of antisera because this species has been used by other workers (Sawada *et al*, 1982; Muraschi *et al*, 1965). However our experiments initially used only one rabbit per serotype and a second rabbit was immunised only if the first failed to respond. The literature is not clear on how many rabbits per serotype were used by other workers, but occasional comments on the difficulties of producing antisera (Wells, 1981; Burrells *et al*, 1983) imply that we are not alone in finding that rabbits may frequently fail to produce antibody to the capsule of *P.haemolytica*.

In contrast to rabbits, domestic hens never failed to produce high titre antisera, although the avian antisera tended to cross-react with antigens of heterologous serotypes to a greater extent than did the rabbit antisera; eg avian antisera to serotype A13 reacted with an unidentified

test and the IHA (Tables I and II). Cross-reactions were resolved by adsorbing the antisera with cells of heterologous serotypes (see Table III). This is discussed below.

Interpretation of IHA Results

In the IHA, cross-reactions were common (Table I) and this required the results to be interpreted on a qualitative rather than a quantitative basis. Thus an isolate was «typed» if the titre with one of the antisera was 4-fold greater than with any other antiserum. While this criterion has the sanction of universal application it does not take into account the variations in homologous titres which may occur (Table I). Thus our antiserum to A11 had a titre of 512 against the A11 serotype and a titre of 8 against the A12 serotype. The homologous titre of A12 antiseum was 8 so any A12 isolate which happened to be identical to the prototype A12 would give a titre of 8 with both A11 and A12 (see Table I) and thus would be of uncertain serotype. This problem could, in theory, be avoided if antisera were «normalized» by dilution so that they all give a titre corresponding to that of the weakest antiserum. To our knowledge no-one has used this approach and it would require that all isolates be typed using low titre antibody. Futhermore the titre of an antiserum can differ when it is tested with a prototype and an isolate. In such cases the isolate usually gave the lower titre. As can be seen in Table V the two isolates typed as A7 gave titres 4-fold lower than the prototype reaction with A7 antisera. Consequently if all antisera were diluted to the titre of the lowest this would almost certainly mean that many isolates would fail to react and therefore could not be typed.

Cross-reactions of antisera with heterologous serotypes are not unexpected since whole organisms are used as immunising antigens. These organisms can share common endotoxins and futhermore, examination of SDS-PAGE patterns (Thompson *et al.*, 1975) indicates that within the A and T biotypes many proteins are common to all serotypes, at least in the sense that they have similar banding patterns. Consequently cross-reactions may occur in the IHA if antigens other than the capsular polysaccharide adsorb to the RBC.

The Ability of the AGID Test to Distinguish Serotypes of Prototype Strains of P.haemolytica.

Cross-reactions may occur in the AGID test because antigens other than the capsular polysaccharide may be released from the cells and some of these antigens will be able to diffuse into the agar. The cross-reacting antigens can be either protein or endotoxin and this point will be considered later. Despite the considerable theoretical potential for cross-reactions in the AGID test, in our hands 9 of the 15 serotypes produced no cross-reactions (Table II). These 9 antisera gave a precipitation line with the homologous prototype antigen but failed to react with the remaining 14 antigens.

Four of the antisera to A biotypes *viz.* antisera to A1, A7, A12 and A13 showed cross-reactions. However with the exception of the A1 antiserum, the cross-reactions were limited to a reaction with only one of the heterologous prototype strains. These cross-reactions (with the exception of A1) were removed by adsorption of the antiserum with a deposit of cells from the cross-reacting serotype (Table III). Antisera to serotype T3

cross-reacted with T15 antigen, and antisera to T15 cross-reacted with T3 antigen. Adsorption of these antisera with cells of the cross-reacting serotype removed both the heterologous and the homologous reactions. This is not surprising as the two serotypes are closely related if not identical (Fraser *et al.*,1982).

Antiserum to A1 cross-reacted with all of the A biotypes (Table II). However it gave two AGID lines with the homologous antigen but only one with the heterologous A biotypes. This antiserum was adsorbed with A7 cells which removed all cross-reactions; and also removed one of the two AGID lines with the homologous prototype antigen (Table III). Adsorbed antisera were then serotype specific in the AGID test, ie. they gave an AGID line with, and only with, the prototype serotype used as immunising antigen (Table III).

These results clearly indicate that the AGID test can be used to distinguish between all prototype strains (taking T3 and T15 as one serotype). However they do not show that the test will reliably identify the serotypes of field isolates. Some such isolates may produce cross-reactions which do not occur with the prototype strains.

When cross-reactions do occur they may make it difficult to interpret both IHA and AGID test results. However one of the advantages of the AGID test over other serotyping methods, especially IHA, is that the immunological relationship between two antigens can be tested by placing them in adjacent wells. If the lines formed are contiguous, this «line of identity» indicates that the two antigens are immunologically identical. If the AGID lines cross this implies that the antigens are unrelated. This is particularly useful when dealing with *P.haemolytica* which has many antigens, only one of which, (the capsular antigen), is serotype specific.

In particular, a line of identity between a test antigen and a purified capsular antigen allows unequivocal identification of the former (see below).

This provided an unequivocal test to confirm the serotype of any field isolate which showed a reaction with more than one antiserum. However it was still important to apply the test to establishing the serotypes of actual field isolates and to demonstrate that it fulfilled it's function in practice.

Purification of Capsular Polysaccharide

Serotypes A1 and A7 were among the most prevalent serotypes isolated from disease conditions (see Chapters 3 and 4) and have also been widely found in other studies (Fraser *et al.*, 1982; Prince, 1985). Antisera to both prototype strains showed cross-reactions with other serotypes (see Table II). This introduced a small element of uncertainty as to the serotype of isolates because it could be argued that the single line of identity observed with the prototype antigen did not represent a capsular polysaccharide line but was instead due to a cross-reacting antigen such as protein or endotoxin. This uncertainty could be resolved if purified capsular polysaccharide was used.

Antisera to A1 was adsorbed with A7 cells. This antisera gave a single line of identity when tested in parallel with the purified capsular polysaccharide and a standard A1 antigen preparation

Antisera to A7 (unadsorbed) gave three gel precipitation lines when tested with crude prototype antigen. One of these gave a single line of

identity when tested in parallel with the capsular polysaccharide and a standard A7 antigen preparation.

When tested with all 15 antisera in the AGID test both purified capsular antigens gave a single line with, and only with, the homologous antiserum. We conclude that an isolate giving a line of identity with a purified capsular polysaccharide provides an unequivocal determination of the serotype of the isolate.

Attempt to Produce Mono-Specific Antisera

To circumvent the difficulties encountered when using antisera raised against whole cells and thus to many antigens, an attempt was made to produce mono-specific antisera. Purified capsular polysaccharide was found to be non-antigenic (Adlam *et al.*, 1984), so two antigen preparations were used.

1/ An AGID line produced by the reaction between purified capsular polysaccharide and the homologous antiserum. This line was excised and thoroughly washed in PBS.

2/ Capsular polysaccharide adsorbed to the hens own RBC.

It was hoped that adsorption to RBC would duplicate the situation of the capsular polysaccharide in the whole cell. The antigenic properties of the capsular polysaccharide appear to be maximised when it is part of the whole cell (Tadyon *et al.*, 1981). The failure of animals to produce an immunological reaction could alternatively be due to lack of phagocytosis of the capsular polysaccharide so it was hoped that the combination of purified capsular polysaccharide and antibody excised from a AGID plate would be phagocytosed more efficiently.

Domestic hens failed to react to either antigen. We conclude that the poor antigenicity of purified capsular polysaccharide was not improved by these procedures.

Field Isolates of P.haemolytica - Confirmation of Typing by IHA and AGID

25 isolates of *P.haemolytica* were obtained from goats with acute pneumonia (Chapter 3). These isolates were typed by IHA and the AGID test. For an isolate to be typed by IHA we initially used the standard criterion that the antigen should show at least a 4-fold preference for one antiserum as compared to the others. By this criterion 17 of the 25 caprine isolates of *P.haemolytica* were allocated to a serotype (Table V).

However 5 isolates showed only a 2-fold preference for one antiserum. These isolates reacted with only one antiserum in the AGID test and these results agreed with the IHA results. If the criterion of a 4-fold preference is relaxed ie. a 2-fold preference is accepted, then 22 of the 25 isolates were typed identically by IHA and AGID.

Two further isolates (1847 and 13287) showed a two fold preference for the A6 and A2 antisera respectively in the IHA. These isolates also reacted with A6 and A2 respectively in the AGID test. But in the AGID test isolate 1847 cross-reacted with A2 antiserum and 13287 cross-reacted with A11 antiserum. However they showed lines of identity only with the A6 and A2 antigens respectively. We therefore conclude that these isolates are serotypes A6 and A2 and within the criteria outlined above, both tests are in agreement. This means that in the case of 24 of the 25 isolates the two tests agreed.

The remaining isolate 12251 showed a 4-fold preference for A2 antiserum in the IHA. However in the AGID test it failed to react with A2 antiserum but reacted with A11 (3 AGID lines) and A7 antisera, (one AGID line). When tested in parallel with the A11 prototype antigen and the A7 purified capsular polysaccharide this isolate gave a line of identity with the A11 antigen, but failed to show a line of identity with the A7 capsule line. We conclude that isolate 12251 is serotype A11. This conclusion does not agree with the IHA test result, but a line of identity in the AGID with the capsular antigen is a more convincing criterion than a reaction in the IHA, which may involve antigens other than the capsular polysaccharide.

On the basis of these results we conclude that the AGID test is a suitable alternative to IHA for typing both prototype and field isolate strains of *P.haemolytica* and on occasions is the more accurate method. It was therefore decided to use the AGID test for all future serotyping of *P.haemolytica*.

CHAPTER 3

Survey of the Prevalence of P.haemolytica in Sheep and Goats in New Zealand

3.1 Introduction

The specificities and relative convenience of IHA and AGID for establishing the serotype of *P.haemolytica* were compared in Chapter 2, using both prototype strains and isolates from field cases of pneumonic pasteurellosis of goats. It was concluded that AGID was an efficient and accurate technique for the serotyping of *P.haemolytica*. In this chapter we use AGID to serotype isolates from CNP of sheep, from pneumonic pasteurellosis of sheep and goats and from the nasal cavities of goats.

The results of this section represent a survey of the prevalence and distribution of *P.haemolytica* in sheep and goats in New Zealand.

3.2 Materials and Methods

3.21 Isolation and Identification of P. haemolytica from Ovine Lungs

Samples of CNP lesions of ovine lungs were taken from 60 sheep in each of four centres: Auckland, Gisborne, Christchurch and Invercargill.

A portion of lesion was smeared over half of a blood agar plate (see Appendix). This inoculum was then streaked over the rest of the plate and incubated at 37°C overnight.

Colonies which had the following charateristics were provisionally identified as *P.haemolytica*. 1-2 mm in diameter, smooth surface, entire

edge, translucent and with a zone of haemolysis directly under the colony. Such colonies were selected and streaked on blood agar and incubated at 37°C overnight to isolate the organism in pure culture. Each such isolate was inoculated on to MacConkey agar (using a heavy inoculum) and on to Triple Sugar Iron Agar slants, and incubated at 37°C for 24 hours.

Isolates were also tested for Gram reaction and oxidase activity.

Isolates which showed the following characteristics were considered to be *P.haemolytica*

1/ Typical colony morphology, as above

2/ Gram negative rod

3/ Growth on MacConkey agar, from heavy inoculum

4/ Oxidase positive

5/ Produced acid throughout TSI slants but neither gas nor H_2S

The production of antisera and its use in AGID and IHA were as described in Chapter 2.

3.22 P.haemolytica Isolated from Pneumonic Pasteurellosis in Sheep and Goats.

Isolates, already identified as *P.haemolytica* were obtained from a number of MAF laboratories throughout New Zealand. They normally arrived by mail on nutrient agar slopes,or in brain-heart-infusion broth (the methods of dispatch were only partially under our control).

A few of the broth cultures were not viable on arrival while all cultures on slopes could be subcultured,so this was the method of choice.

3.23 **Isolation and Identification of Nasal Isolates of P.haemolytica from Goats.**

Twenty goats from each of 4 flocks and 29 goats from 1 flock (a total of 109 goats from 5 flocks), including angora and feral goats were examined. To collect samples of nasal mucus, a sterile cotton swab was inserted about 13cm into a nasal cavity and rotated both clockwise and anti-clockwise. Swabs were immediately broken off into bijou bottles of Amies Transport Media and taken directly to the laboratory. Each swab was smeared over half of the surface of a blood agar plate. This inoculum was then streaked over the rest of the plate and incubated at 37°C for about 24 hours.

Colonies resembling those of *P.haemolytica* were streaked on blood agar and incubated at 37°C overnight. Each isolate was inoculated on to MacConkey agar (using a heavy inoculum) and on to Triple Sugar Iron Agar slants and incubated for 24 hours. Identification of *P.haemolytica* was as described previously.

3.3 **Results**

+ and ++ refer to the AGID lines seen. ++ indicates a strong reaction.

Bold print **+** or **++** indicates the AGID lines which specify the serotype of the isolate. Normal print + reports cross-reactions.

When an isolate reacted with antisera to one serotype only this was deemed to denote the serotype of the isolate. When an isolate reacted with more than one antisera it was retested alongside purified capsular polysaccharide or prototype antigen. A line of identity was seen with (and only with) one serotype and this was deemed to be the serotype of the isolate.

(a) = two AGID lines seen.

(*) = isolate re-tested alongside purified or prototype antigen for a line of identity.

Serotyping of Isolates of P.haemolytica Derived from CNP Lesions, in Auckland, Using AGID

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result
Au4	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	A14
Au8	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au10	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	A14
Au11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au14	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au16	+	-	-	-	-	-	-	-	-	-	-	-	++	-	-	A13*
Au20	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au21	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au27	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
Au29	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
Au31	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
Au32	-	+a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
Au39	++a	-	-	-	-	-	-	-	+	-	-	-	-	-	-	A1 *
Au41	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au44	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
Au46	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
Au54	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au55	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au57	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au59	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
Au60	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1

Table VII

+ and ++ refer to the AGID lines seen. ++ indicates a strong reaction.

Bold print + or ++ indicates the AGID lines which specify the serotype of the isolate. Normal print + reports cross-reactions.

When an isolate reacted with antisera to one serotype only this was deemed to denote the serotype of the isolate. When an isolate reacted with more than one antisera it was retested alongside purified capsular polysaccharide or prototype antigen. A line of identity was seen with (and only with) one serotype and this was deemed to be the serotype of the isolate.

(a) = two AGID lines seen.

(*) = isolate re-tested alongside purified or prototype antigen for a line of identity

(#) = concentrated antigen used as described in Materials and Methods, Chapter 2.

Serotyping of Isolates of P.haemolytica Derived from CNP Lesions, in Gisborne, Using AGID.

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result	
G1	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
G3	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G5	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G7	-	-	-	-	-	-	-	-	-	-	++a	-	-	-	-	A1	
G9	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
G12	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
G19	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
G20	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G21	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G23	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G26	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
G27	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G28	++a	-	-	-	+	-	-	-	-	-	-	-	-	-	-	A5	#
G31	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
G32	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
G35	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G41	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
G43	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	A7	
G44	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
G45	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G48	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
G53	+	++a	-	-	-	-	-	-	-	-	-	+	-	-	-	A2	*
G58	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G59	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
G60	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	

Table VIII

Serotyping of Isolates of P.haemolytica Derived from CNP Lesions, in Christchurch, Using AGID

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result	
C1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	A7	
C2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C4	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C5	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	A7	
C6	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	A7	
C7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	#
C8	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	A2	*
C10	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
C11	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
C13	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	A7	
C15	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C16	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	#
C17	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	#
C18	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C20	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C21	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
C22	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C23	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	A1	*
C25	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	#
C27	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
C29	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	A8	
C31	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
C33	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
C34	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	#
C37	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C38	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C39	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C40	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	A7	
C42	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	
C43	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
C44	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*

Table IX

Table IX continued

C45	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
C46	-	-	-	-	-	-	**+**	-	-	-	-	-	-	-	-	A7	
C47	-	-	-	-	-	-	-	-	-	-	**+**	-	-	-	-	A1	
C48	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
C49	-	-	-	-	-	-	-	-	-	-	**+**	-	-	-	-	A1	
C50	**+**	-	-	-	-	-	-	-	-	-	-	+	-	-	-	A1	*
C51	-	-	-	-	-	-	-	-	-	-	-	+	**+**	-	-	A1	*
C52	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	#
C55	-	-	-	-	-	-	**+**	-	-	-	-	-	-	-	-	A7	
C56	-	-	-	-	-	-	**+**	-	-	-	-	-	-	-	-	A7	
C58	-	-	-	-	-	-	**+**	-	-	-	-	-	-	-	-	A7	
C60	-	-	-	-	-	-	**+**	-	-	-	-	-	-	-	-	A7	
C63	-	-	-	-	-	-	**+**	-	-	-	-	-	-	-	-	A7	

+ and ++ refer to the AGID lines seen. ++ indicates a strong reaction.

Bold print **+** or **++** indicates the AGID lines which specify the serotype of the isolate. Normal print + reports cross-reactions.

When an isolate reacted with antisera to one serotype only this was deemed to denote the serotype of the isolate. When an isolate reacted with more than one antisera it was retested alongside purified capsular polysaccharide or prototype antigen. A line of identity was seen with (and only with) one serotype and this was deemed to be the serotype of the isolate.

(*) = isolates re-tested alongside purified or prototype antigens for a line of identity.

(#) = concentrated antigen used as described in Materials and Methods, Chapter 2.

Serotyping of Isolates of P.haemolytica Derived from CNP Lesion, in Invercargill, Using AGID

Isolate	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	Result
I1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
I3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
I4	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
I5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I6	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I7	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I8	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I10	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
I11	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
I13	+	+	-	-	-	-	++	-	-	-	-	-	-	-	-	A2 *
I15	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
I19	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	A7
I20	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I21	-	++	-	-	-	-	-	-	+	-	++a	+	-	-	-	A9 *
I22	+	-	-	-	-	-	++a	-	-	-	+	-	-	-	-	A11*
I23	+	-	-	-	-	-	++a	-	-	-	-	-	-	-	-	A7 *
I24	+	-	-	-	-	-	++	-	-	-	-	+	++	-	-	A13*
I25	+	+	-	-	-	-	++	-	-	-	-	-	-	-	-	A2 *
I26	+	+	-	-	-	-	+	++	-	-	-	-	-	-	-	A2 *
I27	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	A2 *
I28	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	A2 *
I29	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	A2 *
I30	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2
I31	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
I32	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	A2 *
I33	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	A2 *
I34	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *
I35	+	-	-	-	-	-	-	++	-	-	-	-	-	-	-	A8 *
I36	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	A9 *
I37	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1
I38	+	++a	-	-	-	-	-	-	-	-	-	-	-	-	-	A2 *

Table X

Table X continued

I39	++	**++**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I44	+	**++a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I45	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I46	+	**+a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I47	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I48	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I49	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I50	+	**++a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I51	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A1	
I53	+	**++a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I54	+	**++**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I55	+	**++a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I56	+	-	-	-	-	-	-	**++**	-	-	-	-	-	-	-	A8	*
I57	+	**++a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I58	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I59	+	**++a**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*
I61	+	**+**	-	-	-	-	-	-	-	-	-	-	-	-	-	A2	*

+ and ++ refer to the AGID lines seen. ++ indicates a strong reaction.

Bold print **+** or **++** indicates the AGID lines which specify the serotype of the isolate. Normal print + reports cross-reactions.

When an isolate reacted with antisera to one serotype only this was deemed to denote the serotype of the isolate. When an isolate reacted with more than one antisera it was retested alongside purified capsular polysaccharide or prototype antigen. A line of identity was seen with (and only with) one serotype and this was deemed to be the serotype of the isolate.

(*) = isolates re-tested alongside purified or prototype antigens for a line of identity.

(#) = concentrated antigen used as described in Materials and Methods, Chapter 2.

Comparison of the Recovery Rate of P.haemolytica from CNP Lesions Collected from Sheep in Four Areas of New Zealand.

Centre	No. Tested	No. Positive	Percentage Positive
AUCKLAND	60	21	35%
GISBORNE	60	25	42%
CHRISTCHURCH	60	45	75%
INVERCARGILL	60	49	82%

Table XI

Comparison of the Serotypes of P.haemolytica Isolated from Ovine CNP Lesions from Four Areas of New Zealand.

Serotype	AUCKLAND	GISBORNE	CHRISTCHURCH	INVCARGILL	Totals
A1	12 (57.1%)	11 (44%)	12 (26.7%)	8 (16.7%)	43 (31.7%)
A2	6 (28.6%)	11 (44%)	17 (37.8%)	32 (66.7%)	66 (47.5%)
A5	-	1 (4%)	-	-	1 (0.7%)
A7	-	1 (4%)	11 (24.4%)	2 (4.2%)	14 (10%)
A8	-	-	1 (2.2%)	2 (4.2%)	3 (2.2%)
A9	-	-	-	2 (4.2%)	2 (1.4%)
A11	-	1 (4%)	2 (4.4%)	1 (2.1%)	4 (2.9%)
A13	1 (4.8%)	-	2 (4.4%)	1 (2.1%)	4 (2.9%)
A14	2 (9.5%)	-	-	-	2 (1.4%)
Total	21	25	45	48	139 (100%)

Table XII

Note: There are 11 A-biotypes of *P.haemolytica*. 9 of these 11 were isolated from CNP lesions of sheep.

Serotypes of Isolates of P.haemolytica Derived from the Lungs of Goats which Died from Pneumonic Pasteurellosis.

	Possible Serotypes Determined By:		
Isolate	AGID	IHA	Serotype
4600	A2	A2	A2
3981	A7	A7	A7
4206	A2	A2	A2
12445	A2	A2	A2
5850	A2	A2	A2
2348	A2	A2	A2
2557	A2	A2	A2
2351	A2	A2	A2
655	A2	A2	A2
631	A2	A2	A2
3198	A2	A2	A2
113/20	A2	A2	A2
112/014	A2	A2	A2
2557	A2	A2	A2
3372	A2	A2	A2
3952	A2	A2	A2
2883	A2	A2	A2

Table XIII

Table XIII continued

Isolate	AGID	IHA	Serotype
3068	A2	A2	A2
3452	A2	A2/A11	A2 *
2695	A2	A2/A11	A2 *
17585	A7	A7/A2	A7 *
966	A6	A6/A7/A13	A6 *
1847	A6/A2	A6/A12	A6 *
13287	A2/A11	A2	A2 *
12251	A11/A7	A2/A11	A11*

(*) = Result obtained when the isolate was re-tested alongside purified capsular polysaccharide or prototype antigen to give a line of identity with standard antigens as defined.

Serotypes of Isolates of P.haemolytica Derived from the Lungs of Sheep which Died from Pneumonic Pasteurellosis.

Isolate	AGID	Serotype
16592	A2	A2
02267	A6	A6
2451	A1	A1
16792	A9/A11	A11*
3981	A1/A2	A2 *
4411	A2	A2
13330	A2	A2
6628	A2	A2
6737	A1/A2	A2 *
1115	A1/A2	A2 *
2170	A1/A2/A6	A2 *
2217	A1/A8	A8 *
2434	A1/A2	A2 *
3354	A1/A2	A2 *
4449	A1/A2	A2 *
17514	A1	A1
11994	A11	A11
13246	T10	T10

Table XIV

(*) = Result obtained when the isolate was re-tested alongside purified capsular polysaccharide or prototype antigen to give a line of identity with standard antigens as defined.

The Serotypes of Isolates of P.haemolytica Obtained from the Nasal Cavities of Goats from 5 Different Flocks.

Flock	Isolate	AGID Result
A	Fer 1	A2
A	Fer 2	A2
A	Fer 7	A2
B	Fer 21	A2
B	Fer 25	A2
B	Fer 28	A2
B	Fer 33	A2
C	Fer 68	A2
C	Fer 70	A2
C	Fer 45	A2
D	Ang 16	A11
D	Ang 14	A2
E	Ang 24	A2
E	Ang 35	A2

Table XV

Note: The prefix «Fer» indicates an isolate of *P.haemolytica* from a feral goat and «Ang» indicates an isolate from an Angora goat.

Comparison of the Serotypes of P.haemolytica Obtained from the Acute Pneumonic Lungs of Sheep, CNP Lesions of Sheep, the Acute Pneumonic Lungs of Goats and the Nasal Cavities of Goats.

Serotype	CNP Sheep	Acute Pneumonia Sheep	Acute Pneumonia Goats	Nasal Cavity Goats	Totals
A1	43(31.7%)	2 (11.1%)	-	-	45 (23%)
A2	66(47.5%)	11 (61.1%)	20 (80%)	13 (92.8%)	110 (56%)
A5	1 (0.7%)	-	-	-	1 (0.5%)
A6	-	1 (5.6%)	2 (8%)	-	3 (1.5%)
A7	14 (10%)	-	2 (8%)	-	16 (8.2%)
A8	3 (2.2%)	1 (5.6%)	-	-	4 (2%)
A9	2 (1.4%)	-	-	-	2 (1%)
T10	-	1 (5.6%)	-	-	1 (0.5%)
A11	4 (2.9%)	2 (11.1%)	1 (4%)	1 (7.1%)	8 (4%)
A13	4 (2.9%)	-	-	-	4 (2%)
A14	2 (1.4%)	-	-	-	2 (1%)

Table XVI

Note: This represents the combined results of Tables VII-XV.

3.4 **Disscusion**

The aim of the survey of *P.haemolytica* in CNP lesions of sheep was to answer three questions.

1/ What percentage of CNP lesions yield *P.haemolytica*?

2/ Which serotypes of *P.haemolytica* are present, and in what proportion?

3/ Are the serotypes found related to the geographical source of the CNP lesion?

1/ Overall *P.haemolytica* was isolated from 139 (53.3%) of the 240 pneumonic lungs. This figure is similar to, although slightly less than found in some surveys of CNP lesions in New Zealand. Thus Pfeffer *et al.*, (1983), recovered *P.haemolytica* from 55% of CNP lesions and Prince, (1985) isolated *P.haemolytica* from 67% of lesions.

Our isolation rates from the two South Island samples, Christchurch and Invercargill, were 73.3% and 81.7% respectively, while the two North Island samples, Auckland and Gisborne, were much lower being 35% and 41.7% respectively (Table XI). This difference is probably due to the seasonal nature of the disease. Chronic non-progressive pneumonia occurs in the late summer and early autumn months and the lesions are usually resolved by the coming of winter. Although all our samples were taken at approximately the same time, the CNP season begins and finishes sooner in the North than the South Island. Lesions are healing in the north while the disease is still prevalent in the south. The resolution of lesions is presumably accompanied by the elimination of *P.haemolytica*.

2/ We isolated a total of 9 serotypes from CNP lesions of sheep. All of these were A biotypes. 43 (31.7%) were of serotype A1, 66 (47.8%) A2, 1 (0.7%) A5, 14 (10%) A7, 3 (2.1%) A8, 2 (1.4%) A9, 4 (2.8%) A11, 4 (2.8%) A13 and 2 (1.4%) A14. (Table XII).

The lack of T biotypes is not surprising as CNP is associated primarily with the A biotypes (Smith, 1961; Gilmour, 1978).

The distribution of serotypes is similar to that found in Britain (Thompson *et al.*, 1977; Fraser *et al.*, 1982), where A2 is the predominant serotype and A1, A6, A7 and A9 are also common, making up with A2, 80% of the total. In New Zealand, Prince, (1985), isolated *P.haemolytica* from lesions of CNP from sheep in the southern part of the North Island. Only 6 serotypes were detected. Five of these six serotypes were also detected in our survey. In addition we found serotypes A5, A11, A13 and A14.

The three most common isolates, A1 (31.75), A2 (47.8%) and A7 (10%) make up 89.5% of the total isolates from CNP lesions.

3/ Certain serotypes were found only in one district (A5 solely in Gisborne, A14 only in Auckland) and the proportion of serotypes found varied between centres. Invercargill had a higher proportion of A2 and Christchurch had a high proportion of A7 isolated. These variations however were not dramatic and may reflect only random sampling differences.

We conclude that *P.haemolytica* may be isolated from lesions of CNP with a frequency of between 35% and 82%, depending on the season and the severity of the lesions. 9 of the 11 A biotypes were detected in CNP lesions, although three (A1, A2 and A7) together accounted for 90%. These three serotypes should be included in any vaccine for the control of chronic non-progressive pneumonia and a vaccine containing just these three serotypes would have some relevance in 90%

of the affected animals. There is no major difference in the serotypes found in various areas of the country and this is also of importance for vaccine manufacture, as one vaccine could be used throughout the country.

Survey of P.haemolytica Serotypes Isolated From Acute Pasteurellosis

Sheep: Six serotypes of *P.haemolytica* were isolated from the lungs of sheep which died of pneumonic pasteurellosis (Table XV). A2 was the predominant serotype, with 11 of the 18 isolates (61.1%) being of this serotype. Also present were A1 (11.1%), A6 (5.6%), A8 (5.6%), T10 (6%) and A11 (11.1%). Fraser *et al*, (1982) reported a large survey of serotypes of *P.haemolytica* isolated from cases of ovine pasteurellosis in Britain. This survey included isolates from septicaemic pasteurellosis, a disease not included in our surveys. A2 was by far the most common (35.5%). No other A biotype represented more than 12.5% of the total, hence the distribution of the serotypes in our study is similar to that found in Britian by, Fraser *et al.*, (1982).

Our single isolate of serotype T10 is of particular interest for two reasons. Firstly because a T biotype has not previously been isolated in New Zealand and secondly because, although T biotypes may be isolated infrequently from pneumonic lesions (Gilmour, 1978), they are usually associated with a septicaemia rather than pneumonic pasteurellosis. Septicaemia is not common in New Zealand but since we now know that the T biotype is present in New Zealand it is likely that the rarity of the disease is due to climate and management.

Goats: 25 isolates of *P.haemolytica* were derived from goats with pneumonic pasteurellosis. Of these isolates 80% were serotype, A2. Also present were serotypes A6 (8%), A7 (8%) and A11 (4%). Surveys of serotypes of *P.haemolytica* associated with caprine pneumonia have been undertaken only in tropical

countries where the goats and conditions bear little similarity to New Zealand. However Pegram, (1974), found three isolates of *P.haemolytica* from pneumonic goat lungs in Somali were A2. Ojo (1977) cites a study of *P.haemolytica* from goats in Nigeria (Ojo, (1975)) in which 200 isolates assigned to 6 serotypes distributed as follows: A2 (43%), A6 (20%), A7 (20%), A8 (6.5%), T10 (3.5%) and A11 (5%). Despite the differences in conditions these are similar to our results at least in the predominance of A2.

Survey of P.haemolytica Serotypes From Caprine Nasal Cavities

The survey of *P.haemolytica* in the nasal cavity of goats had several different aims.

1/ To establish the proportion of goats which carried the organism.

2/ To establish which serotypes were present.

3/ To compare the serotypes found in the nasal cavity with those causing pneumonia.

4/ To compare the serotypes found in feral and angora goats

Of the 109 goats tested, 14 (12.8%) carried *P.haemolytica* in their nasal cavity. We are aware of only two similar surveys, one in Mid-West of the USA (Ngatia *et al*, 1984) and one in Nigeria (Ojo, 1976). These studies respectively found 64% and 5% of goats examined carried *P.haemolytica*. Our figure of 12.8% (ie. 14 out of 109 goats) was an intermediate one, though high enough to indicate that *P.haemolytica* is not uncommon in the nasal cavity of goats.

The finding that 92.8% of nasal isolates are of the one serotype, *viz.* A2 clearly demonstrates that this is the most significant serotype of *P.haemolytica* in nasal cavities of goats. This serotype is also the one most frequently isolated from acute pneumonic pasteurellosis of goats.

There appears to be no difference in the serotypes carried in the nasal cavities of feral and angora goats which is not surprising as they are often run together as a single herd.

In conclusion, *P.haemolytica* is found in New Zealand and is associated with acute pneumonic pasteurellosis of sheep and goats and CNP in sheep. The predominant serotype is A2 which accounts for nearly three-quarters of all serotypes isolated from pneumonic pasteurellosis. Together, A1, A2 and A7 made up 81.4% of all isolates. A2 is also the predominant serotype found in the nasal cavities of goats, being 92.8% of the total, although this predominance of A2 may change if goats come into closer contact with sheep which carry a wider range of *P.haemolytica* serotypes in their nasal cavities.

Since the serotypes of *P.haemolytica* causing disease in sheep and goats are the same, any vaccine suitable for control of chronic non-progressive pneumonia in sheep could well be suitable for use in the control of acute pneumonic pasteurellosis in sheep and goats. It may not be economicaly viable to inoculate sheep specificaly against acute pneumonia, but if the vaccine is also protective against CNP it may be economically advantageous. A vaccine for goats against acute pneumonia is more likely to be economically feasible considering the high current value of goats.

CHAPTER 4

Comparison of Total Proteins, Antibiotic Sensitivities and Capsular Antigen Production Of Prototype Strains and Field Isolates of P.haemolytica

4.1 Introduction

This section examines New Zealand isolates of *P.haemolytica* as follows:

1/ Comparison of total proteins.

2/ Antibiotic sensitivities

3/ Capsular antigen production

Comparison of total proteins. Pneumonic pasteurellosis is found in both sheep and goats (Harbourne, 1979; Ojo, 1977). Immunity to *P.haemolytica* is primarily dependant on the presence of antibody to the capsular polysaccharide, but other antigens including proteins may also play a significant role in inducing immunity (Tadayon *et al.*, 1981). If the proteins of isolates of *P.haemolytica* from sheep differed greatly from those of isolates from goats, it may be necessary to produce different vaccines for use in the two species. SDS-PAGE has been used to compare *P.haemolytica* serotypes (Thompson *et al.*, 1975) and we used this method to compare the total proteins of isolates derived from sheep and goats.

Antibiotic sensitivities. The use of antibiotics for the control of pneumonic pasteurellosis has been advocated, but few if any recommendations are given (Whitten, 1971). As well, antibiotic resistance has been reported from the USA (Zimmerman *et al.*, 1980) and the UK (Wray *et al.*, 1983), so it is important to establish the antibiotic sensitivities of isolates of *P.haemolytica* in New Zealand.

Capsular antigen production. Immunity to *P.haemolytica* depends on an immune response to the serotype-specific capsular antigen (Knight *et al.*, 1969). Thus a vaccine against *P.haemolytica* should contain large amounts of the capsular polysaccharide. To determine if fresh field isolates of *P.haemolytica* produced more capsular antigen than did the prototype strains we compared them by Laurell Rocket electrophoresis.

4.2 Materials and Methods

Comparison of Isolates by SDS-PAGE Gels

The proteins of isolates from caprine and ovine sources were compared by SDS-PAGE to see if there existed any discernable differences in isolates from the two species.

The preparation and running of SDS-PAGE gels is described in the Appendix.

Antibiotic Sensitivity Testing

Prototype strains and isolates from 34 fatal cases of pneumonic pasteurellosis were examined. Two strains from goats and one from sheep which had involved unsuccesful treatment with antibiotics were included.

The Minimum Inhibitory Concentration of antibiotics was determined by the agar plate dilution method (Zimmerman *et al*, 1980). Strains to be tested were inoculated into 10mls of BHI broth and incubated at 37°C for 6 hours. 0.75ml was placed in a replicator which was used to inoculate plates containing various concentrations of antibiotics.

Media for the MIC contained, per plate: 17mls of Mueller-Hinton agar, 1ml of sterile defibrinated horse blood and 2mls of antibiotic solution. Plates were used immediatly or stored at 4°C for up to a week. Antibiotic stock solutions were made on the day of media preparation. Control plates containing only agar and blood were inoculated before and after inoculation of antibiotic plates to check the viability of the inoculum.

Plates were read after 18 to 24 hours incubation at 37°C. The MIC was taken as the lowest concentration of antibiotic without growth, a barely visible haze, or the growth of not more than 1-2 colonies.

Comparison of Capsular Antigen Production by Different Strains of P.haemolytica Using the Laurell Rocket Test

Isolates of two serotypes of *P.haemolytica viz*. A11 and A7 and the equivalent prototypes were examined by the Laurell Rocket Test to compare the amount of capsule production.

For each serotype, four isolates from cases of CNP were selected (one from each district), plus two isolates from acute pasteurellosis (one from a caprine, one from an ovine source). These were compared by the Laurell Rocket Test using broth antigen.

The techniques used in the Laurell Rocket test are described in the Appendix.

4.3 **Results**

Comparison of Isolates by SDS-PAGE Gels

Figure 5

Comparison of the total proteins of *P.haemolytica* serotype A2 (left to right),

1/ The prototype strain (tracks 1 and 8)

2/ Three goat isolates (tracks 2-4)

3/ Three sheep isolates (tracks 5-7)

Note the overall similarity between all isolates. Some differences occur in the middle molecular weight proteins. These differences are not related to the source of the isolate *ie*. goat and sheep isolates could not be distinguished by SDS-PAGE.

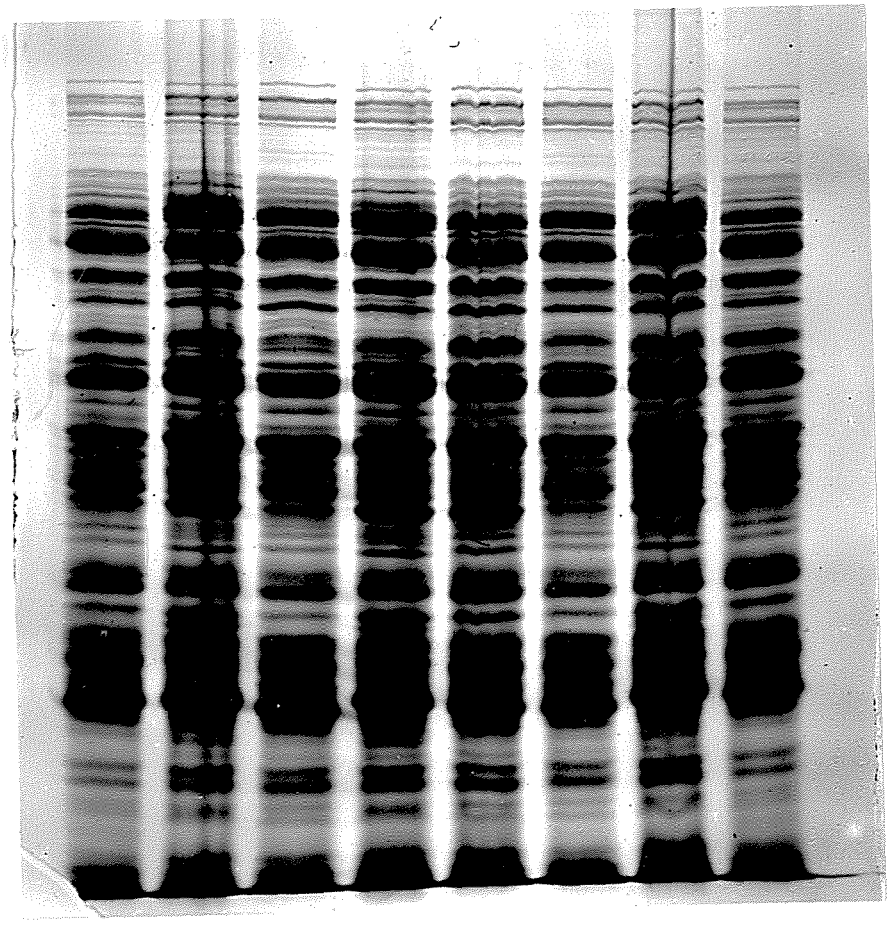


Figure 6

Comparison of the total proteins of *P.haemolytica* serotype A6 (left to right),

1/ The prototype strains (tracks 1 and 6)

2/ Two goat isolates (tracks 2 and 3)

3/ Two sheep isolates (tracks 4 and 5)

All tracks look identical and there appears to be little difference between the proteins of isolates of goat and sheep origin.

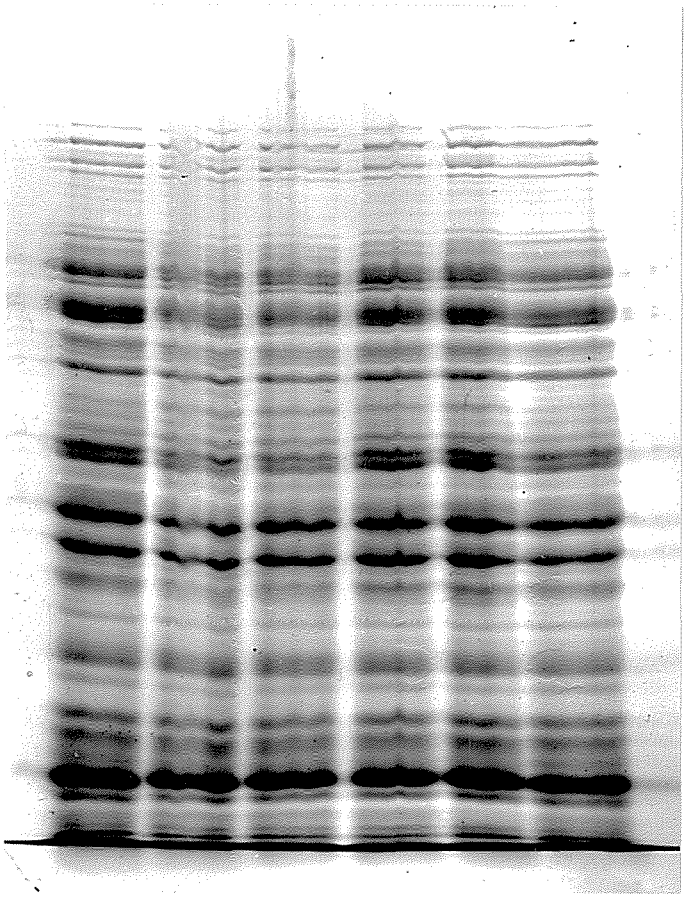


Figure 7

Comparison of the total proteins of *P.haemolytica* serotype A7 (left to right),

1/ The prototype strains (tracks 1 and 4)

2/ Two goat isolates (tracks 2 and 3)

The two goat isolates are very similar to the prototype strains.

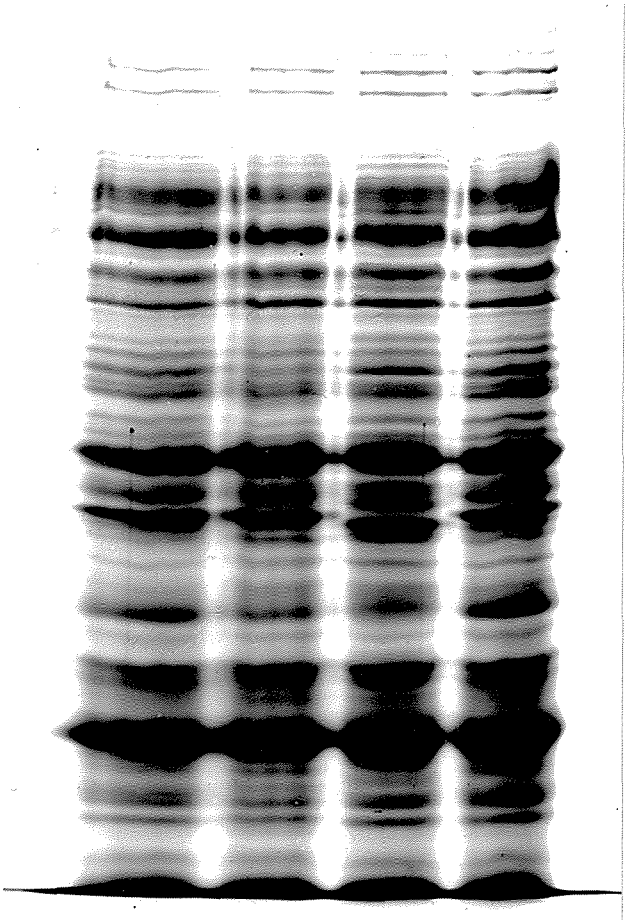


Figure 8

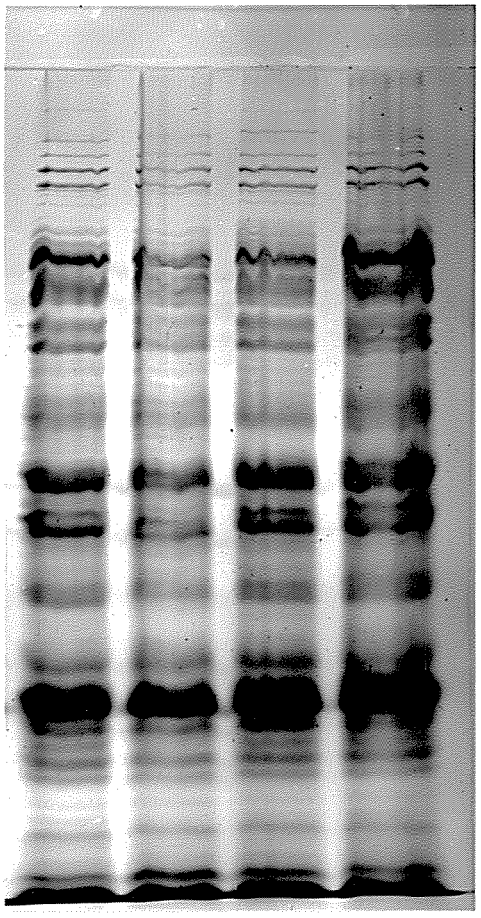
Comparison of the total proteins of *P.haemolytica* serotype A11 (left to right),

1/ The prototype strain (tracks 1 and 4)

2/ One goat isolate (track 2)

3/ One sheep isolate (track 3)

Again there appears to be only minor differences in the proteins of *P.haemolytica* from goat or sheep disease.



Antibiotic Sensitivity Testing

MIC

Isolate	Penicillin	Chloramphenicol	Tetracycline	Streptomycin
16592	<0ø5µg/ml	2µg/ml	2µg/ml	16µg/ml
02267	<0ø5µg/ml	1µg/ml	1µg/ml	8µg/ml
3981	<0ø5µg/ml	<0ø5µg/ml	<0ø5µg/ml	8µg/ml
4411	<0ø5µg/ml	2µg/ml	2µg/ml	8µg/ml
13330	<0.5µg/ml	<0.5µg/ml	1µg/ml	8µg/ml
6628	<0ø5µg/ml	4µg/ml	2µg/ml	8µg/ml
6737	<0ø5µg/ml	2µg/ml	<0ø5µg/ml	8µg/ml
1115	0ø5µg/ml	4µg/ml	4µg/ml	16µg/ml
2170	<0ø5µg/ml	2µg/ml	2µg/ml	8µg/ml
3354	<0ø5µg/ml	1µg/ml	1µg/ml	16µg/ml
4449	2µg/ml	4µg/ml	4µg/ml	8µg/ml
4600	<0ø5µg/ml	2µg/ml	2µg/ml	8µg/ml
3981	1µg/ml	4µg/ml	2µg/ml	8µg/ml
4206	2µg/ml	4µg/ml	4µg/ml	8µg/ml
12445	2µg/ml	4µg/ml	8µg/ml	8µg/ml
5850	1µg/ml	4µg/ml	4µg/ml	8µg/ml
2348	2µg/ml	4µg/ml	2µg/ml	8µg/ml
2557	2µg/ml	4µg/ml	2µg/ml	8µg/ml
2351	2µg/ml	4µg/ml	2µg/ml	8µg/ml
655	1µg/ml	4µg/ml	2µg/ml	8µg/ml
631	2µg/ml	1µg/ml	4µg/ml	8µg/ml
3198	<0ø5µg/ml	<0ø5µg/ml	2µg/ml	8µg/ml
113/20	<0ø5µg/ml	1µg/ml	2µg/ml	8µg/ml
112/014	1µg/ml	4µg/ml	2µg/ml	8µg/ml
3952	2µg/ml	4µg/ml	2µg/ml	8µg/ml
2883	2µg/ml	2µg/ml	2µg/ml	8µg/ml
3068	2µg/ml	4µg/ml	8µg/ml	8µg/ml
3452	<0ø5µg/ml	4µg/ml	2µg/ml	8µg/ml
3056	2µg/ml	4µg/ml	2µg/ml	8µg/ml
17585	<0ø5µg/ml	4µg/ml	2µg/ml	8µg/ml
966	<0ø5µg/ml	2µg/ml	2µg/ml	8µg/ml
1847	<0ø5µg/ml	1µg/ml	2µg/ml	8µg/ml
13287	<0ø5µg/ml	4µg/ml	4µg/ml	>1024µg/ml
12251	<0ø5µg/ml	1µg/ml	4µg/ml	8µg/ml
A1	1µg/ml	4µg/ml	8µg/ml	>1024µg/ml
A2	<0ø5µg/ml	4µg/ml	2µg/ml	8µg/ml
A7	1µg/ml	4µg/ml	2µg/ml	>1024µg/ml
T10	2µg/ml	4µg/ml	8µg/ml	8µg/ml

Table XVII

Antibiotic Minimum Inhibitory Concentrations

Antibiotic	No. Isolates
MIC Penicillin <0ø5µg/ml	20
MIC Penicillin 1µg/ml	6
MIC Penicillin 2µg/ml	12
MIC Penicillin 4µg/ml	-
MIC Penicillin 8µg/ml	-
MIC Chloramphenicol <0ø5µg/ml	3
MIC Chloramphenicol 1µg/ml	6
MIC Chloramphenicol 2µg/ml	7
MIC Chloramphenicol 4µg/ml	22
MIC Chloramphenicol 8µg/ml	-
MIC Tetracycline <0ø5µg/ml	2
MIC Tetracycline 1µg/ml	3
MIC Tetracycline 2µg/ml	22
MIC Tetracycline 4µg/ml	7
MIC Tetracycline 8µg/ml	4
MIC Streptomycin <0ø5µg/ml	-
MIC Streptomycin 1µg/ml	-
MIC Streptomycin 2µg/ml	-
MIC Streptomycin 4µg/ml	-
MIC Streptomycin 8µg/ml	32
MIC Streptomycin 16µg/ml	3
MIC Streptomycin >1024µg/ml	3

Table XVIII

Comparison of Capsule Production by Laurell Rocket Test

Figure 9

Comparison of the relative amount of capsule produced by different strains of *P.haemolytica* serotype A11.

Left to right,

1/ The prototype strains (wells 1 and 6)

2/ Four isolates from ovine CNP (wells 2-5)

3/ One isolate from goat pneumonic pasteurellosis (well 7)

4/ One isolate from sheep pneumonic pasteurellosis (well 8)

The prototype strain produces significantly more capsular material then do the isolates from either CNP or pneumonic pasteurellosis.

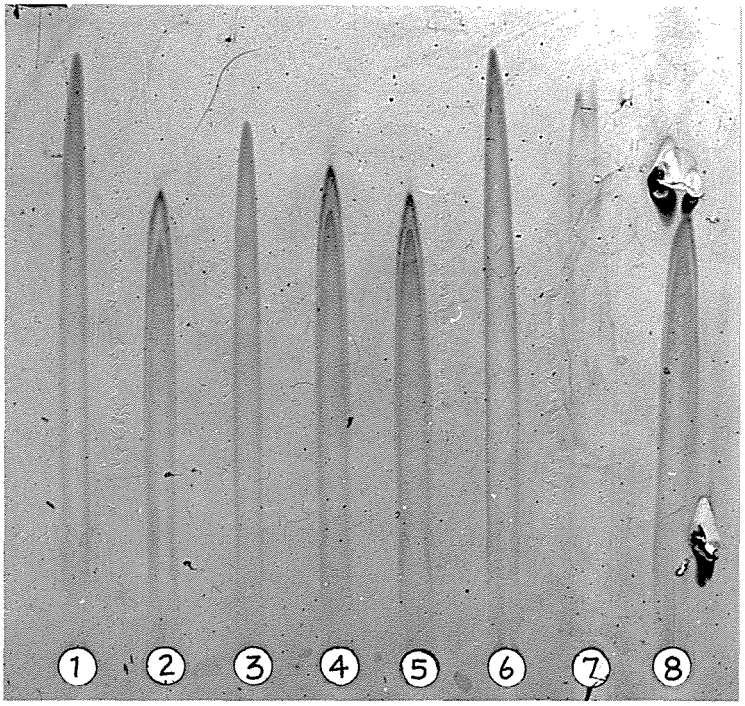

1
2
3
4
5
6
7
8

Figure 10

Comparison of the relative amount of capsule produced by different strains of *P.haemolytica* serotype A7.

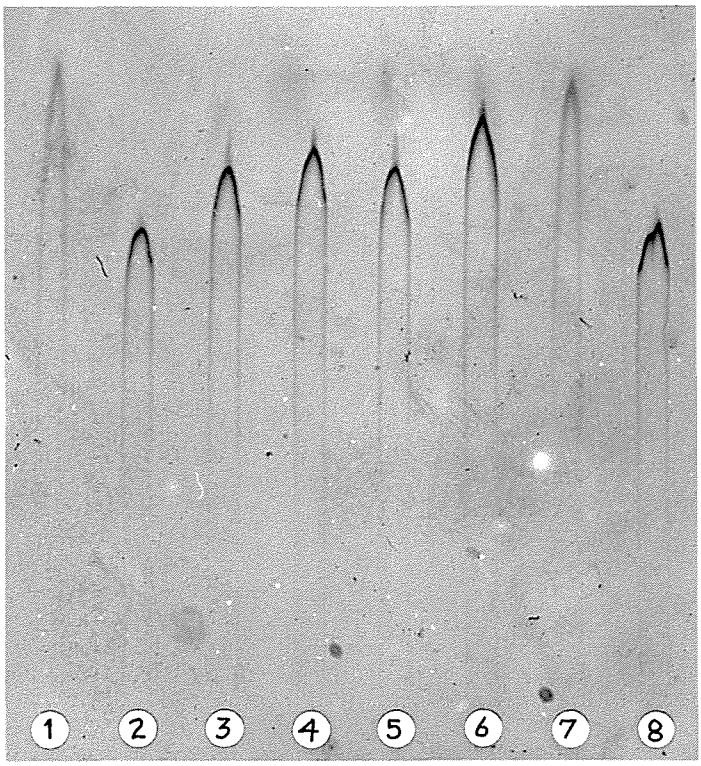
Left to right,

1/ The prototype strains (wells 1 and 7)

2/ Four isolates from ovine CNP (wells 2-5)

3/ One isolate from goat pneumonic pasteurellosis (well 8)

The prototype strains produce significantly more capsular material then do the isolates from either CNP or pneumonic pasteurellosis, although isolate from CNP does come close.


1
2
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8

4.4 Disscusion

Comparison of Isolates by SDS-PAGE Gels

Isolates from sheep and goats were compared by SDS-PAGE to find if the proteins of isolates of a single serotype of *P.haemolytica* from different sources (sheep and goats) differ significantly. It was found (Figures 5-8) that the proteins of isolates from any one species show some variations. However the variations between isolates from the two species (sheep and goats) did not exceed the variations observed within isolates from a single species. This suggests that transfer of *P.haemolytica* between sheep and goats is not only possible but likely, especially in mixed farming ventures. With respect to the efficacy of any vaccine the proteins may be important. However since the proteins of isolates of *P.haemolytica* from sheep and goats are similar it should not be necessary to develop two different vaccines to protect sheep and goats.

Comparison of Capsule Production by Laurell Rocket Test

Capsular antigen is generally believed to be the major protective antigen. It seemed reasonable to expect that fresh field isolates of *P.haemolytica* may produce more capsular material than the, now laboratory adapted, prototype strains. However our results indicate that the most capsular material is produced by prototype strains. We therefore conclude that for vaccine production these prototype strains should not be substituted by any of the isolates which we tested.

Antibiotic Sensitivity Testing

The aims of this were two-fold.

To examine which antibiotics were most effective against *P.haemolytica in vitro* and thus to recommend antibiotics for *in vivo* use.

To assay the level of resistance in *P.haemolytica* isolated from fatal cases of pneumonic pasteurellosis.

Only 3 of the 39 strains of *P.haemolytica* examined showed marked resistance to any of the antibiotics tested. This is not surprising, because those isolates which exhibited a high level of antibiotic resistance were isolated from cattle or pigs (Chang *et al.*, 1976, Zimmerman *et al.*, 1980, Wray *et al.*, 1983), which are more likely to have been treated with antibiotics than the ovine and caprine sources from which the *P.haemolytica* in this study were derived.

All *P.haemolytica* isolates showed some degree of resistance to streptomycin, which is not a drug of choice. Three isolates showed a high level of resistance to this drug alone. This was not correlated with a high level of resistance to other antibiotics. Four strains of *P.haemolytica* were resistant to 8µg/ml tetracycline while we found no strain resistant to more then 4µg/ml of chloramphenicol or penicillin, so these are the drugs of choice. However, treatment with chloramphenicol is not permitted in animals used for human consumption, so its use is limited. In those cases, penicillin, possibly supplemented with tetracycline should be used.

Although there does not seem to be a high incidence of antibiotic resistance in *P.haemolytica*, regular assays of antibiotic resistance patterns would be of interest.

CHAPTER 5

General Discussion

The present investigation was mainly concerned with serotyping *P.haemolytica* and the use of this procedure to establish the prevalence of serotypes in sheep and goats in New Zealand.

Production and Specificity of Antisera.

The first requirement for serotyping *P.haemolytica* is the production of high titre antibody, specific for the capsular polysaccharide of each of the 15 serotypes. Rabbits are the normal species used to prepare *P.haemolytica* antisera and most workers seem to find them satisfactory (Biberstein *et al.*, 1960; Muraschi *et al.*, 1965). However we found that although rabbits produced antibody to the capsular polysaccharide of several serotypes, they consistently failed to produce antisera to others. This problem is not discussed in the literature, but has been briefly commented on by some workers (Wells, 1981; Burrells *et al.*, 1983). We conclude that the failure of rabbits to produce antibody is not unique to this work. The reasons why Biberstein *et al.*, (1960) were able to produce antisera to all serotypes of *P.haemolytica* is not clear, but it could depend on the breed or number of rabbits used. The fact that other workers (Biberstein, 1960; Murasachi *et al.*, 1965) used several rabbits for each serotype does tend to suggest that they did not invariably get a high response in each animal. The breed of rabbits used is not always reported but Fraser *et al.*, (1982) and Sawada *et al.*, (1982) used New Zealand White rabbits whereas with the present study a pure strain of animal was not available.

For those serotypes that in our hands did not produce an adequate immunological response in rabbits, antisera was raised in domestic hens. In our experiments domestic hens never failed to produce high titre antibody to *P.haemolytica*. It might be concluded from this that domestic hens rather than rabbits should be routinely used for the production of antibody to *P.haemolytica*. However cross-reactions with heterologous serotypes were more commonly observed with avian antisera than with rabbit antisera. These cross-reactions were removed by adsorbing the antisera with cells of a heterologous serotype. However, although the results of this procedure are satisfactory, (cross-reactions disappeared), it is desirable to produce mono-specific antisera eg. by the inoculation of purified serotype specific capsular antigen. This point is returned to later.

Serotyping of P.haemolytica by IHA.

In the IHA, cross-reactions were numerous, so an isolate was «typed» only if the titre with one antiserum was at least 4-fold greater than with any other antiserum. This approach is less than satisfactory as it does not take into account the variations in homologous titres that occur. Using this criterion 10 of the 15 prototype serotypes could be correctly identified by IHA. This is insufficient to allow reliable serotyping of isolates of *P.haemolytica*. The main thrust of this thesis was to establish the AGID as a acceptable serotyping test in place of the IHA, so the removal of IHA cross-reactions by adsorption were not investigated.

Serotyping of P.haemolytica by AGID.

Although cross-reactions did occur between serotypes in AGID the majority were removed by adsorption of the antisera by homologous cells. In the remaining case, cross-reactions between T3 and T15 were ignored because the two serotypes are clearly related and may be treated as one (Fraser *et al.*, 1982). By treating T3 and T15 as one serotype, all 15 prototype serotypes could be correctly identified by AGID. One of the advantages of AGID over IHA is that the immunological relationship can be tested for by a «line of identity» between two antigens in adjacent wells. This allowed unequivocal identification of *P.haemolytica* serotypes by observing a line of identity between antigen from an isolate and from the prototype serotype.

Purification of Capsular Polysaccharide.

In the AGID, serotypes, A1 and A7, showed strong cross-reactions. To assess the validity of AGID the capsular polysaccharides of A1 and A7 were purified by organic solvent precipitation. These purified polysaccharides gave a single line when tested by AGID using the homologous antisera and showed a line of identity with the homologous prototype antigen gel precipitation lines. The purified polysaccharides gave no reaction when tested by AGID with heterologous antisera. The use of purified polysaccharide enabled us, by looking for a line of identity to unequivocally identify an isolate even though cross-reactions were also observed. It also demonstrated that the AGID line obtained with the prototype antigen was the «correct» line ie. it represented the capsular polysaccharide and not some irrelevant antigen.

Attempt to Produce Mono-Specific Antisera.

To avoid the complications produced by cross-reacting antisera we attempted to produce monospecific antisera in hens using the two antigen preparations *viz.* 1/ An AGID line produced by the reaction of purified capsular polysaccharide and the homologous antiserum 2/ Capsular polysaccharide adsorbed to the hens own RBC.

Hens failed to react to either of these antigens. This implies that other components of the cell are necessary to stimulate an immune reaction to the capsular polysaccharide or that the attachment of the capsular polysaccharide to the whole bacterial cell is necessary for phagocytosis to occur, which in turn is a prerequisite for an immune response. Adlam *et al.*, (1984), suggested that the capsular polysaccharide is attached to a lipid component of the cell wall which could be necessary for an immune response. The failure of hens to react to purified capsular polysaccharide meant that, even with its shortcomings, antisera raised against whole cells must be used for serotyping.

Comparison of IHA and AGID for the Serotyping of Field Isolates of P.haemolytica.

25 isolates of *P.haemolytica* were serotyped by both IHA and AGID. In 17 cases the two tests agreed. The IHA showed a 4-fold or higher preference for 1 antiserum and AGID showed a precipitation line with only 1 antiserum. A further 5 isolates showed only a 2-fold preference for 1 antiserum in the IHA test. However the AGID result agreed with the IHA result in these cases and showed a precipitation line with one and only one antiserum. A further 2 isolates showed cross-reactions in the AGID.

These were resolved by looking for a line of identity with prototype antigens and those results agreed with the IHA results although only a 2-fold preference was observed. In the case of the final isolate cross-reactions occured in AGID and the results of AGID and IHA did not agree. This conflict was resolved by the unequivocal observation of a line of identity between antigen from the isolate and one, but only one of the prototype antigens.

We conclude that AGID is a suitable alternative to IHA for serotyping *P.haemolytica* prototype strains and field isolates, and is on occasions the more reliable test. It was therefore used for further survey work reported here.

Survey of P.haemolytica in Ovine CNP Lesions.

P.haemolytica was isolated from ovine CNP lesions from 4 widely separate districts in New Zealand and serotyped by AGID. *P.haemolytica* was isolated from 53ø3% of CNP lesions. The isolation rates were higher in the South Island (77ø5%) than in the North (38ø4%). All samples were collected in March or early April. The difference may be due to the seasonal nature of the disease and its relation to climate. CNP starts and finishes sooner in the north than the south and hence the lesions derived from the south probably represented earlier and as yet unresolved lesions.

A total of nine different serotypes were isolated from CNP lesions of sheep. All of these were A biotypes. 43 (31ø7%) were of serotype A1, 66 (47ø8%) A2, 1 (0ø7%) A5, 14 (10%) A7, 3 (2ø1%) A8, 2 (1ø4%) A9, 4 (2ø8%) A11, 4 (2ø8%) A13 and 2 (1ø4%) A14. A2, A1 and A7 together make up 89ø5%. It is interesting to note that the distribution of serotypes is

similar to that found in Britain (Fraser *et al.*, 1982), where A2 is the predominant serotype and A1, A6, A7, and A9 are also common. We found no significant connection between the geographical source of the isolate and the serotypes isolated. No T biotypes were found. This is not surprising as CNP is associated primarily with A biotypes (Gilmour, 1978).

We conclude that an effective vaccine against *P.haemolytica* containing only serotypes A1, A2 and A7 could contribute to the control of ovine CNP.

Survey of P.haemolytica from Ovine Pneumonic Pasteurellosis.

18 isolates of *P.haemolytica* were obtained from sheep with pneumonic pasteurellosis. Six serotypes were found. A2 (61%) was the predominant serotype. A1, A6, A8, T10, and A11 were also present. The distribution of serotypes involved in ovine pasteurellosis in Britain (Fraser *et al.*, 1982) is similar to that in our study, with A2 being the most prevalent.

The isolation of serotype T10 is interesing as no T biotypes have been isolated previously in New Zealand. The T biotypes are associated with septicaemic pasteurellosis which is not common in New Zealand (Hartley *et al.*, 1955). Since we now know that the T biotype is present in New Zealand, the apparent rarity of this disease is presumably due to climate and management, rather than absence of the causative biotype. The presence of the T biotype in the tonsils of normal sheep and its associations, if any, with disease in sheep deserves further investigation.

Survey of P.haemolytica from Caprine Pneumonic Pasteurellosis.

25 isolates of *P.haemolytica* were obtained from goats with pneumonic pasteurellosis. Four serotypes were found. A2 (80%) was the predominant isolate. A6, A7, and A11 were also present. Ojo, (1975) reported a study of *P.haemolytica* from goats in Nigeria. The distribution of serotypes is similar to those in our study with A2 again being the predominant serotype.

Survey of P.haemolytica from the Nasal Cavity of Goats.

14 of the 109 goats tested (12ø8%) carried *P.haemolytica* in their nasal cavity. 11 of the isolates were serotype A2 and the remaining 1 was serotype A11. Other studies (Ojo, 1976 and Ngatia *et al.*, 1984), found between 5% and 64% respectively of goats carried *P.haemolytica* in their nasal cavities. No information is given as to the biotypes or serotypes of these isolates.

Relative Prevalence of P.haemolytica Serotypes in New Zealand.

The predominant serotype associated with pneumonic pasteurellosis in sheep and goats is A2. It is also the most prevalent serotype found in the nasal cavities of goats. Together A1, A2, and A7 are 81ø4% of the total isolates. It follows that any vaccine suitable for the control of *P.haemolytica* in chronic non-progressive pneumonia in sheep could be suitable for use in the control of acute pneumonic pasteurellosis in both sheep and goats.

Comparison of Isolates by SDS-PAGE.

The variation in total proteins seen between isolates derivied from the two species (sheep and goats) did not exceed the variations observed between isolates derived from a single species. Since the proteins of *P.haemolytica* derived from sheep and goats are similar it is probably unnecessary to develop different vaccines to protect sheep and goats from *P.haemolytica* infection.

Comparison of Capsule Production by Laurell Rocket Test.

Our results indicated that prototype strains of *P.haemolytica* produced more capsular antigen than did our fresh isolates from diseased animals. While this was surprising, it suggests that prototype strains should be used for vaccine production rather than local field isolates.

Antibiotic Sensitivity Testing.

All strains of *P.haemolytica* showed a degree of resistance to steptomycin and three strains showed a high level of resistance. No strains were resistant to more than 4µg/ml of chloramphenicol or penicillin so these are the drugs of choice.

APPENDIX

A **Laurell Rocket Test.**

Pre-coating of Glass Plates. Glass plates measuring 8cm by 10cm were cleaned thoroughly with detergent, rinsed in distilled water and allowed to dry.

A 1% agarose solution in distilled water was boiled gently to dissolve the agarose and the hot solution was spread thinly and evenly over one complete face of the clean glass plate, using a cotton swab. The underside of each plate was marked and the plates were dried overnight at 37°C. Once dried the pre-coated plates could be stored, stacked between sheets of filter paper, wrapped in aluminium foil, at 4°C.

Tris-Barbiturate Buffer (pH 8.6)

Diethylbarbituric acid	22.4g
Tris-HCl buffer	44.3g
Calcium lactate	0.533g
Sodium azide	0.65g
Distilled water	up to 1 litre

This gives a 5x concentrated buffer. It is diluted 1:4 prior to use.

Preparation of Agarose Plates. 15ml of a 1% agarose solution in Tris-barbiturate buffer was steamed in a pressure cooker to melt the agarose. This solution was cooled to 56°C and 0.2ml of the appropriate antisera was

added. A pre-coated plate was placed on a level table, coated side up and the edges were enclosed in tape to provide a boundary for the agarose solution. The molten agarose was quickly poured onto the plate, to cover the entire surface. Bubbles were removed with a hot wire.

The plate was covered with a large glass petri dish and left to solidify for 20 minutes at room temperature. The plate was either used immediately or left overnight at 4°C in a humidified chamber.

8 wells were cut, using a template, in one end of the plate, approximately 1.5cm from the edge. The agarose was removed from the wells using a suction pump.

Preparation of Antigen. For each *P.haemolytica* serotype or isolate to be assayed by the Laurell Rocket test, a 10ml aliquot of BHI broth was inoculated with 200µl of thawed, frozen stock culture and shaken at 37°C overnight. This culture was heat-killed at 56°C for 30 minutes

Running the Rocket Test. The buffer chambers of the electrophoresis unit were each filled with 1 litre of Tris-barbiturate buffer. The cooling plate was cooled to 10°C, the temperature at which the test was run. The agarose plate was placed on the cooling plate with the wells at the cathode end. 25µl of heat-killed broth antigen was added to each well. Wicks of double-layer Whatman No.1 filter paper measuring 8cm by 13cm were dampened in the buffer and placed on the last 1cm of each end of the agarose plate to connect the plate to the buffer.

The plate was then electrophoresed at 10 volts/cm for 3 hours. Upon completion of electrophoresis the plate was removed from the electrophoresis chamber and dried.

Thiazine Red Stain

Thiazine Red R powder		1.5g
Glacial acetic acid		5.0ml
Distilled water	up to	1 litre

This stain may be re-used

Washing, Drying and Staining of Plates. The agarose plate was gently rinsed in distilled water with all the troughs being filled. A wet piece of Whatman No.1 fiter paper was laid over the top of the plate and both were dried, either overnight at 37°C or for 20 minutes under a hair-dryer.

When the plate was completely dried the filter paper covering was carefully wetted and removed. The plate was rinsed in distilled water and any filter paper left on the plate was gently rubbed off.

The plate was completely immersed in PBS and gently agitated for 4-5 hours, with the PBS being changed at least twice during that time. The plate was again rinsed in distilled water, for 10 minutes and immersed in Thiazine Red R for 10 minutes.

Plates were decolourised in 1% acetic acid solution for 20 minutes, with the 1% acetic acid being changed twice during that time. Plates were fixed in a 1% acetic acid/1% glycerol solution for 10 minutes, removed and allowed to dry at room temperature. Plates could then be read.

B **Blood Agar Plates.**

Blood agar plates were prepared as double layers. The base was Difco Blood Agar Base No.2 with no blood added. The upper layer was the same, but supplemented with horse defibrinated blood (Gibco) to 5%.

C **Amies Transport Media** (pH 7.2)

Charcoal	10.0g
Sodium chloride	3.0g
Sodium hydrogen phosphate	1.15g
Potassium dihydrogen phosphate	0.2g
Potassium chloride	0.2g
Sodium thioglycollate	1.0g
Calcium chloride	0.1g
Magnesium chloride	0.1g
Davis agar	4.0g
Distilled water	up to 1 litre

All constituents were added to the water and boiled to dissolve the agar. 5ml aliquots were dispensed into bijou bottles with continuous stirring to keep the charcoal evenly distributed. Bottles were sterilised by heating at 121°C for 15 minutes. While cooling bottles were inverted to suspend the charcoal evenly. The media were stored at 4°C.

D **Phosphate Buffered Saline** (PBS)

	NaCl	8.5g
	$Na_2HPO_4.12H_2O$	2.7g
or	Na_2HPO_4 anhydrous	1.07g
	$NaH_2PO_4.12H_2O$	0.39g
	Distilled water	up to 1 litre

E **SDS- Polyacrylamide Gel Electrophoresis**

Preparation of Coomassie Brilliant Blue Protein Reagent.

Coomassie Brilliant Blue G-250	100mg
95% ethanol	50ml
85% (w/v) phosphoric acid	100ml
Distilled water	up to 1 litre

The dye was dissolved in a mixture of the ethanol and phosphoric acid and made up to 1 litre with distilled water. The solution was then fitered through two layers of Whatman No.1 filter paper and stored in the dark at room temperature.

Preparation of Standard Curve. To prepare the standard curve a range of bovine serum albumen solutions from 0-100µg per 0.1ml were made in 0.2M NaOH. These samples were sealed with tape and placed in a boiling water bath for 3 minutes to simulate the treatment of the *P.haemolytica* proteins. When cooled 5ml of Coomassie Brilliant Blue protein reagent was added and

mixed by inversion. The absorbance of the samples was read at 595nm using a Spec 20 spectrophotometer. Each new batch of Coomassie Brilliant Blue protein reagent required a new standard curve to be prepared.

Estimation of Protein Content of Samples. An aliquot of cell suspension was diluted 1/3, 1/10, 1/30 and 1/100 in 0.2M NaOH. 100µl of each dilution was placed in a boiling water bath for 3 minutes to solubilise the proteins. Once the samples had cooled 5ml of Coomassie Brilliant Blue protein reagent was added and the absorbance read at 595nm. The protein content of the diluted sample was then read off the standard curve.

Preparation and Running of SDS-PAGE Gels

Running Gel Acrylamide

Acrylamide	30.0g
Methylene bis acrylamide	0.5g
Distilled water	up to 100ml

Stacking Gel Acrylamide

Acrylamide	30.0g
Methylene bis acrylamide	1.6g
Distilled water	up to 100ml

Acrylamide solutions were prepared by dissolving the acrylamide in 70ml of distilled water. The methylene bis acrylamide was then dissolved and the solution made up to 100ml.

Lower Tris Buffer

Trizma base	18.17g
10% SDS in distilled water	4.0ml
12M HCl	to pH 8.8
Distilled water	up to 100ml

Upper Tris Buffer

Trizma base	6.06g
10% SDS in distilled water	4.0ml
12M HCl	to pH 6.8
Distilled water	up to 100ml

Trizma base was added to 70ml of distilled water and the pH was adjusted to the appropriate value with HCl. SDS was added and the solution was made up to 100ml with distilled water. The pH was checked and adjusted.

Ammonium Persulphate

Ammonium persulphate	0.1g
Distilled water	up to 1.0ml

A fresh solution was prepared immediatly prior to use.

Tris-Glycine Reservoir Buffer (pH 8.3)

Trizma base	6.07g
Glycine	28.8g
SDS	2.0g
Distilled water	up to 2 litres

SDS Sample Buffer

2-mercaptoethanol	10.0ml
SDS	6.0g
Upper tris buffer	25.0ml
Distilled water	up to 100ml

Bromophenol Blue Tracking Dye

Bromophenol blue	0.05g
Glycerol	40.0ml
Distilled water	up to 50ml

Isopropanol Stain

Isopropanol	250ml
Glacial acetic acid	100ml
Coomassie Brilliant Blue R-250	0.4g
Distilled water	up to 1 litre

Storage. The above solutions were stored at 4°C except for the tracking dye and the isopropanol stain which were stored at room temperature.

Preparation of Glass Plates. The apparatus and technique used for SDS-PAGE was that of Ionas, (1983), where the vertical gel slab was polymerised between two glass plates. One of the glass plates was a rectangle measuring 13cm by 17cm and 0.55cm thick. The second was the same size but had a notch 2cm deep and 10cm long cut 1.5cm from the corner along one of the shorter sides. Cleanliness of the glass plates was essential. Immediately before use the plates were scrubbed with Jif, rinsed in hot water and rubbed with alcohol soaked tissue. A thread of petroleum jelly was piped from a syringe along the three straight sides of the notched plate, approximatly 0.5cm from the edge. Three 0.15cm thick perspex spacers were then pressed into position over the threads of petroleum jelly. The thickness of the spacers determines the thickness of the gel. Another thread of petroleum jelly was piped along the spacers, with an extra amount placed at the junctions of the spaces to assist liquid-tightness of the mould. The rectangular glass plate was placed on top of the spacers and pressed firmly down to give a good seal. To facilitate gel pouring the plates were clamped onto a

vertical perspex stand using bulldog clips. The notch in the glass plate was placed uppermost, facing away from the stand.

Preparation and Pouring of Running Gel Acrylamide. 10% running gel acrylamide was prepared by adding the following solutions in the order given, ensuring adequate mixing after each addition.

Lower tris buffer	5.0ml
Running gel acrylamide	6.7ml
Distilled water	8.3ml
Ammonium persulphate	0.3ml
N N N'N'-tetramethylethylenediamine	0.01ml

The resulting solution was poured, avoiding air bubbles, between the two glass plates to a depth of 11cm. Immediately, the solution was gently overlayed with distilled water. This provided conditions sufficiently anaerobic to enable complete polymerisation of the acrylamide solution. The gels were left 30-45 minutes at room temperature for polymerisation to occur.

Preparation and Pouring of Stacking Gel Acrylamide. A stacking gel solution was prepared by adding the following solutions in the order given, ensuring adequate mixing after each addition.

Upper tris buffer	2.5ml
Stacking gel acrylamide	1.5ml
Distilled water	6.0ml
Ammonium persulphate	0.3ml
N N N'N'-tetramethylethylenediamine	0.01ml

Water and any unpolymerised acrylamide were poured off the running gel. The exposed surface was washed twice with aliquots of freshly prepared stacking gel acrylamide and the space above the gel was filled with the remaining stacking gel solution. A perspex comb (with 8, 7.5mm wide teeth, 3mm apart and 17mm long) was inserted between the glass plates into the stacking gel, leaving a gap of 8mm between the running gel and the teeth of the comb. It is essential to avoid catching air bubbles on the teeth of the comb as this would create air bubbles in the stacking gel. Polymerisation of the stacking gel required 5-10 minutes at room temperature. Once polymerisation was complete the comb was removed and the wells washed with Tris-glycine reservoir buffer.

The gel sandwiched between the glass plates, was removed from the vertical stand and the bottom spacer was carefully removed.

A thick thread of petroleum jelly was applied around the buffer portal to the upper reservoir. Another thread was applied midway between the upper and lower reservoirs. This was to provide a liquid-tight seal between the two chambers, so the current would flow through the gel. The gel sandwich

was then pushed onto the electrophoresis apparatus so the notch in the glass plate was next to the buffer portal to the upper reservoir. Bulldog clips were applied to firmly attach the gel onto the electrophoresis apparatus. The reservoirs were topped up with Tris-glycine reservoir buffer so that contact was made with the top and bottom of the gel. Bubbles were eliminated from the lower gel surface using a syringe with a bent needle, full of Tris-glycine reservoir buffer.

Growth of Samples for SDS-PAGE. Cultures were grown overnight in 10ml aliquots of BHI. A 5ml volume was transferred to 100ml of pre-warmed BHI in a 500ml flask. Cells were harvested at maximum turbidity, by centrifugation at 3,000g and 5°C for 30 minutes. To remove all media constituents the cells were washed twice in PBS. The final suspension was 5% cells in PBS. This suspension was stored at -70°C.

Sample Preparation for SDS-PAGE. To prepare the sample for SDS-PAGE the cells were lysed and the proteins solubilised by boiling for 3 minutes, the following:

Cell suspension	100µl
SDS sample buffer	25µl
Bromophenol blue tracking dye	12.5µl

When cooled, samples containing 80µl of protein were applied to each track.

Electrophoresis of the Protein Sample. A current of 10mA was applied to the gel until the tracking dye reached the stacking gel/running gel interface. The current was then increased and maintained at 15mA until the tracking dye was 1cm from the bottom of the gel, at which point the power was disconnected. The protein required 1.5-2 hours to pass through the stacking gel, and a further 4-4.5 hours to pass through the running gel.

Staining Protein Bands in Gels. On completion of the run the gel sandwich was removed from the electrophoresis apparatus. The 2 perspex spacers were pulled out and the glass plates levered apart. A segment of the lower left-hand corner of the gel was removed to enable correct orientation of the gel once stained. The gel was then carefully allowed to fall off the glass plate into a container of isopropanol stain. This was left rocking at least 3 hours at room temperature.

Destaining the Gel. Stain was decanted off the gel and the gel was rinsed in 10% aqueous acetic acid. This was replaced with fresh 10% acetic acid and left agitating. Every 2-3 hours the destaining solution was replaced with fresh 10% acetic acid, until the background of the gel was clear. The gel was then washed with distilled water until no acetic acid smell remained.

F **Purification of Capsular Polysaccharide.** (Adlam *et al.*, 1984).

(i) 2 l of BHI broth inoculated with 0.2 ml of thawed, frozen stock culture.
Incubated at 37°C overnight with shaking.

(ii) Culture heated-killed at 60°C for 1 hour. 4ml (=0.2% v/v) formalin added.

(iii) Culture centrifuged 9,500g, 1 hour, 4°C to remove organisms. Supernatant concentrated 1/50 (final volume 40 ml) by UM10 Amicon filter.

(iv) 3 volumes (120 ml) of -20°C methanol and 1% (1.2g) sodium acetate added slowly with stirring to 4°C supernatant. Precipitate allowed to settle under gravity at 4°C.

(v) Methanol supernatant filtered through Whatman No.1 filter and then through Millipore 5 µm membrane.

(vi) 3 volumes (460 ml) of -20°C acetone added slowly with stirring to methanol supernatant. Precipitate allowed to settle under gravity and supernatant discarded. Precipitate resuspended in water and freeze-dried.

(vii) 1g of freeze-dried crude acetone precipate dissolved in 50 ml of 1/10 saturated aqueous sodium acetate. 25 ml of 77% (v/v) phenol added and suspension shaken by hand for 2 minutes.

(viii) Phases split by centrifugation 5,000g, 10 minutes and the phenol phase re-extracted with another 50 ml of 1/10 saturated sodium acetate.

(ix) Pooled aqueous phase dialysed against 0.1M $CaCl_2$ for 24 hours and then ultra-centrifuged at 100,000g for 3 hours at 5°C.

(x) Supernatant had 3 volumes of -20°C ethanol added slowly with stirring. The resultant precipitate was centrifuged 13,500g for 1 hour at 4°C.

(xi) Precipitate freeze-dried.

(xii) Precipitate re-dissolved in water (1mg/ml) and tested against antisera by AGID.

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