STUDIES OF *Pasteurella haemolytica*:

(i) COMPARISON OF SEROTYPING TECHNIQUES

(ii) PREVALENCE OF SEROTYPES IN NEW ZEALAND SHEEP

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ABSTRACT

P.haemolytica, which is the aetiological agent of pasteurellosis, is the major cause of mortalities in sheep in Great Britain and is a problem in several other countries including New Zealand. Furthermore, P.haemolytica, acting as a secondary invader, exacerbates lesions of chronic non-progressive pneumonia (CNP) initiated by Mycoplasma ovipneumoniae and this disease causes considerable economic loss to the New Zealand sheep farming industry.

P.haemolytica exists as 15 serotypes and immunity is serotype specific. P.haemolytica vaccines are marketed overseas and their use in New Zealand is under active consideration. It is logical however, to establish which serotypes are present in New Zealand before a vaccine is produced, but there is at present, no information on this point. This is largely because of technical difficulties in typing isolates. This situation stimulated the present investigation which has 2 major aims: To develop an improved method of typing P.haemolytica and to gather some data on the prevalence of the various serotypes in New Zealand.

With respect to the first aim, several approaches were taken to the problem of differentiating between serotypes.

(1) Indirect heamagglutination (IHA), the standard method by which P.haemolytica is serotyped, was found to be laborious and gave many cross-reactions.

(2) SDS-PAGE of total protein showed similar patterns for all

serotypes within a biotype, whereas the 2 biotypes had different patterns. Thus, SDS-PAGE cannot be used to identify the serotype, but could be useful for identifying the species and the biotype.

(3) Latex beads, coated with antibody prepared against whole cells, agglutinated homologous cells, but also gave many cross-reactions. This test should, in principle, become type-specific if purified capsule were used as the immunising antigen, but further work is required to prepare capsular polysaccharide free from endotoxin which stimulates the production of cross-reacting antibody.

(4) Gel precipitation was simple to perform and was serotype specific. However, serotype A2 required a concentrated antigen preparation for the detection of a precipitation line. The concentrated antigen could not be routinely used with the other types because of cross-reactions between antigens.

(5) Counter immunoelectrophoresis (CIE) was not serotype specific due to endotoxin causing cross-reactions.

(6) Bacterial agglutination was a rapid and simple test to perform and showed some limited cross-reactions.

Since IHA, gel precipitation and agglutination showed some potential, they were then compared using 40 isolates from the lungs of 60 sheep with CNP These findings reinforced the conclusions drawn when prototype strains were used.

We initially proposed that the serotype of isolates should

be primarily determined by agglutination tests, but since this is not 100% specific, the results must be confirmed by gel precipitation. This approach was investigated using 110 isolates from the nasopharynx of sheep and it was satisfactory for all serotypes except A2. Strains within the A2 serotype showed some heterogeneity at least when examined by gel precipitation and a hypothesis is presented to explain this.

We conclude that at present, the best method of serotyping isolates is by gel precipitation, but that all isolates which are not positively serotyped by this approach should be retested by IHA.

Information concerning the prevalence of serotypes was obtained during the above studies. 110 isolates were obtained from the nasopharynx of 50 sheep from each of 4 farms. All "A" serotypes, except A12 and A14, were isolated. Serotype A2 made up 35% of these isolates. 60 lungs with CNP lesions were obtained from 20 farms in the Manawatu region and 40 isolates of P.haemolytica were obtained. All were serotyped and they were: A1 (25%), A2 (55%), A6 (7.5%), A7 (7.5%), A8 (2.5%), and A9 (2.5%). No "T" types were found in either the nasopharynx or the lungs. The implications of these findings for vaccine production are discussed.

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

P.haemolytica is associated with 2 major disease complexes in sheep : pasteurellosis and chronic non-progressive pneumonia (CNP). Ovine pasteurellosis occurs as 2 distinct clinical entities : pneumonic pasteurellosis and systemic pasteurellosis. The pneumonic form is found throughout the world (Dungal, 1931; Carter, 1956; Salisbury, 1957; Biberstein and Thompson, 1966; Cameron and Smit, 1970; Mwangota, 1975), whereas systemic pasteurellosis has been reported in the United Kingdom only (Stamp et al., 1955). The sole cause of both syndromes is P.haemolytica.

The aetiology of CNP has not been unequivocally settled. However, most workers agree that while the primary agent is probably M.ovipneumoniae the presence of bacteria, especially P.haemolytica, is necessary for the development of lesions of clinical significance (Jones and Gilmour, 1983). The disease is prevalent in 6 to 9 month lambs in New Zealand, and was estimated in 1975 to cause an annual loss of about $26,000,000. This loss is not due to mortalities, but is caused by pleural adhesions (which down-grade carcasses) and by diminution of weight gain.

Treatment of pasteurellosis or CNP by therapeutic means is impractical because pasteurellosis is usually rapidly fatal, whereas CNP is usually subclinical. It follows that pasteurellosis could best be controlled by immunisation with P.haemolytica and futhermore it seems reasonable to assume that immunity to P.haemolytica would ensure that any CNP lesions produced by M.ovipneumoniae would not be exacerbated by

P.haemolytica and hence would be economically unimportant.

Immunity to P.haemolytica requires an immune response to the capsular polysaccharide (Biberstein and Thompson, 1965) of which there are 15 serotypes (Fraser et al, 1982), so theoretically, a vaccine should incorporate all 15 serotypes and hence may be uneconomic.

It is perhaps surprising, that although P.haemolytica can be recovered from a large number of New Zealand sheep (Alley, 1975b; Pfeffer et al, 1983), there are no reports in the literature concerning the serotypes involved. It is possible, even likely, that not all serotypes are present in the country, and of those that are present some may be much more important than others as disease causing agents. Information on this would clearly be relevant if a vaccine were to be made and marketed in New Zealand since such a vaccine need only contain those serotypes which are major pathogens of New Zealand sheep.

The elucidation of this information requires a convenient and reliable method of determining the serotypes of isolates. Indirect haemagglutination (IHA) is the standard technique for serotyping isolates (Biberstein, 1960), but this method is laborious and hence is unsuitable when large numbers of isolates are involved.

Considering the above, the following aims were formulated for this research programme :

1) To isolate and identify P.haemolytica from sheep. This included the use of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as a means of unequivocally identifying P.haemolytica isolates.

2) To compare the convenience and specificity of several serotyping techniques (viz IHA, latex bead agglutination, gel precipitation, counter immunoelectrophoresis (CIE), and bacterial agglutination) using prototype strains of all 15 serotypes.

3) To find whether the conclusion reached when prototype strains were investigated remained valid when determining the serotypes of a large number of fresh isolates of P.haemolytica

4) To survey the prevalence of serotypes of P.haemolytica found in:

(a) The lungs of sheep with CNP

(b) The nasopharynx of apparently healthy sheep.

This survey was confined to sheep in or near the Manawatu region, but should be regarded as an essential preliminary investigation which will facilitate a more comprehensive study assessing the prevalence and importance of serotypes of P.haemolytica in diseased and normal sheep throughout New Zealand.

CHAPTER 1

Historical Review

1.10 Classification of P.haemolytica

The genus Pasteurella consists of 4 main species viz P.multocida, P.pneumotropica, P.ureae and P.haemolytica which are all pleomorphic, gram negative, non-motile rods with a fermentative metabolism. They are distinguished from the Enterobacteriaceae because they are oxidase positive and they produce acidity throughout triple-sugar iron agar without gas or hydrogen sulphide formation. This reaction is also typical of some yersinias, but these,unlike pasteurellas, grow freely on MacConkey agar. Actinobacilli resemble pasteurellas in many respects, but unlike pasteurellas they possess urease activity.

P.haemolytica may be distinguished from other pasteurellas by producing haemolysis on blood agar or by their ability to grow (albeit very sparsely) on MacConkey agar (Biberstein, 1978).

1.11 Biotypes

Biberstein et al., (1958) noticed that P.haemolytica could be divided in to 2 groups on colonial characteristics, crystal violet uptake and serology, but it was not until 1959 that Smith (1959) divided P.haemolytica in to 2 biotypes, "A" and "T", on the basis of biochemical and cultural characeristics. The "T" strains derived their name from trehalose which they fermented after only 2 days incubation. In contrast, the "A" strains did not ferment trehalose within 10 days. Similar distinctions

could be made using the fermentations of salicin (Smith, 1961) and mannose (Shreeve et al., 1969). "A" strains derive their name from arabinose which they ferment within 7 days, while the "T" strains (if they utilise the sugar at all) take not less than 10 days. A similar result is obtained with xylose (Shreeve et al., 1969).

Broth cultures of "A" and "T" strains have similar growth curves up to the end of the exponential phase after which the viable count of the "A" strains declines rapidly while the "T" strains lose viability only slowly. The pH of 24h "A" type broth cultures is consistantly higher than that of the "T" biotype. 24h "T" biotype colonies on blood agar measured 2mm diameter and dark brown centres are seen with transmitted light. However, the colour fades toward the peripheries. Type "A" colonies tend to be smaller and have a raised central area. They look grey when viewed by transmitted light.

Stained smears prepared from "T" types which were dispersed in distilled water usually show the bacteria in a lace-like pattern, whereas smears made with organisms dispersed in saline show evenly dispersed bacteria. The appearance of smears of "A" types does not depend on whether water or saline is used to disperse the bacteria. Even dispersal is obtained with either.

Antibiotic sensitivity, in vitro, can also be used to distinguish "A" and "T" strains. The "A" types are more sensitive to penicillin, ampicillin, tetracycline, cephalothin, furadantin, chloramphenicol, erythromycin and streptomycin (Biberstein and Kirkham, 1979).

Basic fuschin (0.2µg/ml) in brain-heart infusion broth

inhibits "A" strains but not "T" strains. Indeed the 2 biotypes differ so markedly by colonial and biochemical criteria and in their nucleic acid homologies (Biberstein and Franis, 1968) that it has been suggested that they should be classified as separate species.

1.12 Serological Typing

This approach was initiated by Montgomerie et al., (1938) who described 2 serological types, but their results were not confirmed by Bosworth and Lovell, (1944). Later Florent and Godbille (1950) concluded that there were at least 3 serological types, but in contrast Carter (1956) suggested that the species was serologically homogeneous.

The situation has been clarified largely due to the efforts of Biberstein who has examined the problem for nearly 30 years. Initially Biberstein et al., (1960) descibed 10 distinct serological types based on soluble substances binding to untreated RBC's in the IHA test. These soluble substances are probably capsular polysaccharide (Cameron, 1956). Subsequently, 5 more serotypes have been identified (Biberstein and Gills, 1962; Biberstein and Thompson, 1966; Pegram et al., 1979; Fraser et al., 1982), although the fifteenth serotype, T15, resembles T3 serologically. There are however, strains of P.haemolytica which cannot be typed with the existing typing sera and do not even give a positive reaction with homologous serum in the IHA test. These isolates are considered to be untypable (Aarsleff et al., 1970). These are mostly isolated from the nasopharynx of sheep rather than from diseased lung tissue (Biberstein and Cameron, 1966).

1.13 Somatic Antigens

The use of somatic antigens as a means of classifying P.haemolytica has been studied using autoclaved cells in agglutination tests (Biberstein et al., 1960), and it was found that certain of the somatic antigens were widely distributed throughout the species since with only 2 sera a large number of strains, including different serotypes, could be grouped. Group A consisted of IHA serotypes A1, A5, A6, A7, A8, A9, while Group B was composed of mostly type A2 strains. However, some strains reacted with a variety of sera while with others reciprocal reactions did not occur. It was found that one serotype could contain more than one type of somatic antigen. Immunity to P.haemolytica is believed to be serotype specific, so the usefulness of a knowledge of somatic antigens of isolates is less than a knowledge of the distribution of capsular antigens.

1.14 Relationship of Biotype to Serotype

Serotype and biotype are not independant of each other. Biberstein and Gills (1962) investigated 37 isolates revealing that all isolates of serotypes 1, 2, 5, 6, 7, 8, 9, 11 are of biotype "A" while 3, 4, and 10 belong to biotype "T". When types 12, 13, 14 were discovered they were found to belong to biotype "A", while the fifteenth serotype belongs to the "T" biotype. The relationship of biotype to serotype was further confirmed by work incidental to surveys in both Great Britain and the United States (Biberstein, 1978). However, recently a report on cultures from East Africa contained data suggesting that both biotypes could be represented within one serotype (Mwangota et al., 1978). This work remains unconfirmed.

1.2 Commensal Association of P.haemolytica in Sheep

P.haemolytica is a commensal organism isolated from the nasopharynx and tonsils of apparently healthy sheep. The 2 biotypes appear to localise in different anatomical regions. Biotype "A" predominates in the nasopharynx and biotype "T" in the tonsils. In a survey by Gilmour et al., (1974), P.haemolytica was cultured from 95% of tonsils and 64% of nasopharyngeal swabs. 65% of tonsil isolates were biotype "T", whereas only 6% of nasal isolates were of this biotype.This different localisation of the 2 biotypes is believed to be one of the factors contributing to the different disease syndromes caused by each biotype.

1.30 Diseases Associated with P.haemolytica in Sheep

P.haemolytica is associated with several diseases of sheep, the major diseases being pasteurellosis and CNP. Pasteurellosis encompasses 2 distinct clinical entities: pneumonic pasteurellosis and septicaemic pasteurellosis. P.haemolytica can cause other diseases, at however, lower frequency. Biotype "A" has been isolated from ewes with mastitis. This infection occasionally becomes systemic and fatal or ewes may lose a mammery gland (Biberstien, 1981). Arthritis is a common sequel to experimental intravenous inoculation of "T" biotypes and is occasionally diagnosed in the field (Biberstein and Kennedy, 1959). Pasteurella meningitis affects both ewes and lambs sporadically (Gilmour and Angus, 1983). Encephalitis and lesions in the gastrointestinal tract are occasionally attributed to P.haemolytica (Harbourne, 1979). Diagnosis of all these conditions is dependant on the isolation of large numbers of P.haemolytica from the lesions. In general however,

infection of the respiratory tract with P.haemolytica is more important than all others.

1.31 Pneumonic Pasteurellosis

Synonyms: enzootic pneumonia, acute necrotising or exudative pneumonia, acute pneumonia. This disease occurs in all ages of sheep throughout the year, although outbreaks are more common in the spring and autumn (Harbourne, 1979). Usually outbreaks are first recognised only when sheep die or show acute signs of respiratory illness. The signs include dullness, coughing, anorexia, pyrexia above 40.6°C, nasal and occular discharges. Often sheep froth at the mouth just prior to death (Gilmour and Angus, 1983). Animals usually die within 36h of onset of clinical signs (Alley, 1975a). The proportion of clinically affected animals varies, but up to 10% of a flock may be affected. The disease does not always reach epidemic proportions and sporadic cases may be seen.

The gross pathology of pneumonic pasteurellosis is characterised by intense congestion and varying degrees of red or grey consolidation of the ventral portion of both lungs (Alley, 1975a). Usually pericarditis and pleurisy are also seen and the pleural cavity often contains large amounts of straw-coloured fluid with fibrin clots (Gilmour and Angus, 1983). Histopathological examination reveals a cellular exudate consisting of neutrophils, macrophages and detached alveolar epithelial cells. Large numbers of bacteria are present in the lesion. Destruction and damage to the alveolar epithelium occurs throughout the lungs (Alley, 1975a).

P.haemolytica of biotype "A" is isolated, often in pure

culture, from the lesions and exudates associated with pneumonic pasteurellosis. In some cases, "A" types can also be isolated from the liver, spleen, kidneys, lymph nodes and blood. Occasionally a "T" type may be isolated from a pneumonic lesion (Gilmour, 1978).

It is generally believed that environmental factors are important predisposing causes of pneumonic pasteurellosis. Outbreaks appear to be linked to transportation; warm, cold or wet weather; dipping; castration or dosing (Gilmour and Angus, 1983); the feeding of roots and the change from poor to better pastures in the autumn (Harbourne, 1979); and infection with tick-bourne fever caused by Cytoecetes phagocytophila (Gilmour et al., 1982c). P.haemolytica alone can cause acute pneumonic disease (Gilmour et al., 1975), but the severity and frequency of this disease may be increased by other agents such as Parainfluenza 3 virus (PI3) (Hore, 1966; Sharp et al., 1968), and bovine respiratory syncytial virus (Al-Darraji et al., 1982), while these agents alone do not produce significant clinical signs.

1.32 Septicaemic Pasteurellosis

"A" and "T" strains can both cause septicaemic pasteurellosis, but "T" strains are the usual organisms associated. Nonetheless, in lambs under 3 months infection with "A" types tends to be systemic rather than pneumonic, although severe pleurisy and lesions in the pericardium are also seen (Gilmour, 1978).

Systemic infection with "T" types usually occurs in 5 to 12 month lambs during autumn (Dyson et al., 1981). The disease is

characterised by sudden death and affected lambs are seldom seen alive. Those which are alive are usually seen lying down, breathing with difficulty, frothing at the mouth and have a high temperature (Stamp et al., 1955). Predisposing factors seem to be environmental, such as the feeding on rape or turnips, a change to better pastures or a change in weather (Gilmour and Angus, 1983).

On post-mortum examination subcutaneous haemorrhages are seen over the neck and thorax. The lungs are swollen and a frothy, blood tinged fluid exudes from the bronchioles. Lesions may occur in the pharynx and upper alimentary tract, but lesions around the tonsils are especially prominent (Dyson et al., 1981).

Death from septicaemic pasteurellosis is probably due to toxaemia caused by endotoxins since a lethal dose of a heat-killed culture is only slightly lower than the lethal dose of living culture (Smith, 1960). It is postulated that the disease begins when predisposing conditions allow the "T" types present in the tonsils to invade tissue of the upper alimentary tract producing lesions in this area. Bacteria from the lesion then enter the blood stream either through lymphatic or venous drainage. These organisms lodge in the capillary beds of various organs, where they multiply producing embolic lesions. Thus, the disease is probably not a true septicaemia since most multiplication occurs in the tissues rather than in the blood (Dyson et al., 1981).

1.33 Importance of Pasteurellosis

Pneumonic pasteurellosis was first described in Iceland (Dungal, 1931) and has since been noted in most countries in the world that maintain flocks (Salisbury, 1957; Carter, 1967; Cameron and Smit, 1970; Mwangota et al., 1977). Septicaemic pasteurellosis was first described in Scotland (Stamp et al., 1955) and has subsequently been reported in most countries including South Africa. (Cameron and Smit, 1970) and the USA where it was said to cause considerable loss in feeder lambs (Biberstein and Kennedy, 1959). However, the disease is rare in Kenya (Mwangota et al., 1977) and New Zealand (Alley, Personnel communication) although it has been known to occur (Hartley and Boyes, 1955).

Pasteurellosis is the primary killer of sheep in Britain (Gilmour,1978) and is thus of considerable economic importance in that country. In New Zealand, on the other hand, outbreaks of pasteurellosis are sporadic and infrequent, so overall losses are low, despite reports of loses up to 47% in some individual flocks (Sorenson, 1976).

1.34 Chronic Non-Progressive Pneumonia

Synonyms: Hogget pneumonia, summer pneumonia, enzootic pneumonia (New Zealand only), atypical pneumonia, proliferative interstitial pneumonia, sheep pulmonary adenomatosis (Ionas, 1983), apical pneumonia, lobar pneumonia (Jones and Gilmour, 1983).

CNP is prevalent in New Zealand sheep aged 3 to 10 months (in contrast to pneumonic pasteurellosis which affects all ages

of sheep) and as the name suggests is chronic, non-progessive and infrequently fatal. The disease elicits few clinical signs (Gilmour and Brotherston, 1963), although in severe cases coughing and breathing difficulty may be seen particularly following exercise. Affected animals may be depressed, have a nasal discharge, and may lose condition. Resolution of the disease usually occurs in late autumn (Alley, 1975a).

CNP can be clearly distinguished from pneumonic pasteurellosis on the basis of different pathological lesions. The macroscopic lung lesions of CNP vary from grey to red-brown areas of consolidation in the cranio-ventral lobes of both lungs. In addition, narrow branching dull-red bands of collapse are sometimes present and, occasionally, pleural adhesions are seen. On microscopic examination, the disease is characterised by alveolar collapse, infiltration of the alveolar spaces with macrophages and variable numbers of neutrophils, lymphoid and bronchiolar epithelial cell hyperplasia, and the presence of both lymphocytes and macrophages in the alveolar space. (Whereas pneumonic pasteurellosis is characterised by large numbers of neutrophils and universal destruction of the alveolar epithelium). Damage by CNP is localised and less severe than seen with pneumonic pasteurellosis (Alley, 1975a).

The aetiology of CNP has not been unequivocally established. The most likely hypothesis is that mild lung lesion are caused by M.ovipneumoniae alone, but usually the lesions are exacerbated by P.haemolytica.

M.ovipneumoniae colonises the nasopharynx of of lambs before the peak prevalence of CNP (Ionas, 1983) and this organism is invariably isolated from lesions of CNP (Jones et

al., 1979; Alley and Clarke, 1980). The disease may be experimentally transmitted by intratracheal inoculation of homogenates of pneumonic lung which invariably contain M.ovipneumoniae and P.haemolytica (Jones et al., 1978). When pure cultures of M.ovipneumoniae are inoculated intranasally, lung lesions are produced, but these are less severe than those seen in the field (Alley and Clarke, 1979). Lambs placed in contact with those lambs that have had mycoplasma inocula can also develop mild lesions (Sullivan et al., 1978). Lesions resembling those in the field can be induced by inoculation of mixed cultures of M.ovipnuemoniae, P.haemolytica and M.arginini into lambs. M.ovipneumoniae was recovered from most lesions, but P.haemolytica and M.arginini were reisolated less frequently (Jones et al., 1978).

P.haemolytica is isolated from many, but not all, lesions of CNP (Sullivan et al., 1973; Alley and Clarke, 1977). This organism does not alone cause lung lesions except when given to lambs at very high concentrations or to lambs under 2 to 3 months old (Jones and Gilmour, 1983). The lesions seen are typical of acute pneumonia and are readily distinguishable from CNP. However, when low doses of P.haemolytica are administered in combination with M.ovipneumoniae, pneumonia is induced (Jones et al., 1978; Jones et al., 1982). By implication the lesions represent CNP but this is not unequivocally stated. Alley and Clarke (1977) examined lesions from 60 sheep and isolated microorganisms from the lesions. M.ovipneumoniae (without any other organisms) was isolated from some lesions. These lesions were usually associated with chronic proliferative changes. The more severe lesions (i.e. greater neutrophil exudation in alveoli and increased proliferative changes) were associated with the presence of high concentrations of both M.ovipneumoniae

and bacteria of which P.haemolytica was the most common isolate.

The above information indicates that M.ovipneumoniae is capable of causing mild pneumonic lesions in lambs and that P.haemolytica is then able to colonise the lung and increase the severity of the pneumonia. The role of viruses, stress, mycoplasmas other than M.ovipneumoniae and bacteria other than P.haemolytica is the subject of comment and hypothesis. We do not exclude them from playing a part in initiating or exacerbating pneumonic lesions.

1.35 Importance of Chronic Non-Progressive Pneumonia

Reports from Britain indiate that CNP may affect more than 40% of lambs raised indoors, and 30% maintained outside (Jones and Gilmour, 1983). Similar, and perhaps higher percentages of New Zealand lambs are affected. Reports indicate 70 to 80% of some flocks may have the disease (Alley, 1975a). Despite CNP being generally non-fatal, it is believed to cause considerable economic loss to the farming industry in New Zealand. This is for 2 reasons: Firstly, CNP may cause pleural adhesions in some lambs which may result in a down grading, or in extreme cases to a rejection of carcasses for export (Dysart, 1976). A recent report emanating from the Ministry of Agriculture and Fisheries (See Central Districts Farmer, vol 3, January 1985) stated that at the works the most important defects of sheep carcasses were due to sarcocystis (32.5%), pleurisy leading to pleural adhesions(31.4%) and sheep measles (12.6%) The second consequence of CNP which results in loss to farmers is that lambs fail to gain weight at the same rate as healthy lambs (St George et al., 1971; Carmichael et al., 1972; Kirton et al., 1976; Harris and Alley, 1977; Jones et al., 1982). CNP was

estimated to cost New Zealand $26,000,000 per annum in 1983 (Ionas, 1983).

1.4 Immunity to P.haemolytica

Definitive experiments to establish the protective antigen(s) of P.haemolytica and the mechanism of immunity have not been undertaken. However, there is evidence that the protective antigen is the capsular polysaccharide and that protection requires the presence of antibody to this antigen. Other antigens may however play a role. The most significant experiments are outlined below.

Biberstein and Thompson (1965) vaccinated groups of mice with strains of A1, A2, T4 or T10. Mice were challenged with homologous organisms (capsular and somatic antigens in common) or with organisms of the same type, but with different somatic antigens or with organisms which differed with respect to both antigens. The highest degree of protection was achieved when the immunising and challenge strains shared both antigens. However, a higher degree of protection occurred when capsular antigens only were shared rather than when only somatic antigens were shared. This indicates that capsular antigens are the most important antigens for immunity, although not the only ones.

Similar work was carried out by Evans and Wells (1979) who vaccinated mice with a mixture of serotypes A1, A2 and A6. Mice were protected against challenge with A1 and A6, but not against A2 and A9. Evans and Wells concluded that immunity to P.haemolytica is type-specific, but that it is difficult to protect against serotype A2.

Tadayon and Lauerman (1981) vaccinated mice with extracts of P.haemolytica cells including capsule, protein, lipopolysaccharide, lipid A, ribosomal fractions and a potassium thiocyanate extract which would contain a high, although not exclusive, proportion of capsule. When challenged with homologous cells, the potasium thiocyanate extract vaccinates showed the highest degree of protection, and the capsular extract vaccinates the second highest degree of protection. This indicates that the capsule is important for immunity, but is not the only antigen involved. However, the degree of purification of the fractions does not allow an unequivocal interpretation of the results.

Knight et al., (1969) found that mice vaccinated with a strain of A1 were protected better when challenged with a strain of A2 than when challenged with homologous cells. This A2 strain differed from the immunising strain in "O" somatic antigens as well as capsular material. This work, perhaps, serves to indicate that capsular antigens are certainly not the only antigens involved in immunity. Indeed it has been shown that a protein fraction is capable of stimulating immunity in mice, although to a lesser degree than that of capsule (Cameron, 1966; Tadayon and Lauerman,1981). Himmel et al., (1982) showed that a heat labile protein may be isolated from culture supernatants of P.haemolytica. This substance has cytotoxic effects on bovine alveolar macrophages (ovine macrophages were not tested). Antibody to this protein was stimulated by all serotypes of P.haemolytica, but no infomation is available on its protective effect. Tabatabai et al., (1981) isolated, from serotype A1, a soluble extracellular antigen which was shown to have neuraminidase activity. Antibody directed against this inhibited the activity of the enzyme suggesting that it is part

of the antigen complex of P.haemolytica.

Most of the above experiments used mice as the experimental animals, so even if unequivocal results were obtained, it is uncertain if the conclusions can be extrapolated to sheep. However, experiments with sheep also indicate the importance of the capsule in immunity. Gilmour et al., (1979) vaccinated sheep with sodium salicylate extracts of A1 and A6 (containing a high proportion of capsule) and with A2 cells, since extracts of A2 elicit only a poor response. Lambs were protected upon challenge with A1 and A6, but were not significantly protected against A2, nor against infection with heterologous types.

As stated above, the results available indicate that an immune response to the capsule is important for immunity to P.haemolytica, but that other antigens may also play a protective role. The experiments also indicate that it is particularly difficult to induce solid immunity to serotype A2. The reason for this is not clear, but it is interesting to note that A2 is the most prevalent serotype associated with pasteurellosis (Biberstein and Thompson, 1966; Thompson et al., 1977; Fraser et al., 1982).

Immunity to P.haemolytica was traditionally judged by the serological response in sheep determined by the IHA assay. However, the titre of antibody produced did not give an infallible measure of immunity (Cameron, 1966). The ultimate test of immunity is to determine the resistance to challenge of vaccinated sheep, but this requires a method of challenge which consistantly induces pneumonia in non-immune sheep.

Smith (1964) inoculated conventionally reared lambs

intabronchially with large numbers (10^{10} to 10^{11}) of P.haemolytica and produced infections which were usually fatal and resembled pneumonic pasteurellosis. However, the disease was not produced consistantly at lower doses. Gilmour et al., (1975) exposed SPF lambs to aerosols of P.haemolytica, but this also produced erratic results. Similarly, early experiments using sequential PI3 virus and P.haemolytica had only limited success in the sense that acute pasteurellosis could not be reliably induced (Biberstein et al., 1967). Subsequent work with PI3 virus and aerosols of P.haemolytica in SPF lambs, but with a 6 day interval between inoculations, consistantly produced pneumonia in a high proportion of lambs (Sharp et al., 1978).However, since SPF lambs are only seasonally available and are also expensive to maintain, it becomes impractical to carry out tests on large numbers.

It has recently been shown that conventionally raised sheep consistantly develop respiratory infections when injected intravenously with 1.25% agar in PBS followed immediatly by an aerosol of P.haemolytica (Gilmour et al., 1982a). Vaccinated sheep tend to have milder lesions than the unvaccinated controls when challenged by this method (Gilmour et al., 1982b). SPF lambs which were passively vaccinated with serum against P.haemolytica were not protected following challenge, even though mice were protected (Wells et al., 1979). This implies that antibody is not the only important factor in immunity to P.haemolytica in sheep. Cell mediated immunity is probably involved. Studies with lymphocytes from vaccinates show that they proliferate in the presence of a sodium salicylate extract of P.haemolytica (Wells et al., 1979). The proportion of lymphocytes that divide increases markedly if they are collected after exposing lambs to P.haemolytica aerosols and this may

indicate a secondary cellular immune response. However, immune serum from lambs has a bacteriostatic effect on P.haemolytica in vitro (MacDonald et al., 1983) and antibody is necessary for clearance of P.haemolytica from in vitro cultures of bronchio-alveolar cells from both vaccinated and non-vaccinated SPF lambs (Wells et al., 1979). Furthermore, P.haemolytica lyses ovine alveolar macrophages in the absense of antibody (Kaehler et al., 1980; Wells, 1981). Thus, it seems that both humoral and CMI responses contribute to immunity to P.haemolytica.

1.5 Vaccine to P.haemolytica

Treatment of pasteurellosis is not a practical proposition since affected animals are usually found dead. If perchance animals are found in the initial stages of pasteurellosis antibiotics may be administered. However, in the USA, Chang and Carter (1976) found half of the P.haemolytica strains they isolated were resistant to penicillin, streptomycin and terramycin. Treatment of CNP is also impractical because in most cases it is subclinical. Prevention of both pasteurellosis and CNP by eliminating predisposing factors is also unlikely since these are often part of the normal sheep husbandry or are environmental and thus uncontrollable. The best hope then for the control of pasteurellosis and CNP is probably immunisation. A pasteurellosis vaccine would, of course, contain P.haemolytica organisms or extracts. Such a vaccine could also be useful against CNP. While not necessarily eliminating the disease, a reduction in the severity of lesions and the duration of the disease may occur.

Since immunity to P.haemolytica is mostly type-specific

(but see previous discussion) a P.haemolytica vaccine should, in theory, contain capsular antigens from all 15 serotypes. Such a vaccine would probably be uneconomical. However, surveys in Britain and the USA indicate that not all serotypes are of equal importance in the production of disease (Biberstein and Thompson, 1966; Thompson et al., 1977; Fraser et al., 1982). Thus, only those serotypes frequently associated with the pathological condition need to be included in a vaccine.

Currently in Britain, Hoechst are marketing 3 vaccines containing P.haemolytica components. They are:-

Heptavac-P (8 clostridial and 8 P.haemolytica components)
Ovivac-P (4 clostridial and 8 P.haemolytica components)
Ovipast (8 P.haemolytica components only).

The P.haemolytica components consist of capsular extracts of serotypes A1, T3, T4, A6, A7, A9, T10 plus heat killed A2 cells since this induces a stronger response than A2 capsular material alone (Gilmour, 1979). However, the ability of P.haemolytica vaccines to protect animals from disease has been questioned (Stamp et al., 1955; Cameron and Smit, 1970; Gilmour, 1978; Mwangota et al., 1978; Gilmour et al., 1979). One of the reasons for the lack of efficacy of P.haemolytica vaccines was that serotypes not included in the vaccine were causing the outbreaks. Thus, more extensive surveys are required to elucidate which types should be included in the vaccine. A minimal number of surveys have been undertaken in Britain (Biberstein and Thompson, 1966; Thompson et al., 1977; Fraser et al.,1982) and, to date, no such surveys have been performed in New Zealand.

1.61 Indirect Haemagglutination

The main reason for the lack of information regarding serotypes is due to technical problems with the standard method of serotyping isolates, the IHA test. The test was first described by Biberstein et al., (1960) and later modifications have included the use of a microtitre method (Shreeve et al., 1972), using glutaraldehyde-fixed RBC's (Sawada et al., 1982), and recently using colonies from agar as antigen which speeds up the test (Fraser et al., 1983). The test involves the absorbing of antigen from every isolate on to RBC's which are then added to 15 serial dilutions of antisera. Not only is the test laborious, but cross-reaction are occasionally seen (Biberstein, 1965; Burrells et al., 1983). Furthermore, RBC lysis often occurs at low serum dilutions and haemolysis of A12 sensitised RBC's can occur (Frank and Wessman, 1978; Sawada et al., 1982). Thus, IHA is not ideal as the standard method of determining the serotype of isolates. In this thesis we examine alternative methods to the IHA test by which isolates may be serotyped.

1.62 SDS-PAGE

The major proplem in typing P.haemolytica is the presence of high titre antibody producing cross-reactions. Examination of the proteins of P.haemolytica by SDS-PAGE does not require the use of typing antisera. Admittedly, to hope to determine the capsular polysaccharide type by examining the proteins by SDS-PAGE might be regarded as the ultimate serendipity, but it is at least possible that differences may occur in the proteins of serotypes and that those differences may provide protein "markers" which detect the presence of a particular serotype.

The proteins of each serotype have previously been compared by electrophoresis in polyacrylamide gels (Thompson and Mould, 1975). The "A" and "T" strains were found to be quite different and while the protein patterns within a biotype were similar, some differences did occur. However, only one strain per serotype was examined, so it is not known whether the minor variations in proteins seen when different serotypes are examined represent random fluctuations or the differences are conserved and are consistant within a serotype, thus allowing P.haemolytica to be typed by an examination of the protein patterns by SDS-PAGE. Thus, in the present work we examine the use of SDS-PAGE to find if it has any potential for determining the serotype of P.haemolytica isolates.

1.63 Antibody-Coated Latex Bead Agglutination

Antibody-coated latex beads have been routinely used in diagnostic laboratories to detect polysaccharide antigens of Neisseria meningitidis, Streptococcus pneumoniae and Haemophilus influenzae in human body fluids(Newman et al., 1970; Severin, 1972). Thus, the test has the potential to detect and discriminate between P.haemolytica capsular polysaccharides. Antisera raised against polysaccharide antigens contains a high proportion of IgM antibody. This is partially purified by precipitation with sodium sulphate and absorbed on to latex particles. To detect antigen the sensitised beads are mixed with the antigen and if the antigen and antibody are homologous the beads rapidly agglutinate. Its use as a means of determining the serotypes of large numbers of isolates, provided a specific test is obtained, is extremely attractive.

1.64 Gel Precipitation

Soluble antigens may be detected by gel precipitation (Brock, 1979) and prior to the introduction of latex beads, the capsular polysaccharides of N.meningitidis, Strep.pneumoniae and H.influenzae were detected by gel precipitation and more recently by CIE (Coonrod and Rytel, 1972; Dorff et al., 1971; Tugwell and Greenwood, 1975). Gel precipitation has also been used in the serological typing of various organisms (Muraschi and Tompkins, 1963).

Muraschi et al., (1965) examined the use of gel precipitation for serotyping strains of P.haemolytica using ether extracted antigens. These they beleived to be somatic. Each of the 11 serotypes available at the time, displayed a characterisic major precipitation line which allowed the strains to be readily differentiated, although, some serotypes shared a common minor precipitation line. This degree of type specificity using "somatic" antigens is surprising since a large proportion of serotypes possess common somatic antigens (Biberstein, 1960). The degree of specificity seen suggests that the ether extraction method of Muraschi et al., (1965) did not extract the "O" antigens, but rather mostly type-specific capsular material. Although the use of gel precipitation appears to give mostly type-specific results when used to serotype P.haemolytica there have been no reports in the literature of researchers taking advantage of this for serotyping isolates. In this work we investigate the use of gel precipitation for serotyping isolates.

1.65 Counter Immunoelectrophoresis

Gel precipitation is simple to perform, but requires overnight incubation. CIE also relies on antigens and antibodies moving through agar, but in this case they are forced towards each other in an electric field. Thus, only an hour is required before results may be read, and since antigen and antibody travel towards each other rather than randomly diffuse through the agar, the test should be more sensitive than gel precipitation.

CIE has been used to identify the capsular material of N.meningitidis, Strep.pneumoniae and H.influenzae (Coonrod and Rytel, 1972; Dorff et al., 1971; Tugwell and Greenwood, 1975). Since P.haemolytica antigens can be detected by gel precipitation (Muraschi et al., 1965) it seems reasonable to expect that they would also be detected by CIE. With this in mind, in the present work we examine the potential of CIE as a tool for serotyping isolates of P.haemolytica.

1.66 Agglutination

Direct bacterial agglutination has been studied as an alternative method of serotyping isolates of P.haemolytica (Frank and Wessman, 1978). The prototype strains reacted specifically with their homologous sera in most cases. Only one major cross-reaction was seen. This was between serotype A7 cells and A12 antiserum. However, this cross-reaction was also noted in the IHA test. Minor cross-reactions occurred between serotypes T3 and T4 cells and T10 antiserum, but these could be eliminated by dilution leaving the homologous reaction. None of the cross-reactions led to confusion since all were one-way.

103 isolates of P.haemolytica were obtained and serotyped by agglutination and by IHA. Of the 103, 95 were typed the same by both methods, 5 were untypable by both methods and 3 typed by agglutination as A2 or A9 but did not react in the IHA test.

Further studies with 10 isolates (Frank,1980) which were untypable by IHA found they could be grouped into 3 types by agglutination tests. These isolates were not members of an established serotype and simply lacked the specific capsular material, but belonged to 3 distinct serotypes.

Frank (1982) collected 275 isolates of P.haemolytica from sheep in the mid-western USA and serotyped them by agglutination. This further supports the suggestion by Frank and Wessman (1978) that agglutination tests are a viable alternative to the IHA test. However, workers in other laboratories obtained many cross-reactions by agglutination and concluded that IHA is a more useful test for establishing the serotype of isolates. The present work examines the relative merits of the above tests: IHA, latex bead agglutination, gel precipitation, CIE and agglutination.

CHAPTER 2

Isolation and Identification of P.haemolytica

2.1 Introduction

The present work is mainly concerned with two topics: The development of a technique for serotyping isolates of P.haemolytica and the use of this for surveying the prevalence of the various serotypes in sheep. We therefore need to standardise a method of isolating P.haemolytica from sheep and identifying the organism.

Biberstein et al.,(1970) states that P.haemolytica may be identified in mixed bacterial cultures on blood agar plates with "virtually complete accuracy on the basis of colonial appearance and haemolytic pattern alone." However, colony morphology is to some degree a subjective criterion, so other criteria were also used in this thesis.

2.2 Materials and Methods

Isolation of P.haemolytica from Ovine Lungs. Samples of lungs from 3 sheep with CNP from each of 20 farms in the Manawatu region were obtained from the abbotoir by Ionas in 1981 and stored at -80°C.

A portion of lung was thawed and smeared over half of the surface of a 5% horse blood agar plate (See Appendix). The inoculum was streaked over the rest of the plate and incubated at 37°C for 24h. Characteristic features of 24h P.haemolytica colonies are : 0.1 to 0.2cm diameter, smooth surface, entire

edge, low convex shape, translucent, and a zone of beta haemolysis directly underneath the colony (See Figure 1).

Colonies showing these characteristics were subcultured on blood agar and incubated at 37°C for about 18h. Each isolate was then inoculated on MacConkey agar, triple sugar iron agar and urea agar and incubated for 24 to 48h. Isolates were also tested for Gram reaction (counterstaining with carbol fuschin), and oxidase activity.

P.haemolytica Isolated from the Ovine Nasopharynx. 50 sheep from each of 4 flocks, differing in either geographic location or managment practice, were sampled.(See Table I)

FLOCK	LOCATION	AGE	BREED	MANAGEMENT
A	Pahiatua track (Hill country)	Mixed 2-6yr	Perendale	50/hectare Controlled grazing
B	Masterton (Low land)	3-5yr	Romney	Controlled grazing
L	Woodville	Mixed	Romney	Set stocked Low rate Free range
R	Woodville	Mixed	Romney	Set stocked High rate (Rotationally grazed)

Table I

Four flocks of sheep sampled for P.haemolytica in the nasopharynx.

Samples of nasal mucus were obtained by inserting a sterile 15cm cotton swab about 13cm into a nostril and rotating the swab both clockwise and anticlockwise. Swabs were immediately broken off into bijou bottles containing Amies Transport Media (See Appendix), and taken to the laboratory. Each swab was smeared over half of the surface of a blood agar plate and incubated at 37°C for about 24h.

Colonies resembling P.haemolytica (see above) were

subcultured on to blood agar. The serotype of each isolate was determined.(See Chapter 5 and 6) In cases where isolates were not of an established serotype further tests were performed to confirm that they were P.haemolytica (see below).

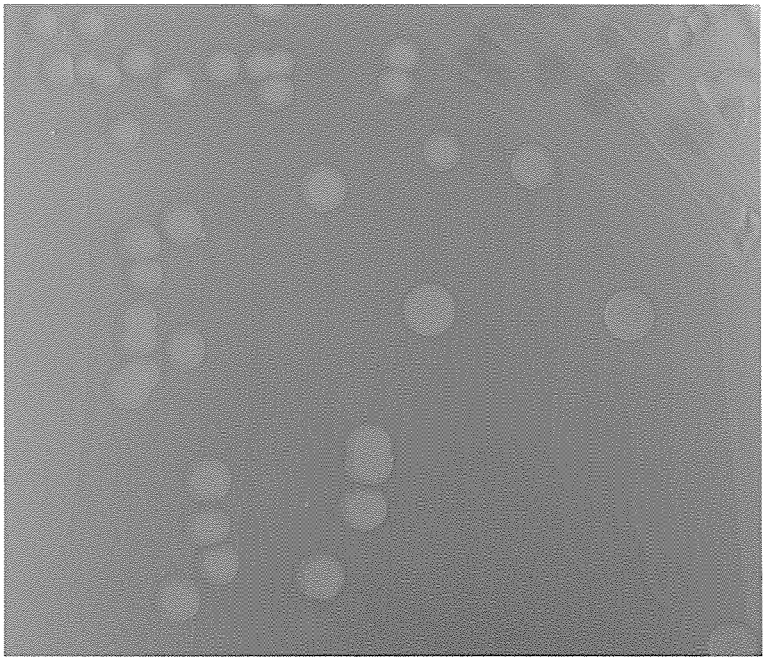
Isolates, obtained from the ovine respiratory track, which showed the following characteristics were considered to be P.haemolytica ;

(i) Typical colony morphology (as above),
(ii) Gram negative rod,
(iii)Growth on MacConkey agar from a heavy inoculum,
(iv) Oxidase positive,
(v) Urease negative,
(vi) Produces acid throughout TSI agar, but no gas, nor H_2S.

In practice isolates were also identified by serotyping (Chapter 5 and 6), and by SDS-PAGE (Chapter 3) which divided P.haemolytica into two groups related to the "A" or "T" biotypes.

Figure 1

Colonies of P.haemolytica serotype A1. Note: in the right hand corner colonies have been pushed aside revealing zones of haemolysis.



2.3 Results

Numbers of samples from which P.haemolytica was isolated are recorded in Table II.

SAMPLE ORIGIN	NO. SAMPLED	NO. OF ISOLATES	% POSITIVE
Lungs with CNP lesions	60*	40	67%
Nasopharynx of healthy sheep			
FARM : A	50	28	56%
B	50	24	48%
L	50	33	66%
R	50	25	50%

Table II

Number of samples of ovine lung or nasopharngeal mucus from which P.haemolytica was isolated.

* 3 lungs from each of 20 farms.

2.4 Discussion

P.haemolytica has been isolated in New Zealand from ovine lungs which showed lesions associated with CNP (Alley, 1975b; Pfeffer et al., 1983). They recovered P.haemolytica from 59% and 55% of lungs respectively. Our recovery rate of 67% (See Table II) is slightly higher than that of other New Zealand workers.

Alley, (1975b) also examined the nasopharynx and recovered P.haemolytica from 135 of 184 (73%) of the healthy sheep tested. We recovered 110 isolates of P.haemolytica from the nasopharynx of 200 sheep (55%). Our lower recovery rate may be attributed to seasonal variation in the carriage of P.haemolytica which was shown to occur in Scotland (Biberstein et al.,1970). These workers found the recovery rate varied from 40 to 95% and peaked twice throughout the year,once in early summer and then in late autumn. The lowest rate occurred in winter during which we sampled the nasopharynx.

We conclude that P.haemolytica can be readily isolated from sheep and that the organism is ubiquitous throughout the regional and probably the national flock.

CHAPTER 3

COMPARISON OF ISOLATES BY SDS-PAGE

3.1 Introduction

Proteins of P.haemolytica have been compared by electrophoresis in polyacrylamide gels (Thompson and Mould, 1975). These workers utilised a phenol-acetic acid-water extract of the sedimented organisms to compare serotypes. They concluded that P.haemolytica "A" and "T" strains could be readily differentiated, but within the "A" or "T" biotypes isolates were "virtually similar." However, only one isolate of each serotype was examined.

It is now generally accepted that solubilisation of protein is best achieved by heating samples to 100°C in the presence of sodium dodecyl sulphate (SDS) in reducing conditions. Futhermore, SDS-PAGE gels give good resolution of protein bands on the basis of molecular weight. This section establishes the optimum conditions for examining P.haemolytica by SDS-PAGE and compares "A" and "T" biotypes including several isolates from each of three serotypes. To investigate the use of SDS-PAGE gels as a means of identifying P.haemolytica we compare several isolates of P.haemolytica with some other gram negative rods recovered from the lungs of sheep.

To make comparisons valid the phase of growth of the cultures was standardised. This was achieved by harvesting cells in the late logarithmic phase. To facilitate this, initial experiments investigate the growth curves of two

serotypes(A1 and T4).

3.2 Materials and Methods

Origin of strains

A. Prototypes. A lyophilised culture of each serotype was received from ICI-Tasman, Upper Hutt. These were reconstituted by the addition of a few drops of BHI and streaked on blood agar plates which were then incubated at 37°C. Several colonies from each plate were inoculated in to 8.4ml BHI and incubated at 37°C for about 4h by which time turbidity was readily detectable. 1.6ml of sterile glycerol was then added to give a final concentration of 16% glycerol. 1.0ml aliquotes were stored at -80°C.

B. Local Isolates. See Chapter 1.

Investigation of Growth Curves

Two 30ml volumes of Brain-Heart Infusion (BHI) in side-arm flasks were inoculated with 1ml of P.haemolytica serotype A1 or T4 and incubated at 37°C on a shaking platform. The absorbance at 500nm was recorded at intervals.

Estimation of Protein Content of P.haemolytica

The method of Bradford (1973) as modified by Ionas (1983) was used. This involved initial solubilisation of proteins in NaOH. See Appendix.

Optimum Protein Loading For Maximum Resolution of SDS-PAGE Gels

The preparation and running of SDS-PAGE gels is described in the Appendix. Protein loadings of 20,40,60,80,120,160,200,240µg per track of P.haemolytica serotype A1 were compared.

Comparison of Isolates

(a)The prototype strain of each serotype of P.haemolytica was examined by SDS-PAGE.

(b)Seven isolates of P.haemolytica serotype A1, obtained from pneumonic lung tissue of sheep from various farms in the Manawatu region, (Chapter 2) were compared to the prototype A1 strain.

(c)Thirteen isolates of P.haemolytica serotype A2, obtained from pneumonic lungs, were compared to the prototype strain of A2.

(d)P.haemolytica serotype A6 isolates, obtained from pneumonic lungs, were compared to the prototype A6 strain.

(e)

Isolate	Gram neg rod	Haemolysis	MacConkey	Oxidase
6B	+	-	+	+
11A	+	+	+	-
17A	+	+	-	+
18C	+	-	+	+
Typical P.haem	+	+	+	+

Table III

Comparison of some characteristics of four gram negative rods, isolated from the lungs of sheep with chronic non-progressive pneumonia, and a typical P.haemolytica.

Four gram negative rods, recovered from pneumonic lung tissue, which were not P.haemolytica (see Table III), were compared to P.haemolytica serotypes A1 and T4 prototype strains.

3.3 Results

(a) Growth curves of P.haemolytica serotypes A1 and T4 are shown in Figure 2.

(b) Varying loads of P.haemolytica serotype A1 protein were electrophoresed through a polyacrylamide gel. See Figure 3.

(c) The 15 prototype strains of P.haemolytica were compared by SDS-PAGE. See Figures 4 and 5.

(d) Several isolates of P.haemolytica serotype A1, A2 and A6 were compared to their respective prototype strains by SDS-PAGE. Figures 6,7 and 8a and 8b.

(e) Isolates of gram negative rods, which although recovered from the lungs of sheep with CNP, were not P.haemolytica, were compared to the prototype strains of P.haemolytica serotypes A1 and T4. See Figure 9.

Figure 2

Growth curves of P.haemolytica serotypes A1 and T4.

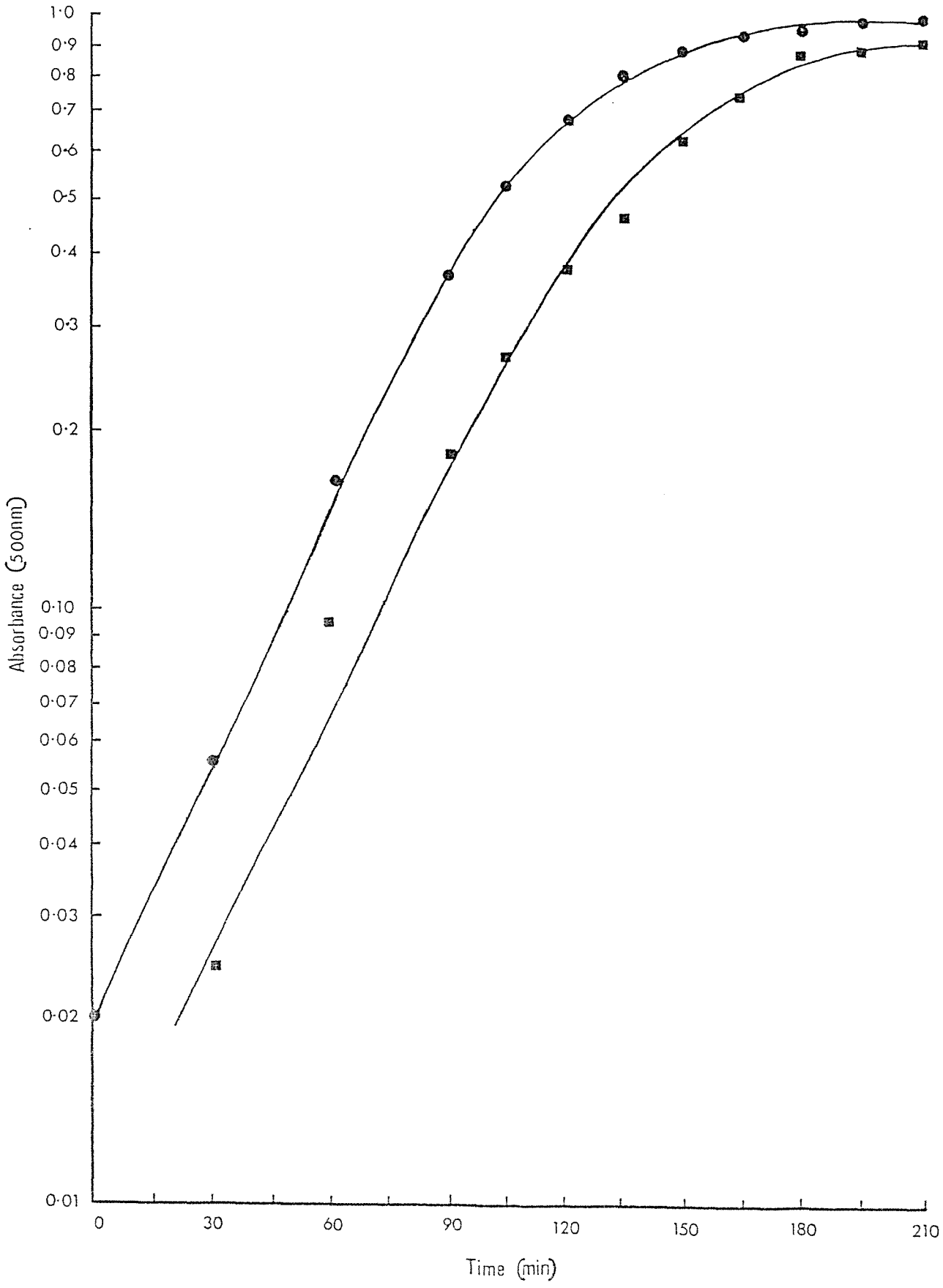

1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·10
0·09
0·08
0·07
0·06
0·05
0·04
0·03
0·02
0·01
Absorbance (500nm)
0
30
60
90
120
150
180
210
Time (min)

Figure 3

Total proteins of P.haemolytica serotype A1 separated by SDS-PAGE. To investigate the optimum protein load per track, 20,40,60,80,120,160,200,240µg protein were applied to tracks (left to right). A loading of 80µg protein per track was selected as the optimum for the comparison of isolates.

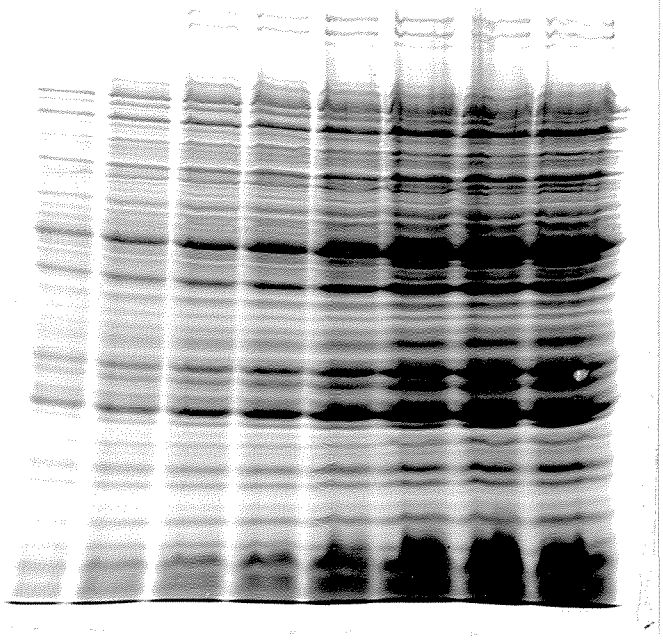


Figure 4

Comparison of the total proteins of P.haemolytica serotypes (left to right) A11,A9,A6,A5,A7,A2,A14,A1 by SDS-PAGE.Note the overall similarity of the serotypes. However, some differences are detectable in the proteins of the middle molecular weight range. These differences may be quantitative rather than qualatative.

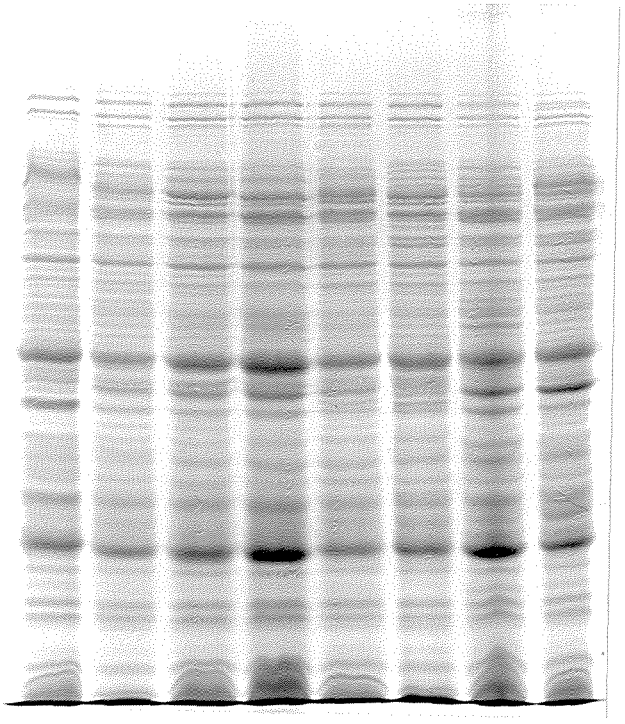


Figure 5

Comparison of the total proteins of P. haemolytica serotypes (left to right) A1, A12, A8, A13 and T3, T10, T4, T15 by SDS-PAGE.

Note (i) There is an overall similarity of the protein of different serotypes within the "T" biotype. However, some minor variations occur (Arrow A).

(ii) The protein patterns of the four "A" biotype strains are markedly different from that of the "T" strains. However, there is one major common band (Arrow B).

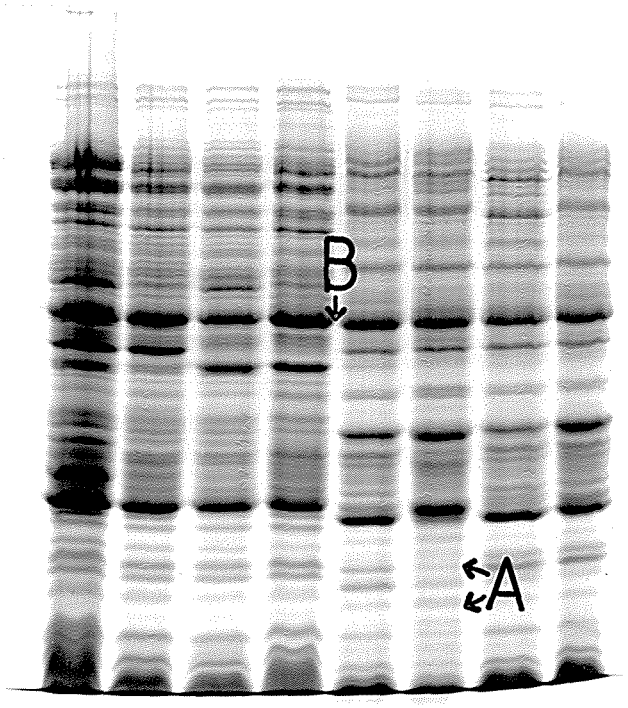

B
A

Figure 6

Comparison of the total proteins of isolates of a single serotype (A1) of P.haemolytica. Isolates are (from the left) 3B,15C,16B,4A,7B,2C,10C. These were recovered from lungs of sheep with CNP. The prototype A1 strain is shown in the extreme right hand track. Note: The protein patterns are almost identical. However, some minor differences occur.(Arrowed)

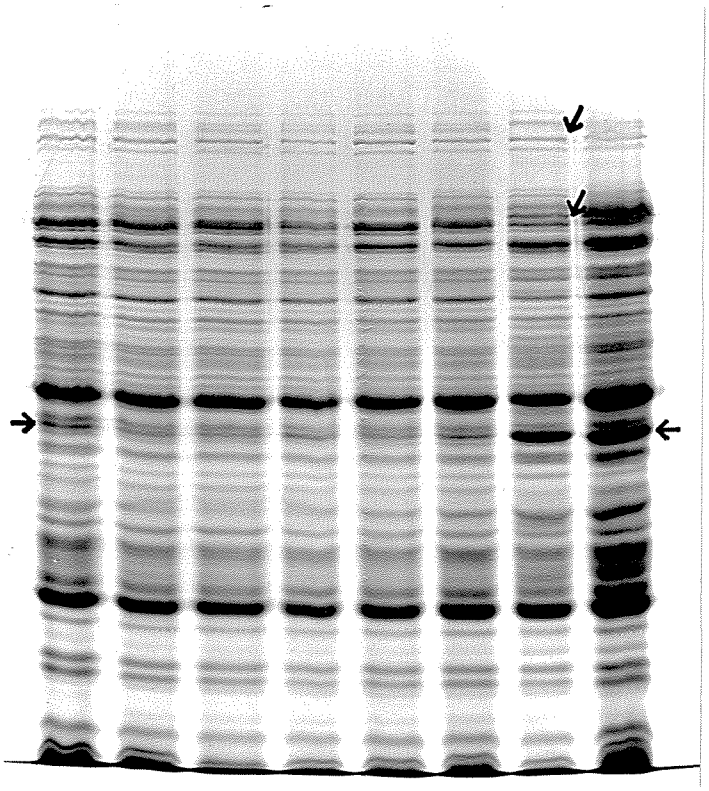


Figure 7

Comparison of the total proteins of isolates of a single serotype (A6) of P.haemolytica. These were recovered from lungs of sheep with CNP. The prototype A6 strain is shown in the extreme right hand track. Note the isolates are almost identical. However, there are differences (Arrowed) which appear quantitative rather than qualatative.

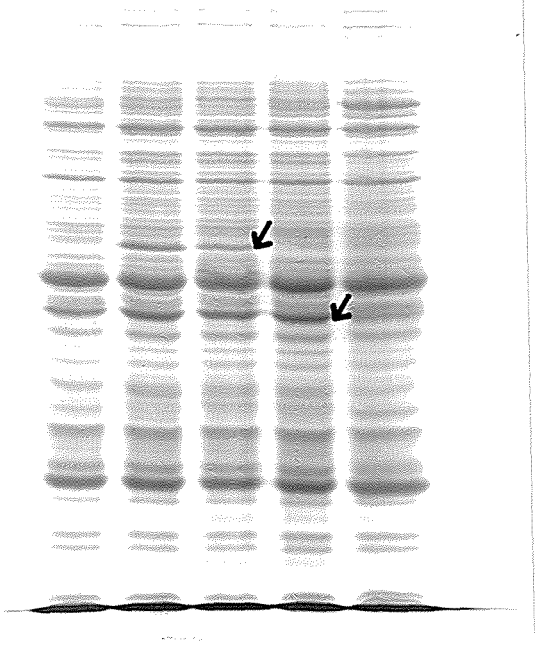


Figure 8a

Comparison of the total proteins of a single serotype (A2) of P.haemolytica. Isolates are (from the left) 9B,9C,10C,12A,14A, 19A,1B recovered from lungs of sheep with CNP. The prototype A2 strain is shown in the extreme right hand track. Note the overall similarity. However, there are differences in the proteins of the middle molecular weight which are probably quantitative rather than qualatative.

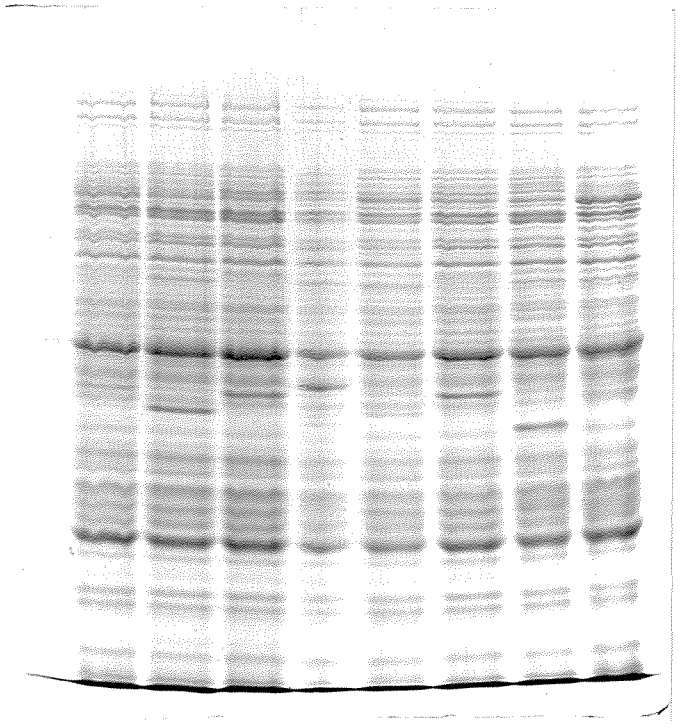


Figure 8b

Comparison of the total proteins of a single serotype (A2) of P.haemolytica. Isolates are (from the left) 1B,1C,2A,4C,6C,8A,8C recovered from lungs of sheep with CNP. The prototype A2 strain is shown in the extreme right hand track. Note the overall similarity. However, there are differences in the proteins of the middle molecular weight which are probably quantitative rather than qualatative.

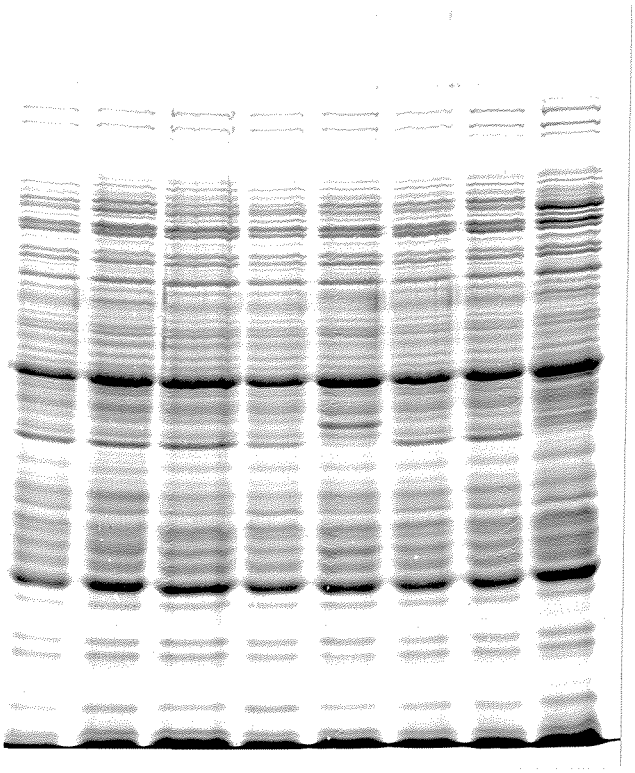
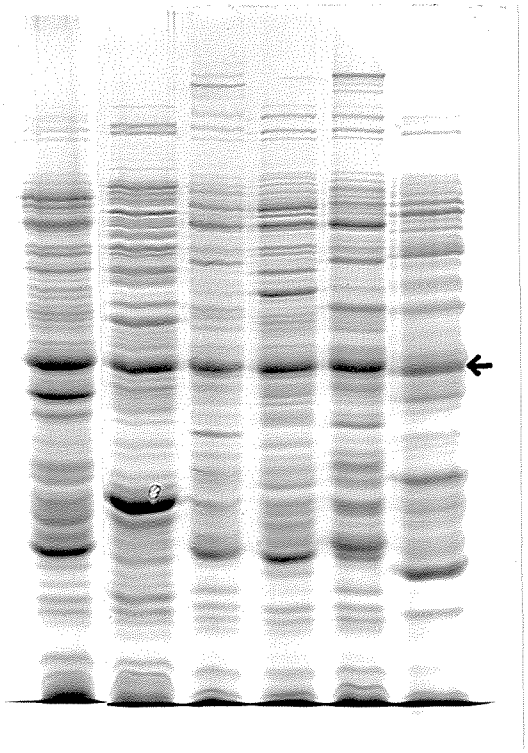


Figure 9

Comparison of four isolates of gram negative rods, (not P.haemolytica) recovered from the lungs of sheep with CNP,with two strains of P.haemolytica. The central tracks contain the four gram negative isolates bounded by P.haemolytica serotype A1 on the left and P.haemolytica serotype T4 on the right. Note that all patterns are markedly different. However, one major common band occurs.(Arrowed)



3.4 Discussion

Growth Curve. Serotypes A1 and T4 were selected to represent the two biotypes in growth curve experiments. From Figure 2 we conclude that in both cases cultures harvested at an optical density of 0.40, at 500nm, are in the late logarithmic phase. All comparisons of total protein in this present work used such cells.

Loading of Gel. Since individual proteins of any species of bacterium differ in their relative concentrations it follows that what is optimal for one protein band would not be ideal for all other bands. Comparison of different track loadings of P.haemolytica (Figure 3) suggest that the best compromise is achieved at a loading of 60 or 80µg per track. Subsequent protein loadings were standardised at 80µg.

Comparison of serotypes of P.haemolytica. P.haemolytica serotypes of biotype "A" have substantially the same protein pattern when examined by SDS-PAGE (Figure 4). For example, serotypes A5,A6, and A9 are identical, as are A1,A12, and A14. However, some minor variations were detected among bands of the middle molecular weight proteins. These differences may be quantitative rather than qualatative.Similar conclusions apply to serotypes within the "T" biotype (Figure 5).

In contrast to the homogeneity within a biotype, when the protein patterns of "A" and "T" strains are compared (Figure 5) they are seen to be quite different. Only one major band appears to be common to both biotypes. Thus, it is possible to distinguish "A" and "T" strains on the basis of protein pattern alone.

Comparison of Isolates of the Same Serotype. Several isolates from each of three serotypes were compared by SDS-PAGE and isolates within a serotype were found not to be identical (Figures 6, 7 and 8). However, the observed differences were minor and probably quantitative rather than qualatative.

Protein differences between different serotypes of the same biotype were compared in Figures 4 and 5. Since serotypes are distinguished by capsular polysaccharide antigens rather than by protein antigens it is not surprising that the minor protein differences observed cannot be correlated with particular serotypes. Hence, SDS-PAGE cannot be used to identify serotypes.

Use of SDS-PAGE to Identify P.haemolytica. The protein patterns of P.haemolytica serotypes A1 and T4 were compared to those of four isolates of gram negative rods obtained from pneumonic lungs (Figure 9). These isolates although not fully identified, were not P.haemolytica (Table 3), and showed quite different protein patterns compared to those of the "A" or "T" biotypes. However, the one major band, common to the "A's" and "T's", was seen with these non-P.haemolytica lung isolates.

We conclude that SDS-PAGE gels may be used to identify isolates as P.haemolytica and futhermore can be used to establish the biotype of an isolate, but cannot be used to establish the serotype.

CHAPTER 4

Assessment of Serotyping Techniques and the Production of Antisera using the Prototype Strains of P.haemolytica

4.1 Introduction

IHA described by Biberstein et al., (1960) is the standard technique for identifying the serotype of an isolate of P.haemolytica. Antigen from the isolate is absorbed on to RBC's, which are then added to serial dilutions of antisera to each of the 15 serotypes of P.haemolytica. The antiserum showing the highest titre in relation to the homologous titre is judged to be the serotype of the isolate. This is a laborious technique, especially if large numbers of isolates are involved.

Antibody coated latex bead agglutination has been successfully used to detect the capsular polysaccharide of Haemophilus influenzae (Newman et al., 1970); Neisseria meningitidis (Kaldor et al., 1977) and Cryptococcus neoformans (Goodman et al., 1971). The use of this test could be extended to include the capsular polysaccharide of P.haemolytica and, at least in principle, may allow serotypes to be distingished.

Polysaccharide antigens can give lines in gel precipitation tests, so in principle gel precipitation can be used to type P.haemolytica. Muraschi et al., (1965) extracted "somatic antigens," which presumably would be bacterial endotoxins, for use in gel precipitation. The reactions showed a limited degree of "type specificity," so it is unclear as to whether the antigens detected were capsular polysaccharide (type specific) or endotoxin (not type specific) or possibly both. It is not

surprising that the technique used by Muraschi et al., (1965) has not been adopted by other workers. Nevertheless, gel precipitation has some advantages and in this chapter we examine its potential for typing P.haemolytica.

One disadvantage of gel precipitation is that the test usually takes about 24h to complete. This delay may be overcome using counter immunoelectrophoresis (CIE). CIE forces antigen and antibody together, because at the pH used (pH=8.6) the antigen is negatively charged and moves toward the anode, while immunoglobulin moves toward the cathode due to endosmosis. Where antigen and antibody meet in optimum proportions, a precipitate forms.

Direct bacterial agglutination is a rapid method for identifying serotypes of P.haemolytica (Frank and Wessman, 1977). These workers utilised bacterial agglutination as the standard method for establishing the serotypes of isolates in surveys of the prevalence of serotypes (Frank, 1980). However, other workers (Fraser et al., 1983) found the test to be unsatisfactory because of an unacceptable number of cross-reactions.

This chapter assesses IHA, latex bead agglutination, gel precipitation, CIE, and bacterial agglutination for specificity and convenience using the 15 prototype strains of P.haemolytica.

All of these techniques require the production of anti-capsular antibody to the 15 prototype strains. This initially appeared to require only the routine inoculation and bleeding of rabbits. However, as became clear in the course of this study, rabbits did not consistantly produce the required

immune response, so other species, viz rats and domestic hens, were used for antisera production to some serotypes.

4.2 Materials and Methods

4.21 Antiserum Production to the Capsular Polysaccharide in Different Species of Animal

Rabbits. To prepare antisera in rabbits using all 15 prototype strains of P.haemolytica as antigens, cultures were grown overnight in BHI, centrifuged and resuspended in PBS to half the original volume. Strains of the "A" biotype were used immediatly. The "T" serotypes were heated to 56°C for 30min. Inoculation schedule : 0.5ml subcutaneously, then 1.0ml, 2.0ml, 3.0ml, 3.0ml, 3.0ml, 3.0ml, 3.0ml, 3.0ml intravenously at 3 to 4 day intervals. Rabbits were bled 10 days after the last injection and the sera were titrated using gel precipitation and IHA tests. If the titre was ≧8 by gel precipitation the rabbits were exsanguinated by cardiac puncture. However, if a serum did not reach this titre the rabbit was given a further 2 inoculations of 3.0ml intravenously, and exsanguinated 10 days later.

Rats. Attempts were made to prepare hyperimmune serum in rats using P.haemolytica serotypes A9 (which gave high titre antibody in rabbits) and A6, A7, and A12 (which did not produce detectable antibody in rabbits). Cells were grown on blood agar plates, and removed with PBS containing 0.3% formalin. The suspensions were diluted to an absorbance of 1.0 at 500nm. Two rats were inoculated with each serotype. Inoculation schedule : 0.25ml intraperitoneally and then 7 doses of 0.5ml

intraperitoneally at 3 to 4 day intervals. Rats were exsanguinated by cardiac puncture 7 days after the final inoculation. Sera were titrated by gel precipitation.

Domestic Hens. Antisera were prepared in hens using P.haemolytica serotypes A2, T3, A6, A7, T10, A11, A12, and A13. Cells were grown on blood agar plates and removed with PBS to a turbidity equal to MacFarland Barium Sulphate Tube No.3. Inoculation schedule : 1.0ml intravenously and 0.5ml incorporated into Freunds Complete Adjuvant administered subcutaneously, then 4 subsequent doses of 1.0ml intravenously at weekly intervals. Hens were bled 7 days after the last inoculation and the sera were titrated using gel precipitation and IHA tests. If the titre was ≧8 by gel precipitation the hens were exsanguinated. However, if a serum did not reach this titre the hen was given a further 2 inoculations and exsanguinated 7 days later.

4.22

Indirect Haemagglutination

Preparation of Glutaraldehyde-fixed Red Blood Cells(RBC). Sheep blood from the jugular vein was collected in heparinised vacutainers. The RBC's were washed 3 times in PBS by centrifuging for 5min at 500g. The final deposit was resuspended in PBS to 5 times the volume of packed cells. An equal volume of this 20% cell suspension was mixed with 0.2% glutaraldehyde in PBS and incubated at 37°C for 15min. The cells were then washed 5 times in PBS and resuspended to 10 times the packed cell volume of PBS containing 0.1% sodium azide and stored at 4°C.

Preparation of Antigens for Sensitising RBC's. 10ml aliquots of BHI were each inoculated with one of the 15 prototype strains of P.haemolytica and incubated at 37°C overnight. The cultures were heated at 56°C for 30min.

Coating Glutaraldehyde Fixed RBC's with Antigen. Supernatant was decanted from the stock glutaraldehyde fixed RBC's and the cells resuspended in PBS to 5%. This was mixed with 9 times its volume of heat treated antigen and incubated at 37°C for 30min with occasional mixing. Excess antigen was removed by washing the cells 3 times in PBS. The RBC's were resuspended in PBS to the original volume of antigen.

Indirect Haemagglutination Assay. Two-fold dilutions in PBS of 50µl of antisera to each of the 15 P.haemolytica serotypes were prepared in microtitre plates. 50µl of sensitised RBC suspension was added to each cavity so that each antiserum was titrated against RBC's sensitised with each of the 15 prototype strains. Wells containing 50µl PBS were included as negative controls. Each serum was also tested against RBC's which were fixed with glutaraldehyde, but not treated with antigen. Agglutination of RBC's indicated a positive reaction. The titre was taken as the reciprocal of the highest dilution showing total agglutination.

4.23

Latex Bead Agglutination

Type specific agglutination of the latex particles was not ultimately achieved, so details of the materials and methods are relegated to the appendix. However, the following is an outline of the preparation of "sensitised" beads and the variations used

in attempts to achieve type-specific agglutination.

Antibody to the capsular polysaccharide of serotypes A1, T3, T4, A5, A8, A9, A11 were precipitated from their respective sera using 28% sodium sulphate. This precipitate was dissolved in PBS and reprecipitated with 28% sodium sulphate, then redissolved in PBS and dialysed against PBS. The resulting solution contained the type-specific antibody which could be detected by gel precipitation. This antibody was adsorbed on to the beads and excess bovine serum albumen (BSA) was added as a "blocking agent" and the beads were washed again.

Each batch of antibody coated beads was tested for specificity using antigen from all serotypes. Varying antigen preparations were used. This included a range of concentrations, phenol extraction and periodate or pronase treatment. For antibody precipitation a range of sodium sulphate concentrations were used. Some sera used were pre-adsorbed with heterologous antigen. Coated beads were mixed with heterologous antigen and the resulting agglutinated beads were sonicated in an attempt to separate the beads again. Finally, the capsular polysaccharide was partially purified by ultracentrifugation of pronase treated antigen and used to inoculate rabbits and hens.

4.24

Gel Precipitation

Agar Plates for Gel Precipitation Test

Sodium chloride	80g
Sodium azide	1g
Noble agar	10g
Distilled water to	1000ml

This was heated to 100°C to dissolve the agar and 25ml aliquots were dispensed into petri dishes. The lids were left off the petri dishes while the agar solidified to dry the surface. Wells were cut using a template.

Antigen Preparation and Testing - Standard Antigen. The 15 prototype strains of P.haemolytica were inoculated into BHI and incubated overnight at 37°C. The cultures were heated at 56°C for 30min and used as antigen. Each was tested by gel precipitation against antisera to the 15 serotypes.

Antigen Preparation and Testing - Concentrated Antigen. The 15 prototype strains were streaked on blood agar and incubated for 18h at 37°C. Cells were washed off the agar surface with 3ml PBS and heated at 56°C for 30min. This concentrated antigen was tested by gel precipitation against antisera prepared to P.haemolytica serotype A2 which was either untreated or had been adsorbed with A6 and A8 cells. Absorption was achieved by mixing 1.0ml of A2 sera with 0.5ml A6 cells and leaving them at room temperature for 1h. The mixture was centrifuged to remove the cells. 0.5ml of A8 cells was then mixed with the serum, and the procedure repeated.

Effect of a Variety of Treatments on the Gel Precipitating Antigen. A concentrated P.haemolytica serotype T3 antigen was prepared (as above) and aliquots were treated in the following ways :

(i) Extracted with phenol by mixing it with an equal volume of a saturated solution of water in phenol. After 2h the preparation was centrifuged and the aqueous layer was removed. The residual phenol in the aqueous solution was removed by several extractions with ether.

(ii) Periodic acid treated. 0.9ml of sample was mixed with 0.1ml of 2.0% periodic acid and heated for 1&1/2h at 56°C.

(iii) Pronase treated. 5µl of 1.0% pronase was added to 1.0ml of antigen and incubated at 37°C for 3h.

(iv) Ultracentrifuged at 100,000g for 4h.

Antiserum to P.haemolytica serotype T3 was tested by gel precipitation against untreated and treated antigen as described above.

4.25

Counter Immunoelectrophoresis

Veronal-acetate Buffer 0.05M, pH=8.6

Sodium diethylbarbiturate	5.4g
Sodium acatate trihydrate	4.4g
0.1M HCl to pH 8.6	58.2ml
Distilled water to	1000.0ml

Antigen Preparation. Aliquots of BHI were inoculated with the 15 serotypes of P.haemolytica and incubated overnight at 37°C. The cultures were then heated at 56°C for 30min.

Optimum Running Time for CIE. Microscope slides were washed with detergent and rubbed with paper towels dampened with alcohol. A precoating of 1.0% Noble Agar in veronal-acetate buffer was applied by smearing the slides with a cotton swab soaked in the agar. This was dried at 37°C and 5ml of 1.0% Noble Agar in Veronal-acetate buffer was pipetted on to the slides avoiding the formation of ripples and bubbles in the agar. Once the agar had solidified the slides were stored in a humidified chamber at 4°C for at least 18h.

Six pairs of wells, 4mm in diameter and 4mm apart, were made on each of 7 slides. Antigen preparations of P.haemolytica serotypes A1, T3, A5, A6, A8, or A9 were added to one well of each pair. Homologous antiserum was added to the other well of each pair. The slides were placed on the LKB electrophoresis apparatus with the antibody wells of each pair nearest the anode. 1000ml of Veronal-acetate buffer was added to each reservoir, wicks of Whatman No.1 filter paper were applied, and the system was run at a constant current of 7mA per slide. At intervals of 15, 30, 45, 60, 75, 105, 135min a slide was removed for staining.

Staining CIE Gels. When the slides were removed they were washed with distilled water. Wet Whatman No.1 filter paper was laid on top of the slides, avoiding air bubbles. The slides were dried using a hair dryer, and the filter paper was removed after wetting it with distilled water. The slides were washed in PBS for at least 12h to remove soluble proteins, and rinsed

for 10min in distilled water. They were immersed in an aqueous solution of 0.3% thiazine red for 10min and destained with 1.0% acetic acid, followed by immersion in 1.0% acetic acid and 1.0% glycerol for 10min.

CIE of Prototype Strains. Glass plates measuring 9.2cm by 8.3cm were prepared as with the microscope slides, except that 12ml of agar was used for each plate. The preparation was electrophoresed across the 8.3cm axis for 1h using a current of 27mA per plate. Antigen preparations of each prototype strain of P.haemolytica were electrophoresed against antisera to all 15 serotypes.

Analysis of Non Type-specific Antigen-Antibody Precipitates. Antisera prepared to P.haemolytica serotypes A1, T4 and A5 were electrophoresed against samples of the following antigens :

(i) Heat treated P.haemolytica serotype A1 broth culture (Control standard preparation).

(ii) Heated P.haemolytica serotype A1 broth culture treated by adding 5µl of 1.0% pronase to 1ml of sample, and incubating at 37°C for 3h. (This presumably destroyed the proteins).

(iii) Heated P.haemolytica serotype A1 broth culture from which the lipopolysaccharide component had been removed by ultracentrifugation at 100,000g for 4h.

(iv) The resuspended sediment from (iii) above. This procedure concentrates the lipopolysaccharide.

4.26

Agglutination of the Prototype Strains of P.haemolytica

A small amount of bacterial colony from blood agar was removed using a bacteriological loop and mixed with 10µl of antiserum on clean glass slides. Each of the 15 serotypes of P.haemolytica were tested with antisera to each of the 15 types.

A positive reaction was indicated by clumping of the cells and a clearing of the sera within 3 seconds. Negative results remained turbid.

4.3 Results

4.31 Antisera Production. IHA and gel precipitation antiserum titres with the homologous antigen are recorded in Table IV.

	Gel Precipitation Titre (IHA Titre)					
	RABBITS					
Serotype	1	2	3	4	RATS	HENS
A1	32(3200)	32(>4096)	16(512)	16 (1024)	ND	ND
A2	- (<2)	- (<2)	- (<2)	- (4)	ND	8(2048)
T3	- (ND)	- (ND)	- (ND)	- (ND)	ND	32(256)
T4	- (<2)	16 (64)	16(128)	16(>4096)	ND	ND
A5	- (<2)	4 (16)	16 (32)	16(2048)	ND	ND
A6	- (16)	- (16)	ND	ND	-	8(>4096)
A7	- (<2)	- (<2)	ND	ND	-	32(2048)
A8	2 (ND)	32 (512)	ND	ND	ND	ND
A9	32(2048)	32 (ND)	32(4096)	ND	-	ND
T10	- (ND)	- (32)	ND	ND	ND	16(512)
A11	2 (<2)	- (ND)	ND	ND	ND	>32(1024)
A12	- (<2)	- (4)	- (<2)	- (<2)	-	32(512)
A13	- (<2)	- (<2)	ND	ND	ND	32(1024)
A14	32(2048)	16 (512)	16(128)	ND	ND	ND
T15	16 (8)	16 (8)	8 (1028)	ND	ND	ND

Table IV

Gel precipitation and IHA titres of antisera prepared in rabbits, rats and hens against homologous antigen.

ND = Not done

- = Gel precipitation line was not detected

4.32 IHA Table V records the IHA titres of antisera to each serotype titrated against RBC's sensitised with antigen from each of the 15 prototype strains. An example of a titration of one antiserum (to P.haemolytica A2) against RBC's sensitised with antigen from each of the 15 serotypes is shown in Figure 10.

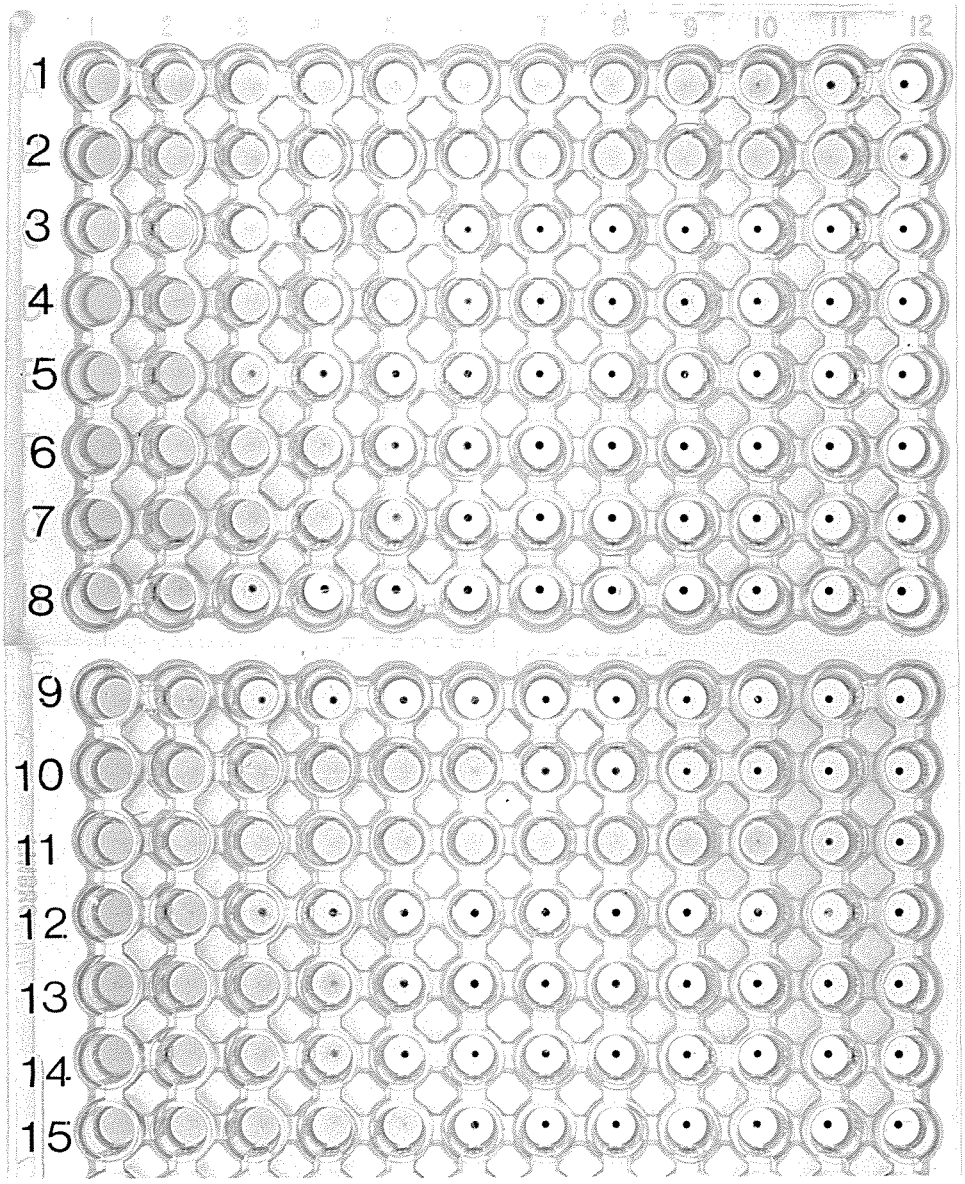
Antisera		Antigen (sensitised RBC)														
Rab	Hen	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15
A1		2048	<2	2	2	<2	<2	<2	2	<2	<2	16	<2	<2	<2	<2
	A2	1024	2048	32	32	4	16	16	4	4	64	1024	4	8	8	32
	T3	128	16	256	128	8	16	16	32	64	1024	4	4	32	8	512
T4		<2	<2	2	>4096	4	8	4	4	<2	32	<2	<2	<2	<2	2
A5		2	2	2	8	2048	8	8	4	<2	2	<2	<2	4	2	2
	A6	128	16	8	8	8	>4096	8	8	2	8	64	8	8	8	8
	A7	64	32	16	32	8	32	2048	4	8	16	128	64	8	64	8
A8		2	<2	<2	2	4	<2	<2	512	<2	2	2	<2	<2	<2	<2
A9		<2	<2	2	2	<2	<2	<2	<2	2048	<2	4	<2	<2	<2	<2
	T10	32	16	8	32	8	8	8	4	2	512	256	4	32	8	8
	A11	16	>4096	16	32	4	16	>4096	8	4	128	1024	4	32	16	16
	A12	128	16	4	16	8	16	32	4	<2	8	256	512	8	8	8
	A13	64	32	32	1024	16	32	16	16	2	256	256	8	1024	16	32
A14		<2	<2	<2	<2	<2	<2	<2	4	<2	<2	<2	<2	<2	2048	<2
T15		<2	<2	2048	8	2	2	4	4	<2	2	<2	<2	2	<2	2048

Table V

IHA titres of antisera to each of the 15 serotypes of P.haemolytica tested against RBC's sensitised with antigen from the 15 prototype strains. Defining specificity as not less than a 4 fold preference for homologous serum over heterologous reactions,the following were specific: A1, T4, A5, A6, A7, A8, A9, A13, A14. However, A2,T10, A11, A12 showed cross-reactions between strains which were not related. Reciprocal cross-reactions also occurred between T3 and T15. These 2 serotypes are known to be closely related (Fraser et al., 1982) and probably should not be recognised as separate serotypes.

Figure 10

IHA of antisera to P.haemolytica A2 against RBCs sensitised with antigen from each of the 15 serotypes. Each row contains a 2 fold dilution series of antisera starting (at the left) with a 1/2 dilution, and RBCs sensitised with one of the 15 serotypes (as indicated). The homologous titre is 2048. A high level of cross-reaction is seen with RBCs sensitised with P.haemolytica A1 and A11 antigen (titre = 512).


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

4.33 Latex Bead Agglutination. Table VI indicates the agglutination of antibody coated beads when mixed with antigen from each of the 15 serotypes of P.haemolytica. Type specific reactions occurred with beads sensitised with T3, T4, A5 and A11 antibody, whereas A1, A8, A9 showed multiple cross-reactions. In attempts to eliminate cross-reactions, a range of antigen concentrations, phenol extraction and periodate or pronase treatment of the antigen was tried. However, specificity did not increase (except with a loss of the homologous reaction). The cross-reactions elicited by phenol-extracts were similar to the "O" antigen groups of Biberstein (1960). Variations in the antibody by either precipitation with a range of sodium sulphate concentrations or by pre-adsorbing with heterologous antigen also did not improve the specificity. Coated beads that were agglutinated by heterologous antigen and then sonicated, reagglutinated. No detectable antibody was raised in rabbits inoculated with partially purified capsular polysaccharide. However, hens produced antibody detectable by gel precipitation to both capsule and endotoxin.

	ANTIGEN (Supernatant of broth culture)														
BEADS	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15
A1	+++	-	-	-	++	-	++	+	+	-	++	+++	+	+	-
T3	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-
T4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
A5	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
A8	-	+	-	-	+	-	+	+++	-	-	-	-	-	-	-
A9	++	-	-	-	-	-	++	+	+++	-	-	++	+	+	-
A11	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

Table VI

Latex beads sensitised with A1, T3, T4, A5, A8, A9, or A11 antisera agglutinated by broth culture of the 15 prototype strains.

4.34 Gel Precipitation. Table VII records which antisera gave gel precipitation lines when antisera to each serotype were tested against broth cultures of each of the 15 serotypes. However, Table VIII records the gel precipitation lines seen when A2 antisera, either untreated or absorbed by A6 and A8 cells, was tested against concentrated antigen preparations of each serotype.

The chemical nature of the antigens involved in gel precipitation was investigated by treating the antigen with phenol (Figure 11), periodate (Figure 12) and pronase (Figure 13); and also by ultracentrifugation of the antigen (Figure 14).

	Antigen (heated broth culture)														
Sera	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 VII

Gel precipitation using a heated broth culture of each of the 15 prototype strains of P.haemolytica as antigen and antisera to each of the 15 serotypes.

Note : Lines of precipitation were obtained with all homologous antigens and antibodies except A2. Reciprocal cross-reactions are seen with T3 and T15.

Sera	Antigen (heated plate washings)														
	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15
A2	+	+	-	-	+	+	+	+	+	-	+	+	+	+	-
Absorbed A2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Table VIII

Gel precipitaton of antisera to P.haemolytica serotype A2 which was either untreated or absorbed with A6 and A8 cells. The sera was tested using concentrated antigen preparations of each the 15 serotypes.

Note : A2 sera gave lines of precipitation with antigen from all of the "A" strains of P.haemolytica. These non type-specific lines did not occur once the serum had been absorbed with A6 and A8 cells. The homologous reaction still occurred with the absorbed serum.

Figure 11

Gel precipitation to P.haemolytica T3 (central well) against P.haemolytica T3

A standard antigen preparation

B concentrated antigen

C concentrated antigen extracted with phenol.

Note : The concentrated antigen, both untreated and extracted with phenol, shows a strong line of identity with the standard antigen preparation. An additional line is seen, close to the central (antibody) well, with the concentrated antigen. This s removed by phenol extraction and is probably due to a protein antigen. The phenol extract (C) shows an additional line of precipitation close to the antigen well. This is probably due to endotoxin.

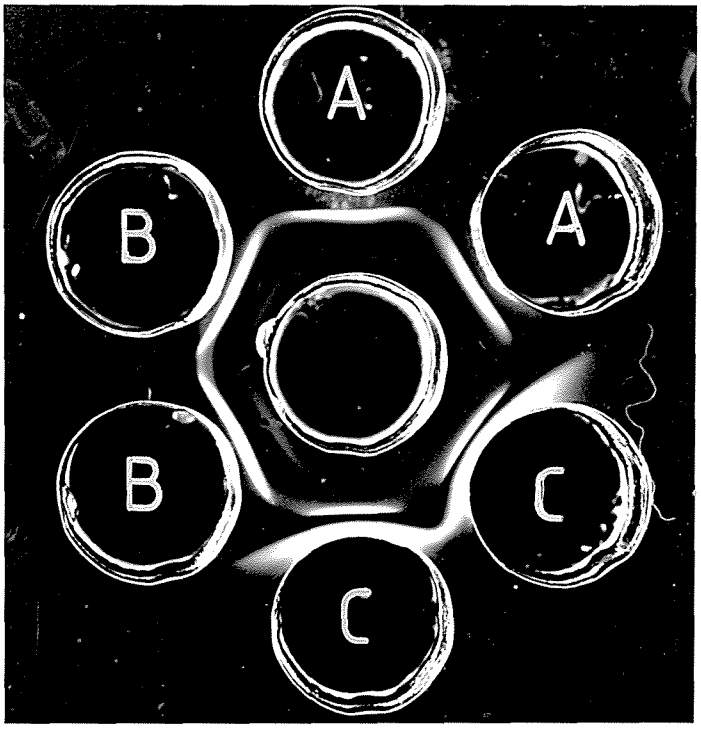

A
A
B
B
C
C

Figure 12

Gel precipitation of antibody to P.haemolytica T3 (centre) against a phenol extract of pf T3 concentrated antigen which was otherwise untreated (A) or periodate treated (B).

Note the preiodate treatment totally destroys the precipitation line close to the antigen well. The inner line still occurs but became slightly more diffuse when the antigen was treated with periodate.

Fig 12

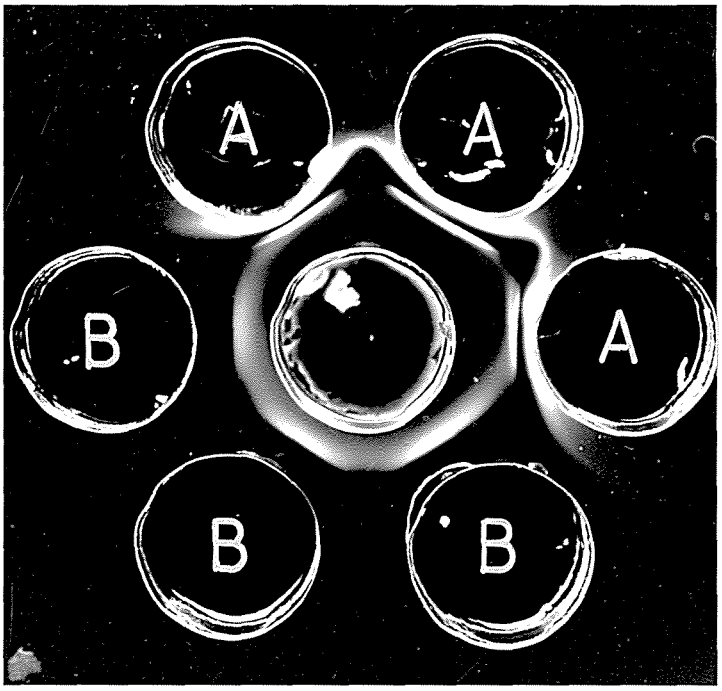


Figure 13.

Gel precipitation of antibody to P.haemolytica T3 (centre) tested against P.haemolytica T3 concentrated antigen which was otherwise untreated (A) or pronase treated (B).

Note : Pronase treatment destroyed the antigen which gave a line of precipitation close to the central (antibody) well. However, the other line is unaffected by pronase treatment.Thus, the inner line is probably due to a protein antigen. The outer line is probably due to capsular polysaccharide.

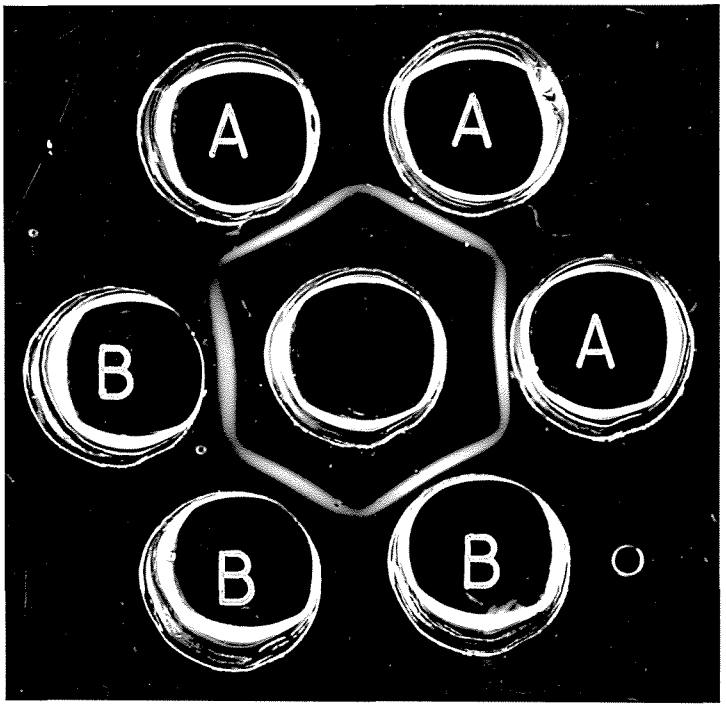
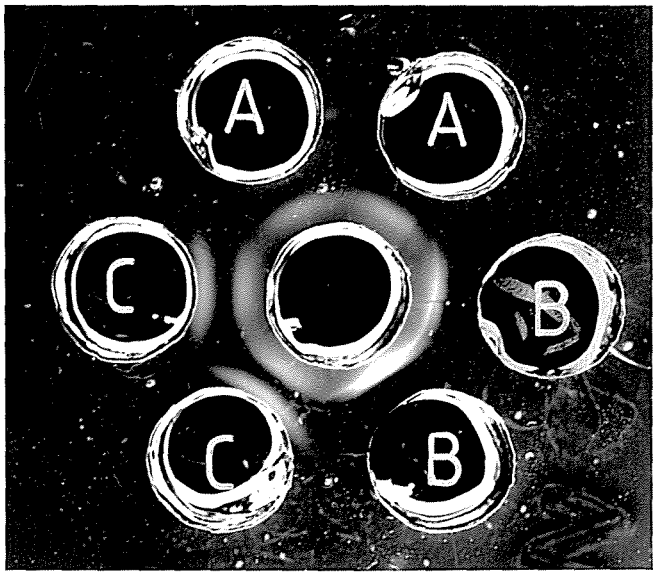

A
A
A
B
B
B

Figure 14

Gel precipitation of antibody to P.haemolytica T3 (centre) against P.haemolytica T3 standard antigen preparation (A) and concetrated antigen which was ultracentrifuged (Supernatant (B), sediment (C)).

Note : Ultracentrifugqation sediments an antigen which gives a line of precipitation close to the antigen well. This is probably endotoxin (see Disscussion).


A
A
C
B
C
B

4.35 CIE. Antigen-antibody systems were electrophoresed for varying periods. The results are shown in Figure 15.

Results of CIE of each of the 15 serotypes is shown in Table IX. Some of these results are illustrated in Figure 16 which shows CIE of antisera to P.haemolytica serotypes A1, A2, T3, T4, A5 against antigen to the 15 prototype strains.

CIE of antibody to P.haemolytica sero types A1,T4 and A5 against heated broth culture of P.haemolytica serotype A1 which had been treated as follows :
(i)No additional treatment (standard preparation),
(ii)Pronase treated,
(iii)Ultracentrifuged supernatant,
(iv)Ultracentrifuged deposit is seen in Figure 17.

	Antigen														
Antisera	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 IX

CIE of antigen to the 15 prototype strains of P.haemolytica against antisera prepared to each serotype.

- \- indicates no line of precipitation was seen.
- \+ indicates a line of precipitation.
- ++ indicates a strong line of precipitation.

We concluded CIE of P.haemolytica serotypes produced numerous lines of precipitation due to cross-reacting antigens.

Figure 15

Investigation of the optimum time required for CIE of P.haemolytica. The 7 slides are identical except for the for which they were electrophoresed. These were (top to bottom) 15, 30, 45, 60, 75, 105, 135 min. Each slide contains 6 antigens paired with homologous antibody. These are A8, A5, A1 (left to right, top row of each slide) and A9, A6, T3 (left to right, bottom row of each slide). The antibody is in the left well of each pair.

60min (fourth slide down) was judged to be the shortest period which gave an optimum precipitate for all 6 antigen-antibody systems.

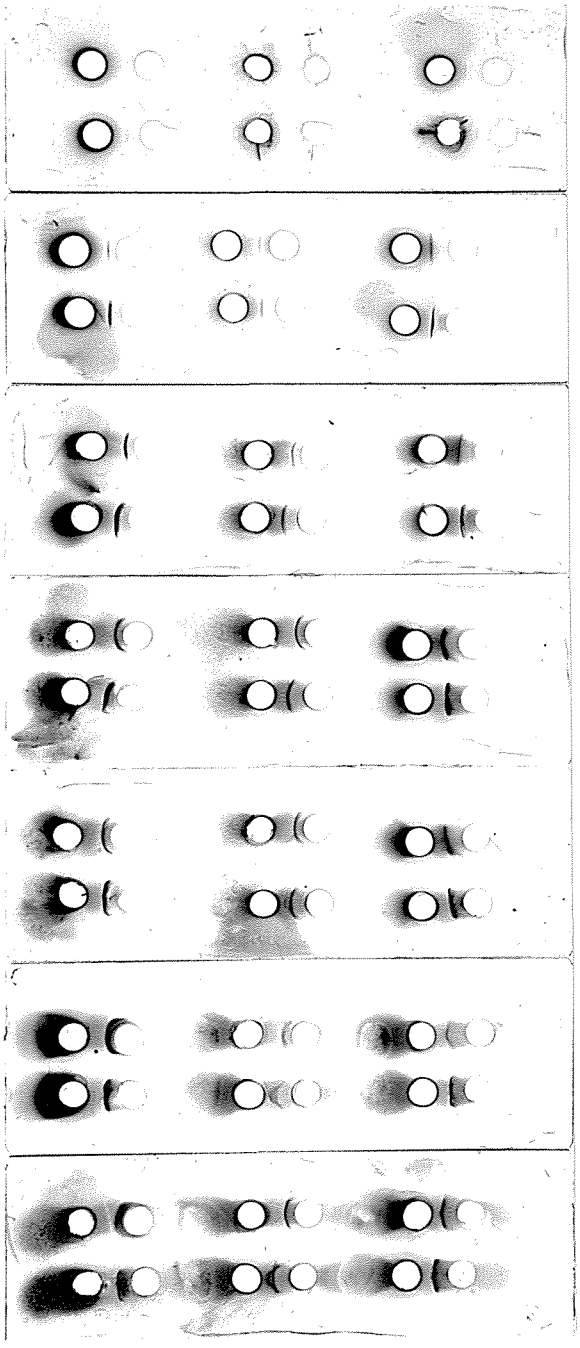


Figure 16

CIE of antisera to P.haemolytica serotypes A1, A2, T3, T4, and A5 against antigens of the 15 prototype strains.

A1 antibody shows a strong line of precipitate with the homologous antigen, and a barely visable line with A2 antigen. No other lines were detected. A2 antibody shows barely visable lines of precipitation wiht some antigens such as T4 and T10, but not wiht the homologous antigen. T3 antibody shows a precipitation line close to all antibody wells. An additional cross-reacting antigen line is seen with A2. a clear precipitation line is seen with homologous antigen and with the closely related T15 prototype strain. T4 antibody shows a strong line of precipitation with homologous antigen and weak lines with A6, A9, A11, A12 and A14 antigen. A5 antibody shows a strong line of precipitation with homologous antigen and weak lines with A2 and A14 antigen.

Data for the remaining 10 antisera is not illustrated, but is recorded in Table 8.

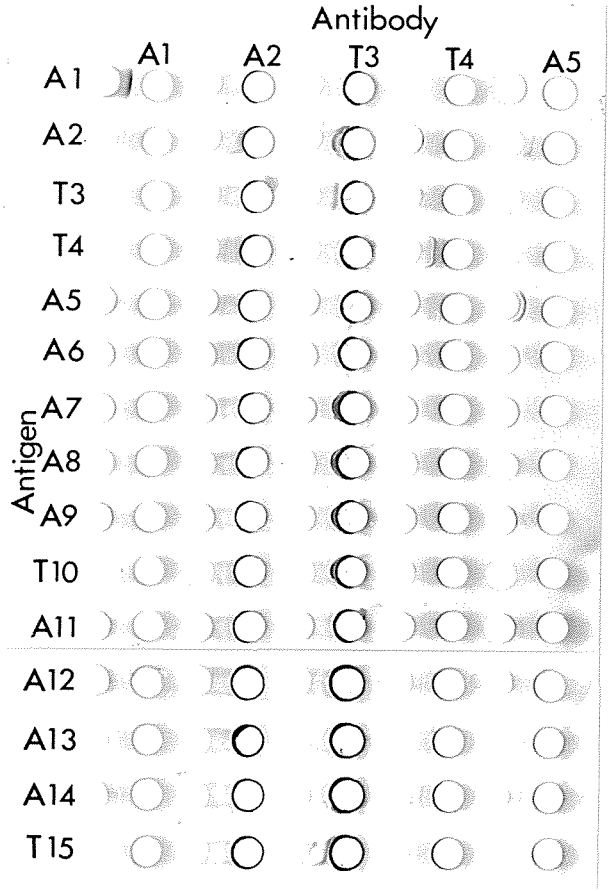

Antibody
A1
A2
T3
T4
A5
Antigen
A1
A2
T3
T4
A5
A6
A7
A8
A9
T10
A11
A12
A13
A14
T15

Figure 17

Investigation of the removal of cross-reacting antigens of P.haemolytica A1 following treatment of the antigen with :

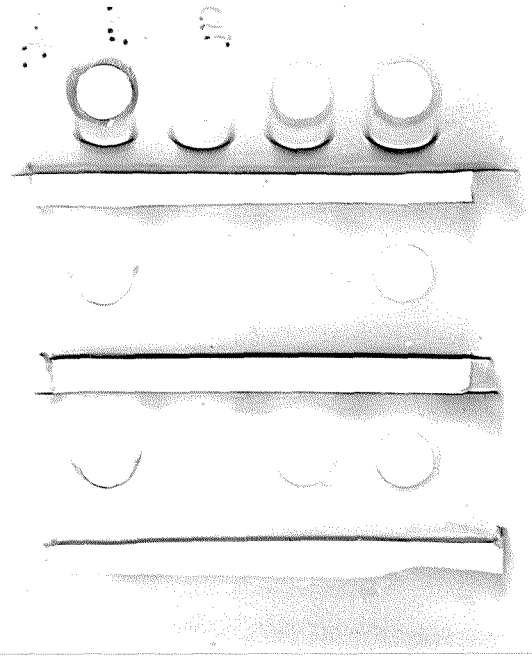
1 No treatment (Control, top row)

2 Pronase

3 Ultracentrifugation (supernatant)

4 Ultracentrifugation (sediment, bottom row).

The homologous reaction with all 4 antigen preparations is seen at the left. Weak cross-reactions are seen as precipitation lines close to many circular antigen wells, especially with the A5 antiserum. The cross-reaction, but not the homologous reaction, is totally removed when the antigen used is an ultracentrifuged supernatant (third row down). The cross-reacting line is increased when the sediment of an ultracentrifuged preparation is used (bottom row). Pronase treatment did not remove the cross-reating antigen (second row down). We conclude the cross-reaction is not due to a protein, but is due to endotoxin.



4.36 Bacterial Agglutination. Table X records which antisera agglutinated bacteria when antisera to each serotype was mixed with cells of each prototype strain.

		Antigen														
Sera		A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15
	A1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
absorbed	A2	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
	T3	-	-	++	-	-	-	-	-	-	-	-	-	-	-	++
	T4	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-
	A5	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	A6	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-
	A7	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
	A8	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-
	A9	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-
	T10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
1/32	A11	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-
	A12	-	-	-	-	-	-	++	-	-	-	-	++	-	-	-
1/5	A13	-	-	-	-	-	-	-	-	-	-	++	-	++	-	-
	A14	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
	T15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	=

Table X

Bacterial agglutination of the 15 prototype strains of P.haemolytica against antisera to all serotypes.

++ strong agglutination.

\+ agglutination.

\- no visable agglutination.

4.4 Discussion

Antisera Production

Serological typing of P.haemolytica isolates requires the production of antisera to the 15 serotypes, and this is usually achieved using rabbits (Biberstein et al, 1960). However, it has been noted that in practice serological testing is often limited because of the restricted availability of typing sera. (Biberstein and Kirkham, 1979). This is due to the frequent failure of rabbits to produce a significant titre of antibody following immunisation. The problem occurs to a greater or lesser extent with all serotypes (Liardet, personnel communication), however, it is a persistent problem with certain serotypes, especially A2. This information is not clearly stated in the literature and only became apparent to us some time after this work was undertaken. For this reason we did not attempt to undertake a detailed comparison of the relative abilities of rabbits, rats and domestic hens to produce typing antisera to P.haemolytica.

Initially 15 rabbits were inoculated with one of each of the 15 prototype strains of P.haemolytica. Only those rabbits inoculated with serotypes A1, A9, A14 and T15 responded by producing antisera of a titre suitable for serotyping studies. In the course of the present work, further rabbits were inoculated with these 4 serotypes and the rabbits usually produced antibody adequate for serotyping studies. Hence these serotypes are probably more antigenic in rabbits than the other 11. These 11 were inoculated into at least one more, and in some cases 3 more rabbits. Serotypes T4, A5 and A8 each produced antibody in at least one of these rabbits (Table IV).

However, the other 8 serotypes produced little, or no detectable antibody in any rabbit. We conclude that these 8 serotypes are of low antigenicity, at least in the rabbits available to us.

Another species of animal may however have the genetic potential to produce antibody to the capsular polysaccharides of all serotypes, and since rats were readily available in this laboratory, we inoculated 4 with serotypes A6, A7 and A12 which were non-antigenic in rabbits, and A9 which was comparatively highly antigenic in rabbits. Two rats were inoculated with each of the strains and no detectable antibody was produced. We conclude that capsular polysaccharide of P.haemolytica is even less antigenic in rats than in rabbits.

Corstvet et al., (1982) and Gentry et al., (1982) successfully used "chickens" for raising antibody to P.haemolytica, so with this in mind serotypes A2, T3, A6, A7, T10, A11, A12, A13 were each inoculated into hens (one hen per serotype). Antibody to the homologous type as determined by IHA and gel precipitation was produced with all serotypes except A2 and A7. Two more hens were inoculated with each of these serotypes and high titre antibody was produced. The highest response to types A2 and A7 is recorded in Table IV.

We conclude that P.haemolytica capsular polysaccharide is comparatively antigenic in hens. However, as will be discussed later in this section, when rabbits produced antibody it was relatively specific, whereas antibody produced in hens gave more cross-reactions.

IHA

IHA is generally accepted as the standard method by which serotypes of isolates of P.haemolytica are determined. However, IHA has disadvantages: It is laborious and requires the absorption of antigen from each isolate on to washed RBC's. Antigen coated RBC's are then added to serial dilutions of antisera to the 15 serotypes of P.haemolytica.

Another disadvantage is that in practice cross-reactions between different serotypes frequently occur. This means that titres of antisera to the 15 serotypes must be compared and it becomes necessary to set an arbitrary standard to interpret the test. The standard used in this thesis is that for an isolate to be "typed" the putative homologous antiserum must show at least a 4 fold or higher titre for the isolate over the other 14 antisera.

While investigating the specificity of antisera by IHA using the prototype strains it became apparent that the antisera could be divided into 3 main categories: the first group consisted of antisera to serotypes A1, T4, A5, A8, A9, A14 and T15. These showed very few cross-reactions and furthermore, any cross-reaction which did occur were of low titre in comparison to the homologous titre. The strongest cross-reaction within this group had a titre which was 128 fold less than the homologous reaction. All of the antisera which were prepared in rabbits, but none that were prepared in hens, fell within this group.

The second category of antisera, (prepared in hens to serotypes A6 and A7), showed cross-reactions with all serotypes,

although the cross-reactions usually occurred at a much lower titre than the homologous reaction. The strongest cross-reaction within this group had a titre which was 8 fold less than the homologous reaction.

The third category of antisera (prepared in hens to serotypes A2, T3, T10, A11, A12 and A13) showed cross-reactions with all serotypes of which at least one had a titre within 2 fold of the homologous titre. This precluded the use of these antisera from typing isolates by IHA.

It is interesting that the antisera prepared in rabbits showed fewer and weaker cross-reactions than antisera prepared in hens. One possible reason is suggested here. The capsular polysaccharide, which is generally accepted as the determinant of type-specificity, may have a determinant which is common to all of the serotypes but is not antigenic in rabbits. If this determinant was antigenic in hens, then avian antisera but not rabbit antisera would cross-react.

Latex Bead Agglutination

Antibody coated latex beads were agglutinated by homologous antigens, but unfortunately, many cross-reactions also occurred. The cross-reactions were not due to proteins since since treatment of the antigen by proteolytic enzymes did not remove them. Furthermore, the cross-reactions occurred with phenol extracts which implied that polysaccharide antigens were involved. This was confirmed by the removal of both heterologous and (in most cases) the homologous reaction when the antigen was treated with periodate. The cross-reacting serotypes correlated to a considerable extent with the "O"

antigen types of P.haemolytica. We conclude that the antibody-coated beads detected mainly the endotoxins common to the various serotypes. This is perhaps not surprising since whole antigens were used for immunisation.

Attempts to produce "pure" anticapsular antisera using polysaccharide partially freed from endotoxin by ultracentrifugation failed, since an antibody response to both capsular polysaccharide and endotoxin was elicited. Other attempts to eliminate heterologous reactions were persued (see Results), but as these were unsuccessful they are not discussed.

The use of antibody-coated beads was not further persued in this present work, although it may ultimately be the most satisfactory approach for serotyping isolates. It requires however, either that the capsular polysaccharide be totally freed from endotoxin or the use of monoclonal antibodies to make the test specific.

Gel Precipitation

The antigen used in this test was prepared by heating broth cultures of each of the 15 serotypes. This gave lines of precipitation with all homologous types except for serotype A2 (Table VII). No cross-reactions were seen except a reciprocal reaction between T3 and T15 which are closely related organisms (Fraser et al.,1982).

When concentrated antigens were tested with A2 antisera, lines of precipitation were seen with all of the "A" types. The cross-reacting antibody was removed by adsorbing the A2 antisera with A6 and A8 cells . This left the homologous line (Table

VIII).

Thus, to establish the serotype of an isolate by gel precipitation it is necessary to use unconcentrated antigen in the case of all serotypes except A2. This avoided cross-reactions. Whereas, if the serotype was A2, it could only be typed by gel precipitation if a concentrated antigen were used. This point is returned to and discussed in relation to the results obtained with agglutination tests which allow a rapid, although not 100% reliable, identification of A2 isolates.

Chemical nature of gel precipitating antigens. Since the type-specific antigen of P.haemolytica is probably the capsular polysaccharide (Cameron, 1966), it follows that the antigen giving a type-specific line by gel precipitation is probably the capsular polysaccharide. However, the production of 2 gel precipitation lines when a concentrated antigen was used (Figure 11) led us to obtain further evidence as to the chemical nature of the antigens involved in gel precipitation.

The "extra line" seen when a concentrated antigen is tested by gel precipitation, was destroyed when the antigen was treated with pronase and so, is probably a protein (Figure 13).

Phenol treatment destroyed one cross-reacting antigen and solubilised another which gave a line of precipitation close to the antigen well (Figure 11). Endotoxin is extracted by hot phenol. To test if this antigen was endotoxin, it was treated with periodate which cleaves the bond between adjacent carbons with hydroxyl groups. Thus, most polysaccharides, including endotoxin, are destroyed. This "third" antigen was destroyed by

periodate (Figure 12). When ultracentrifuged the antigen was sedimented (Figure 14).i.e. it separated from the supernatant and could be detected in increased concentrations in the sediment. Ultracentrifugation is known to sediment endotoxin (Sutherland and Wilkinson, 1971), so we conlude that the antigen giving a line close to the antigen well is endotoxin.

Periodate treatment did not destroy the type-specific antigen (Figure 12). However, the precipitation line became more diffuse and closer to the antigen well.

Counter Immunoelectrophorisis

Optimum running time. 6 antigen-antibody systems of P.haemolytica were electrophoresed for varying periods up to 135min (Figure 15). 60min was judged to be the shortest period showing the strongest lines. Subseqently, 60min was used as the standard running time for CIE.

CIE of prototype strains. CIE of prototype strains of P.haemolytica showed lines of precipitation with all homologous antigens and antibodies except serotypes A2, A7 and T10 (Table IX). A lack of a line for these serotypes is surprising since lines occurred by gel precipitation and CIE is usually a more sensitive test than gel precipitation. The observed absence of a precipitate was not investigated.

Many lines of precipitation which represented cross-reactions between serotypes were also seen in the CIE test. The antigen of serotype A1 which cross-reacted with antisera to A5 was investigated (Figure 17). Pronase did not destroy this antigen, so it probably is not a protein.

Ultracentrifugation sedimented the cross-reacting antigen, so we conclude the antigen is probably endotoxin.

The absence of a homologous line in some cases and the presence of many lines representing cross-reactions preclude CIE from being used to serotype isolates. Thus, CIE was not further investigated.

Serotyping Prototype Strains by Agglutination

Agglutination yielded type-specific results for antisera to types A1, T4, A5, A6, A7, A8, A9, T10, A11 and A14. However, it should be noted that A11 antisera was diluted 1/32 to dilute out cross-reacting antibody. Antiserum to serotype T15 was the only antiserum which failed to agglutinate homologous cells.

Antisera to A2, T3, A12 and A13 each agglutinated the homologous serotype and one other. Antisera to A2 which had been adsorbed with A6 and A8 cells still agglutinated A7 cells. T3 antiserum cross-reacted with T15 cells which is not surprising since they are closely related. A12 antiserum cross-reacted with A7 cells which was the strongest cross-reaction noted by Frank and Wessman, (1978).

A13 antiserum was diluted 1/5 eliminating many cross-reactions, however the diluted antiserum still reacted with A1 cells. Diluting the antiserum further removed the homologous reaction.

Since cross-reactions were "one-way" only, it is possible to distinguish the 15 prototypes by agglutination. The main advantage of the test is that it is simple and rapid and

furthermore colonies can be tested directly from the agar plate used for isolation. Plates may even be stored for several weeks at 4°C without interfering with results. However, agglutination results need to be confirmed using another technique because of cross-reactions. The use of this approach for establishing the serotype of fresh isolates is considered in Chapter 6.

CHAPTER 5

Comparison of Techniques using P.haemolytica Isolated from Ovine Lung

5.1 Introduction

The specificity and relative convenience of IHA, gel precipitation, and bacterial agglutination were compared in Chapter 4 using the 15 prototype strains of P.haemolytica. All 3 techniques could identify the serotype of most prototype strains. However, to compare the techniques for use in field studies, many fresh isolates should be typed using all 3 techniques. This section therefore compares IHA, gel precipitation, and agglutination using 40 isolates of P.haemolytica obtained from the lungs of 60 sheep with CNP. These sheep were from 20 different farms (see Chapter 2).

The results of this section also represent a survey of the prevalence of serotypes of P.haemolytica recovered from the lungs of sheep with CNP.

5.2 Materials and Methods

The preparation of antisera and its use in IHA, gel precipitation and agglutination is descibed in Chapter 4. The recovery of isolates is described in Chapter 2. They represent 40 isolates obtained from the lungs of sheep with CNP. The sheep came from 20 different farms.

5.3 Results

The serotypes of isolates, derived from the lungs of sheep with CNP, were investigated by IHA. Antisera titres for all 15 serotypes of P.haemolytica are recorded for each isolate in Table XI. The serotype of each isolate, as inferred from IHA titres, is also shown.

These isolates were also investigated by gel precipitation and agglutination. Table XII records the results of both of these tests and the IHA conclusions.

Table XIII presents the results from this section as the prevalence of isolates from each serotype which were isolated from the lungs of sheep with CNP.

Farm	Lung	IHA titre of antibody to serotype indicated															Conclusi TYPE
		A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	
1	B	<2	1024	4	<2	<2	2	4	<2	<2	2	2	4	8	<2	<2	A2
1	C	<2	1024	4	<2	<2	4	8	<2	<2	4	2	4	4	<2	<2	A2
2	A	<2	512	8	<2	2	8	16	2	2	4	2	8	16	<2	<2	A2
2	B	128	16	32	<2	<2	32	32	<2	<2	16	<2	8	8	<2	<2	A1
2	C	64	8	16	<2	<2	8	16	2	<2	16	<2	16	8	<2	<2	A1
3	B	128	16	<2	8	<2	16	16	2	<2	2	<2	16	16	<2	<2	A1
3	C	256	32	16	<2	<2	32	32	2	2	16	2	16	16	<2	<2	A1
4	A	256	8	16	<2	2	32	32	2	<2	16	<2	16	16	<2	<2	A1
4	B	<2	8	8	8	<2	2	128	<2	<2	<2	8	16	16	<2	<2	A7
4	C	<2	256	<2	4	<2	4	8	<2	<2	<2	<2	4	4	<2	<2	A2
5	C	<2	8	<2	8	<2	2	128	<2	2	<2	8	16	16	<2	2	A7
6	A	<2	16	16	2	2	4	256	2	<2	4	<2	16	8	<2	<2	A7
6	C	<2	256	2	4	<2	4	4	2	<2	<2	2	2	<2	<2	<2	A2
7	A	<2	256	<2	4	<2	2	2	<2	<2	<2	<2	4	4	<2	<2	A2
7	B	2048	64	64	4	<2	32	64	<2	4	32	4	32	32	2	4	A1
8	A	<2	256	2	<2	<2	4	4	<2	<2	4	2	4	8	<2	<2	A2
8	B	<2	64	2	<2	<2	<2	2	<2	<2	2	4	2	4	4	<2	A2
8	C	<2	256	4	<2	<2	4	4	<2	<2	4	2	4	8	<2	<2	A2
9	A	<2	256	<2	4	<2	8	8	<2	<2	<2	<2	4	4	<2	<2	A2
9	B	2	512	8	<2	2	4	16	2	2	4	2	8	16	<2	<2	A2
9	C	<2	128	4	<2	<2	8	8	<2	<2	8	2	4	4	<2	<2	A2
10	C	512	16	<2	16	<2	16	16	<2	<2	4	2	16	16	2	2	A1
11	B	<2	128	4	<2	<2	4	4	<2	<2	4	2	4	4	<2	<2	A2
11	C	<2	128	2	<2	<2	4	8	<2	<2	4	2	4	8	2	<2	A2
12	A	<2	512	<2	4	<2	4	8	<2	<2	<2	<2	2	2	<2	<2	A2
12	B	8	8	4	8	4	128	16	<2	2	<2	<2	4	4	<2	<2	A6
13	A	<2	512	<2	8	<2	8	8	<2	4	<2	4	8	8	<2	<2	A2
13	B	<2	256	<2	4	<2	2	4	<2	<2	<2	2	4	4	<2	<2	A2
14	A	<2	256	2	<2	<2	4	4	<2	<2	4	2	4	8	<2	<2	A2
14	C	4	8	8	16	4	256	16	2	<2	<2	<2	8	8	<2	<2	A6
15	C	256	16	16	<2	<2	16	16	2	<2	16	<2	16	16	<2	<2	A1
16	A	<2	4	<2	4	<2	<2	2	<2	512	<2	128	2	<2	<2	<2	A9
16	B	2048	32	64	2	4	64	128	4	16	32	16	32	32	4	4	A1
17	B	2	16	2	8	2	512	16	<2	<2	<2	<2	8	8	<2	<2	A6
17	C	4	8	8	<2	4	128	16	2	<2	4	<2	16	16	<2	<2	A6
18	A	<2	512	4	<2	2	4	8	<2	<2	4	2	4	8	<2	<2	A2
18	B	<2	16	8	<2	2	8	64	512	<2	4	<2	4	8	<2	<2	A8
19	A	<2	256	8	<2	<2	8	16	<2	<2	8	2	4	16	<2	<2	A2
19	C	4	512	8	32	4	16	256	4	2	16	4	32	32	4	4	A2/A7
20	B	<2	256	<2	8	<2	8	32	<2	4	<2	8	8	8	<2	<2	A2
Homolog. prototype		2048	1024	128	> 4096	128	512	512	256	256	256	1024	512	1024	2048	> 4096	

Table XI

40 isolates of P.haemolytica (from ovine lungs with CNP lesions obtained from 60 sheep from 20 different farms) were tested by IHA using antisera to each of the 15 serotypes. The isolates reacted with many antisera but (except in one case viz 19C) showed at least a 4 fold preference for one serum over the others. This was regarded as the homlogous serum and the inferred serotype is recorded on the right.

FARM LUNG	+ve sera by: IHA	Gel ppt.	Aggl
1 A	N		
1 B	A2	A2	A2
1 C	A2	A2	A2
2 A	A2	A2	A2
2 B	A1	A1	A1
2 C	A1	A1	A1
3 A	N		
3 B	A1	A1	A1
3 C	A1	A1	A1
4 A	A1	A1	A1
4 B	A7	A7	A7
4 C	A2	A2	A2
5 A	N		
5 B	N		
5 C	A7	A7	A7
6 A	A7	A7	A7
6 B	N		
6 C	A2	A2	A2
7 A	A2	A2	A2
7 B	A1	A1	A1
7 C	N		
8 A	A2	A2	A2
8 B	A2	A2	A2
8 C	A2	A2	A2
9 A	A2	A2	A2
9 B	A2	A2	A2
9 C	A2	A2	A2
10 A	N		
10 B	N		
10 C	A1	A1	A1
11 A	N		
11 B	A2	A2	A2
11 C	A2	A2	A2
12 A	A2	A2	A2
12 B	A1	A1	A1
12 C	N		
13 A	A2	A2	A2
13 B	A2	A2	A2
13 C	N		
14 A	A2	A2	A2
14 B	N		
14 C	A6	A6	A6
15 A	N		
15 B	N		
15 C	A1	A1	A1
16 A	A9	A9	A9
16 B	A1	A1	A1
16 C	N		
17 A	N		
17 B	A6	A6	A6
17 C	A6	A6	A6
18 A	A2	A2	A2
18 B	A8	A8	A8
18 C	N		
19 A	A2	A2	A2
19 B	N		
19 C	A2/7	A2	A2
20 A	N		
20 B	A2	A2	A2
20 C	N		

Table XII

The serotype of 40 isolates of P.haemolytica (obtained from sheep from 20 different farms) by IHA, gel precipitation and agglutination. Only the positive antisera are recorded. NOTE: IHA, gel precipitation gave comparable results for all isolates except 19C.

N = P.haemolytica not isolated

Serotype	No. ISOLATED FROM LUNGS	% PREVALENCE
A1	10	25
A2	22	55
A6	3	7.5
A7	3	7.5
A8	1	2.5
A9	1	2.5
TOTAL	40	100

Table XIII

Prevalence of serotypes of P.haemolytica isolated from ovine lungs with CNP lesions. Origin of isolates: 3 lungs with CNP lesions were collected from each of 20 farms in the Manawatu region. 40 isolates were recovered with at least one isolate originating from each farm.

5.4 Discussion

For an isolate to be typed by IHA we arbitrarily chose that one antiserum should show at least a 4-fold preference for the antigen coated RBC's over the other 14 antisera (Chapter 4). By this criterion 39 of the 40 isolates of P.haemolytica, obtained from the lungs of sheep with CNP, were allocated to a serotype (Table X). However, one isolate, viz 19C, showed only a 2-fold preference for type A2 antiserum. By the criterion above, the serotype of this isolate was not determined, but it might be expected to be an A2 isolate. All 40 isolates tested by gel precipitation with antiserum to the 15 serotypes showed a line of identity with only one antigen-antibody system (Table XII). This test confirmed that isolate 19C is an A2 serotype.

Similar results were obtained with agglutination tests. i.e. All 40 isolates agglutinated with one (and only one) antiserum. The inferred serotype was the same as determined by IHA and gel precipitation tests for each isolate. Thus, isolates were allocated into the same serotypes when tested by

IHA, or gel precipitation, or agglutination.

If all tests give the same results then it is logical to use the most convenient test or tests to investigate the serotype of field isolates. Agglutination tests are convenient, rapid and they worked with isolates from the lungs of sheep with CNP. The next chapter investigates if this conclusion (and the typing scheme outlined in Section 4.4) can be extrapolated to nasal isolates.

The results from this section are also a survey of the prevalence of P.haemolytica serotypes associated with CNP in sheep (Table XIII). This is discussed in Chapter 7.

CHAPTER 6

Serotyping Nasal Isolates of P.haemolytica

6.1 Introduction

IHA, gel precipitation and agglutination have been compared for their ability to distinguish the serotype of both prototype strains of P.haemolytica (Chapter 4) and isolates from the lungs of sheep with CNP (Chapter 5). This led us to suggest that a convenient approach would be to serotype isolates by agglutination and confirm the result by gel precipitation using only the homologous antiserum. This was satisfactory when lung isolates were involved.

However, this present section assesses this approach using isolates obtained from the nasopharynx of healthy sheep and as will be seen, difficulties arose with a significant proportion of these isolates. Thus, although 70 of 110 isolates were "serotyped," the remaining 40 isolates gave either negative or contradictory results with the test. Consequently, these isolates were further investigated.

The results obtained in this section also represent a survey of the prevalence of serotypes of P.haemolytica recovered from the nasopharynx of healthy sheep.

6.2 Materials and Methods

Isolates of P.haemolytica obtained from the nasopharynx of healthy sheep from 4 different farms (See Chapter 2) were tested by bacterial agglutination using antisera to P.haemolytica serotypes in the following order: A2, A7, A1, A6, A8, A9, A11, A13, A12, A14, A5, T3(T15), T4, T10. Once an isolate agglutinated with an antiserum, thus provisionally identifying the serotype, no further agglutination tests were performed with the isolate. However, confirmation of the serotype of the isolate was sought by a gel precipitation test using only the putative homologous antiserum. Isolates showing a line of identity were regarded as being unequivocally identified with regard to their serotype and were not examined further.

40 isolates that gave gel precipitation results that disagreed with agglutination results and isolates that were negative in both tests were tested by IHA, and were further examined by gel precipitation and agglutination using antisera to all 15 serotypes.Of these 40 isolates 18 were determined to be serotype A2 by IHA. Highly concentrated antigens of these 18 A2's were prepared as with the concentrated preparation (See Section 4.23) except that cells were removed with 1ml PBS only. The highly concentrated preparations were tested against antisera to prototype A2 by gel precipitation using the standard precipitin agar and also 0.5% agar.

6 of these 18 A2 isolates (2 randomly selected from each of 3 farms) viz 84B, 61B, 3L, 39L, 4R and 24R, were inoculated into hens to produce immune sera as descibed in Section 4.21. Isolates 3L and 24R were also inoculated into rabbits as descibed in Section 4.21 to produce antisera. The antisera

raised was tested by gel precipitation against concentrated antigen from each of the 6 isolates and also against concentrated A2 antigen. The 40 isolates were tested by gel precipitation with rabbit antisera to isolate 3L.

6.3 Results

110 isolates were provisionally serotyped by agglutination and the results confirmed by gel precipitation. The results are recorded in Table XIV which shows of the 110 tested, 70 gave agglutination results which were confirmed by gel precipitation with the (provisionally identified) homologous antiserum.

The remaining 40 isolates were retested by agglutination and gel precipitation, but for this retesting all 15 antisera were used. These isolates were also examined by IHA. Gel precipitin tests were negative in all cases; in some cases agglutination tests were positive with one antiserum only (22 of 40), in others all 15 failed to agglutinate (10 of 40), and in the remaining (8 of 40) the agglutination test was positive for more than one serotype. This is recorded in Table XV which also records the IHA results in detail. 18 of the 40 isolates showed a 4 fold or greater preference for A2 antiserum over all other antisera. These isolates did not give a gel precipitation line with antiserum to prototype A2 despite using highly concentrated antigen and/or weak agar (0.5%) to facilitate diffusion. 14 of the 40 isolates did not show a 4 fold preference for any one serum over the rest, and 8 of the 40 isolates were untypable by IHA since RBC's absorbed with their antigen autoagglutinated.

6 randomly selected isolates of the 18 were inoculated into hens, and 2 of the 6 were also inoculated into rabbits to

produce antisera. The resulting sera were tested by gel precipitation against antigen from each of the 6 isolates and also with A2 antigen (Table XVI). 3 of the 40 isolates (viz 39L, 4R, 27L) gave a line of identity with rabbit antiserum to isolate 3L and 3L antigen.

The numbers of isolates of each serotype are recorded in Table XVII.

FLOCK A		
Isolate	Aggl	Gppt
1	A7	A7
3	A7	-
4*	A1	A1
5a	A8	A8
5b	A7	A7
6	A11	A11
7	A2	-
9	A2	-
10*	A2	-
11*	A1	A1
12	A7	-
13	A2	A2
14	A7	-
15a*	A7	A7
15b	A6	A6
17	A2	A2
18	A7	-
23*	A2	-
26*	A8	-
27	A2	A2
29	A8	A8
31*	A6	A6
35	A7	-
38	A2	A2
41*	A2	-
42	A8	A8
43	A2	A2
45	A7	A7

FLOCK B		
Isolate	Aggl	Gppt
47*	A7	A7
48	A8	A8
49	A7	A7
50	A11	A11
57*	A2	A2
59*	A1	A1
60*	A2	A2
61*	A2	-
62	A1	A1
64*	A7	A7
65*	A1	A1
66*	A2	A2
67*	A9	A9
69	A2	A2
70*	A2	A2
72	A2	A2
73*	A2	A2
74	A6	A6
75	A11	A11
80*	A7	A7
81	A2	A2
82*	A8	A8
83*	A2	A2
84	A2	-

FLOCK L		
Isolate	Aggl	Gppt
1a	A7	-
1b	A11	A11
2	A6	A6
3	-	-
5	A8	-
6*	A13	-
7	A2	A2
8*	A11	-
9*	A2	-
10*	A7	-
11	A2	A2
13	A2	A2
14	A6	A6
16	A6	A6
17	A6	A6
18*	A11	A11
19*	A1	A1
22	A13	-
24	-	-
26	A11	A11
27	A12	-
28	A11	A11
29	A2	-
30	-	-
31*	A11	A11
32	T3	-
33a	T3	-
33b	A1	A1
34	-	-
37	A2	A2
38	A1	A1
39	-	-
41	A6	A6

FLOCK R		
Isolate	Aggl	Gppt
1	A2	A2
2	A11	A11
3	A7	A7
4	A7	-
5	A1	A1
6*	A11	A11
9*	A13	A13
10	A1	A1
11*	A7	-
12	A2	A5
13a	A2	A2
13b	-	-
15	A7	-
16*	A11	A11
18*	-	-
19	-	-
22	A11	A11
23*	A2	A2
24*	T3	-
25*	A7	-
28*	-	-
29*	A6	A6
41	A2	A2
42	A9	-

Table XIV

The serotype of isolates of P.haemolytica, obtained from the nasopharynx of sheep from 4 different flocks (A, B, L, R) determined initially by agglutination and followed by gel precipitation with the antiserum with which it agglutinated. Note: The serotype as determined by agglutination and gel precipitation concur with 70 of the 110 isolates.

* In these cases 2 separate colonies were subcultured from the original agar plate used for isolation and these two cultures showed identical features, whereas isolates designated "a" or "b" were from the same sample but did not show identical features.

Isolate	Agglut	Gppt	IHA TITRES														IHA TYPE
			A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	
3 A	A7	-	2	512	4	<2	<2	2	64	<2	2	4	256	4	16	2	2	-
7 A	A2,A11	-	Autoagglutinated															-
9 A	A2	-	<2	>4096	2	<2	<2	2	16	<2	2	4	64	4	16	<2	<2	A2
10 A	A2	-	<2	512	4	4	2	4	16	<2	4	4	128	4	16	2	2	A2
12 A	A7	-	<2	512	4	<2	<2	4	16	<2	2	4	512	8	64	2	2	-
14 A	A7	-	<2	512	16	4	2	8	32	<2	4	8	512	8	128	2	2	-
18 A	A7	-	<2	512	4	<2	<2	2	16	<2	4	4	256	2	64	<2	<2	-
23 A	A2,A11	-	8	1024	64	4	<2	16	512	128	8	16	512	8	512	8	2	-
26 A	A8	-	<2	512	4	<2	<2	2	32	<2	8	8	256	8	32	<2	<2	-
35 A	A7	-	<2	512	32	2	<2	16	64	2	2	16	128	8	64	2	2	A2
41 A	A2	-	<2	1024	8	2	2	8	32	2	4	8	64	2	16	2	<2	A2
61 B	A2,A6	-	<2	>4096	4	<2	<2	2	16	<2	4	4	256	8	32	2	2	A2
84 B	A2	-	<2	>4096	2	<2	<2	2	8	<2	2	4	256	8	64	4	2	A2
1 L	A7	-	<2	2048	8	<2	<2	<2	<2	<2	64	2	<2	<2	<2	<2	<2	A2
3 L	-	-	<2	>4096	8	<2	<2	<2	8	<2	<2	4	8	4	4	32	<2	A2
5 L	A8	-	2	32	16	<2	4	16	32	64	<2	16	4	16	32	2	4	-
6 L	A13	-	<2	1024	4	2	2	4	16	<2	4	4	128	4	4	2	2	A2
8 L	A11/12	-	Autoagglutinated															-
9 L	A2	-	<2	128	4	<2	<2	<2	16	<2	2	4	128	4	32	2	2	-
10 L	A7	-	Autoagglutinated															-
22 L	A13	-	Autoagglutinated															-
24 L	-	-	<2	512	2	<2	<2	<2	128	128	8	8	512	4	32	<2	<2	-
27 L	A12/13	-	<2	128	4	<2	<2	2	8	<2	<2	4	<2	8	8	8	<2	A2
29 L	A2	-	<2	512	8	<2	2	8	32	2	<2	16	8	16	32	2	8	A2
30 L	-	-	2	1024	8	2	<2	2	8	2	2	16	256	4	8	<2	<2	A2
32 L	T3	-	<2	>4096	8	16	<2	8	64	16	8	16	256	16	32	32	8	A2
33 L	T3	-	32	>4096	4	2	2	2	16	2	4	4	256	2	64	32	32	A2
34 L	-	-	Autoagglutinated															-
39 L	-	-	<2	>4096	2	<2	<2	2	4	<2	<2	4	4	<2	2	16	<2	A2
4 R	7,11,13	-	<2	>4096	8	<2	<2	8	32	<2	8	8	256	8	32	4	2	A2
11 R	A6,A7	-	<2	256	64	<2	2	8	512	<2	<2	32	2	32	512	4	8	-
13 R	-	-	<2	32	16	<2	<2	8	16	<2	<2	2	16	2	<2	<2	<2	-
15 R	A7	-	Autoagglutinated															-
18 R	-	-	Autoagglutinated															-
19 R	-	-	<2	256	8	<2	<2	<2	16	<2	<2	8	512	4	32	<2	<2	-
24 R	T3	-	8	>4096	8	16	16	16	32	2	2	8	128	4	32	<2	<2	A2
25 R	7,10 11	-	Autoagglutinated															-
28aR	-	-	<2	256	2	<2	<2	4	64	<2	2	4	256	4	64	2	2	-
28bR	-	-	<2	1024	2	2	2	4	16	<2	4	8	128	4	16	2	2	A2
42 R	A9	-	4	64	32	4	16	8	32	4	2	8	8	32	64	8	8	-

Table XV

40 isolates of P.haemolytica (obtained from the nasopharynx of sheep) which did not show lines of identity when tested by gel precipitation with antisera that agglutinated the cells, retested by agglutination, gel precipitation and IHA with antisera to the 15 serotypes. Only antisera that gave positive results by agglutination and gel precipitation with each isolate are indicated. IHA antibody titres for the 15 serotypes are recorded for each isolate. The serotype of the isolate inferred from the IHA titres is on the right.

SERA		ANTIGEN						Prototype
Rab	Hen	3L	39L	4R	84B	24R	61B	A2
3L		+	+	+	-	-	-	-
	84B	+	+	+	+	+	-	-
	61B	-	-	-	-	-	+	-
	24R	+	+	+	+	+	+	-

Table XVI

Antisera to 4 isolates which were negative by gel precipitation with antisera to the prototype strains, tested by gel precipitation against antigen from 6 strains and prototype A2 antigen.

+ a single line of precipitation

	No. of Isolates from Farm:					
Serotype	A	B	L	R	Total	(%)
A1	1	3	3	2	9	(8)
A2	9	11	11	7	38	(35)
A5	0	0	0	1	1	(1)
A6	2	1	5	1	9	(8)
A7	4	4	0	1	9	(8)
A8	3	2	1	0	6	(5)
A9	0	1	0	0	1	(1)
A11	2	2	5	4	13	(12)
A13	0	0	0	1	1	(1)
untyped	7	0	8	8	23	(21)
TOTAL	28	24	33	25	110	(100)

Table XVII

Prevalence of serotypes of P.haemolytica isolated from the nasopharynx of sheep from 4 different flocks.

	Prevalence	
Serotype	Lung	Nose
A1	25	8
A2	55	35
A5	-	1
A6	7.5	8
A7	7.5	8
A8	2.5	5
A9	2.5	1
A11	-	12
A13	-	1
untyped	-	21

Table XVIII

Comparison of the percentage prevalence of serotypes isolated from ovine lungs with CNP lesions and the prevalence of serotypes isolated from the nasopharynx of healthy sheep.

6.4 Discussion

Of the 110 isolates obtained from the nasopharynx of healthy sheep, 70 which were provisionally allocated into serotypes by agglutination had their serotype confirmed by gel precipitation using the putative homologous antiserum (Table XIV). This result was unexpected because all 40 isolates of P.haemolytica recovered from the lungs of sheep with CNP could be positively identified by agglutination and gel precipitation.

The 40 untyped nasal isolates were examined by IHA and 18 were serotyped as A2, whereas the remaining 22 were "untypable". This raised the question as to why the 18 A2 strains failed to give a precipitation line with the prototype A2 antisera. Two hypotheses were considered: The concentration of antigen involved was too low or the antigen failed to diffuse through the agar. However, increasing the antigen concentration and decreasing the agar concentration (to facilitate diffusion) were unsuccessful in producing a line.

To attempt to cast further light on the negative gel precipitation results, several of the 18 A2 isolates were used to immunise hens and rabbits. Antibody to isolate 3L was found to give a precipitation line with the homologous antigen and with 3 others of the 40 isolates. This antiserum failed to give a gel precipitation line of identity with the prototype A2 antigen, despite cross-reacting with it in the IHA test. The A2 isolates (as defined by the IHA test) derived from the nasopharynx of sheep could therefore be divided into 3 groups (a, b, and c):

Desciption	Number	Determinants
A2 by IHA and gel precipitation	21	I,IIa
A2 by IHA (but not by gel precipitation)		
-line with antisera to 3L	4	I,IIb
-no gel precipitin line with any	14	I, ?

Table XIX

The 3 groups of A2 isolates obtained from the nasopharynx

We propose that the A2 antigen is heterogenous, consisting of 2 determinants. Determinant I which is functional in the IHA test only and all A2 isolates have this. Determinant II which gives a line by gel precipitation and exists as one of several possible allelles which do not cross-react. For example, the prototype strain has allelle IIa and isolate 3L has allelle IIb. Further studies may reveal a series of allelles of this nature within the A2 serotype.

The interpretation of results from this section as a survey of the prevalence of serotypes in the nasopharynx healthy sheep will be discussed in Chapter 7.

CHAPTER 7

General Discussion

The present investigation is concerned with serotyping P.haemolytica and the use of this procedure to establish the prevalence of serotypes in New Zealand. This required a standard approach for the isolation and identification of P.haemolytica from sheep. Other investigators invariably isolate the organism using blood agar and in practice identification is based on colonial morphology and the presence of haemolysis. The present work took this approach for both the isolation and provisional identification of P.haemolytica.

In contrast to studies of bacteria which infect the human population, P.haemolytica has not been intensely investigated. Most work has been carried out by Biberstein (1978), whose description of the species is summerised as follows: gram negative rods which are oxidase positive; grow in TSI agar producing acidity throughout without any gas production; grow consistantly but sparsely on MacConkey agar; and are urease negative. All the lung isolates provisionally identified as P.haemolytica conformed to the above criteria. Further proof of the identity of the isolates was gained by serotyping since all of the isolates were identified as belonging to one or other serotype of P.haemolytica.

Isolates obtained from the nasopharynx of sheep were also identified by colonial morphology and haemolytic pattern. The serotype of 64% of these isolates was then established by gel precipitation (Chapter 6) which confirmed that they were indeed

P.haemolytica. The remaining 36% of isolates were "untypable" by gel precipitation. However, these isolates all conformed to the criteria of Biberstein, (1979) for the identification of P.haemolytica. This is consistant with the statement by Biberstein et al., (1970) that "it is possible to pick P.haemolytica from cultures of nasal swabs with virtually complete accuracy on the basis of colonial appearance and haemolytic pattern alone."

Comparison of P.haemolytica Serotypes by SDS-PAGE

Serotyping P.haemolytica is a laborious process. It is generally accepted that the serotype is determined by the capsular polysaccharide although this has not been vigorously demonstrated. The possibility exists that serotypes could be identified by an examination of proteins and if so this could be advantageous. i.e. it would not require the production of antisera. This possibility was persued by the examination of proteins of P.haemolytica by SDS-PAGE.

The "A" and "T" biotypes and each serotype within them were compared by SDS-PAGE. The 2 biotypes gave markedly different protein patterns but within each biotype the protein patterns were almost identical. Nevertheless, some minor variations occurred within biotypes (Figures 4 & 5). These minor variations between the prototype strains could be used to distinguish some serotypes when only the prototype strains were considered. We therefore investigated if this could be extrapolated to the identification of serotype of isolates by comparing several isolates from each of 3 serotypes viz A1, A2 and A6 (Figures 6, 7 & 8). The variations which occurred within each serotype were almost as great as the variations between

serotypes of the same biotype.

We conclude that SDS-PAGE can readily identify the biotype ("A" or "T") of an isolate and it follows that SDS-PAGE can identify the species. The use of SDS-PAGE for species identification was further investigated by comparing isolates (which were not P.haemolytica) from ovine lungs with CNP lesions to the P.haemolytica patterns. All non-P.haemolytica isolates differed markedly from both the "A" and "T" biotype patterns (Figure 9). Thus, SDS-PAGE can be used to identify the species but not the serotype. This is not surprising since the serotype depends not on protein differences, as was suggested by Thompson and Mould (1975), but on the capsular polysaccharide.

Antisera Production

Serotyping of P.haemolytica ideally requires the production of high titre antibody to all 15 serotypes. In this context a "high titre" could be defined as a titre of $\geq$ 8 by gel precipitation or $\geq$ 128 by IHA.

Rabbits are the usual species used to prepare P.haemolytica antisera and by implication they seem to be entirely satisfactory. i.e. there have been no clear statements in the literature of rabbits failing to produce the desired response, although Biberstein and Kirkham (1979) do immunise rabbits and mention, without explaination, that the availability of typing sera is restricted. However, we found that rabbits frequently failed to produce detectable antibody and later learned, through personnel discussion, that other workers have encountered similar difficulties. In particular, most laboratories have problems producing adequate antibody to serotype A2 (Liardet,

Personnel communication). We do not imply that rabbits should not be used to produce P.haemolytica antisera because those antisera which were of adequate titre were also highly specific.

The reason for the low response of the rabbits used in our experiments is uncertain, but could be due to a genetic lack in ability of the available animals to respond to the antigen rather than an intrinsic lack of antigenicity of the capsular polysaccharide. With this in mind, rats were used in an attempt to raise antisera to several serotypes. However, no detectable antibody was produced.

Domestic hens were successfully used by Gentry et al. (1982) and by Corstvet et al. (1983) to produce P.haemolytica antibody, so we resorted to this species and antibody was produced of the required titre to all serotypes tested. However, this antibody showed many cross-reactions in the IHA test. This point clearly impinges on which approach must be used to unequivocally establish the serotype of isolates and is discussed further in the next section.

Comparison of Techniques using Prototype Strains

IHA is the standard method by which isolates of P.haemolytica are serotyped. It has at least 2 disadvantages: it is laborious and cross-reactions between serotypes may occur. In our hands major cross-reactions occurred with antisera raised in hens, whereas only low degree cross-reactivity occurred with rabbit antisera. Clearly, rabbits are the animal of choice for producing "typing" antisera, but in practice it was necessary to use antisera raised in hens as the rabbits available to us failed to produce detectable antibody to the capsular

polysaccharide of over half of the 15 serotypes. This has been the major technical problem encountered in the present work.

Published work (Biberstein, 1960; Fraser etal., 1982) indicates that antibody to all 15 serotypes have been produced in rabbits. Nevertheless, as referred to earlier, personnel communications to Mr D. Liardet who (with a veiw to making a commercial vaccine) has had world wide discussions on the immune response to P.haemolytica capsular polysaccharide, indicate that other laboratories have problems producing antibody to P.haemolytica polysaccharides in rabbits.

The difficulty in producing IHA "typing" sera in rabbits and the cross-reactions obtained in this test when avian sera was used might be resolved by one of the following: a major investigation of the ability of different strains of rabbits to produce antibody to capsular polysaccharide of all 15 serotypes of P.haemolytica (in practice this approach was unavailable to us), or the use of a test which gives specific results with avian sera. It might be expected that antisera that cross-reacts in the IHA test would cross-react in other tests, but the following considerations allow at least the possibility that other tests may give more type specific results than IHA.

In the IHA test P.haemolytica is propagated in broth, heat-killed and the supernatant is mixed with sheep RBC's. The capsular polysaccharide (but ideally not other antigens) attaches to the RBC's which are then washed free of unadsorbed antigens. Antibody to the capsular antigens of the homologous type only should agglutinate the coated RBC's. This is the ideal situation, but since cross-reactions occur, they must be due to either: (a) common, non-capsular antigens adhering to

the RBC's. Note: most protein antigens are common to serotypes within a biotype (See Figures 4 & 5); or (b) antibody to the capsular polysaccharide may in some instances cross-react with other capsular polysaccharides. This variation in specificity could be dependent either on the individual animal, or the species used to produce antibody. In particular it is possible that antisera to capsular polysaccharide produced in hens may show greater cross-reactivity with other capsular polysaccharides than does rabbit antibody.

If (b) above were causing the cross-reactions, typing of P.haemolytica by using avian antisera would not be possible. However, if the cross-reactions were due to "reason (a)" a test which differed from IHA may not detect the non-capsular antigens.

These considerations led us to examine the specificity of the following tests using rabbit and hen antisera (as above) and using the prototype strains as antigen: latex bead agglutination, gel precipitation, CIE and direct bacterial agglutination. However, it must be realised that establishment of one of these techniques as type-specific does not necessarily prove correct the premise on which this approach was based.

Serotyping of P.haemolytica by Adsorption of Antibody on to Latex Beads

This technique did not in our hands give serotype specific results due (See Chapter 4) to cross-reactions of "O" antigens common to various serotypes. Nevertheless, the rapidity and convenience with which antigens can be detected using this technique suggests that the production of serotype specific

antisera (free of antibody to all other P.haemolytica antigens) would be a worthwhile exercise. However, capsular polysaccharide may be difficult to free from endotoxin and furthermore, purified capsular polysaccharide may have low antigenicity. These problems could be avoided by the production of monoclonal antibodies, but this is not within the scope of the present work.

Serotyping of P.haemolytica Prototype Strains by Gel Precipitation

Antisera to the 15 serotypes were tested with all 15 antigens (heated broth culture) using gel precipitation tests. Only type-specific lines of precipitation were observed even with those avian antisera which caused cross-reactions in the IHA test.

The major problem with this technique was that antigen of serotype A2 gave no precipitate when broth culture was used. This problem was overcome by using a concentrated antigen (viz a concentrated cell suspension scraped from the surface of a blood agar plate). To simplify testing isolates it seemed reasonable to use such an antigen routinely for all isolates, but when this concentrated antigen was used cross-reactions between many types were detected. This problem could be overcome by using 2 antigen preparations for each isolate. Despite this duplication, gel precipitation is more convenient than the IHA test and is highly specific. The use of this test for serotyping isolates is discussed at a later stage.

Serotyping P.haemolytica Prototype Strains by Counter Immunoelectrophoresis

In contrast to gel precipitation, CIE moves antigen and antibody in opposing directions along one axis of the gel, whereas antigen and antibody diffuse radially in the gel precipitation test and become diluted in the process. This implies that CIE would be more sensitive than gel precipitation and in practice many cross-reactions were observed to occur. These were shown, in some cases, to be due to endotoxin (See Figure 17). However, some antisera showed a decrease in sensitivity by failing to produce a precipitation line with the homologous antigen. Since CIE showed little promise as a method of serotyping P.haemolytica, the reason for some antisera failing to produce a line was not investigated, but could be due to a variation in the electrophoretic mobility of the antigens involved.

Serotyping of P.haemolytica Prototype Strains by Agglutination

Direct bacterial agglutination showed considerable specificity when the prototype strains were investigated, but cross-reactions occurred between 4 serotypes. This degree of specificity was higher than expected since agglutination tests with other bacterial species usually detect "O" or "H" antigens rather than capsular antigens (Brock, 1979). The reason for the observed specificity may be that the antibody directed against, for example, the "O" antigens is either unable to penetrate the capsule or else once penetrated is unable to cross-link cells due to the width of the capsule.

Since agglutination is rapid and reasonably specific with

prototype strains, we conclude it could be used to provisionally identify the serotype of isolates. The serotype inferred could then be confirmed by gel precipitation which is the most specific test available. This approach has 2 major benefits: antisera would be conserved since if agglutination with one antiserum occurred, further agglutination tests would not normally be necessary. Furthermore, a further saving of antisera occurs because only the putative homologous antiserum need be used in the confirmatory gel precipitation test. The second advantage is that only one preparation of antigen is necessary for gel precipitation rather than 2 needed if gel precipitation alone were used. i.e. If the antigen is provisionally identified as A2 a concentrated antigen is required, but if it is one of the other 14 serotypes a broth culture is used as antigen.

Comparision of Techniques for Serotyping P.haemolytica using Isolates from the Lungs of Sheep

The above conclusions are based on the results obtained when only the prototype strains of P.haemolytica were compared by the various serotyping techniques. Extrapolation of these findings to field isolates may be invalid. To ascertain the validity of such extrapolations 40 isolates of P.haemolytica obtained from the lungs of 60 sheep with CNP were serotyped using 3 techniques viz IHA, gel precipitation and agglutination. Each isolate was tested by the 3 methods using antisera to the 15 serotypes.

The IHA titres for each isolate were compared and a ≥ 4 fold preference of one serum over another for the adsorbed RBC's was arbitrarily chosen as the requirement to "type" an isolate. By

this criterion 39 of the 40 isolates were "typed". If only a 2 fold preference was used, then all 40 isolates would have been serotyped. IHA is generally accepted as the standard method by which the serotype of P.haemolytica isolates is determined, so these results were used to establish the specificity of other tests.

Both gel precipitation and agglutination tests yielded only one positive result for each isolate when tested with all 15 antisera. Thus, these tests showed a high degree of specificity in the sense that cross-reactions did not occur. Furthermore and most encouragingly, the serotypic identification of each isolate using gel precpitation and agglutination agreed with IHA results in all cases. This implies that any of the 3 tests involved could be used to serotype lung isolates giving identical results, so the choice of serotyping technique to be used could reflect practical considerations such as the conservation of antisera and the labour involved in executing the test.

When prototype strains were investigated using various serotyping techniques, as above, we concluded that the prototype strains could be tentatively identified by agglutination and the results confirmed by gel precipitation. Comparison of these techniques with isolates from the ovine lung suggest that these conclusions may be extrapolated to include isolates, at least from the ovine lung.

Use of Agglutination Tests and Gel Precipitation Tests to Identify the Serotype of Nasal Isolates

Studies comparing various serotyping techniques using prototype strains and isolates from the lung led to the conclusion that the serotype could be provisionally determined by agglutination tests and then confirmed by gel precipitation tests using only the homologous antiserum. This approach was used to serotype isolates from the nasopharynx, but it soon became clear that not all isolates could be typed by this method. Thus, of the 110 isolates examined, 70 typed by agglutination had their serotype confirmed by a gel precipitin test. 40 isolates remained. These either did not "type" by agglutination or did not produce a gel precipitation line to confirm the provisional agglutination results. These untyped isolates were further tested by agglutination, gel precipitation and IHA using all 15 antisera for all tests. Most of these isolates were agglutinated by one or two antisera, but none gave a precipitation line with any antisera even when concentrated antigen was used with the A2 antiserum.

Using the IHA test, 18 of the 40 isolates were typed by the standard criteria. i.e. showed a 4 fold or greater preference for one antiserum as compared to all others. All 18 were serotyped as A2. These were again retested by gel precipitation using concentrated antigen, but no gel precipitation line was detected with any of them. This result is puzzling, but since IHA is universally accepted as the standard typing method, the 18 isolates were regarded as typed for survey purposes. The remaining 22 isolates were regarded as "untypable." These 22 isolates conformed to Biberstein's definition of P.haemolytica and it should be noted that surveys involving serotyping of

P.haemolytica from the nasopharynx almost invariably included untypable strains (Biberstein et al., 1970).

The time available for the present work did not allow an exhaustive study of why the 18 isolates identified as A2 strains by IHA failed to give a gel precipitation line with antisera against the A2 prtotype strain, but nevertheless we have some further, though preliminary, information on this point and a working hypothesis.

To summerise the results: Antisera raised against any A2 strain reacts in the IHA test with any other A2 strain. In contrast, when a gel precipitation test was used, antibody raised against the A2 prototype strain did not react against all A2 isolates (e.g. it did not react with 3L). Conversely, antibody to 3L reacted in the gel precipitation test against 3L and 3 other of the 18 A2 isolates but not against the prototype A2 isolate. Other A2 isolates failed to react in the gel precipitation test with either the prototype or 3L antibody.

We postulate that 2 determinants are present in the A2 capsular polysaccharide: Determinant "I" which reacts in the IHA test, but not in the gel precipitation test and is common to all A2 strains. Determinant "II" which reacts in the gel precipitation test. Determinant "II" exists as 2 or more allelles which do not cross-react. Further investigation of this hypothesis is in progress, but is not within the scope of this thesis.

To return to the practical problem of serotyping isolates of P.haemolytica, we conclude that at present the most efficient method is to initially examine isolates using gel precipitation

tests because the test is simple and where a positive result is obtained, the identification is unequivocal. Furthermore, in our hands the gel precipitation test allows all strains except some A2 and "untypable" strains to be identified. Those isolates which are not positive by gel precipitation should then be tested by the IHA test to find whether they are "untypable" strains or variants of serotype A2.

Prevalence Studies of P.haemolytica in Sheep

P.haemolytica has been isolated from sheep in several areas of New Zealand (Alley, 1975; Pfeffer et al., 1983), but the prevalence of the various serotypes has never been determined. However, the present work which involved a comparison of various serotyping techniques provided information which can be regarded as 2 separate, although limited, surveys of the prevalence of the serotypes of P.haemolytica.

Survey of Serotypes from the Lungs of Sheep with CNP

40 strains of P.haemolytica were isolated from CNP lung lesions, originating from 3 sheep from each of 20 farms in the Manawatu region. 55% of the isolates were serotype A2, 25% were serotype A1 and types A6, A7, A8 and A9 together totalled 20% (See Table XIII). These 6 serotypes were also the most prevalent of the "A" biotype isolated from pasteurellosis in Britain (Fraser et al., 1982). No serotypes of the "T" biotype were isolated from the lungs. This is not surprising since pneumonic pasteurellosis and CNP mainly involve the "A" biotype (Gilmour, 1978; Gilmour et al., 1982), and disease associated with "T" types is rarely found in New Zealand (Alley, M.R., Personnel communication). No untypable isolates were obtained

from the lungs and (in interesting contrast to the result with nasal isolates) all of the A2 isolates gave a gel precipitation line of identity with antiserum prepared against the prototype A2 strain.

Survey of Serotypes from the Nasopharynx of Sheep

The nasal isolates were derived from healthy sheep from 4 different flocks (A, B, L, R). Each of the 4 flocks were similar in that only "A" types were isolated (See Table XVII). This is not surprising since "T" types are known to predominantly colonise the tonsils, while "A" types mainly colonise the nasopharynx (Gilmour et al., 1974). Flocks L and R showed a similar distribution of serotypes. These flocks were kept on contiguous blocks of land, but were maintained as separate experimental mobs. Flock L was in the foothills of the Ruahines and had a low stocking rate. Flock R was rotationally grazed at high density on flat land at the bottom of the foothills. The absence of differences with respect to P.haemolytica seems to imply that stocking rate is not an important factor in either the proportion of sheep from which P.haemolytica could be recovered or the serotypes involved. Flocks A and B had a high prevalence of serotype A7 (14 and 16%) in comparison to flocks L and R (0 and 4%). No untypable strains were isolated from flock B whereas 24 to 32% of isolates from farms A, L and R were untypable. All "A" serotypes except A12 and A14 were isolated. The absence of these 2 types was not unexpected since serotype A12 is rare in Britain and the USA (Biberstein and Thompson, 1966) and A14 has only been recovered in Ethiopia (Pegram et al., 1979).

Notwithstanding the above results which have pointed out

variations between farms, the overall distribution of P.haemolytica was not markedly different in any of the 4 farms and A2 predominated in all cases. This is similar to the situation in Britain, but contrasts with the USA where A9 is the most prevalent serotype in the nasal cavity (Biberstein and Thompson, 1966).

Comparison of the Prevalence of Serotypes from the Nasopharynx and Lungs

When the prevalence of each serotype from the nasopharynx and from diseased lung are compared, some interesting points arise (See Table XVIII):

1. The prevalence of serotypes A1 and A2 is markedly higher in the diseased lungs than is associated, in a commensal relationship, in the nasopharynx. This suggests that these 2 serotypes are more pathogenic than other serotypes. In Britain serotype A2 and A1 (in that order) are by far the most prevalent serotypes associated with pasteurellosis in sheep.

2. The low prevalence of types A6, A7, A8, A9 in the diseased lung reflects the low prevalence of these types in the nose.

3. Untypable isolates and serotype A11 were not found in the lungs with CNP lesions whereas they made up 21% and 12% respectively of nasal isolates. The lack of serotype A11 and untypable strains associated with pasteurellosis was noted in Britain by Biberstein (1978) who suggested that these strains lack pathogenicity.

The above epidemiological information (although limited in extent) has implications for vaccine production for New Zealand sheep. A pasteurella vaccine should protect sheep against pasteurellosis. Furthermore, such a vaccine should, ideally, protect those sheep with CNP against the development of severe lung lesions caused by P.haemolytica exacerbating lesions initiated by M.ovipneumoniae (See Chapter 1). If the data collected in the Manawatu region reflects the general New Zealand situation, a P.haemolytica vaccine could contain types A1, A2, A6, A7, A8 and A9. However, a vaccine containing A1 and A2 only, may protect lambs 80% as effectively as the 6 strain vaccine and may be preferable for reasons of economy.

APPENDIX

A

Blood Agar Plates

Blood agar plates were prepared as double layers. The basal layer was Difco Blood Agar Base No.2 to which no blood was added. The upper layer was supplimented with horse defibrinated blood to 5%.

B

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
Magnesiun chloride	0.1g
Davis agar	4.0g
Distilled water to	1000ml

All constituents were added to 1000ml water and boiled to dissolve the agar. Aliquots of 5ml were distributed into bijou bottles with continual stirring to keep the charcoal suspended. Caps were screwed down firmly and bottles sterilised by heating at 121°C for 15min. While cooling the bottles were inverted to distribute the charcoal evenly. The media were stored at 4°C.

C

Estimation of Protein Content of P.haemolytica

Preparation of Coomassie Brilliant Blue Dye

Coomassie Brilliant Blue G-250	100mg
95% ethanol	50ml
85%(w/v) phosphoric acid	100ml
Distilled water to	1000ml

The dye was dissolved in a mixture of the ethanol and phosphoric acid and made up to 1000ml with distilled water. The solution was then filtered through two layers of Whatman Number 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 prepared in 0.2M NaOH. These samples were sealed with Gladwrap 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 dye was added and mixed by inversion. The absorbance of the samples was read at 595nm using a SP1800 specrtphotometer. Each new batch of Coomassie Brilliant Blue dye required a new standard curve.

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 3min to solubilise the proteins. Once

the samples had cooled 5ml of Coomassie Brilliant Blue Dye was added and the absorbance read at 595nm. The protein content of the diluted sample was then read off the standard curve.

D

Preparation and Running of SDS-PAGE Gels

Preparation of Stock Solutions for Polyacrylamide Gels

a(i) Running gel acrylamide

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

a(ii) Stacking gel acrylamide

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

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

b(i) Lower tris buffer

Trizma base	18.17g
10% SDS in distilled water	4ml
12N HCl	pH 8.8
Distilled water to	100ml

b(ii) Upper tris buffer

Trizma base	6.06g
10% SDS in distilled water	4ml
12N HCl to	pH 6.8
Distilled water to	100ml

Trizma base was added to 70mls distilled water and the pH was adjusted to the

appropriate value with HCl. SDS was then added and the solution was made up to 100mls with distilled water. The pH was checked and adjusted.

c Ammonium persulphate

Ammonium persulphate	0.1g
Distilled water	1.0ml

A fresh solution was prepared immediatly prior to use.

d Tris-glycine reserve buffer (pH 8.3)

Trizma base	6.07g
Glycine	28.8 g
SDS	2.0 g
Distilled water to	2000ml

e SDS sample buffer

2-mercaptoethanol	10.0ml
SDS	6.0g
Upper tris buffer	25.0ml
Distilled water to	100.0ml

f Bromophenol blue tracking dye

Bromophenol blue	0.05g
Glycerol	40ml
Distilled water to	50ml

g Isopropanol stain

Isopropanol	250ml
Glacial acetic acid	100ml
Coomassie brilliant blue R-250	0.4g
Distilled water to	1000ml

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

Preparation of Glass Plates

The apparatus and technique used for SDS-PAGE was that described by Ionas (1984), 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, 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 distilled water and finally rubbed with paper towels dampened with alcohol. A thread of petroleum jelley 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 jelley. Thickness of the spacers determines the thickness of the gel. Another thread of petroleum jelley was

piped along the spacers, with an extra amount placed at the junctions of the spacers 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(TEMED)	0.01ml

The resulting solution was poured, avoiding air bubbles, between the two glass plates,to a depth of 11cm. Immediatly, 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 overnight at room temperature for polymerisation to occur.

E

Preparation and Pouring of Stacking Gel Acrylamide

A stacking gel solution was prepared by mixing the following solutions, in the order given, stirring well after each addition.

Upper Tris Buffer	2.5ml
Stacking Gel Acrylamide	1.5ml
Distilled water	6.0ml
Ammonium persulphate	0.03ml
TEMED	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 eight 7.5mm wide teeth, 3mm apart and 17mm long) was inserted between the glass plates into the stacking gel, leaving a space 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 pockets in the stacking gel. Polymerisation of the stacking required 30 to 60 minutes at room temperature. Once polymerisation was complete the comb was removed and the wells washed with Tris glycine reserve buffer.

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

Apparatus for electrophoresis was positioned and a thick thread of petroleum jelley 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 on to the electrophoresis apparatus so that the notch in the glass plate was next to the buffer portal to the upper reservoir. Bull-dog clips were applied to firmly attach the gel onto the electrophoresis apparatus. The reservoirs were topped up with Tris glycine reserve 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 reserve buffer.

Growth of Samples for SDS-PAGE

Cultures were grown overnight in 10ml volumes of BHI in Universal bottles. A 5ml aliquot was transferred to 100ml of BHI, which had been prewarmed to 37°C, in a 2l conical flask, and incubated on a shaker at 37°C. Cells were harvested by centrifugation when the optical density was 0.4 at 500nm. To remove any media constituents the cells were washed twice with PBS, making the final suspension up to 5% cells in PBS. The 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 80µg protein were applied to each well.

Electrophoresis of the Protein Sample.

A current of 10mA was applied through the gel until the Bromophenol blue tracking dye reached the stacking gel- running gel interface. The current was then increased and maintained at 15mA until the Bromophenol blue tracking dye was 2 to 5mm from the bottom of the gel, at which point the power was disconnected. The protein required 1&1/2 to 2h to pass through the stacking gel, and a further 4 to 4&1/2h through the running gel.

Staining Protein Bands in the Gels.

On completion of the run the gel sandwich was removed from the electrophoresis apparatus. The 2 perspex spaces were pulled out and the glass plates were levered apart with a spatula. A segment of the lower left corner of the gel was removed to enable correct orientation of the gel once stained. The gel was then carfully allowed to fall off the plate on to which it had adhered, into a container of Coomassie brilliant blue stain.

This was left rocking for at least 3h at room temperature.

Destaining the Gel.

Stain was decanted off the gel and the gel was rinsed in 10% aqueous gacial acetic acid. This was replaced with fresh 10% acetic acid, and left agitating. Every few hours the destaining solution was replaced with fresh 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

Antibody-coated Latex Beads

Serum Fractionation Sodium sulphate was dissolved in 3ml volumes of antisera to types A1, T3, T4, A6, A8, A9, T10, A11 to a final concentration of 28% sodium sulphate. The resultant precipitate was separated from the supernatant by centrifugation at 2,500g for 15min at room temperature and the supernatant discarded. The precipitate was redissolved in PBS to the original volume of serum and sodium sulphate was added to 28%. This was centrifuged as above and the precipitation with 28% sodium sulphate was repeated as before. Finally, the precipitate was resuspended to the original volume of serum and dialysed against 2l of PBS at 4°C for 24 to 48h with 3 or 4 buffer changes. This was then tested for the presence of antibody by gel precpitation.

Conjugation to Latex Beads Latex particles were DOW polystyrene particles, diameter 0.807μm, in a 10% solution.

Glycine Buffered Saline

Glycine	7.3g
Sodium chloride	10g
Sodium azide	1g
Distilled water to	1l

Adjust pH to 8.2 with NaOH

An equal volume of latex particles was mixed with the serum fraction and incubated at 37°C for 2h. Then an equal volume of glycine buffered saline containing 0.5% bovine serum albumen (BSA) was added and incubated a further 30min at 37°C. The suspension was centrifuged at 12,000g for 15min at 4°C, and the pellet resuspended in glycine buffered saline, then centrifuged again and the pellet resuspended in glycine buffered saline plus 0.5% BSA to twice the original volume of latex particles.

Latex Agglutination The latex suspension was shaken well and 10µl was pipetted on to a clean glass slide. 25µl of the antigen under test was then added. This was mixed by rotating the slide by hand at room temperature. Each batch of latex beads was also tested with a saline control.

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