PASTEURELLA HAEMOLYTICA IN SHEEP : SOME STUDIES ON PATHOGENESIS.

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TO

MY WIFE NABBA AND MY CHILDREN

WITH LOVE AND DEEP ADMIRATION.

CONTENTS

DECLARATION.

This is to certify that I, Imad Ibrahim Al-Sultan have carried out the investigations reported herein and have composed this thesis. Fields of collaboration have been fully identified.

SUMMARY.

Pasteurella haemolytica, a common commensal of the nasal passages and tonsils of many healthy sheep, can, under circumstances still imperfectly defined, invade other tissues and cause severe and often fatal disease. Two distinct syndromes are recognized : acute pneumonia due to biotype A serotypes and acute systemic infection of 6 - 12 month old sheep with biotype T serotypes. For neither syndrome is the pathogenesis of disease properly understood and experimental reproduction of disease has been achieved only with pneumonic pasteurellosis. The main objective of the work described in this thesis was to contribute to knowledge of the pathogenesis of ovine pasteurellosis, particularly the systemic disease caused by T serotypes.

By repeated tonsillar swabbing of 37 lambs from birth to 12 weeks of age it was established that the tonsils can be colonized within hours of birth, presumably through intimate contact between lambs and their commensally infected dams. Initially isolations were exclusively of A serotypes and untypable strains while T serotypes first appeared at 3 weeks but were the main isolations (>80%) by 9 weeks of age. In lambs under 2 months old a single topical infection of the tonsils with a mixed T serotype culture provoked a definite but transient local inflammatory response.

On the basis of the foregoing result further attempts were made to reproduce systemic pasteurellosis in groups of 6 sheep aged between 7 and 9 months. Superficial abrasion of tonsillar and pharyngeal mucosa caused a moderate, local inflammatory response but did not modify the activity of the pre-existing commensal population of T serotypes. Supplementary infection of abraded tonsils with a mixed T serotype culture did not exacerbate the local inflammatory changes. Conversely, when infection preceded abrasion more severe local lesions developed but there was no dissemination of infection.

Experiments in mice demonstrated that the LD_{50} of T serotypes of P. haemolytica could be reduced significantly ($p<0.01$) by giving the mice ionic iron (ferric ammonium citrate) intravenously immediately before intraperitoneal infection. Similarly, sheep injected with iron developed a severe local reaction to experimental tonsillar infection with subsequent systemic spread of T serotypes.

Verification of these findings was obtained in a single comparative experiment using 7 - 10 week old lambs. Within the experiment a gradation in severity of response to tonsillar infection was observed between:-

a) infection of intact tonsils

b) infection of abraded tonsils

c) infection of abraded tonsils in iron-treated lambs.

Only in the latter group was there evidence of systemic spread of infection.

Antisera produced by immunizing chickens with live bacteria of selected individual serotypes were rendered serotype-specific and used for indirect immunofluorescence study of tissues from normal, diseased and experimentally infected sheep. In normal animals T serotypes were distributed only over the tonsillar surface and in relatively small numbers but in diseased and experimentally infected sheep P. haemolytica had invaded the tonsillar crypts, breached the epithelial barrier and been disseminated to other organs. These findings support the hypothesis that in systemic pasteurellosis the tonsils may act as a primary focus of infection from which bacteria are disseminated to other organs. However, the factors which result in the conversion of commensal P. haemolytica into invasive microorganisms remain undetermined.

Though producing neither the full range nor severity of lesions encountered in natural systemic pasteurellosis, infection and abrasion of the tonsils of iron-treated sheep does result in a form of disease similar in onset and general pathology to that encountered in the field. Therefore it is a useful model for further pathogenesis studies.

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ABBREVIATIONS USED IN TEXT.

b.w.	=	Body weight
B RBC	=	Bovine red blood cell(s)
CIE	=	Counter immunoelectrophoresis
EDTA	=	Ethylenediaminetetra acetic acid
FAC	=	Ferric ammonium citrate
FBS	=	Formol buffered saline
FCA	=	Freund's complete adjuvant
FIA	=	Freund's incomplete adjuvant
HKO	=	Heat killed organisms
hr	=	Hour(s)
ID	=	Immunodiffusion
IHA	=	Indirect haemagglutination
IIF	=	Indirect immunofluorescence
i.p.	=	Intraperitoneal(ly)
IPA	=	Immunoperoxidase
i.v.	=	Intravenous(ly)
IVAA	=	Intravenous agar-aerosol
min.	=	Minute(s)
NB	=	Nutrient broth
PBS	=	Phosphate buffered saline
PI3	=	Parainfluenza type 3 virus
PWE	=	Phenol water extraction
RBCs	=	Red blood cells
s.c.	=	Subcutaneous(ly)

Abbreviations continued

SSE	=	Sodium salicylate extract
U	=	Untypable
um	=	Micron, micrometre
2-ME	=	2-Mercaptoethanol

GENERAL INTRODUCTION.

Of the several species of the genus Pasteurella two in particular, Pasteurella multocida and Pasteurella haemolytica have been associated with disease of sheep (Buxton and Fraser, 1977). The former can cause a form of haemorrhagic septicaemia especially in northern Africa and has also been isolated from sporadic cases of ovine pneumonia (Al-Sultan, 1976; Namioka, 1978). However, in temperate countries P. haemolytica is by far the commoner species isolated (Gilmour, 1980). In Britain, the general term "ovine pasteurellosis" is used for two forms of disease resulting from acute infection of sheep with P. haemolytica.

One is predominantly an acute respiratory disease affecting sheep of all ages, although in lambs under 3 months of age the infection is usually septicaemic. The other is an acute and often fatal systemic disease of older lambs (6 - 12 months old) that occurs most commonly in late summer and autumn. Each of the two forms of pasteurellosis is associated with a distinct biotype of P. haemolytica, biotype A organisms being involved in respiratory disease and biotype T in the systemic disease of older lambs.

The importance of ovine pasteurellosis arises from the very considerable economic losses which it causes. Returns from Veterinary Investigation Centres throughout the country identify

pasteurellosis as the most prevalent infection diagnosed in sheep for the years 1976 - 1978 (Ministry of Agriculture, Fisheries and Food, 1979). Clearly therefore, disease due to P. haemolytica is a consistent and continuing problem for the sheep industry. Indeed, throughout those parts of the world where sheep are farmed pasteurellosis is acknowledged to be a disease of major significance (Alley, 1975; Gilmour, 1978). For this reason much investigative and research effort has been directed towards an understanding of the disease so that appropriate methods of control and prevention can be developed and applied. Although much has been learned about pasteurellosis and its causative organism the disease remains of major concern to sheep farmers as no fully effective control measures have yet been devised.

One of the inherent problems in studying pasteurellosis is the fact that many healthy sheep carry P. haemolytica as commensals in the nares (biotype A) and tonsils (biotype T). For this reason it has long been suspected that factors other than simple infection are involved in causing the disease. This belief has been borne out by the difficulties experienced in reproducing the diseases experimentally (Gilmour, 1978).

However, the nature of the predisposing, precipitating or contributary causes have not been fully identified yet. Only fairly recently has a method been developed for consistent experimental reproduction of pneumonia caused by biotype A P. haemolytica

in a high proportion of experimental animals (Sharp, Gilmour, Thompson and Rushton, 1978). The procedure requires intratracheal infection of specific pathogen free (SPF) lambs with parainfluenza virus type 3 (PI3 virus) seven days before exposure to an aerosol of P. haemolytica.

Availability of a successful model system enabled some pathogenesis studies to be carried out and provided a challenge system for evaluating candidate vaccines against pneumonic pasteurellosis (Gilmour, Martin, Sharp, Thompson and Wells, 1979).

As far as systemic disease due to T biotype P. haemolytica is concerned attempts to reproduce systemic disease experimentally have been strikingly unsuccessful. A recent reassessment of naturally-occurring T biotype disease indicated that the tonsils are the primary focus of infection (Dyson, Gilmour and Angus, 1981) but the route of access of the organisms in the disease process and the way in which the disease is initiated are still unknown. Dyson and colleagues suggested that the organism spreads from the tonsillar epithelium via the blood or lymph vessels to other tissues and organs, but there is no direct evidence that this is so.

Thus, there are several aspects of ovine pasteurellosis which require detailed investigation and study. A better understanding of pathogenetic mechanisms should help to identify host-associated

predisposing or precipitating factors and thus lead to the development of improved methods of control.

LITERATURE REVIEW.

Pasteurella haemolytica has been subjected to extensive studies in the past twenty years, and thorough reviews have been presented recently by Carter (1967), Thompson (1973), Biberstein (1978) and Evans (1979). Only those aspects of relevance to the present study will be presented in this literature review.

The organism : Pasteurella haemolytica.

(a) Nomenclature and characteristics.

During the latter part of the nineteenth century a considerable variety of gram-negative bacilli were isolated from cases of haemorrhagic septicaemia in different types of wild and domesticated animals. Trevisan (1887) recommended that these organisms be identified as the genus Pasteurella, in recognition of the work of Louis Pasteur who identified a bipolar-staining microorganism as the cause of fowl cholera. Lignières (1900) advocated the use of a specific name which referred to the species of animal from which the organism had been isolated. Thus, there developed such binomials as Pasteuralla oviseptica, P. aviseptica, P. suiseptica, P. lepiseptica and P. boviseptica. Also included in the Pasteurella group in the past were certain other bacteria e.g. B. pseudotuberculosis (Pfeiffer, 1890), P. pestis, the plague bacillus (Kitasato, 1894; Yersin, 1894) and an organism, referred to as Pasteurella X by Knapp and Thal (1963) and called Yersinia enterocolitica by Frederiksen (1964), which was isolated from

cases of a disease resembling tuberculosis in man and animals.

A bacterium of special interest in relation to this thesis is that isolated from a pneumonic condition in cattle by Jones and Little (1921). Whilst clearly belonging to the Pasteurella group it possessed an haemolytic activity not shown by other members of the group and was referred to as Bacillus bovisepticus (Jones, 1921). In his study of 16 strains of this organism Jones (1921) was able to distinguish three groups on the basis of fermentation, serological reactions and colonial morphology. His group I, consisting of eight strains, fermented glucose, sucrose, maltose and mannitol but not salicin and differed from groups II and III in being haemolytic on horse blood agar and fermenting lactose but failing to produce indole. Spray (1923) described two types of similar microorganisms and found them to be highly pathogenic for mice and guinea pigs, but less so for rabbits. These two types were separated by a slight difference in their reaction with glycerol and by the results of the direct agglutination and agglutinin-absorption tests. They were distinguished from the other pasteurellae by their action on blood agar, maltose and glycerol. A nonvirulent atypical organism was reported by Giltner (1923) from a case of sheep pneumonia and was regarded as a pasteurella variant.

Tweed and Edington (1930) reported a type of bovine pneumonia in England due to infection with a bacterium which they referred to

as Pasteurella boviseptica. This organism belonged to Jones' group I.

Dungal (1931) gave a complete account of a disease of sheep which occurred in Iceland, and in which the bacteria isolated had a marked resemblance to those of Jones' group I. The organisms were encapsulated, haemolytic on blood agar, fermented glucose, saccharose, maltose and mannitol and were indole negative.

Newsom and Cross (1932) studied a group of strains of gram-negative coccobacilli isolated from sheep and cattle and defined a "typical" and an "atypical" group. The atypical haemolytic group was similar to Jones' group I and they considered it to be sufficiently different from the "typical" group to be regarded as a separate species for which they suggested the name Pasteurella haemolytica. The typical group was similar to groups II and III of Jones (1921) which have since been classified as Pasteurella multocida.

Montgomerie, Bosworth and Glover (1938) described an enzootic form of pneumonia in sheep in North Wales and East Anglia. These workers isolated an organism closely related to the Pasteurella group. The strains thus obtained were encapsulated, haemolytic, indole negative and with low pathogenicity for experimental animals. On cultural and biochemical grounds they closely resembled group I pasteurellae described by Jones and Little (1921).

Rosenbusch and Merchant (1939) differentiated three types of non-haemolytic pasteurellae and a haemolytic group by agglutination tests and noted a correlation of the serological types with dulcitol, arabinose and xylose fermentation.

Smith (1959a, 1961) first reported the recognition of two types of P. haemolytica on the basis of their morphology, penicillin sensitivity, growth curves and fermentation reactions. Strains fermenting trehalose after 2 days but not arabinose after 10 days were designated T strains, and those fermenting arabinose after 7 days but not trehalose after 10 days, A strains. In his studies he showed that biotype A strains were associated with septicaemia of young lambs and pneumonia in all ages of sheep, while biotype T strains were associated with toxaemic infection of feeder lambs.

No further non-serological characterization of P. haemolytica was reported until recently. The production of bacteriocins by P. haemolytica and P. multocida isolated from natural cases of ovine and bovine pneumonia was reported by Iordache and Ungureanu in 1979. Within and between each species a range of activity was noted but the sensitivity of P. haemolytica exceeded that of P. multocida.

More recently Frank and Tabatabai (1981) described an investigation into a possible virulence marker. These workers detected

neuraminidase activity in culture fluid of a bovine pulmonary isolate of P. haemolytica and were aware that this enzyme had been implicated as a virulence factor in certain other bacterial species (Ray, 1977; Smith, 1977; Milligan, Baker, Straus and Mattingly, 1978). Accordingly, they measured neuraminidase activity in 12 type isolates and in serotyped isolates recovered from healthy and pneumonic lambs and calves. Several untypable isolates of bovine origin were also screened. Considerable variation in neuraminidase activity was recorded and it was noted that no measurable activity could be detected in any biotype T isolate and only low levels were present in biotype A isolates commonly associated with disease. The authors concluded that neuraminidase activity of P. haemolytica did not directly correlate with virulence.

(b) Serology.

Early workers encountered difficulties in investigating the serology of P. haemolytica and the wide range of antigenic types was not recognised for some time.

Jones (1921) observed that his Pasteurella species could be distinguished as three groups or varieties by an agglutination procedure using rabbit serum. Newsom and Cross (1932) pointed out after using agglutination and biochemical tests that their P. haemolytica was similar to Jones' group I. Tweed and Edington (1930) revealed one antigenically homogeneous type of P. haemolytica

by the agglutination reaction. Montgomerie *et al.* (1938) divided *P. haemolytica* into 3 serotypes by the same method. Florent and Godbille (1950) classified *P. haemolytica* strains, which they recovered from pneumonias of sheep and cattle, into two types by agglutination reactions. Carter (1956) applied an haemagglutination procedure for *P. multocida* using human red blood cells (RBC) to study the serology of 51 strains of *P. haemolytica* isolated from pneumonic lungs of cattle in Canada. He concluded that the 51 strains were serologically homogeneous. Included in his tests were six strains of *P. mastitidis* from mastitis in ewes, two strains from septicaemia in lambs in Scotland, and two bovine strains from Belgium. Thus early workers failed to appreciate the diversity of serotypes of *P. haemolytica*.

Biberstein, Gills and Knight (1960) examined 98 strains of *P. haemolytica* by means of a modified indirect haemagglutination (IHA) test. Eleven types were identified on the basis of differences in capsular substances and 18 strains were not typable. They also examined the same strains by the agglutination procedure employing autoclaved bacteria and the same sera as used for the IHA tests. Thirteen somatic groups were identified and designated A, AB, AD, B, BD, C, D, E, F, G, H, I and U. The division into somatic groups on the basis of agglutination was not as clear as the division into types based on the IHA test. There was considerable variation within each cross-agglutinating series and frequently no reciprocity of reactions, suggesting the presence of haptens

rather than complete antigens in the somatic portion. Using all known representative serotypes of P. haemolytica, Biberstein and Gills (1962) divided them into the types (biotypes) described by Smith (1959a, 1961) and found on serotyping that 1, 2, 5, 6, 7, 8, 9 and 11 were biotype A whereas 3, 4 and 10 were biotype T. They thought that the two biotypes might also vary in virulence. In a later report, Biberstein (1965) demonstrated the existence of minor antigens which were responsible for low titre cross-reactions between capsular types. These minor antigens were shown to be distinct from type specific antigens by reciprocal absorption experiments, and it was concluded that they did not seriously interfere with typing. Biberstein and Thompson (1966) added another A serotype to make a total of twelve serotypes of P. haemolytica. Cameron (1966) modified the IHA test by using guinea pig RBC instead of the Human RBC used by Carter (1956) or bovine red blood cells (B RBC) used by Biberstein et al. (1960). He found that clear agglutination patterns were obtained if the red cells, after treatment with antigen, were suspended in dilute normal rabbit plasma.

Muraschi, Lindsay and Bolles (1965) demonstrated a serological method by which they could identify and type P. haemolytica using a gel diffusion technique. They found that results were clear-cut and free from complicating serotype-or species-cross reactions but the method proved extravagant on sera. Frank and Wessmann (1978) found that P. haemolytica could be serotyped by a rapid plate

agglutination procedure. The technique did not require special antigen preparation and yielded essentially the same results as the IHA procedure.

Pegram (1974) in his study on the serological types of P. haemolytica isolated from sheep and goats in the Somalia Democratic Republic claimed that a new A type of P. haemolytica had been found which differed serologically from the 12 known types. Later Pegram, Roeder and Scott (1979), using the IHA test in their study of 96 strains of the bacterium isolated from sheep in Ethiopia, recorded two new A serotypes (13 and 14) of P. haemolytica. Recently, in the course of screening a number of untypable strains received at Moredun Research Institute from cases of systemic pasteurellosis Fraser, Laird and Gilmour (1982) recognised a new biotype T serotype, which provisionally has been designated T15.

Thus, to date, 15 serotypes of P. haemolytica have been recognised by the IHA typing procedure (Table 1) and many untypable strains are known to exist. It is probable that additional serotypes will be identified as study of currently untypable strains progresses.

Table 1. Serotypes of P. haemolytica identified by the indirect haemagglutination procedure.

A biotype	1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14
T biotype	3, 4, 10, 15

(c) Classification.

Biberstein and Francis (1968) drew attention to the differences in genetic constitution between the two biotypes of P. haemolytica. In their attempt to study DNA - RNA hybridization between strains of biotypes A and T, they had concluded that there was a low degree of genetic relationship between the representatives of the two biotypes and suggested that the differences between the types were equivalent to those between different species in other bacterial genera. They observed a high degree of nucleic acid homology between two strains of biotype A, viz. serotypes A1 and A2.

Smith and Thal (1965) in their study of 25 cultures of Pasteurella species attempted to classify them using a numerical (Adansonian) technique. The authors differentiated two main groups, group 1 (oxidase positive) which comprised the two related species P. multocida and P. pneumotropica together with the two types of P. haemolytica A and T, which they suggested differed markedly from each other, and P. haemolytica var. ureae. In group 2 (oxidase negative) they included the species formerly designated P. pseudotuberculosis, P. pestis and "X" which they suggested should form the genus Yersinia. Mráz (1969a) examined a total of 86 diagnostic features on 46 strains of P. haemolytica originating from pathological specimens. Finding no difference in the characteristics of P. haemolytica he advocated more detailed specification of the bacterium. From the results of agglutination and precipitin tests on biotypes A and T of P. haemolytica already

investigated he concluded that they show marked independence from each other. In a second report Mráz (1969b) carried out a taxonomical comparison on 99 strains of Actinobacillus lignieresii and 46 strains of P. haemolytica, and found only three essential differences between the two bacteria. These were (i) haemolytic activity on agar with lamb's blood, (ii) ability to degrade urea and (iii) the guanine and cytosine content of the DNA molecule. In contrast, strains of P. haemolytica usually differ from the type species of the genus, P. multocida, by the pleomorphism of their cells, and by their growth on MacConkey agar with bile salts, by haemolysis on agar with lamb's blood and by other criteria. Therefore, Mráz suggested that species of P. haemolytica be excluded from the genus Pasteurella and transferred to the genus Actinobacillus under the name Actinobacillus haemolyticus. This suggestion is supported by the antigenic relationship, the guanine and cytosine content of the DNA, the character of the infection caused by this organism and differential diagnostic consideration.

Frederiksen (1973) in his study based on 34 characters in 193 strains of P. haemolytica, P. multocida, P. pneumotropica, P. gallinarum, P. ureae, Actinobacillus lignieresii, A. equuli and A. suis pointed out that the differences between biotypes A and T of P. haemolytica are so pronounced that they could be regarded as two separate species.

However, Pohl (1981) investigated DNA - DNA hybridization of about 70 collection cultures representing 32 species belonging to the genera Actinobacillus, Haemophilus, or Pasteurella. Using the optical method of recording the initial reassociation kinetics, she found that most of the strains studied were genotypically interrelated at or above the 30% DNA-binding level and several clusters of strains linked at 40%, 50% or higher DNA-binding levels. From the data presented in her work Pohl concluded that Actinobacillus, Haemophilus, and Pasteurella are interrelated and that the genera Haemophilus and Pasteurella are heterogeneous. She also confirmed the observation of Mráz (1969b) that P. haemolytica is more related to the type species of the genus Actinobacillus than to P. multocida by the DNA - DNA binding clusters.

The taxonomic categories of the interrelated genera of Actinobacillus, Haemophilus and Pasteurella were considered by Mannheim (1981). He studied the DNA relatedness of the majority of taxa in these three genera and proposed their reclassification into a new family called Pasteurellaceae. As far as P. haemolytica is concerned in this study Mannheim suggested that the proposed family should be subdivided into a number of tribes and that P. haemolytica biotype A should be included in one which he named Actinobacilleae and which would contain the species of the genus Actinobacillus (A. lignieresii, A. equuli, A. suis, A. capsulatus, A. species (Ross, Hall, Orning and Dale, 1972) and A. actinomycetem-comitans), together with Haemophilus

pleuropneumoniae and the Pasteurella species described by Bertschinger and Seifert (1978), P. ureae and P. mastitidis. P. haemolytica biotype T with some other organisms were considered not to be affiliated to these although it showed DNA-binding at a level above 30%.

It is evident from the foregoing that classification of P. haemolytica is still unresolved.

(d) Antigenic composition.

So far, the antigenic composition of P. haemolytica has received little attention but it is likely that the serologically specific substances absorbed on to the red cells in the IHA test are lipopolysaccharide (Carter, 1967). Biberstein and Thompson (1965) concluded from their study of the specificity of immunity to P. haemolytica in mice that capsular antigens play the major role in determining the specificity of the immunity conferred and that the somatic antigens are only secondarily involved. Cameron (1966) reported that the outer surface polysaccharide is responsible for immunizing properties and is necessary to elicit haemagglutinating antibody formation. However, he suggested that a protein fraction of the cell is also capable of stimulating the production of protective antibodies and that sera with low haemagglutination titres may be capable of conferring immunity in mice. It was his view that immunity to P. haemolytica is not dependent on a single type of antibody. The work of Tadayon and

Lawerman (1981) showed that capsular antigens of P. haemolytica, particularly the potassium thiocyanate extract, are more immunogenic than other cell fractions when examined by protection tests in mice and hamsters. Their results also indicated that the method of capsular extraction has some influence on whether or not a protective immunity is stimulated. Keiss, Will and Collier (1964) extracted endotoxin from a bovine strain of P. haemolytica. The product was evaluated for its dermotoxic effect in rabbits and its haemodynamic effect on sheep, but was not studied serologically or immunologically.

Strains of P. haemolytica which cannot be serotyped by the IHA test, where the reaction depends on the utilization of specific soluble surface antigens, are called "untypable strains". There has been speculation that such strains are deficient in capsular antigens but their carbohydrate fermentation pattern is similar to that of biotype A strains (Gilmour, 1978). They constitute approximately 10% of the strains isolated from nasal swabs taken from normal animals (Biberstein and Thompson, 1966; Aarsleff, Biberstein, Shreeve and Thompson, 1970; Biberstein, Shreeve and Thompson, 1970). Aarsleff et al. (1970) studied 99 untypable strains and concluded that, although these strains had all the characteristics of P. haemolytica, their relationship to the typable strains of biotype A and T of P. haemolytica was uncertain. Frank (1980) was able to group 10 untypable strains of P. haemolytica, isolated from the nasal passages of cattle, by the

IHA procedure into 3 groups using the rapid plate agglutination method but did not relate his groups to A and T biotypes.

A unique phenomenon has been recorded by Mwangota, Muhammed and Thomson (1978) in Kenya. They tested a large number of P. haemolytica isolates and noted the occurrence of both A and T biotypes of P. haemolytica within each of the serotypes 3, 4, 10, 6 and 12. On the basis of this observation, Biberstein (1978) considered the possibility of mixed serotypes but felt such a condition would be unlikely on statistical grounds alone. No evidence of mixed serotypes arose during tests made on large numbers of strains in Britain and the United States. However, he suggested that in Kenya a special situation which needed further elucidation could exist.

It is evident that for P. haemolytica much has still to be learned about the nature and chemical composition of the bacterial cell surface, both for the 15 serotypes already established and for untypable strains. Such information is needed for a clearer understanding of serotype grouping and virulence attributes as well as for the identification of antigens best suited for stimulating protective immunity in the host. Studies along these lines are known to be in progress in several laboratories.

The disease : ovine pasteurellosis.

In Britain, each of the two biotypes of P. haemolytica is associated with a distinct form of pasteurellosis. Biotype A serotypes are responsible for an enzootic pneumonia which can occur in all types and age of sheep at any time of year although in lambs under 2 months old the disease is most commonly septicaemic. Biotype T strains, on the other hand, cause an acute and usually fatal toxaemia in a well defined age group (6 - 12 months). This form of pasteurellosis almost always occurs from August to November.

(a) Pneumonic pasteurellosis caused by biotype A serotypes.

The disease occurs as pneumonia in flocks and sporadically in individual sheep. As well as being recognized in Britain this form of pasteurellosis has been encountered in many other countries including North America (Newsom and Cross, 1932; Hamdy, Pounden and Ferguson, 1959), Australia (Beveridge, 1937), Iceland (Dungal, 1931), Norway (Mohn and Utklev, 1974), New Zealand (Salisbury, 1957), South Africa (Cameron, 1966), Ethiopia (Pegram, Roeder and Scott, 1979), Somalia Democratic Republic (Pegram, 1974), Kenya (Mwangota et al., 1978), Iraq (Al-Sultan, 1976), India (Ramachandran and Sharma, 1969).

Indeed, it is probable that P. haemolytica is a component of acute ovine respiratory disease in all sheep-rearing countries. In Britain, the term enzootic pneumonia was applied by Montgomerie

et al. (1938) who described an acute exudative pneumonia affecting a flock of sheep and associated with P. haemolytica. The same kind of acute exudative pneumonia was also observed by Woxholtt, Naerland and Hoff (1952), Stevens (1957) and Salisbury (1957). In a flock affected with acute disease of pneumonic pasteurellosis, morbidity and mortality rates rarely exceed 10% Animals which recover from the disease may develop chronic lung lesions and remain unthrifty thereafter (Gilmour, 1978, 1980). The pathological lesions of enzootic pneumonia may vary considerably in degree but not in type (Stamp and Nisbet, 1963). In acute cases of the disease the most striking findings are pleurisy and pericarditis. There is usually a greenish, gelatinous exudate over the pericardium extending into the anterior mediastinum and often large volumes of pale yellow pleural fluid with fibrin clots. In hyperacute cases the lungs are enlarged and oedematous with large areas, particularly of the ventral parts, bright or purplish red; when cut, such areas are solid, oedematous and haemorrhagic, and frothy exudate or even blood-stained fluid can ooze from the cut surfaces. Extensive adhesions can be found between the visceral and parietal pleura.

Histologically, the peracute lung lesions show alveolar necrosis with alveolar spaces filled with fluid and bacteria. The interlobular septa are widened by oedema and capillaries are distended with fluid and red blood cells. Lymphoid cell

aggregations as well as fibrin deposition can be seen. Hyperplasia of bronchiolar epithelium and bronchiolitis are common findings. Spindle-shaped cells with intensely basophilic nuclei, called "oat cells", are seen in large numbers in the lesions. These cells are found lining the alveoli and are frequently present with an infiltration of mononuclear cells. A characteristic finding in this disease is that thrombosed capillaries are devoid of lining endothelium (Dungal, 1931; Montgomerie et al., 1938; Gilmour, 1980).

In the consolidated lung lesions and the inflammatory exudate P. haemolytica biotype A is present in large numbers and usually can be isolated in pure culture. The organism can be grown from most internal organs (liver, spleen, kidney, heart blood and lymph nodes) in the very acute cases in adult sheep and young lambs.

(b) Experimental reproduction of biotype A disease.

For many years it was considered that P. haemolytica was the primary cause of enzootic pneumonia of sheep since the micro-organism was consistently isolated from the lesions. Then the view was favoured that P. haemolytica is not likely to be the genuine primary pathogen but can be a significant or even essential secondary invader, capable of giving the disease a characteristic stamp and poor prognosis. This characteristic picture is the fibrinous nature of the inflammation. This conclusion is

supported by the fact that the organism can frequently be found in the upper respiratory tract of normal animals and this makes P. haemolytica no more than an important secondary bacterium (Shreeve, Biberstein and Thompson, 1972).

Furthermore, there has been only limited success and no uniformity in producing pneumonia with P. haemolytica alone (Dungal, 1931; Florent and Godbille, 1950; Salisbury, 1957; Downey, 1957). Indeed, some workers who were unable to produce the disease in ewes and lambs with broth cultures of the organism suggested the possible aetiological involvement of a virus (Montgomerie et al., 1938; Downey, 1957).

By the intratracheal inoculation of a large number of organisms Smith (1960a) was able to produce pneumonia and generalized infection in lambs. Similar results were obtained in 3-week old lambs inoculated intraperitoneally (i.p.) with type A strains isolated from adult sheep with enzootic pneumonia. Adult sheep withstood the doses used.

Smith (1964) stated that the way in which natural infection is initiated was not known. Hore (1968) showed that P. haemolytica is normally present in the respiratory tract of sheep in many flocks but again stressed that the problem is to know the way in which the natural disease is initiated.

Although experiments by some workers (Smith, 1964; Biberstein, Nisbet and Thompson, 1967) were successful in reproducing the characteristic lesions of so-called enzootic pneumonia in sheep following the administration of P. haemolytica by intrabronchial catheters, these results should be treated with reserve as very large numbers of this organism deposited in the lungs might be expected to produce a pathological change (Gilmour, 1978).

The suggestion of Montgomerie and his colleagues (1938) that P. haemolytica exists as an opportunist pathogen in the nasopharynx of normal sheep, the isolation of PI3 virus from the respiratory tract of sheep (Hore, Stevenson, Gilmour, Vantsis and Thompson, 1968) and the experimental demonstration of the pathogenic significance of this virus in sheep (Hore and Stevenson, 1969) led to the belief that dual infection with PI3 virus and P. haemolytica is necessary to produce the disease.

Biberstein, Shreeve, Angus and Thompson (1971) found that experimental pneumonia induced by P. haemolytica was markedly more severe clinically when PI3 virus was inoculated 3 days previously. The greater severity was shown by earlier deaths, higher mortality and more prolonged febrile periods in the survivors. The authors concluded that the pathological changes also tended to be more severe in animals receiving dual infection but there was no evidence of synergism between the two agents. Biberstein et al. (1971) stated that the extent of the lesions in survivor animals

showed no relationship to the infecting dose of P. haemolytica. They also thought that there was insufficient evidence to suggest a key role for the virus in the pathogenicity of enzootic pneumonia.

Alternative methods of infection with small numbers of organisms have been sought. Exposure to an aerosol of P. haemolytica can cause pneumonia indistinguishable from the field pneumonia in about 40% of SPF lambs and death in up to 10% (Gilmour, Thompson, Smith and Angus, 1975). However, it was found subsequently that if an aerosol of P. haemolytica was given four or seven days after an intratracheal inoculation of PI3 virus, typical pasteurella pneumonia could be produced in up to 90% of lambs (Sharp et al., 1978).

This challenge system has been successfully exploited to assess the relative pathogenicity of A serotypes and to test vaccines (Gilmour et al., 1979). Unfortunately, the system is not readily adapted to conventional lambs as most acquire an early natural infection with PI3 virus which renders them resistant to experimental infection. However, some success with conventional lambs has been reported by workers in New Zealand (Davies, Dungworth, Humphreys and Johnson, 1977; Davies, Herceg, Jones and Thurley, 1981).

The most recent development in producing pneumonic pasteurellosis experimentally has been reported by Gilmour and colleagues (Gilmour, Angus, Donachie and Fraser, 1982a; 1982b). These workers administered a fine particulate suspension of 1.25% agar intravenously (i.v.) to sheep and immediately exposed recipient animals to an aerosol of biotype A strains of P. haemolytica. Necropsies carried out within 7 days of infection disclosed in all animals pneumonic lesions histologically indistinguishable from those of the natural disease. Further studies are in progress to evaluate the reliability of the procedure for experimental reproduction of pneumonic disease (N.J.L. Gilmour, personal communication).

The conclusion to be drawn from the experimental work done up to the present time is that PI3 virus infection in a susceptible animal may be an important factor which can pave the way to pasteurella pneumonia, though the mechanisms involved have not been defined. The intravenous agar-aerosol (IVAA) method is a more artificial approach to induction of disease but initial results are encouraging.

(c) Systemic pasteurellosis caused by biotype T strains.

This form of disease, characterized generally by sudden death with signs of septicaemia and toxaemia, was first described by Stamp, Watt and Thomlinson (1955) and subsequently investigated by Biberstein and Kennedy (1959) and Smith (1960b). Little

further work was done until reassessment of the pathology and bacteriology of the naturally occurring disease was undertaken by Dyson et al. (1981).

Although the disease may occur in individual sheep of any age and at any time of year it is most prevalent in late summer and autumn affecting sheep born earlier in the same year. This period coincides with movement of sheep from poorer hill or upland grazing to improved low-ground pasture or to the start of folding on rape or turnips. The change to improved diet has been considered to be a predisposing factor (Stamp et al., 1955) while adverse weather and intercurrent infection may also be implicated as precipitating causes (Dyson et al., 1981). However, there is still no firm evidence on any of these points.

In a typical outbreak the first deaths often occur within a short time of a flock being moved and continue sporadically over a few days before ceasing (Gilmour, 1980). Overall mortality in an affected group is usually low but may reach 20% on occasion and deaths tend to be among sheep in good condition. Clinical signs of depression and dyspnoea may be seen in a few animals and it is probable that some recover (Gilmour, 1978).

Lesions found at necropsy involve the respiratory and digestive tracts. Morbid pathological changes include congestion of trachea and bronchi which contain blood-stained frothy fluid

in the lumen, and distension of the lungs due to accumulation of oedematous fluid. However, the lungs are seldom pneumonic. The lymph nodes tend to be enlarged, soft and oedematous and sometimes haemorrhagic. Patches of haemorrhagic inflammation are found on the abomasal mucosa, and there is blood splashing on the visceral peritoneum, in the neck and beneath the parietal pleura. Large numbers of T serotypes of P. haemolytica can be cultured from the visceral organs and heart blood as well as lung.

In their more recent study on naturally infected animals Dyson et al. (1981) drew attention to previously unreported necrotic lesions of the pharynx, tonsils and oesophagus and congestive changes in regional lymph nodes. In addition, brain histology disclosed serum protein leakage in cerebral meninges indicative of toxaemia and infiltration of choroid plexuses by mononuclear cells. Biotype T organisms were recovered consistently from lesions in the tonsils and upper alimentary tract, frequently from liver, lung and spleen but only rarely from kidney or brain. The consistently uneven recovery of P. haemolytica from different organs of the same animal caused the authors to doubt if the disease involved a true septicaemia and to advocate "systemic pasteurellosis" as a more apt descriptive term.

Although systemic dissemination of T biotypes is an invariable feature of this form of pasteurellosis it is important to emphasise that T serotypes of P. haemolytica are normally found

as commensals in the tonsils of healthy sheep (Gilmour, Thompson and Fraser, 1974). As yet, no convincing explanation has been offered as to why this organism should suddenly become pathogenic. An earlier view was that P. haemolytica entered the blood via the respiratory tract, perhaps as a secondary invader, but Stamp et al. (1955) found no evidence to support that suggestion. In several cases where pneumonic lesions were present they appeared secondary to bacterial thrombi in the pulmonary vessels. On the basis of their findings Dyson and colleagues (1981) proposed that, in certain undefined conditions, T serotype commensals in the tonsils are able to replicate and penetrate surrounding tissue, producing necrotic lesions in the pharynx and oesophagus. From these initial lesions bacteria enter the bloodstream either directly or via lymphatics and lodge in pulmonary capillaries forming emboli in which further multiplication occurs. From these emboli bacteria are intermittently seeded via the blood stream to liver, spleen and perhaps kidney.

As lesions were also encountered in the omasum and abomasum the possibility of systemic infection from the gut was considered but thought to be unlikely because of the generally inimical conditions for survival of P. haemolytica. Dyson et al. were of the opinion that it might be possible to induce T type disease by establishing infection in the throat, the site at which they considered that initial bacterial multiplication took place.

(d) Experimental reproduction of biotype T disease.

Septicaemia of feeder lambs as described by Stamp et al. (1955) in Scotland, and Biberstein and Kennedy (1959) in California was shown to be caused by biotype T strains of P. haemolytica (Smith, 1959b, 1960b) and was recently defined as a generalised systemic disease (Dyson et al., 1981). Experimental reproduction of the disease in lambs over 3 months of age was attempted by i.v. inoculation of diseased tissue suspensions, primary surface culture suspension and broth cultures (Stamp et al., 1955; Biberstein and Kennedy, 1959). These procedures were successful in producing septicaemia but the pathological findings were not identical to those in the natural disease process. Moreover, heat-killed broth cultures injected i.v. also killed lambs (Smith, 1960b). Doses of live bacteria required to set up the toxaemic phase and kill experimental lambs have been shown to be very high especially in adult sheep, and approximate the number of killed bacteria which could cause death. Accordingly, it seems likely that as yet unidentified predisposing factors are involved in the natural disease (Gilmour, 1978). Gilmour, Angus and Sharp (1980) described a method for infecting sheep i.v. with P. haemolytica biotype T incorporated in rabbit fibrin or agar emboli. This procedure resulted in pulmonary infections with embolic lung lesions, bacterial multiplication and the death of a number of animals. Infection and death could be achieved with as few as $10^{7.9}$ organisms although there was no similarity between the experimentally induced lesions and those in the natural disease.

It is known that biotype T strains of P. haemolytica are normal inhabitants in the tonsils of adult animals and in the nasal cavity of young lambs (Gilmour et al., 1974; Shreeve and Thompson, 1970). The question of how this microorganism changes to become pathogenic and the method by which it spreads between animals and within the individual animal are not yet answered and the mechanism of infection in the natural disease is still obscure. It is therefore appropriate to try other methods of inducing T biotype disease experimentally in sheep.

AIMS OF THE INVESTIGATION.

Experimental work on the pathogenic mechanisms of P. haemolytica has made progress in the last 10 - 15 years but much still remains to be understood. This is particularly true for T biotypes as there is still no satisfactory means of producing the disease experimentally.

The purpose of the experimental work to be presented and discussed in this thesis was to study some aspects of the pathogenesis of ovine pasteurellosis. Opportunity was afforded to study lung tissue from animals experimentally infected with single A serotypes of P. haemolytica. This material was made available by colleagues in the Institute undertaking vaccine challenge experiments and the author is indebted to them for their cooperation in this respect. In addition, attempts were made to test the hypothesis of Dyson et al. (1981) by establishing infection in the tonsils of young and adult sheep.

A recent approach to investigation of the pathogenicity of bacteria such as Escherichia coli and Vibrio cholerae has involved study of the tissue localization of the microorganisms using immunohistological methods (Drees and Waxler, 1970 ; Schrank and Verway, 1976). As no such study has been reported for P. haemolytica, this procedure was adopted to investigate the tissue distribution of P. haemolytica in both the systemic and

pneumonic forms of pasteurellosis. For this purpose it was essential to be able to detect and differentiate between individual serotypes of P. haemolytica lodged in tissues. The standard method of serotype identification by isolation and IHA testing was clearly inappropriate for this type of work. Immunofluorescence has been the most widely used biological tool for this type of study. Hence, part of this thesis describes the production and testing of serotype-specific antisera and their application in immunofluorescence procedures by which different serotypes of P. haemolytica could be revealed in situ in tissue sections.

The serotypes selected for study were those most commonly associated with cases of natural disease. For pneumonic pasteurellosis they were serotypes A1, A2 and A6, while for the systemic disease serotypes T3, T4 and T10 were studied.

GENERAL MATERIALS AND METHODS.

Bacteriological Techniques

(a) Pasteurella haemolytica serotypes

All known serotypes and one untypable strain of Pasteurella haemolytica were used in this study. P. haemolytica biotype A strains were isolated from cases of ovine pneumonia and septicaemia in lambs and biotype T strains were derived from cases of systemic infection in sheep. They were stored either freeze-dried or in 0.5 ml aliquots of broth culture at -70°C. Table 2 provides information about the origin of these strains.

(b) Methods of cultivation

Either a freeze-dried ampoule or an aliquot from -70°C was inoculated into nutrient broth (NB)* in a volume suitable for the purpose of the experiment or preparation to be made. The culture was streaked out on to sheep blood agar (Oxoid blood agar base with 5% citrated ovine blood) and incubated overnight at 37°C. When pure homogenous growth cultures were obtained a few colonies were inoculated into NB and the IHA test was applied to check serotype purity. Only when these screening procedures had been satisfactorily concluded was the organism used as inoculum for further cultures.

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* Oxoid Ltd., Basingstoke, Hampshire, England.

Table 2. Origin of ovine *P. haemolytica* strains used in the study.

Serotype	Reference No.	Tissue	Source*
A1	Q478	Lung	MRI.
	S130A	Lung	MRI.
A2	Q641	Lung	SB.
	S530	Lung	MRI.
T3	M430A	Lung & liver	SB.
	S329A	Brain	SB.
	M30A	Lung	MRI.
T4	Q141	Lung	SB.
	L655/61	Lung	HUK.
	L647	Lung	MRI.
A5	R341B	Nasal swab	MRI.
A6	Q146	Lung	SB.
A7	K115	Lung	SB.
A8	M368	Lung	SB.

Table 2 (contd.) Origin of ovine P. haemolytica strains used in the study.

Serotype	Reference No.	Tissue	Source*
A9	D1127	Lung	B.
T10	M430B	Lung, liver & spleen	SB.
	M30B	Tonsils	MRI.
	M424B	Lung	SB.
A11	L457	Tonsils	SB.
A12	N498	Lung & trachea	MRI.
A13	N513/3163	?	E.
A14	N513/2916	?	E.
U	E3939	Pericardial fluid	N.

* MRI : Moredun Research Institute.

SB : St. Boswells Veterinary Investigation Laboratory.

B : Bangor Veterinary Investigation Laboratory.

N : Newcastle Veterinary Investigation Laboratory.

HUK : Hoescht (U.K.).

E : Ethiopia.

U : Untypable.

(c) Bacterial count

The numbers of viable organisms in suspensions were measured by the method of Miles and Misra (1938). Ten-fold dilutions were made in peptone water (Oxoid) and duplicate 20 µl samples of these dilutions spotted on to sheep blood agar plates. Viable counts were read after incubation overnight at 37°C.

(d) Strain of Mycobacterium butyricum

The strain of Mycobacterium butyricum was kindly supplied by the National Collection of Type Cultures, London (NCTC No. 333) as a freeze-dried ampoule. Nutrient broth (0.5 ml) was added to the ampoule contents and the suspension was subcultured on glycerol Dorset-egg and Lowenstein-Jensen slopes and incubated for 7 days at 37°C. Growth of the organism was visible 3-5 days after sub-culturing. Cultures of the organism were stored at 4°C and used when required.

Antigens and Vaccines

In addition to live organisms two other types of antigen were used.

(a) Sodium salicylate extract (SSE)

Preparation of SSE of P. haemolytica used as antigen in raising hyperimmune sera against serotypes A1, A2, A6, T3, T4 and T10 in laboratory animals was performed according to the method of Wells, Gilmour, Burrells and Thompson (1979). Briefly, bacterial

cells recovered from 6-hour broth cultures were suspended in sterile 1.0M sodium salicylate and agitated for 3 hours at 37°C to strip the outer layers from the cells. Centrifugation (28,000g) was used to remove the cell bodies and then to clarify (40,000g) the supernatant before it was dialysed for 48 hours against phosphate buffered saline (PBS), pH 7·4 (Appendix 1). After concentration (10 - 20 fold) by ultrafiltration the SSE was freeze-dried for storage.

The reactivity of SSE prepared from the individual P. haemolytica serotypes was assessed by IHA using a double dilution method and microtitre plates. Dilutions of SSE were made in test tubes with formol buffered saline (FBS) (Appendix 1). Washed B RBC, made up to 5% suspension (v/v), were added to all the dilutions to give a final concentration (v/v) of 0.05%. Following incubation for 30 min. at 37°C to allow adsorption of the antigen, the RBC's were washed 3 times in FBS and made up to original test tube volumes and 0.025 ml of each dilution with the sensitized RBC's was added to the same volume of a standard antiserum in a microtitre plate. Plates were allowed to stand at room temperature for 2 hr before the haemagglutination reactions were read.

(b) Heat-killed organisms (HKO)

P. haemolytica serotypes were grown in 1·5 litre bottles of NB prewarmed overnight at 37°C. These were left for 6 hours on the orbital shaker in an incubator at 37°C. Organisms were

pelleted by centrifugation and resuspended in a minimal volume of PBS pH 7·4 and incubated at 60°C in a water bath for 90 min. The preparation was then checked for sterility and freeze-dried.

(c) Vaccine preparation

In several experiments an emulsion of antigen with oil adjuvant was required to make the vaccine. All vaccines were prepared immediately before the start of experiments.

A suspension of 2 ml SSE of each individual serotype of *P. haemolytica* was mixed with an equal volume of Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA). A water-in-oil emulsion was made by forcing small amounts of the antigen below the surface of the adjuvant with a needle and syringe. This procedure was continued and followed by homogenization of the emulsion until the correct consistency was obtained. The emulsion was tested by allowing a drop to fall on the surface of water in a wide mouthed container. If the drop remained discrete and did not disperse on the surface of the water it was considered that the prepared vaccine was a stable water-in-oil emulsion.

Serological and Immunohistological Techniques

The following procedures were used to assay the specificity and potency of antisera produced in laboratory animals. Some of the tests were also used to measure the antibody response of sheep to experimental infection.

(a) Indirect haemagglutination (IHA) test

An IHA test has been used widely to assess the efficacy of immune sera prepared by various P. haemolytica vaccines in different animals. The procedure followed was that of Shreeve et al. (1972). The test was run in microtitre plates in which 0.05 ml of undiluted serum was added to the first well and serial two-fold dilutions made in FBS. The same volume of B RBCs sensitized with HKO antigen was added to each well and results read after standing for 2 hr at room temperature and again after holding overnight at 4°C. Titres were expressed as the highest dilution giving clear agglutination of RBCs.

(b) Immunodiffusion (ID) test

This was carried out in a similar manner to the procedure described by Ouchterlony (1953). For testing rabbit and mouse sera the ID test was done with a variety of well patterns in 1% agarose (B.D.H.)* or purified agar (Oxoid) dissolved in PBS and layered in 10 - 11 ml volumes on to glass plates (8 x 8 cm) or Petri dishes. For testing chicken sera, agar containing 8% NaCl was used (Aitken and Parry, 1974). Plates were held in a humid box at room temperature for 24 - 48 hr. Slides or plates were then washed with distilled water and dried under damp filter paper

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* B.D.H. Chemicals Ltd., Poole, England.

at 37°C overnight. The dried agar slides were covered with Coomassie blue stain (Appendix 1) for 2 min. and then decolorized by two changes of methanol-glacial acetic acid (9:1) over 4 min.

(c) Counter-immunoelectrophoresis (CIE) test

Agar plates were prepared as previously described for the ID test with 1% agar dissolved in 0.05M barbital Ca-lactate buffer pH 8.6 (Appendix 1). A standard row pattern of wells was cut. Sera were added to the bottom set of wells and antigens applied to the upper row. A Shandon* electrophoresis unit (model 077) was used for electrophoresis. Runs were carried out for 1 hr at a constant voltage of 100v.

(d) Indirect immunofluorescence (IIF) test on bacterial smears

Clean glass microscope slides were used to prepare bacterial smears by the method of Murcia and Rubin (1979). Slides with 8 wells, each of approximately 5mm diameter, were prepared by placing 8 discrete uniform drops of glycerol on to each slide, using a standard pipette, and then spraying the slides with a fluorocarbon bonding agent (Fluoro Glide).** Once the fluorocarbon had set the slides were rinsed with tap water to remove the glycerol and then air dried.

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* Shandon Scientific Co. Ltd., London, U.K.

** Chemplast Inc., Wayne, New Jersey, U.S.A.

All fourteen P. haemolytica serotypes and an untypable strain were grown overnight at 37°C on sheep blood agar plates. Colonies were removed from the agar surface by rinsing with PBS pH 7·4 and the various serotype suspensions were standardized to Brown's opacity tube 2. The wells on the coated slides were filled with single serotype suspensions of P. haemolytica using a bacteriological loop. Slides were air dried, fixed in cold acetone (4°C) for 10 min. and then stored at 4°C until tested. Antisera were double diluted in PBS pH 7·4 to a final dilution of 1:2048 using a compu-pet.* One drop of the appropriate dilution was added to consecutive wells and the slides incubated in a moist chamber for 30 min., after which three 10 min. washes in PBS were carried out. This was followed by further treatment for 30 min. with the appropriate fluorescein-conjugated anti-immunoglobulin at recommended working dilutions. After repeated washes in PBS 0.05% Evans blue was applied as a counterstain for 50 seconds to reduce background fluorescence (Tessler, Stone and Page, 1979). Slides were washed again in PBS and examined with a Leitz Orthoplan UV Microscope immediately after mounting in buffered glycerol. In all IIF tests negative and positive controls as well as blocking reagents were used for purposes of comparison.

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* Div. Warner Lambert Co., Morris Plains, U.S.A.

(e) Immunoperoxidase (IPO) test on bacterial smears

The procedure used was essentially the same as the IIF test. Bacterial smears of P. haemolytica serotypes were prepared as described above. Antisera were diluted as before and one drop of the appropriate dilution used in each well. Slides were placed in a humid chamber for 90 min. and then washed in 3 changes of PBS each of 10 min. duration. The peroxidase-labelled antispecies antiserum was added and slides returned to the humid chamber for 90 min. Following three 10 min. washes in PBS the slides were stained for 5 min. with 3-amino-9-ethylcarbazole (Graham, Lundholm and Karnovsky, 1965) or with 3-3-diaminobenzidine (Straus, 1964). Cover-slips were applied and slides examined with an ordinary light microscope.

(f) Indirect immunofluorescence (IIF) test on tissue sections

Infected tissues were examined by the method of Sainte-Marie (1961). Blocks were prepared from ethanol-fixed tissues, dehydrated in absolute alcohol, treated with xylene and embedded in paraffin wax at 56°C. Sections 3-4 μm thick were dewaxed in xylene and alcohol and hydrated in PBS, and treated with primary and conjugated antisera in the same way as bacterial smears. When not in use blocks and sections were stored at 4°C. Sections were examined under UV light after mounting in buffered glycerol.

Histopathological Techniques

Samples of tissues were taken from experimental animals subjected to necropsy. The samples were mainly of lungs and tonsils but on occasions pieces of liver, kidney, spleen and oesophagus were also taken. All were fixed in 10% formal saline, dehydrated through ascending grades of ethyl alcohol, cleared in chloroform and embedded in paraffin wax. Sections were cut at 4-6 μm thickness and stained routinely by Mayer's acid alum haematoxylin and eosin (Carleton, 1957). Selected sections were stained by a special staining technique for iron according to Carleton (1957).

Laboratory Animals

Three species of laboratory animal were used in these studies.

(a) Mice

The two strains of mice used, Swiss albino mice and C57 black mice, were bred at the Moredun Research Institute. Mice of both sexes were used and were at least 3 - 4 weeks old at the start of each experiment. They were fed commercial Rat and Mouse Diet* ad libitum.

(b) Rabbits

New Zealand White rabbits, 7 - 9 months old were used. They were all females and received a standard rabbit diet.

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*Oxoid Ltd., Basingstoke, Hampshire, England.

(c) Chickens

Leghorn type chickens aged 5 weeks were obtained from the Royal (Dick) School of Veterinary Studies, Edinburgh. During the experimental period they were fed a standard grower's diet.

All the laboratory animals were maintained in caged accommodation in the Institute's small animal unit.

Sheep

Different ages and breed of sheep were used to investigate the pathogenesis of pasteurellosis. Details are given in the relevant chapters.

CHAPTER 1.

THE OCCURRENCE OF T SEROTYPES OF P. HAEMOLYTICA IN THE TONSILS OF LAMBS.

Introduction

The circumstances under which systemic pasteurellosis of feeder lambs arises are not clear but biotype T strains of P. haemolytica are held to be responsible for the disease (Smith, 1961). In an epidemiological study based on nasal swabs Biberstein and Thompson (1966) isolated only a few biotype T strains and considered them to be selectively pathogenic in contrast to biotype A strains which they encountered commonly as commensals rather than as pathogens. Subsequent work (Gilmour et al., 1974) showed that biotype T strains are localised in the tonsils, rather than in the nasal cavity of healthy adult sheep and thus these organisms cannot be regarded as selective pathogens.

Studies of the nasal carriage of P. haemolytica in lambs showed that a range of serotypes, both A and T, could be isolated, the number of serotypes carried by individual animals increasing with age (Shreeve and Thompson, 1970). However, in that study no swabs were taken from tonsils, the tissue for which T biotypes seem to have a preference. It was deemed of importance to obtain this information to assist further studies on the pathogenesis of T serotype disease. Knowledge was needed of the age at which T

serotypes can first be recovered from the tonsils of lambs and the relationship of organisms isolated to those carried by the dams or other in-contact ewes.

This chapter is concerned with the isolation and serotyping of P. haemolytica from the tonsils of clinically healthy young lambs from birth to 12 weeks of age.

Materials and methods

Animals : In a preliminary investigation lambs were used as they became available throughout the months of February, March, April and May 1980. All were born at the Institute and reared with their dams and with other in-contact ewes and lambs.

In an experiment subsequently undertaken to obtain more information on the age at which P. haemolytica could first be isolated from the tonsils of lambs, 28 ewes and 37 lambs were used. The group contained 25 Finnish Landrace ewes and 3 Dorset Horn ewes and their cross-bred progeny. They were housed together as one group in an open yard.

Sampling : Standard procedures were used for collecting nasal and tonsillar swabs for bacteriological study from healthy lambs and in-contact adult sheep. In addition, tonsils were extracted from lambs which died or were killed by pentobarbitone in the course of unrelated experimental work (e.g. enzootic abortion and

hypothermia) and placed into sterile universal bottles or plastic bags.

For the sequential study tonsillar swabs were taken every 1 - 3 weeks on 6 successive occasions.

Isolation and typing of P. haemolytica : Tonsils removed from dead lambs were macerated aseptically by means of a 'Colworth stomacher' in a sterile bag containing 10 ml NB. One ml of each tonsillar suspension was inoculated on to a 7% sheep blood agar plate and a further 1 ml added to 10 ml NB. Tonsillar swabs taken from live lambs were smeared over half a blood agar plate then placed in NB.

The plates and NB bottles were incubated at 37°C for 18 hr and then examined. If primary plate cultures were negative the NB was subcultured to blood agar. From positive blood agar plates colonies morphologically resembling P. haemolytica were transferred to NB, incubated overnight and the serotype determined by the IHA test (Shreeve et al., 1972). Fermentation tests were carried out when necessary, especially with untypable strains, to confirm the characteristics and identification of P. haemolytica.

Results

Preliminary investigation : In this chapter the results given are merely a summary of the findings in the examination of tonsillar

swabs and homogenates from individual lambs for the isolation of P. haemolytica. The detailed findings are laid out in Appendix 2.

In this investigation P. haemolytica was isolated from 51% of the animals examined (Table 3). Even in lambs under 1 day of age one third of those sampled were found to carry the organism in the tonsils. A similar isolation rate was achieved in lambs up to 4 days old, while all animals over that age yielded P. haemolytica either from tonsils (lambs) or nasal swabs (ewes). In most cases (32/39) only a single serotype or untypable strain was recovered and in no case were more than two serotypes isolated from the same individual. In addition to untypable strains only 5 serotypes were recovered and of these A11 was isolated most frequently. Surprisingly, the sole T serotype isolated was T10 and that only in 5 instances from the tonsils of three 6-week old lambs and from the nasal swabs of two adults. In lambs under 1 week of age the most common isolation was of untypable strains although both A2 and A11 were recovered from a few animals. All 14 untypable isolates from these lambs fermented arabinose but not trehalose and so may be regarded as biotype A organisms.

Table 4 shows the prevalence of biotypes A and T and of untypable strains isolated from the extracted tonsils of lambs up to 7 weeks of age.

Table 3. Serotypes of P. haemolytica isolated from tonsils of lambs and nasal cavities of ewes.

Sample	Animals			Frequency of serotype isolation						Infection	
	Age	No. sampled	No. positive	A2	A7	A9	A11	T10	U	Single	Plural
Tonsil tissue	1d*	25	8	-	-	-	1	-	7	7	1
Tonsil tissue	1 - 4d	31	11	1	-	-	4	-	7	10	1
Tonsil tissue	1 - 7w**	7	7	-	1	-	6	1	2	4	3
Tonsil swabs	6 - 7w	7	7	-	2	2	2	2	2	5	2
Nasal swabs	adult	6	6	-	-	2	2	2	2	6	0

* days

** weeks

Table 4 : Prevalence of biotype A and T and untypable strains of P. haemolytica in extracted tonsils of lambs.

No. of animals examined	No. positive	No. (and percentage) of animals with		
		biotype A	biotype T	U*
63	26	13 (50)	1 (4)	16 (62)

* untypable

The 6 adults studied were the dams of the 7 lambs from which tonsillar swabs were taken and it may be noted in Table 5 that these swabs yielded two serotypes, A7 and T10, which were not recovered from the nasal swabs of their dams.

Further investigation : The results of the preliminary study indicated that lambs can become infected with P. haemolytica at a very early age and that the number of infected animals increases with age. To expand the information gained a further investigation was carried out in which tonsillar swabs were taken on six occasions from a group of 28 ewes and their 37 lambs between birth and 12 weeks of age. Details of the isolation of P. haemolytica from each animal at every swabbing are given in Appendices 3 and 4.

Lambs : P. haemolytica was isolated from the tonsils of all the lambs surveyed in this investigation on at least one of the 6 sampling times. The number of lambs yielding positive swabs at

Table 5. Isolations of P. haemolytica serotypes by nasal swabbing of 6 ewes and tonsillar swabbing of their 7-week old lambs.

Ewe No.	Isolate	Lamb No.	Isolate
1	U	1	U, A11
2	U	2	A7
3	A9	3	T10
4	A9	4	A9, T10
5	A11	5	A7
		6	A11
6	A11	7	U, A9

each sampling time is given in Table 6 and the serotype specificity of the isolates recovered is illustrated in Figure 1.

At the first sampling time (1 - 3 days of age) tonsillar swabs from 14 lambs were positive, rather fewer were positive at 3 weeks of age but the numbers increased again at 6 weeks. By 9 and 12 weeks of age the tonsils of half the lambs in the group were found to harbour P. haemolytica. The frequency with which P. haemolytica could be isolated from individual lambs throughout the period of sampling is indicated in Table 7. The majority (83%) of lambs yielded P. haemolytica on 1, 2 or 3 occasions and from only 6 lambs was the bacterium recovered more frequently.

Altogether, 88 (40%) of the 222 swabs collected during the survey were positive for P. haemolytica, with more than one serotype being recovered from 24 of the 88 positive swabs (Table 8). Plural isolations were made from all ages of lambs but were most common in 12-week old lambs. Most plural isolations involved 2 serotypes only but 3 serotypes were recovered from 3 individual lambs at one sampling and from a fourth lamb at the 5th and 6th samplings.* Of the 117 isolates made from the 88 positive swabs, 70 (60%) were of biotype T, 31 (26%) were of biotype A and 16 (14%) were untypable.

From Figure 1 it can be seen that untypable strains were particularly evident in lambs of 1 to 3 days of age, were still

* see Appendix 4

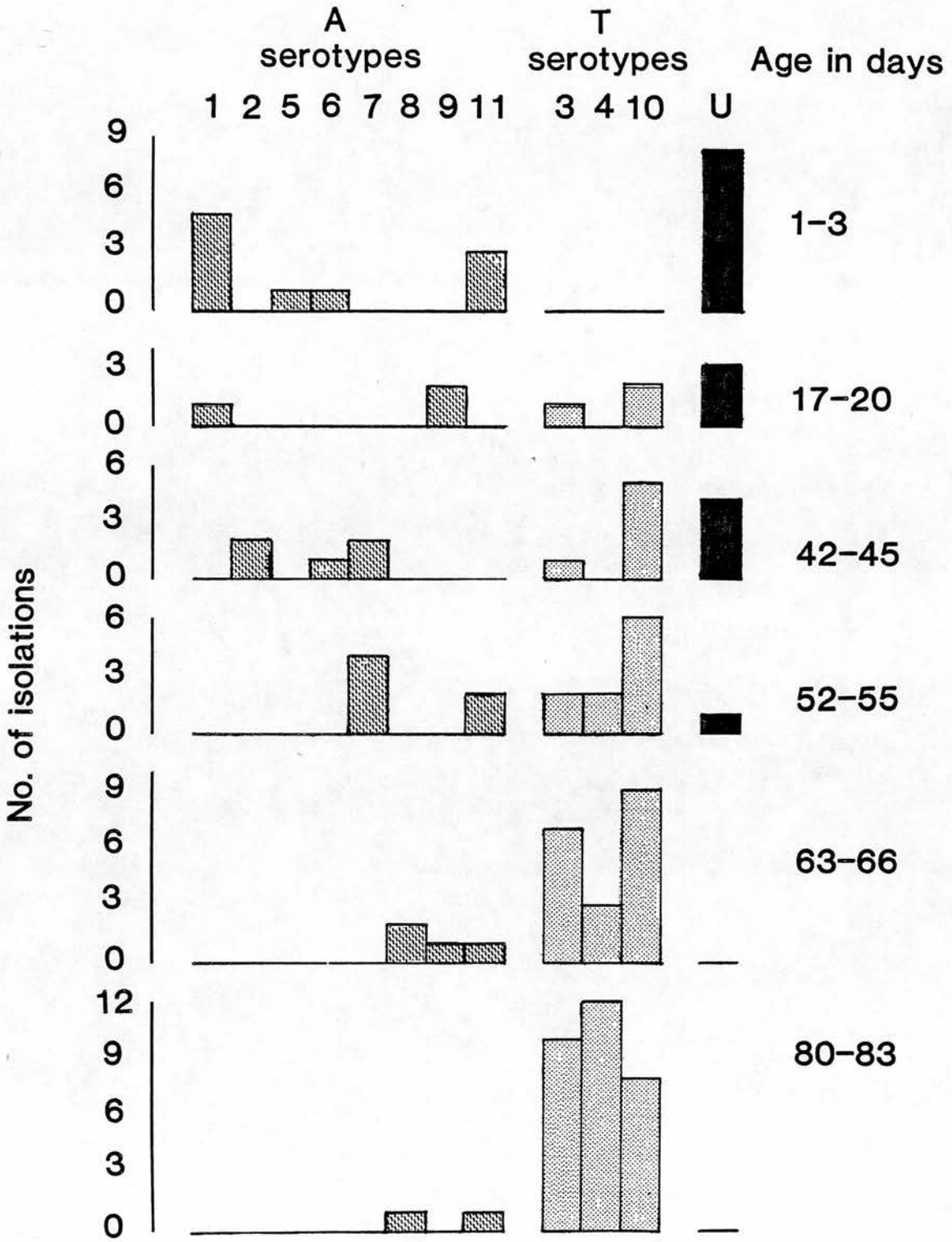
A serotypes
1 2 5 6 7 8 9 11
T serotypes
3 4 10
U
Age in days
No. of isolations
1-3
17-20
42-45
52-55
63-66
80-83

Table 6. Number and percentage of lambs yielding positive tonsillar swabs at different sampling times.

	Sample No.					
	1	2	3	4	5	6
Age of lambs (days)	1-3	17-20	42-45	52-55	63-66	80-83
Number positive	14	8	13	15	19	19
Percentage positive	38	22	35	41	51	51

Table 7. Frequency of positive tonsillar swabs from individual lambs over the 6 sampling times.

	Number of positive swabs					
	1	2	3	4	5	6
Number of lambs	9	12	10	5	1	0
Percentage	24	32	27	14	3	0

Table 8. Number of lamb tonsillar swabs yielding single or plural isolates of P. haemolytica at each sampling time.

Sample No.	Age of lambs (days)	Isolates		
		Single	Plural (2)	Plural (3)
1	1 - 3	11	2	1
2	17 - 20	7	1	-
3	42 - 45	10	3	-
4	52 - 55	14	1	-
5	63 - 66	16	2	1
6	80 - 83	6	10	3
Total		64	19	5

recoverable at 3 and 7 weeks of age but not thereafter. In contrast, T serotypes were absent from the tonsils during the first few days of life but were isolated with increasing frequency between 3 and 12 weeks of age, although it was noticeable that T4 was not detected in lambs under 7 - 8 weeks old. Biotype A serotypes were present from birth and were isolated at every sampling time but without consistency in the serotypes isolated. For example, A1 was found in 5 lambs at the first sampling but was never recovered from lambs over 3 weeks of age, A8 was not isolated before 9 weeks of age while A11, the commonest isolate, was not recovered at 3 or 6 weeks of age.

Examination of the 11 untypable strains isolated from lambs up to 3 weeks of age and 2 of 4 strains from six-week old lambs were adjudged by arabinose fermentation to be A biotypes. The 5 other untypable isolates of lamb origin fermented neither arabinose nor trehalose. The single untypable isolate from ewe tonsils fermented trehalose but not arabinose.

Ewes : Isolations of P. haemolytica were readily made from 25 of the 28 adult animals used in this survey. However, the number of animals positive at any one sampling varied between 6 (21%) and 19 (68%) with the lower frequency of isolation occurring at the later sampling times (Table 9). It should be noted that P. haemolytica was not recovered from 3 ewes in the survey, but the majority (65%) yielded P. haemolytica on 1, 2 or 3 occasions (Table 10).

Table 9. Number and percentage of ewes yielding positive tonsillar swabs at different sampling times.

	Sample No.					
	1	2	3	4	5	6
No. positive/No. sampled	19/28	9/26	16/24	7/28	8/28	6/28
Percentage positive	68	35	67	25	29	21

Table 10. Frequency of positive tonsillar swabs from individual ewes over the 6 sampling times.

	Number of positive swabs						
	0	1	2	3	4	5	6
No. of ewes	3	3	8	7	4	3	0
Percentage	11	11	29	25	14	11	0

Figure 2 outlines the serotype specificity of the P. haemolytica isolates. It is evident that T serotypes were isolated much more frequently than either A serotypes or untypable strains. The actual number of isolations made was 93 T serotypes (81%), 21 A serotypes (18%) and 1 untypable strain (1%). In total 65 (40%) of the 162 swabs collected were positive and the numbers of single and plural isolations were comparable (Table 11). It was unusual to isolate more than 2 serotypes from any one swab but on 3 occasions triple isolations were made. A single ewe from which only a single serotype had been recovered at earlier swabbings yielded 4 serotypes at the 5th swabbing and reverted to a single serotype at the final swabbing.

When isolations made from individual ewes and their lambs over the 12 week period were considered it was found that 14 individual lambs yielded serotypes which were at no time recovered from their dams, 9 lambs gave serotypes identical to those isolated from their mothers and 14 lambs had serotypes of each category. Within the group there were 7 sets of twins and 4 sets of triplets. Non-identical serotypes were recovered from siblings in 4 pairs of twins and 1 set of triplets. Individuals within the other twin and triplet units shared one or two serotypes.

Over the whole time period seven A serotypes, three T serotypes and a single untypable strain were isolated from ewes. No obvious trend was apparent in the profile of serotype isolations

Fig. 2 : Histogram showing the frequency of P. haemolytica serotypes isolated on 6 different occasions from ewes. Note that no isolations were made of serotypes A6, A12, A13 or A14.

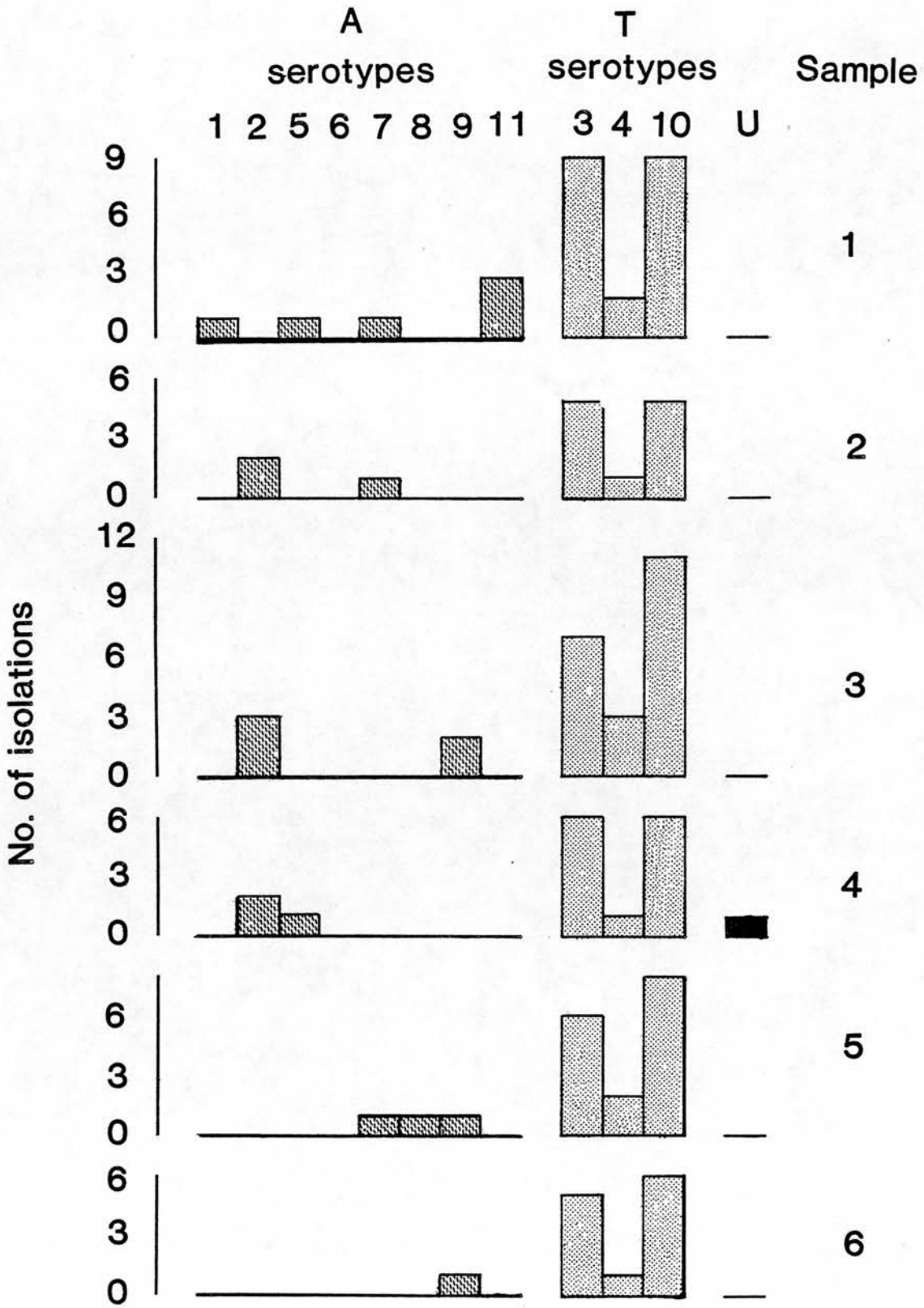
A
serotypes
1 2 5 6 7 8 9 11
T
serotypes
3 4 10
U
Sample
No. of isolations
9
6
3
0
1
6
3
0
2
12
9
6
3
0
3
6
3
0
4
6
3
0
5
6
3
0
6

Table 11. Number of tonsillar swabs from ewes yielding single or plural isolates of P. haemolytica at each sampling time.

Sample No.	Isolates			
	Single	Plural (2)	(3)	(4)
1	13	5	1	-
2	5	4	-	-
3	6	10	-	-
4	4	3	-	-
5	2	4	1	1
6	2	3	1	-
Total	32	29	3	1

at different swabbings (Fig. 2). The only serotype isolated from lambs which was not recovered from ewes was A6. This serotype was present in 2 unrelated individual lambs at under 1 week and at 6 weeks of age respectively. At the first swabbing untypable strains and A serotypes predominated but with successive swabbings their relative numbers increased. The first recovery of T serotypes was at 3 weeks, thereafter the frequency of their isolation increased and from 8 weeks onward they represented more than 50% of the isolations made (Fig. 1). The frequencies with which each individual serotype was isolated from lambs or ewes are given in Table 12. It is evident that T serotypes were the commonest isolates in each age group.

Discussion.

As a means of recovering bacteria from animal tissues swabbing has the major limitation that only those organisms on the surface being swabbed are likely to be isolated. Thus a negative result does not exclude the possibility that the organisms being sought were present within the tissue at sites not accessible by standard swabbing procedures. Conversely, removal of the entire tissue or a major portion of it followed by culture of the macerated tissue makes it highly probable that the organism, if present, will be detected. It was therefore reassuring that in the two investigations the isolations of P. haemolytica made from macerated tonsillar tissue and by tonsillar swabbing of live lambs were generally similar. By both procedures P. haemolytica was

Table 12. Frequencies of isolation of individual serotypes of *P. haemolytica* from the tonsils of lambs and ewes, expressed as percentages of total isolations from each age group.

Serotype	Lambs	Ewes
A1	5.4	1.0
A2	2.7	7.1
A5	0.9	2.0
A6	0.9	-
A7	6.4	3.0
A8	0.9	1.0
A9	1.8	2.0
A11	6.4	5.1
T3	20.0	33.0
T4	11.8	8.1
T10	28.2	36.4
U*	14.5	1.0

* untypable

recoverable from one third of lambs under 4 days of age (Tables 3 and 6) and the range of serotypes encountered was similar in so far as untypable strains of A biotype were the most common isolates and no T serotypes were recovered (Table 3 and Fig. 1). It can be assumed therefore, that findings obtained by swabbing do indicate the P. haemolytica carrier status of lamb tonsils.

From the results of both the preliminary and follow-up investigations it is clear that the tonsils of some (approximately 35%) lambs are susceptible to colonization by P. haemolytica shortly after birth. The factors which influence successful colonization at this early age are not known but it may be noted that carrier status was not consistent between individual sibling lambs, a fact which could argue against any inhibitory effect of passive immunity. Also of note was the predominance of untypable strains of A biotype and of A serotypes in lambs sampled during the first week of life. This suggests a preferential colonization at an age when fatal septicaemic disease due to A serotypes commonly occurs.

The first recovery of T serotypes from tonsils in 3-week old lambs (Fig. 1) parallels the finding of Shreeve and Thompson (1970) that T biotypes could not be recovered from the nasal passages of lambs until the fourth week of life. Taken together these observations imply delayed colonization by T serotypes. Why this should be so is not known. Passive immunity is unlikely

as conventional ewes have only low serum antibody titres to T serotypes of P. haemolytica (Gilmour, 1978) but the antibody status of ewe's milk has not been examined. Alternatively, competition by an already established oro-pharyngeal flora could limit colonization by T serotypes. The element of selectivity which permits establishment of biotype A untypable strains and of A serotypes could operate via a biotype specific surface receptor mechanism. This could be an interesting area for further study and might reveal receptor differences between the two biotypes of P. haemolytica.

As the lambs increased in age colonization of the tonsils by T serotypes increased from low levels at 3 weeks of age to predominance at 12 weeks (Fig. 1). The more frequent isolation of T serotypes was accompanied by a progressive loss of untypable strains and A serotypes. As no similar trend was evident in samples taken from ewes (Fig. 2) it is unlikely that the changing pattern of serotype isolation was a seasonal phenomenon such as occurs in the prevalence of A and T biotypes isolated from cases of ovine pasteurellosis (Fraser, Gilmour, Laird and Donachie, 1982). Thus it is probably a true age effect. It would seem likely that between 3 months and maturity there is again some increase in colonization of tonsils by A serotypes as they generally represent 25 - 30% of isolates from adult tonsils (Gilmour et al., 1974).

P. haemolytica was isolated from only 40% of all the lambs swabbed in this investigation (Table 8) compared to 95% isolation achieved by Gilmour et al. (1974) in their survey of adult sheep. These workers, however, were examining separated sheep heads collected from an abattoir and were able to remove entire tonsils for cultural studies. Nonetheless, in the study reported here, isolations were being made from 50% of 9-and 12—week old lambs and in ewes up to 68% of samples taken at one swabbing were positive (Table 9).

In their study of nasal carriage of P. haemolytica by lambs Shreeve and Thompson (1970) concluded that the number of serotypes carried by individual animals increased with age over the 6-week period which they studied. A similar trend was evident in isolations made from the tonsils of the 37 lambs in the follow-up investigation but not until the final swabbing at 3 months of age (Table 8). In ewes, on the other hand, the number of plural isolations made was variable over the 6 successive swabbings (Table 11).

In both lambs and ewes between 4 and 6 different serotypes and untypable strains were recovered at separate swabbings (Figs. 1 and 2), which is rather more than were isolated at any one time by Shreeve and Thompson (1970). Over the whole period the numbers of different serotypes isolated were 11 and 10 for lambs and ewes respectively, together with untypable strains and again

this exceeded the 7 different serotypes isolated by Shreeve and Thompson (1970). However, the occurrence of a broad spectrum of P. haemolytica serotypes within healthy sheep flocks was reported by Biberstein et al. (1970) who detected between 5 and 10 different serotypes in 7 flocks studied. These workers also noted a narrowing of the spectrum as the nasal carriage rate within flocks increased, as it did in a cyclic fashion not correlated to the health status of the flock or to any definitive environmental influence. A similar phenomenon was encountered in association with outbreaks of pasteurellosis when single serotypes tended to predominate (Shreeve et al., 1972). Though speculation on the basis of fluctuating serotype predominance was offered the reasons for it are still unresolved.

As previously postulated by Shreeve and Thompson (1970) the most probable source of infection for very young lambs is the frequent and intimate contact with their mothers at that age. Lamb-to-lamb spread is also likely as the lambs begin to mingle as a group. However, variation in the nature and number of serotypes isolated was so great that no obvious patterns of infection could be detected. The basis of the changing serotype profile in individual lambs is unknown. In recent experiments in SPF lambs in-contact transmission of P. haemolytica serotype A2 was achieved within 5 days of exposing recipients to infected donor lambs (N.J.L. Gilmour, personal communication).

Conclusions.

Though the two surveys undertaken were based on relatively small numbers of animals, three firm conclusions are drawn from the results obtained.

(1) Under natural conditions the tonsils of lambs can be colonized by P. haemolytica within a very short time of birth.

(2) Initially tonsils are preferentially colonized by untypable strains of biotype A and by A serotypes.

(3) Colonization of tonsils by T serotypes is first evident at 3 weeks of age. Thereafter T serotypes comprise an increasingly greater proportion of isolates recovered and by 9 weeks are the dominant types of P. haemolytica in that tissue.

CHAPTER 2.

ATTEMPTS TO PRODUCE OVINE SYSTEMIC DISEASE BY TONSILLAR INFECTION WITH T SEROTYPES OF P. HAEMOLYTICA.

Introduction

Several attempts have been made to produce experimentally T serotype systemic disease in lambs resembling the natural disease, but none has been successful (Stamp et al., 1955; Biberstein and Kennedy, 1959; Smith, 1960b; Gilmour et al., 1980).

The site of initial infection and the involvement of intrinsic and extrinsic factors assumed to participate in establishment of the disease remain undetermined. However it has been recognised that occurrence of the disease is often associated with a managemental change and has a seasonal incidence. For the purpose of reproducing the systemic form of pasteurellosis in lambs and achieving an understanding of the pathogenicity of T serotype infection, it is necessary to investigate how the infection originates and where the initial multiplication of the organism occurs. It is the generation of massive numbers of P. haemolytica in tissues which causes the death of the host due to toxaemic shock (Smith, 1959b).

The theory of Dyson et al. (1981) on the pathogenesis of ovine systemic pasteurellosis postulates that the disease begins

when commensal T serotypes of P. haemolytica already present in the tonsils invade the surrounding tissue and are disseminated to other sites either directly via the blood stream or after passage through regional lymphatics. Alternatively, P. haemolytica may be conveyed to the various internal organs by the portal system after invasion of the gut mucosa, though this is less likely on account of the inimical conditions in the gut.

This chapter and the succeeding one are concerned with attempts to develop a means of testing the hypothesis of Dyson et al. (1981). Several approaches were adopted in efforts to bring about conditions appropriate to the establishment of an initiating focus of T serotype infection in the tonsillar region. Essentially these involved disturbance of host-parasite interaction and broadly consisted of either (a) altering local microenvironmental conditions in a manner which might favour the local growth and multiplication of P. haemolytica and so facilitate its spread to other tissues or (b) systemically modifying the host in such a way as to potentiate the infectivity and virulence of P. haemolytica.

The former approaches are described in this chapter and the latter procedures comprise the substance of Chapter 3.

Materials and methods

Preparation of inocula : Freeze-dried cultures of T serotypes of P. haemolytica originally isolated from lambs which had died of a natural systemic infection were grown in shake-flasks of NB for 18 - 24 hr at 37°C. Five ml of each T serotype culture were mixed and centrifuged for 15 min at 5000 g (4°C). The sediment was resuspended in 7.5 ml of PBS, pH 7.4. Initially 1.0 ml, in later experiments 0.5 ml, of the suspension was pipetted into each of 6 sterile graduated test tubes, and from each test tube approximately 0.1 ml was used to infect one lamb by means of a cotton swab. (The volume of bacterial suspension carried by a standard disposable cotton swab* was established in preliminary tests. The procedure adopted as routine involved immersion of the swab in the suspension so that it became fully saturated. The swab was then withdrawn, pressed lightly against the inner wall of the test tube to remove excess suspension and then used to infect a lamb. From the volume of suspension remaining in the test tube the amount carried by the swab could be estimated. Over several trials this volume was found to be close to 0.1 ml).

The method of Miles and Misra (1938) was used to determine the viable count of each individual T serotype in broth culture, and of the mixture of the three T serotypes after suspension in PBS.

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* Exogen Ltd., Clydebank, Scotland.

Method of infection : Animals were infected with the mixed T serotype inocula by applying the soaked swabs to the tonsils and the surrounding pharyngeal tissue, using a gentle rotary motion. A separate swab was used to infect left and right tonsils.

By trial and error it was found that in lambs under 8 weeks old access to the tonsils could readily be afforded by having the upper and lower jaws held apart. In young adult sheep (6 months and older) the use of a variable aperture mouth gag was essential. For animals of both ages a metal spatula was used to depress the tongue. Good illumination of the area was obtained by means of a torch secured to an adjustable head band (Figs. 3, 4a,b).

Abrasion of tonsils : Prior to treatment sheep were sedated by i.v. injection of a 2% solution of Rompun* (which contains Xylazine at a rate of 20 mg/ml) at a dose of 1 mg/kg body weight (b.w.).

Abrasion was carried out using a hard bristled nylon tooth brush** which was rubbed against each tonsil and adjacent area in a rotary fashion. The same brush was used to abrade right

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* Bayer-Levar Kusen

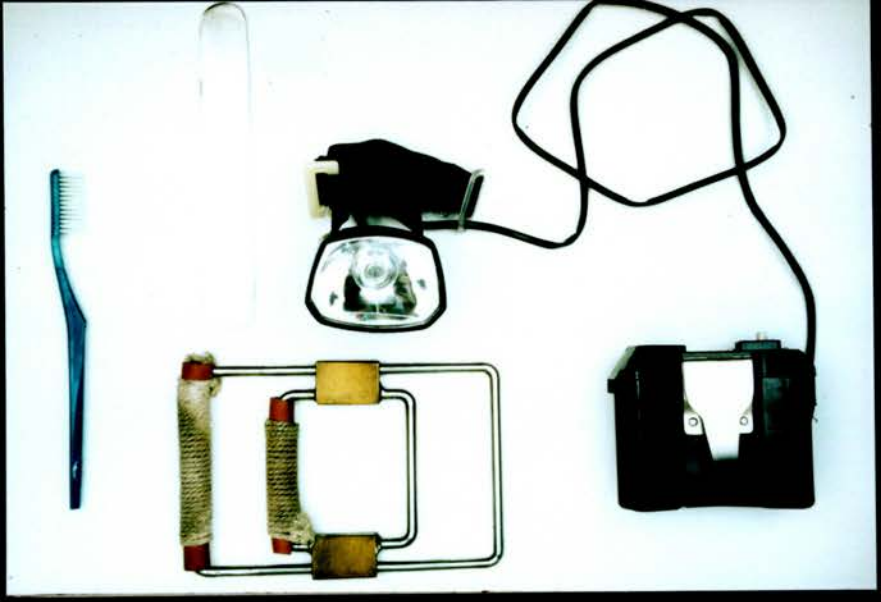
** Jordan, Norway.

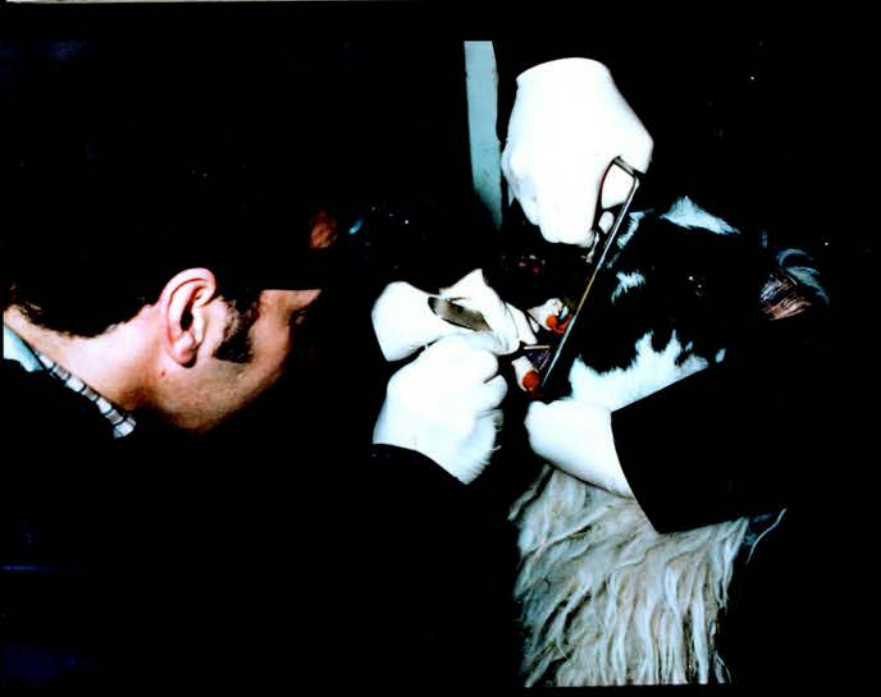
Fig. 3 : Equipment used in abrasion and infection of tonsils. Arrow indicates a metal spatula.

Fig. 4 : The procedure of experimental tonsillar abrasion and infection of sheep.

a : Handling the animal and topical application of the infective inoculum.

b : Tongue depressed with a spatula for easy access to the tonsils.





and left tonsils and the head of the brush was then immersed in 5.0 ml of sterile saline in a universal bottle. The brush was agitated in the saline to release any attached sheets of epithelium and then withdrawn. After centrifugation (5000 g) at room temperature for 5 min. the sedimented cells were used to prepare wet smears on microscope slides which were examined by phase contrast microscopy to verify removal of surface epithelium (Fig. 5).

Before re-use in further experiments each brush was thoroughly washed and then sterilised under UV light.

Bacteriology : In all experiments tonsillar swabbing was undertaken before infection and at days 7, 14 and 21 after infection, or as appropriate, to detect initial commensal P. haemolytica colonization and to assess changes resulting from infection. The methods of isolation and identification of P. haemolytica have been described previously (p.47).

Blood samples were drawn from the jugular vein 24, 48 and 72 hr after tonsillar abrasion and whenever isolation of the organism from blood was being attempted. One ml of blood was spread over the surface of each replicate blood agar plate and 4 ml of the same blood sample were also inoculated into 10 ml NB. Blood plates and NB were incubated for 24 hr at 37°C aerobically and the incubated NB subcultured on blood agar plates after 24 and 48 hr .

Serology : Samples of blood were taken from sheep and lambs before the start of experiments and at weekly intervals to detect any rise in serum antibody titre against the T serotypes using the standard IHA test.

Clinical assessment : All experimental animals were clinically healthy and normal at the start of the experiments. Rectal temperatures were recorded before the start of each experiment and at regular intervals throughout the course of the experiment. Changes in animal behaviour and clinical signs were noted and daily examinations made of the tonsillar-pharyngeal region.

Experiment 1 : The response of lambs to tonsillar infection with mixed T serotypes of P. haemolytica.

This experiment was carried out to investigate the effect of localised infection of the tonsils of 3- and 7-week old lambs with a culture of P. haemolytica containing equivalent numbers of serotypes T3, T4 and T10.

Experimental design : Two groups of Scottish Blackface lambs were used; group 1 consisted of 6 lambs (3 weeks old), 3 of which were infected and 3 were controls. Group 2 consisted of 5 lambs (6 - 7 weeks old), 3 of which were infected and 2 were controls. Both groups were kept separately with their dams. Infection of the tonsils with mixed T serotypes of P. haemolytica was performed as described. The numbers of individual T serotypes per ml of mixed culture were 5×10^9 T3, 3×10^9 T4 and 4×10^9 T10.

Results

Clinical observations : Figs. 6a,b show the temperature response of inoculated and control lambs throughout the course of the experiment. Some of the infected lambs showed a rise in temperature 24 hr after inoculation with signs of distressed breathing and coughing which lasted about 7 days. One lamb (968) in group 2 died 10 days after infection. Only in this animal was the rise in temperature significantly greater than that in controls.

Fig. 6a : Temperature responses of 3-week old lambs to tonsillar infection with T serotypes of P. haemolytica and of uninfected control lambs.

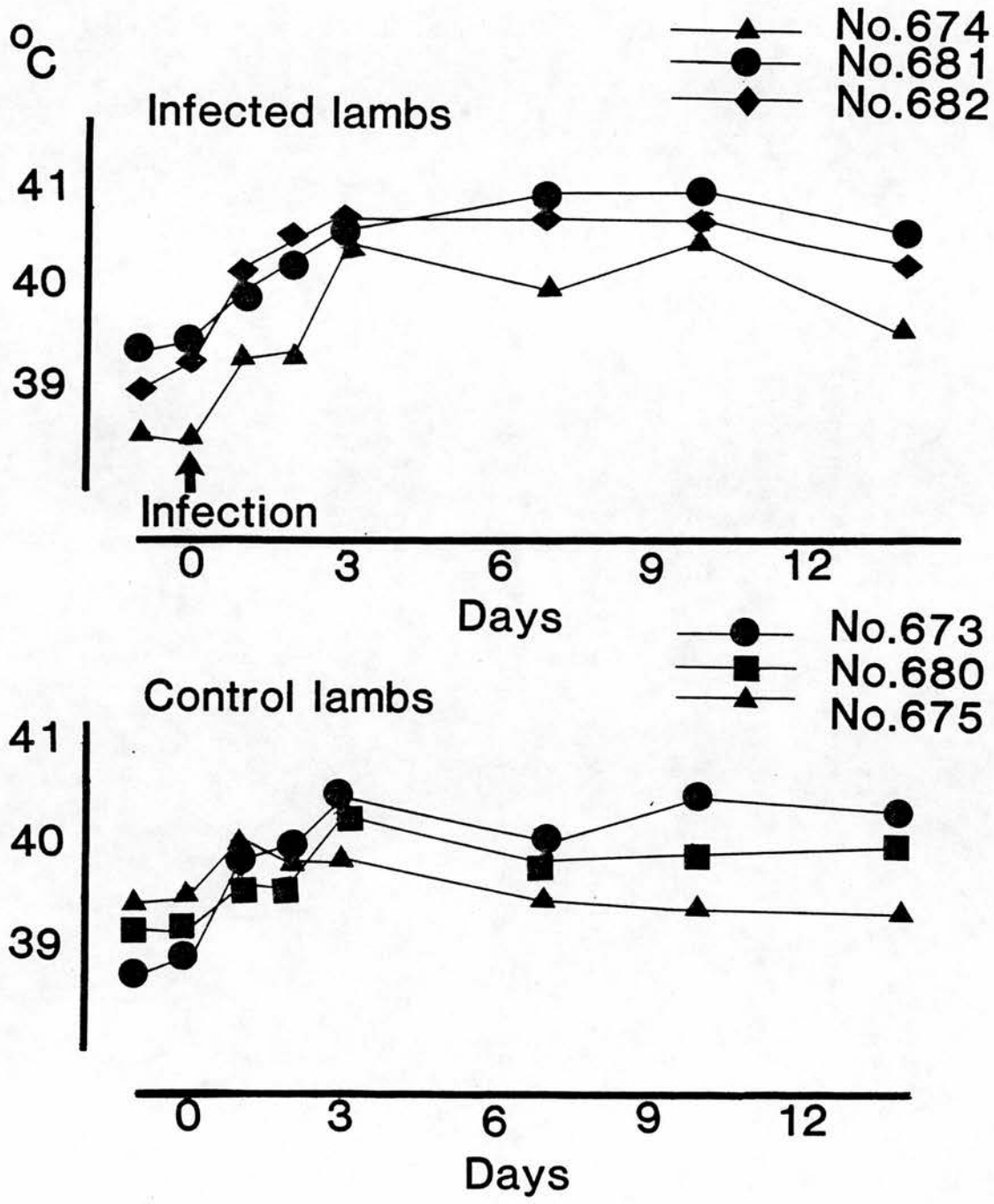
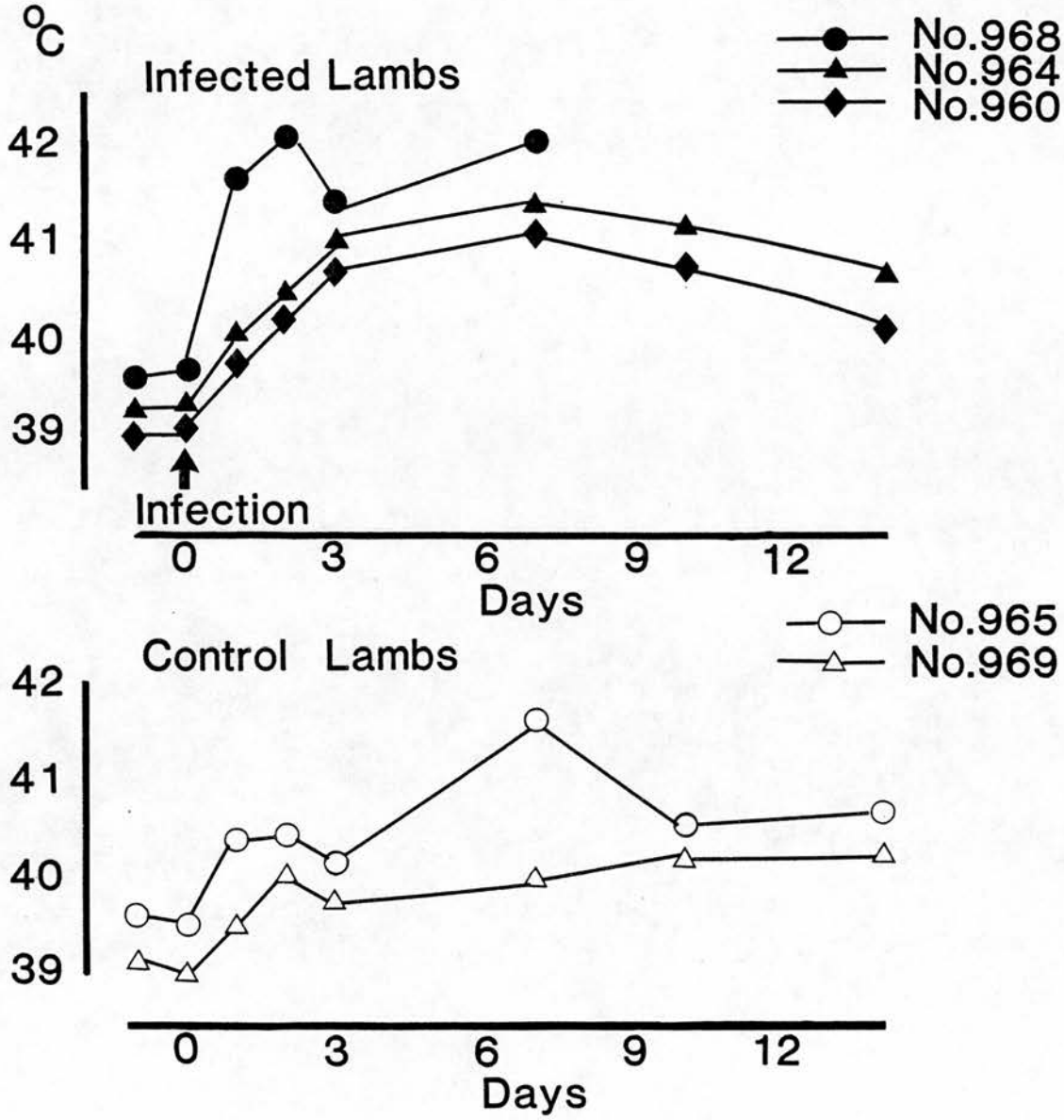

°C
Infected lambs
No.674
No.681
No.682
41
40
39
Infection
0
3
6
9
12
Days
Control lambs
No.673
No.680
No.675
41
40
39
0
3
6
9
12
Days

Fig. 6b : Temperature responses of 7-week old lambs to tonsillar infection with T serotypes of P. haemolytica and of uninfected control lambs.


°C
Infected Lambs
No.968
No.964
No.960
42
41
40
39
Infection
0
3
6
9
12
Days
Control Lambs
No.965
No.969
42
41
40
39
0
3
6
9
12
Days

Daily examination of tonsils indicated a severe inflammatory reaction manifested by enlargement and congestion of the tonsils. In most lambs this reaction had subsided by day 6 after infection.

Pathology : In the lamb which died on day 10 after infection the main changes detected at post-mortem examination were in the lungs and tonsillar area. In the lungs extensive consolidation was present in the apical, cardiac and the anteroventral portion of the diaphragmatic lobes with multiple focal suppurative abscesses (Figs. 7a, b). Frothy exudate filled the trachea and bronchial lymph nodes were greatly enlarged and inflamed. The pharynx was severely inflamed and tonsils were enlarged and congested. There was patchy subcutaneous congestion and slight congestion of the whole intestine. Both kidneys showed faint pinpoint necrotic foci. Profuse pure cultures of P. haemolytica were obtained from several organs (Table 13). The predominant serotype isolated was A9.

Bacteriology : The T serotypes isolated from the tonsillar area of infected and control lambs throughout the experiment are reported in Table 14. Before infection only 2 lambs (964 and 965), both 7-weeks old, were found to carry a T serotype, T10 in both cases.

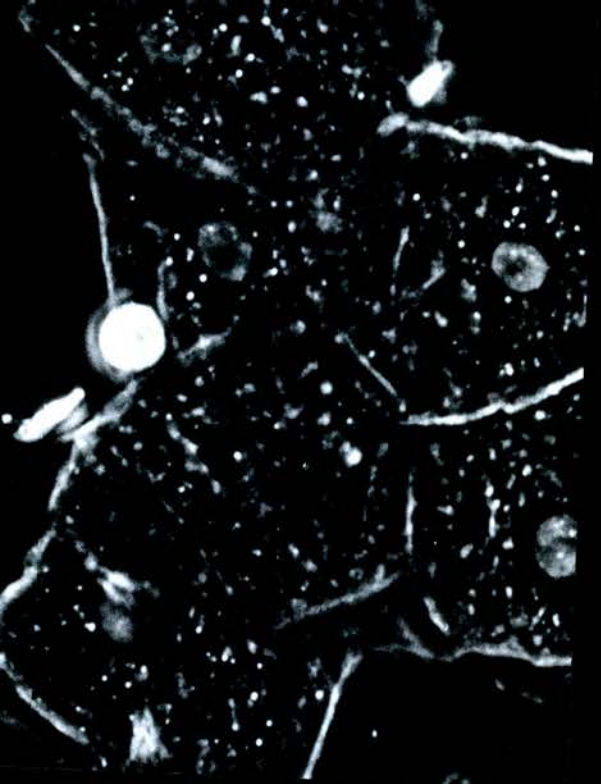
One day after infection all but one (674) of the 6 infected lambs yielded T serotypes and all were positive on subsequent

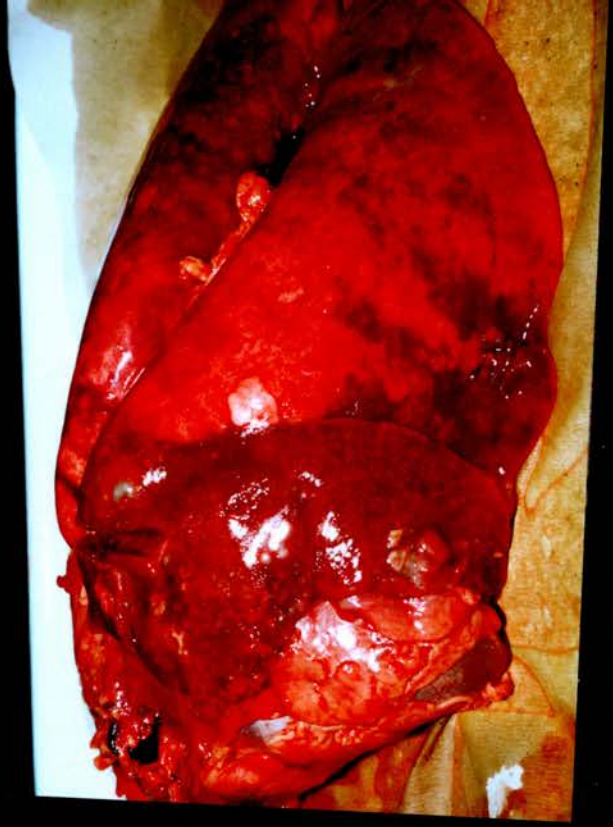
Fig. 5 : Superficial epithelial cells removed by abrasion of the tonsillar-pharyngeal area with a tooth brush. phase contrast X 1360.

Fig. 7 : Lung of Lamb No. 968 which died 10 days after single tonsillar infection with a mixed T serotype culture of P. haemolytica.

a) Dorsal aspect showing consolidation of apical and lower parts of the diaphragmatic lobes.

b) Ventral aspect showing consolidation of the lower parts of all lobes with abscess formation (arrows).





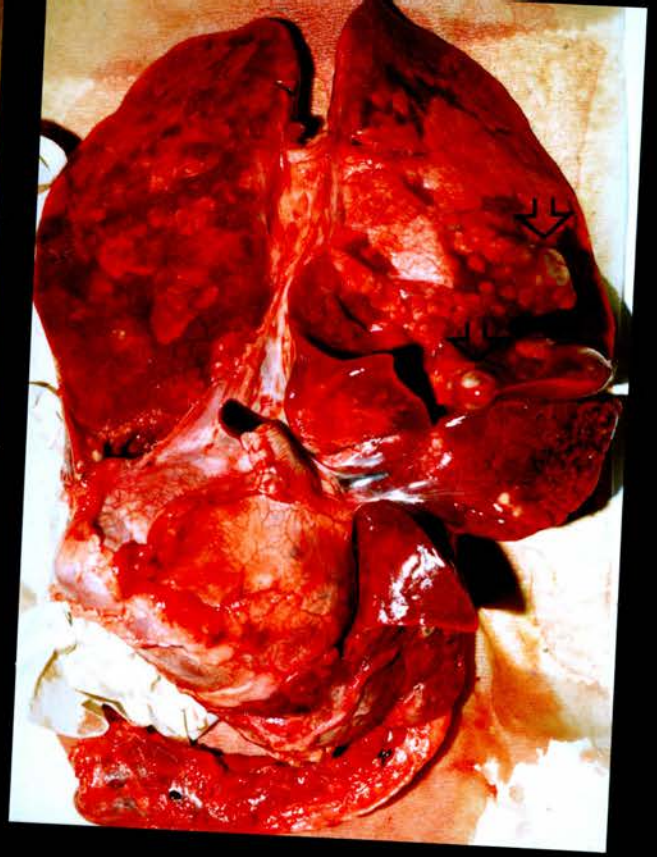


Table 14. Isolations of T serotypes of P. haemolytica made from tonsillar swabs taken before and after infection of lamb tonsils with a mixed T serotype culture.

Lamb No.	Infected	Serotype isolated days after infection				
		Before infection	1	7	14	21
3-week old lambs						
681	Yes	-	4	4 10	3 4	3 4
682	Yes	-	4 10	3 10	10	3 10
674	Yes	-		4 10	4	4 10
673	No	-				
675	No	-		4	4	4
680	No	-		4	4	4
7-week old lambs						
960	Yes	-	3 4 10	3 4 10	3 4	10
968	Yes	-	3 10	3 10	D* D D	D D D
964	Yes	T10	10	10	4 10	10
965	No	T10	10	10	10	10
969	No	-				

* Dead

Table 13. Serotypes of P. haemolytica isolated from sites of lamb (968) which died 10 days after infection with three T serotypes.

Tissue*	Serotype isolated
Lung	A9, T3
Heart	A9
Bronchial L.N.**	A9
Adrenal	A9
Tonsils	A9, T3

* No isolation were made from liver, spleen and kidney.

** Lymph node.

swabbings. However from only one 7-week old lamb (960) were all three T serotypes recovered at a single swabbing; this occurred on days 1 and 7 after infection. Double isolates were obtained from 12 of the other 19 swabs of infected lambs. After infection the numbers of isolations of individual serotypes were 9, 12 and 16 for T3, T4 and T10 respectively.

Two of the 5 control lambs (673 and 969) were negative at all swabbings and one (965) persistently yielded T10. In addition serotype T4 was recovered from 2 lambs (675 and 680) from which no T serotype had been isolated before cohort animals were infected. When those T4 isolations are included with isolations from infected animals the ratio of T3:T4:T10 becomes 9:18:16.

Serology : Only slight rises in IHA antibody titres to T3 and T10 were detected in infected lambs but similar increases occurred in 4 of the 5 controls (Table 15).

Table 15. IHA test results for serum samples collected before and after infection of the tonsils with a mixed T serotype culture of P. haemolytica. (Experiment 1).*

Lamb No.	Infected	Days after infection		
		14	21	
		T3	T3	T10
3-week old lambs				
681	Yes	8	8	-**
682	Yes	-	8	16
674	Yes	-	8	-
673	No	-	-	-
675	No	-	8	-
680	No	8	16	-
7-week old lambs				
960	Yes	32	32	8
968	Yes	D***	D	D
964	Yes	64	64	32
965	No	-	-	-
969	No	-	-	-

* All serum samples taken before infection and at 7, 14 and 21 days after infection were tested against all three T serotypes. Those results not shown were negative (titre $\leqslant 4$).

** IHA titre $\leqslant 4$.

*** Dead.

Experiment 2 : The effect of tonsillar abrasion in young adult sheep.

This experiment was conducted to investigate the effect of tonsillar abrasion in animals with a naturally occurring serotype population in the tonsils. The experiment sought to determine whether a commensal T serotype population of P. haemolytica in the tonsils can cause systemic infection and a clinical response following superficial damage to the pharyngeal-tonsillar mucosa.

Experimental design : Twelve Scottish Blackface hoggs of 6 - 9 months of age were used. Originally it was intended to identify those which were carrying T serotypes in their tonsils in order to compare responses in "carrier" and "non carrier" animals. However, from 3 successive swabbings at weekly intervals it became obvious that such segregation was not possible because of the changing pattern of isolations with time. Accordingly, the 12 animals were randomly divided into two groups of 6 and held in separate loose boxes. The tonsils of 3 sheep in each group were abraded as described previously, while companion animals served as in-contact controls. The sole difference in treatment between the groups was in tonsillar abrasion.

Results

Clinical observations : Twenty four hours after tonsillar abrasion coughing was noted in three individual sheep (167, 720 and 755) particularly after they were disturbed. Coughing of

similar intensity was also noted in one non-abraded sheep (678). In all cases coughing subsided within 5 - 7 days. The mean temperature responses of abraded and non-abraded sheep are shown in Fig. 8. Only one animal (720) in the former group had a rectal temperature greater than 40.5°C (41.1°C on day 3 after abrasion).

All 12 sheep continued to eat during the experimental period.

Examinations of the oropharynx daily for the first 3 days after abrasion showed varying degrees of localised inflammatory change and catarrhal exudation. These were most evident in the three animals in which coughing was a feature. By the fourth day signs of healing and mucosal restoration were evident in abraded animals.

Mucosal congestion and swelling were the only observed changes in the one non-abraded animal which experienced a transient period of coughing. In all animals the inflammatory changes regressed fully within 7 days.

Bacteriology : Isolations of T serotypes from tonsillar swabs taken before and after abrasion are given in Table 16. The commonest isolation made was T10, irrespective of the particular box in which the sheep were held and of whether or not they were

Fig. 8 : Average daily temperature responses of young adult sheep to tonsillar abrasion and of control sheep. Range is indicated by bar.

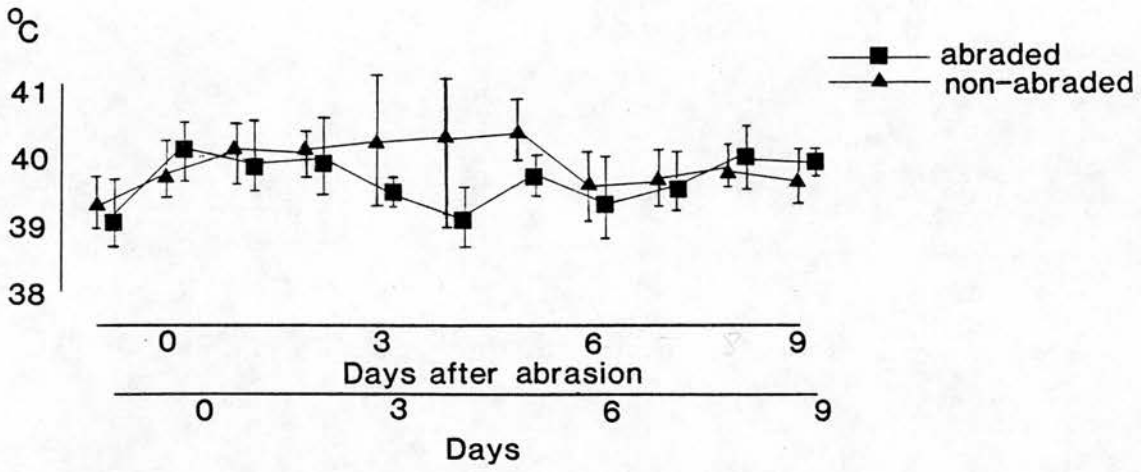

°C
41
40
39
38
abraded
non-abraded
0
3
6
9
Days after abrasion
0
3
6
9
Days

Table 16. Isolations of T serotypes of P. haemolytica made from tonsillar swabs from normal hoggs and those with abraded tonsils.

Animal No.	Days						
	Before abrasion			After abrasion			
	-18	-7	0	6	10	14	20
Animals with abraded tonsils							
756		10	10	10	10		
167	10	10	10	10	10		4 10
173	4		4	4 10	10	10	10
755	10			10		10	
720				10	10	10	
765							
Animals with non-abraded tonsils							
724			10	10		10	3
751		10	10	10			
682			3 10	10			
612	10	10			10	10	10
678	10	10				10	
754	4	4					

subjected to tonsillar abrasion. A slight increase in isolations was achieved from those animals that had been abraded. P. haemolytica could not be isolated from any of the blood samples taken during the experiment.

Serology : As assessed by the IHA test no significant serological response to any T serotype followed tonsillar abrasion.

Experiment 3 : The effect in young adult sheep of tonsillar infection with mixed T serotypes of P. haemolytica twenty four hours before or after local abrasion.

This experiment was designed to investigate the influence of infection with mixed T serotypes on abraded or intact tonsillar mucosa.

Experimental design : Twelve Scottish Blackface hoggs of 6 - 9 months of age were randomly separated into 2 groups and housed in loose boxes. The tonsils of animals in Group A were abraded and then infected 24 hr later with a mixed T serotype culture each ml of which contained 7×10^8 T3, 2×10^8 T4 and 5×10^8 T10 serotypes of P. haemolytica. Tonsillar infection of hoggs in group B was carried out at the same time as for animals in group A. One day after infection the tonsils of the animals in group B were abraded. Animals in both groups were bled 24, 48 and 72 hr after the abrasion for bacteriological study.

Results

Clinical observation : In group A there was no significant rise in temperature after abrasion or following infection. Twenty four hours after abrasion 4 of the animals in the group showed a moderate local inflammatory response with engorgement and swelling of tonsils and pharyngeal mucous membranes. In one of the 4 harsh breathing and coughing were present on the day

following infection but the animal returned to clinical normality within 4 days. At that time the inflammatory changes had subsided in all animals in the group. In 4 of 6 animals of group B clinical signs of restlessness and harsh breathing were present 24 hr after infection and continued for 5 to 6 days. The changes were most apparent in one animal (690) which, on the fifth day after infection, experienced a rise in temperature (to 41°C) that persisted for 48 hr. Local inflammatory reactions affecting the pharynx and tonsils were evident during the first 2 days after infection but had subsided by day 4.

Bacteriology : From tonsillar swabs it was apparent that organisms which colonized the tonsils at infection were able to persist for at least 21 days (Table 17). The serotype most frequently isolated, T10, was recovered three times more often than T3, the least commonly isolated serotype. From neither group could P. haemolytica be cultured from blood samples taken 24, 48 or 72 hr after abrasion.

Serology : The sera of all animals were tested against the three T serotypes of P. haemolytica by the IHA test. Only in 3 individual sheep were there significant (4 fold) rises in antibody titre to T3 (743 and 690) and T4 (735) (Table 18). No animal responded serologically to serotype T10.

Table 17. Isolation of T serotypes of P. haemolytica from tonsillar swabs taken before and after infection of hoggs' tonsils with a mixed T serotype culture (Experiment 3).

Animal No.	Serotypes isolated			
	Before infection	Days after infection		
		7	14	21
	Group A - abraded, then infected			
738		10		
735	4	4 10		4 10
653		10		
728				3 4 10
174		4 10	4 10	4
761		10		
	Group B - infected, then abraded			
823	ND*	10	3 4 10	3 4 10
769	ND			
743	ND	3 10	3 4 10	3 10
690	ND		4 10	
733	ND	10		10
736	ND	3	4 10	10

* Not done.

Table 18. IHA test results for serum samples collected before and after infection of the tonsils with a mixed T serotype culture of *P. haemolytica** (Experiment 3).

Animal No.	IHA titre							
	Before infection		Days after infection					
			7		14		21	
	T3	T4	T3	T4	T3	T4	T3	T4
	Group A - abraded, then infected							
738	-**	-	-	-	-	-	8	8
735	-	8	-	8	8	32	8	16
653	-	8	-	8	8	8	16	8
728	8	16	16	32	16	32	16	16
174	-	8	-	16	-	8	8	8
761	8	-	-	-	8	-	8	-
	Group B - infected, then abraded							
823	16	8	-	-	8	16	16	8
769	-	16	-	8	-	16	16	8
743	8	8	8	16	16	16	32	8
690	16	8	16	8	64	16	64	8
733	8	8	8	8	8	8	16	8
736	-	8	8	8	-	16	16	8

* All serum samples taken before infection and at 7, 14 and 21 days after infection were tested against all three T serotypes. Those results not shown were negative (titre ≤ 4).

** IHA titre ≤ 4.

Experiment 4 : The effect in young adult sheep of repeated tonsillar infection with a mixed T serotype culture of P. haemolytica followed by abrasion of tonsils and further infection.

The purpose of this experiment was to determine whether clinical disease could be precipitated by repeated infections of an intact or abraded tonsillar surface. The experiment was performed sequentially on the same group of animals so as to provide maximum opportunity for establishment of local infection.

Experimental design : Six Scottish Blackface hoggs aged 6 - 9 months were kept in a loose box. They were infected in the tonsillar area on each of 5 successive days. On the seventh day their tonsils were abraded and 24 hr later a series of 4 reinfections at daily intervals was begun. The numbers of organisms of each individual T serotype in the infecting inocula are given in Table 19. In general, for the series of infections which followed abrasion at least a 10-fold greater number of organisms was used. Those sheep with severe local lesions were killed and samples of affected tissues and tonsils were taken for histological study.

Results

Clinical observation : In one animal in the group (945A) a rise in temperature began 72 hr after infection and persisted for 48 hr (Fig. 9). This animal coughed and had signs of an upper respiratory infection. All animals developed congestion of

Fig. 9 : Temperature responses of 2 young adult sheep to repeated tonsillar infection with T serotypes of P. haemolytica interrupted by abrasion.

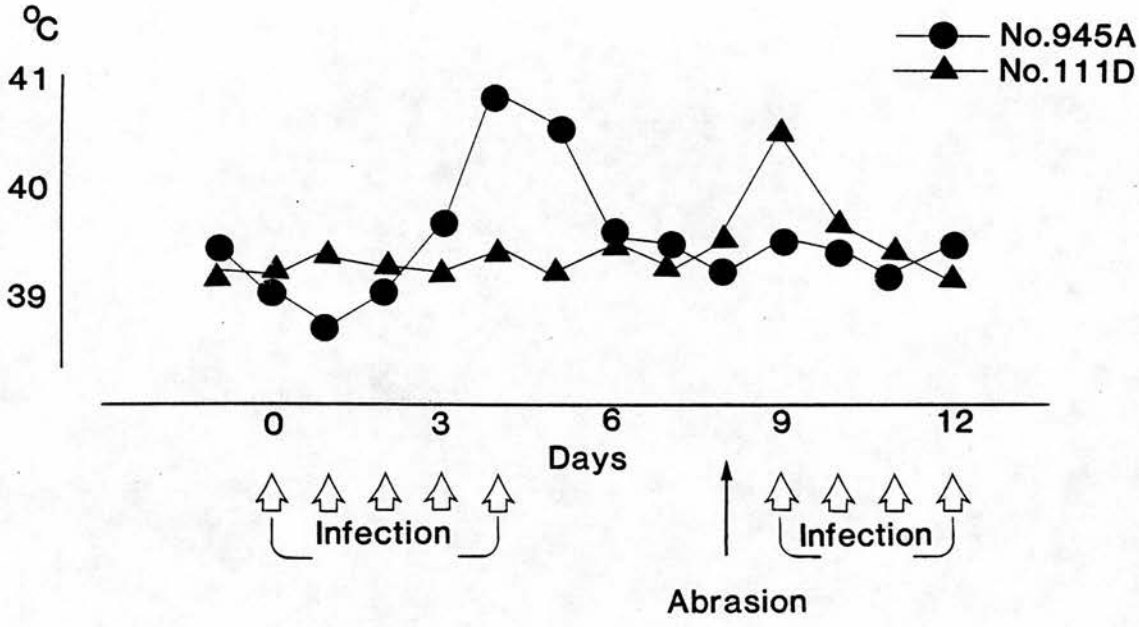

°C
41
40
39
No.945A
No.111D
0
3
6
9
12
Days
Infection
Infection
Abrasion

Table 19. Numbers of organisms of individual T serotypes of P. haemolytica in infective inocula (Experiment 4).

Day of inoculation	Serotypes of P. haemolytica		
	T3	T4	T10
	No. of organisms x 10^9		
0	5.0	0.3	0.3
1	0.2	0.2	0.3
2	0.2	0.4	0.3
3	9.5	25.0	17.5
4	4.0	47.5	35.0
	No. of organisms x 10^{10}		
7	15.6	20.0	30.0
8	1.0	4.0	13.0
9	5.0	4.5	12.0
10	18.0	6.3	6.0

pharyngeal mucosa and tonsillar enlargement. After abrasion of the tonsillar area a second animal (111D) also developed a transient febrile response (Fig. 9). Coughing and difficulty in breathing were noted over 3 days. Four animals, including the 2 which were febrile, developed ulcers and erosions in the peritonsillar area. Similar but milder lesions and tonsillar enlargement were present in the 2 other members of the group. In these cases healing commenced within 7 days of abrasion but was delayed in the more severely affected animals.

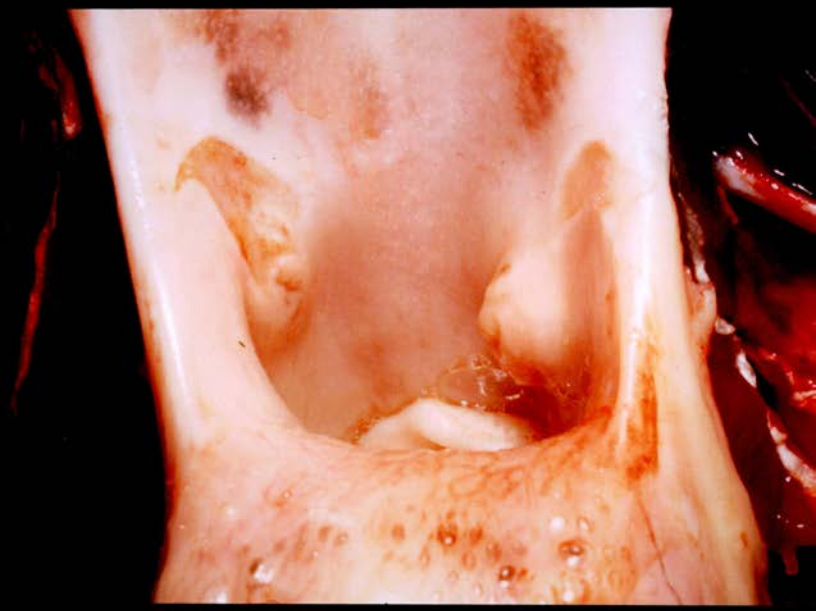
Pathology : Two animals (945A and 111D) with severe lesions in the pharyngeal and tonsillar region (Figs. 10a, b) were killed on day 15 after infection. Post-mortem examination revealed no distinct pathological changes in the tissues and organs apart from softening and moderate enlargement of retropharyngeal, submandibular and smaller peripharyngeal lymph nodes.

Histopathological examination of the lesions showed defined ulcers and a severe inflammatory reaction in the area with complete loss of or damage to the epithelium covering the tonsils (Fig. 11). The inflammatory reaction extended into the tonsillar tissue. The lymphoid tissue of the tonsils were very reactive with prominent germinal centres indicating a vigorous immune response. Dead and degenerate leucocytes and mononuclear cells and epithelial debris were present in the crypts (Fig. 12).

Fig. 10 : Lesions on both sides of pharynx resulting from tonsillar abrasion followed by repeated tonsillar infection with a mixed culture of T serotypes of P. haemolytica.

a) : Ulcers on both sides of the pharynx (Animal No. 111D)

b) : Ulcers involving the enlarged and swollen tonsils (Animal No. 945A)



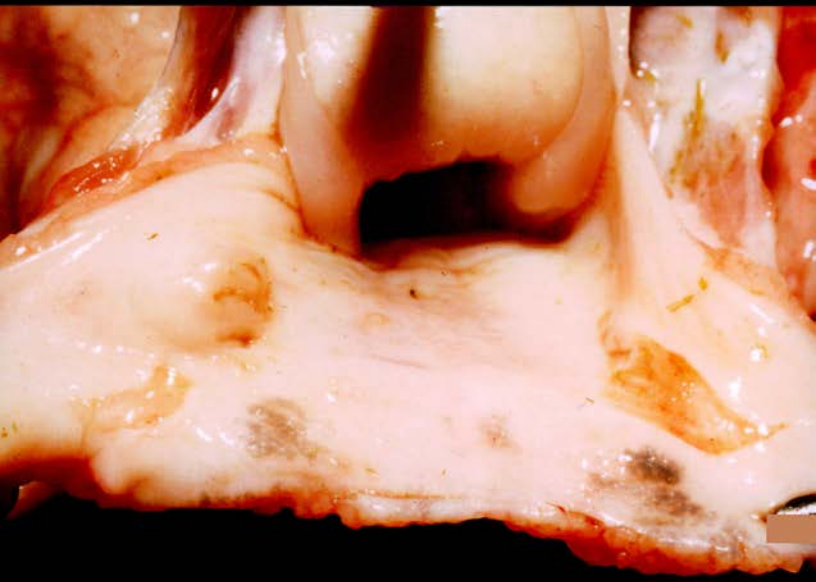
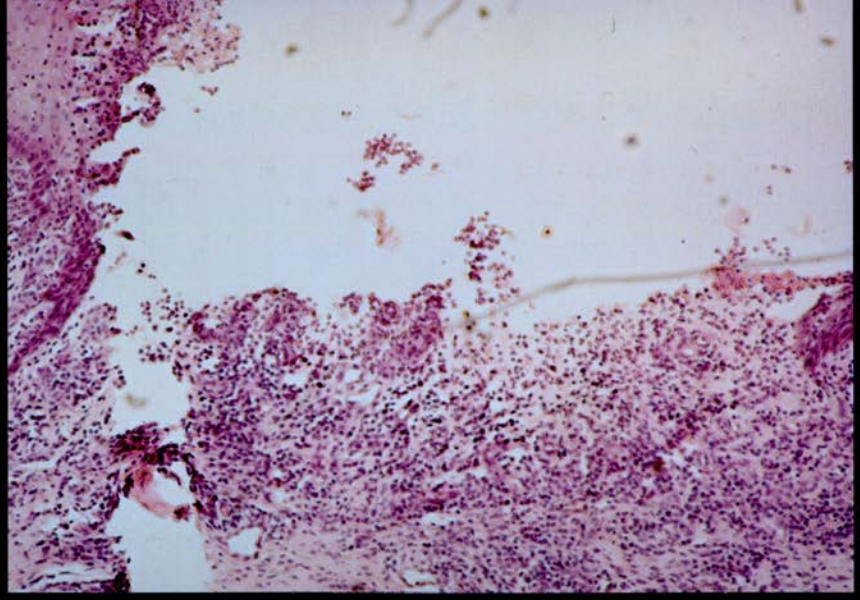


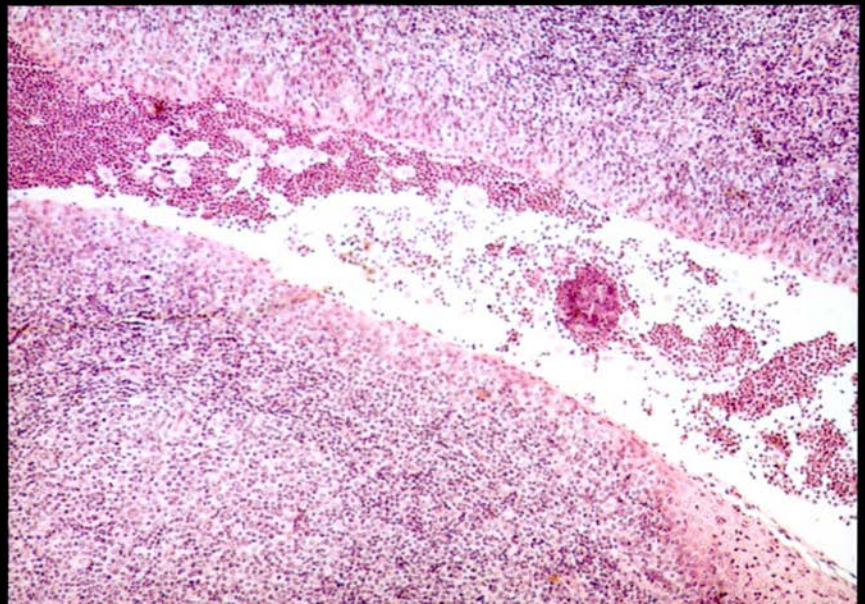
Fig. 11 : Tonsil : showing complete loss of epithelium. Severe inflammatory reaction and necrosis of subepithelial tissue in the ulcerated area.

H & E X250

Fig. 12 : Tonsil : crypt lumen containing dead and degenerated inflammatory and epithelial cells with masses of bacteria.

H & E X250





Bacteriology : Table 20 records the T serotypes isolated from the tonsils before and after infection. In this experiment T3 was the most frequently isolated serotype. Blood samples taken from the 2 animals showing a rise in temperature were negative on culture.

Serology : No significant increase in IHA titre to any T serotype was detected in serum samples collected 7, 14 and 21 days after initial infection.

Discussion.

The aim of the work described in this chapter was to develop an experimental model for studying the pathogenesis of T biotype disease in sheep. One of the difficulties in achieving this aim is finding a suitable method for establishing infection in the pharynx and upper alimentary tract particularly the tonsils. In the natural disease the sites of initial infection have been presumed to be the tonsils, pharynx and oesophagus (Dyson et al., 1981).

It is most likely that development of a focus of infection in the pharyngeal-tonsillar region may follow physical assault to the mucous membrane and epithelial covering of the tonsils. Such local damage could alter the local micro-environment, allowing the commensal T serotypes of P. haemolytica to grow and invade surrounding tissues.

Table 20. Isolation of T serotypes of P. haemolytica from tonsillar swabs taken before and after infection of hoggs' tonsils with a mixed T serotype culture. (Experiment 4).

Animal No.	Serotypes isolated.			
	Before infection	Days after infection		
		15	22	28
878A	10	4	3	3 4
945A		3 4	D* D D	D D D
104B		3	3	3
111D	4	10	D D D	D D D
150		3 4 10	3 10	3 10
943		3 10	3	3

* Dead

Mechanical damage of the type envisaged occurs not uncommonly as a consequence of careless use of drenching guns when sheep are being dosed orally with anthelminthic. Each year there are reports of fatal pasteurellosis in sheep which have been injured in this way. Damage may also be caused by some forms of foodstuff e.g. large, hard and jagged pieces of turnip or sharp bits of stubble. It is possible too for small foreign bodies, including ears of grain to become embedded in the tonsils and set up a focus of infection. For experimental purposes a hard-bristled tooth-brush causes more consistent removal of surface epithelium than any of several test tube brushes that were tried. However, in animals with no more than a commensal T serotype population the ensuing local reactions were always mild and transient.

The results of experiment 1, which involved 3- and 7-week old lambs, do suggest that in the special circumstances of animals without an established commensal population tonsillar infection with T serotypes of P. haemolytica can induce some clinical illness. All 6 lambs developed a marked pharyngeal inflammation and tonsillar engorgement and, in some, respiratory symptoms and elevation of temperature were also manifest. However, the death of one lamb (968) and the finding of coincident septicaemic infection with serotype A9 in that animal suggested that the clinical signs may have resulted from exacerbation of a pre-existing A9 infection. This view gains

some support from the high temperature detected in one control lamb (965), but in none of the control or experimental animals was there a serological reaction to A9 or any other A serotype.

Experiments 2, 3 and 4 were conducted in a breed of sheep known to be susceptible to systemic pasteurellosis and within the age group most commonly affected under natural conditions. The outcome of experiment 2 indicated that tonsillar abrasion was insufficient to alter an established commensal population of T serotypes (Table 16). This may be because the commensal microorganisms are normally present in small numbers and are of low virulence and without invasive capacity. In the subsequent experiment the commensal population was reinforced by infection of the tonsils of carrier animals either before or after abrasion. Little effect was obtained by infecting tonsils that were already abraded but a distinct response was achieved in animals in which infection preceded abrasion. The difference between the groups was reflected in the greater number of isolations made from the group given the latter sequence of treatment (Table 17).

Experiment 4 was designed to exploit the findings of the previous experiment by establishing infection of the tonsils before they were abraded. In an attempt to achieve even better results the weight of infection was increased and supplemented in the period following abrasion. This regime of treatment resulted in lesions of greater severity including the development

of pharyngeal ulcers (Figs. 10a, b). Thus it appeared that repeated challenge with large numbers (10^{10} - 10^{11}) of viable organisms provided the best opportunity for colonization i.e. the lodgement and persistence on the epithelial surface needed to initiate the disease process (Pass and Thomson, 1971).

While this experiment was successful in so far as a clinical response and local lesions were produced, in none of the animals was there any bacteriological or serological evidence of systemic spread of any of the T serotypes used for tonsillar infection. Though distinct, the pharyngeal lesions were not wholly typical of those encountered in the natural disease.

Thus for the experimental reproduction of systemic pasteurellosis some factor additional to those already employed is necessary.

As far as the author is aware there are no published accounts of attempts to induce T-type pasteurellosis by establishing tonsillar infection. However, Smith (1957) referred to unpublished experiments by McEwen in which oral infection with large numbers of untyped P. haemolytica did result in clinical disease and septicaemic death in some 15% of cases, a result which, in spite of repeated attempts, was achieved on one occasion only.

Conclusions.

(1) In lambs under 2 months of age, a single infection of the tonsils with three T serotypes of P. haemolytica causes a severe but transient local inflammatory reaction.

(2) In young adult sheep tonsillar abrasion alone does not modify the behaviour of commensal T serotypes of P. haemolytica.

(3) Repeated infection of the tonsils of young adult sheep with large numbers of T serotypes, followed by abrasion, produces local lesions without systemic spread of the organism.

CHAPTER 3.

INVESTIGATION INTO THE ENHANCEMENT OF PATHOGENICITY OF P. HAEMOLYTICA BY IRON.

Introduction

A valuable criterion in distinguishing P. haemolytica from other Pasteurella species is its low virulence for laboratory animals. On the other hand, fatal infections can be produced in mice by inoculation of large numbers of viable P. haemolytica (Smith, 1958) and the fatal effect is attributed to the toxicity of these large inocula. Smith (1958) devised a method for reducing the number of viable P. haemolytica needed to kill mice by mixing the organisms with mucin and administering them i.p. This procedure can reduce substantially the numbers of organisms needed to kill mice. It is accepted that mucin enhances the virulence of many weakly-or non-pathogenic bacteria. Although mucin is assumed to lower the resistance of the host, its infection-promoting action is of special biological interest as very little is known about the mechanisms by which the onset and progress of bacterial infection occur. Mucin-enhanced P. haemolytica infection, originally demonstrated in 1958 by Smith, is the only method currently available for assessing the pathogenicity of P. haemolytica serotypes and has proved to be a valuable model for the study of immunity to this microorganism (Evans, 1979).

As part of the study of the pathogenesis of infection with T serotypes of P. haemolytica consideration was given to factors which, rather than affecting the host, may act upon the organism to enhance its virulence. In this regard, a factor of particular importance is iron, the metal whose availability in host body fluids is probably the most crucial in enabling invading micro-organisms to become established (Weinberg, 1971, 1978; Anon., 1974; Payne and Finkelstein, 1978; Woolcock, 1979). Most body iron occurs intracellularly and, with the exception of those infectious diseases in which iron storage tissues or erythrocytes are specific targets, invading microorganisms are dependent for their growth requirements (0.3 - 4.0 μM) on the small quantity of ionic iron in body fluids (10 - 50 μM). However, this iron is complexed to high affinity iron-binding proteins such as serum transferrin and lactoferrin in secretions and the fact that these proteins are normally unsaturated greatly limits the availability of iron in body fluids and on mucosal surfaces.

Many bacterial species, including several which produce septicaemic and disseminating infections, themselves produce iron-binding substances (siderophores) which enable them to compete more successfully with the host for iron. In this way their pathogenicity is enhanced. Increasing the supply of exogenous iron also alters the host-parasite balance in favour of the microorganism.

In-vivo experiments have demonstrated that administration of cationic iron i.v. to laboratory animals (dose 2-5 µg/g. b.w.) can reduce by a factor of 10^{-2} to 10^{-3} the LD_{50} of several bacteria, e.g. Klebsiella pneumoniae, Pseudomonas aeruginosa, Listeria monocytogenes and Yersinia enterocolitica (Martin, Jandle and Finland, 1963; Sword, 1965; Smith, Carey, Damare, Hetrick, Johnston and Lee, 1980). Also bacteria which normally are relatively avirulent can be rendered pathogenic (Jackson and Burrows, 1956). Accordingly, this approach was used in mice to test the pathogenicity of a mixture of T serotypes of P. haemolytica. Much of the information obtained proved relevant to subsequent studies on the pathogenesis of T serotypes in sheep.

Materials and Methods.

Inocula : A suspension of mixed T serotypes was prepared as described on page 72. For experiments 1 and 2 this mixed suspension was serially diluted at 10^{-1} intervals in PBS, pH 7.4. In experiment 3 the undiluted suspension and ten-fold dilutions to 10^{-6} were incorporated into 5% suspensions of hog gastric mucin* in PBS, pH 7.4 at a ratio of 1 part bacteria to 4 parts gastric mucin. For each dilution the volume of inoculum was 0.5 ml per mouse.

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* Gastric-mucin-Bacteriological, I.C.N. Pharmaceuticals Inc., Cleveland, U.S.A.

Iron : The inorganic iron compound used was ferric ammonium citrate (FAC)* containing approximately 25% w/w ferric iron.

An aqueous solution of FAC was prepared by dissolving 10g of the iron salt in 100 ml of distilled water and sterilizing the solution by Millipore membrane filtration. The ferric iron content of the filtered solution was determined by atomic absorption spectrophotometry and suitable dilutions prepared for inoculation of mice and sheep. For mice the iron concentration of the inoculum was 0.6 mg/ml and each mouse received 0.1 ml (2 µg/g b.w.). The inoculum used for sheep had an iron concentration of 100 mg/ml and individual sheep were injected with a volume containing a dose equivalent to 2 mg/kg b.w.).

Measurements of iron in serum : Blood samples collected from the jugular vein of sheep were allowed to clot in sterile universal bottles. The serum was separated by centrifugation and stored at -20°C. Serum iron levels were measured in the Department of Biochemistry at Moredun Research Institute by Mrs. E. Herbert, using the atomic absorption spectrophotometer procedure. Briefly, a stock solution of ferric nitrate (BDH), containing 1 mg Fe/ml was diluted with 5% hydrochloric acid to give an

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* B.D.H. Chemicals Ltd., Poole, England.

appropriate standard range. Standards and serum samples were diluted 1/5.2 with 6% Butanol and assayed using the IL ISI* atomic absorption spectrophotometer against 5% Butanol, blank solution. Readings were recorded for each serum sample.

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*IL Instrumentation Laboratory U.K. Ltd., Warrington, Cheshire, England.

Experiment 1 : Determination of the lethality for mice of a T serotype mixture of P. haemolytica.

A pilot experiment was designed for the purpose of determinating the lethal effect of a T serotype mixture in mice.

Experimental design : The neat and diluted suspensions (to 10^{-4}) were inoculated i.p. into mice at a dose of 0.5 ml/mouse, using 2 mice per dilution. The animals were observed 24, 48 and 72 hr after infection. The experiment was terminated on day 3 when survivors were killed and their livers and spleens removed for attempted isolation and viable counts of T serotypes. The numbers of individual T serotypes per ml of mixed culture were 2.8×10^{10} T3, 1.5×10^{10} T4 and 2.4×10^{10} T10.

Results.

Only the undiluted T serotype mixture killed both mice. Death occurred within 12 hr of inoculation and P. haemolytica was readily recovered from the livers and spleens of these animals at titres of 10^{8} to 10^{10} per gram of tissue. Organisms were also isolated from the heart and lungs but not from the kidneys. No organisms were isolated from any of the surviving mice which were killed 3 days after infection.

Experiment 2 : The effect of injecting mice with iron on the LD_{50} of a mixed T serotype culture of P. haemolytica.

Experimental design : Two experiments were undertaken using mixed T serotype cultures prepared as already described. The numbers of individual T serotypes per ml of the mixed cultures used in these experiments were 1.5 and 7.5 x 10^9 T3, 2.0 and 5.0 x 10^9 T4 and 2.3 and 4.0 x 10^9 T10. In each experiment serial 10-fold dilutions of the mixed cultures were inoculated i.p. into 2 sets of mice, using 8 animals per dilution. One set of mice received 0.1 ml of FAC i.v. at a dose of 2 µg/g b.w. immediately prior to infection and the other was given no pre-treatment. The mice were observed at intervals after infection, the number of deaths occurring by 48 hr was recorded and LD_{50} values calculated and analysed statistically by the computational method of Finney (1978). The author is indebted to Mr. M. McLauchlan for handling the data in this way.

Results.

The LD_{50} values of the mixed T serotype cultures of P. haemolytica are shown in Table 21. The results indicate clearly that the values were significantly lower ($p<0.01$) when the mice were treated with iron prior to infection.

Table 21. The effect of prior injection of iron into mice on the LD_{50} of mixed T serotype cultures of P. haemolytica.

Experiment No.	No. mice/ dilution	Injection with		No. of deaths per dilution group.							LD_{50}	Significance p.
		org. alone	FAC + org.	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
1	8	+	-	8	6	0	0	1	0	0	2.87×10^{8}	<0.01
	8	-	+	8	8	5	6	6	4	0	4.71×10^{5}	
2	8	+	-	7	3	0	0	0	0	0	6.62×10^{9}	<0.01
	8	-	+	7	8	6	5	5	3	0	3.27×10^{6}	

Experiment 3 : The effect of iron and mucin alone or together in enhancing the virulence of T serotypes of P. haemolytica in mice.

This experiment was undertaken to compare the effects of iron and mucin in enhancing the virulence of a mixed T serotype cultures of P. haemolytica in mice and to study the effect of combined treatment.

Experimental design : C57 black mice of both sexes were divided randomly into 4 groups each of 56 mice. Eight mice were used for each dilution of inocula. Group A mice were inoculated i.p. with the mixed cultures alone, Group B was pretreated with FAC i.v. and then infected i.p. with the mixed culture of T serotypes. The third group (C) was injected i.p. with the mixture of T serotypes incorporated in mucin. In Group D mice were pretreated with FAC i.v. and then injected i.p. with the mixed culture in mucin. Death of mice was recorded 24 and 48 hr after infection, and LD_{50} values were calculated.

The numbers of individual T serotype per ml of mixed culture used were 2.8×10^{10} T3, 1.3×10^{10} T4 and 5×10^{10} T10.

Results.

The LD_{50} values of the mixed T serotype cultures of P. haemolytica in the different treatment groups are shown in Table 22. Pretreatment with iron or administration of bacteria in

Table 22. The effect of different treatments on the mouse LD_{50} values of a mixed T serotype culture of P. haemolytica.

Group	Inoculation procedures	No. of mice/ dilution	Dilution of inoculum							LD_{50} values*
			Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
A	Org. alone	8	8	6	0	0	0	0	0	5.43×10^{8}
B	Org. in mucin	8	8	8	4	4	3	0	0	1.18×10^{7}
C	Org. with iron	8	8	8	5	3	1	0	0	2.02×10^{7}
D	Org. in mucin with iron	8	8	8	8	7	5	3	2	2.06×10^{5}

* Statistical analysis

A vs B, C or D : $p<0.01$

B or C vs D : $p<0.01$

mucin resulted in comparable significant reductions in LD_{50} by a factor of between 2 and 5×10^{-1}. A much greater effect was achieved when the treatments were combined as the LD_{50} in group D was 2.6×10^{-3} times less than that in group A.

Experiment 4 : Estimation of iron concentration in serum of normal sheep after i.v. injection of iron.

This pilot experiment was carried out to observe the effect of i.v. iron injection in normal sheep and to determine the disappearance of injected iron from the circulation.

Experimental design : Three adult sheep were bled before the start of the experiment and FAC was injected i.v. at a dose of 2 mg/kg b.w. The animals were observed for any signs of abnormality and blood samples withdrawn 10 min., 2 hr, 6 hr and 24 hr after iron injection. The treatment was repeated on the following day. Measurement of iron in serum was performed as described (p.118).

Results.

The effect of injecting an iron solution into normal sheep : The injection of FAC equivalent to 2 mg/kg b.w. i.v. caused lip-licking which commenced within 30 seconds of injection and persisted for about 1 minute. No other abnormal signs or behaviour were seen.

The concentration of iron in serum after i.v. injection : Before injection the amount in the serum of the 3 sheep was between 200 and 250 µg/100 ml. Within minutes of injection of FAC the serum iron concentration had increased 10-fold to 2500 - 2900 µg/100 ml

(Fig. 13a). During the next 6 hours the concentration fell to about 1000 µg/100 ml and by 24 hours was almost back to normal. Injection of a further dose of iron was followed by a similar rapid rise and fall in serum iron concentration.

A very similar pattern of response occurred in each of the 3 animals

Fig. 13a : The effect of repeated intravenous injection of ferric ammonium citrate (2 mg/kg b.w.) on serum iron level in a sheep.

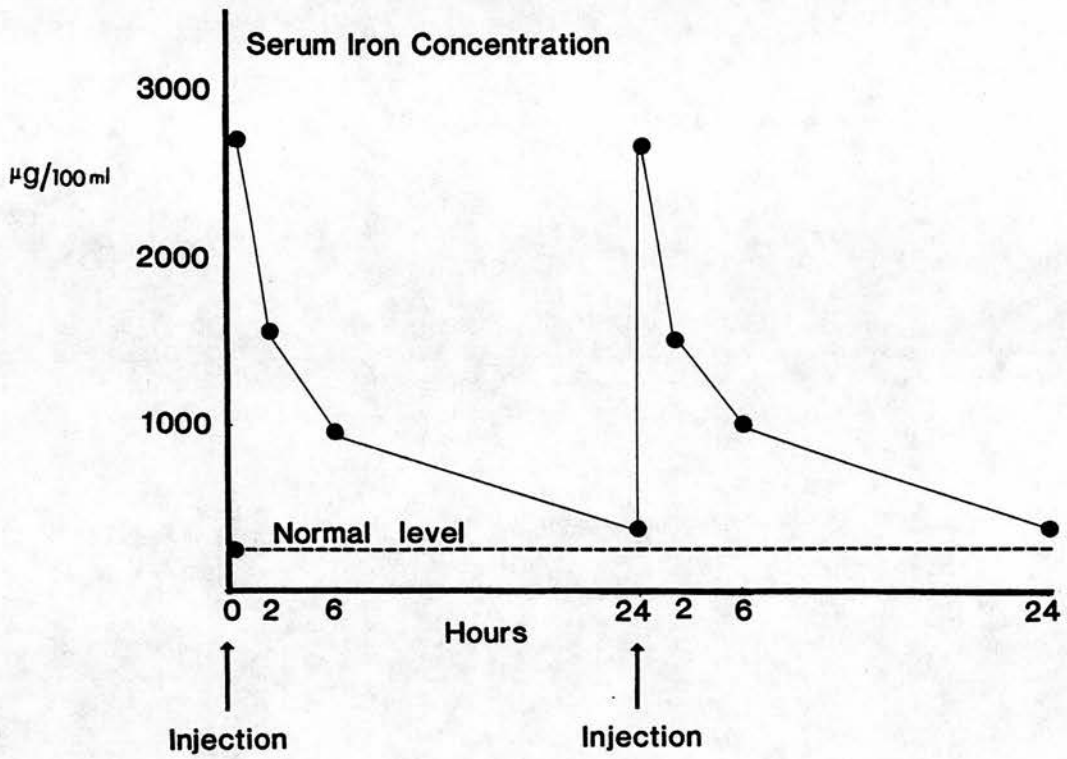
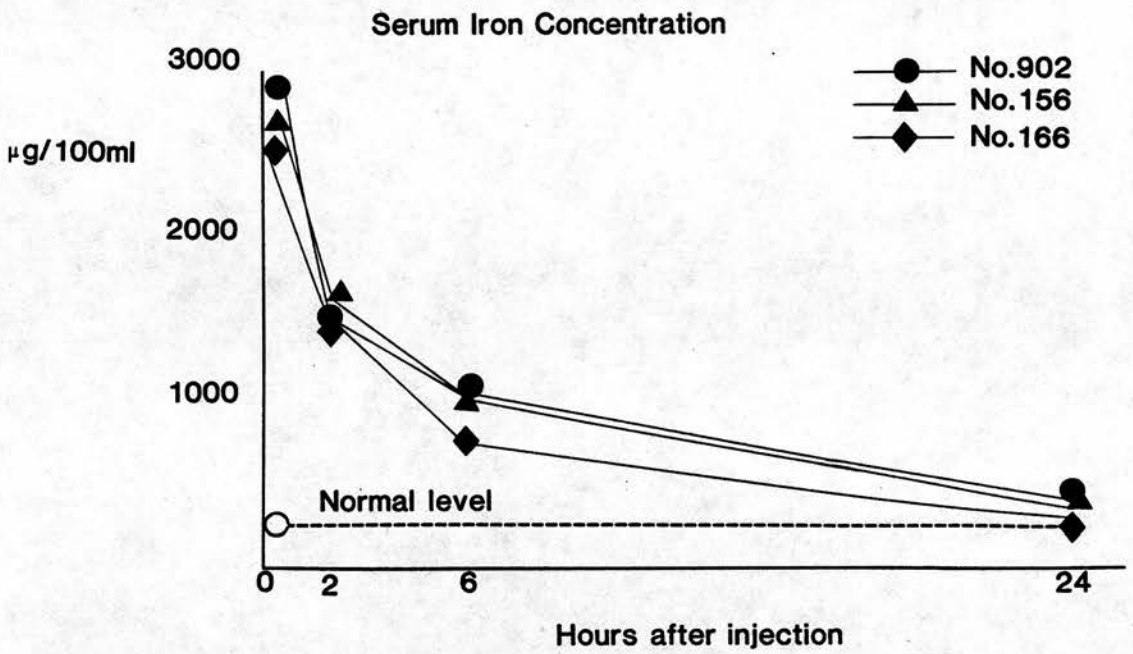

Serum Iron Concentration
3000
µg/100 ml
2000
1000
Normal level
0 2 6
Hours
24 2 6
24
Injection
Injection

Fig. 13b : Serum iron concentration in 3 individual sheep after intravenous administration of ferric ammonium citrate at a dose of 2 mg/kg b.w.


Serum Iron Concentration
3000
2000
1000
μg/100ml
No.902
No.156
No.166
Normal level
0
2
6
24
Hours after injection

Experiment 5 : Tonsillar infection of young adult sheep with a mixed T serotypes culture of P. haemolytica after iron injection.

This experiment was performed for the purpose of attempting to produce T serotype disease in young adult sheep treated with excess iron.

Experimental design : Nine Blackface sheep aged 6 - 9 months old were used. All animals, on day 1 of the experiment were given FAC i.v. at a dose equivalent to 2 mg/kg b.w. This procedure was followed immediately by tonsillar infection of six sheep with a mixed T serotype culture. The remaining 3 sheep were kept as controls. On day 2, following i.v. injection of the infected and control animals with the same volume of FAC, tonsillar abrasion was performed and the tonsils were reinfected. Treatment with FAC and infection of tonsils were repeated on days 3, 4 and 5 for the 6 infected sheep while the controls received iron only i.v. over the same period. Animals which showed severe clinical signs and/or developed lesions in the tonsillar area were killed and samples from different organs were processed for bacteriological and histopathological studies. The numbers of individual organisms in the mixed cultures inoculated on to the tonsils are presented in Table 23.

Table 23. Number of viable microorganisms in the mixed T serotype cultures of P. haemolytica used to inoculate sheep tonsils. (Experiment 5)

Serotype	Number of viable microorganisms (Log 10^{10}) per ml of culture used on Day				
	1	2	3	4	5
T3	1.2	2.0	8.2	0.4	8.0
T4	2.5	1.2	6.3	0.3	9.8
T10	1.2	6.5	5.0	5.0	2.3

Table 25. T serotypes of P. haemolytica isolated from various organs of two sheep killed 8 days after infection.

Sheep No.	Organs						
	Liver	Spleen	Lung	Heart	Lymph node	Kidney	Tonsils
920	-	3, 4, 10	4	-	-	-	3, 4, 10
970	-	3, 4, 10	3, 4	-	-	-	3, 4, 10

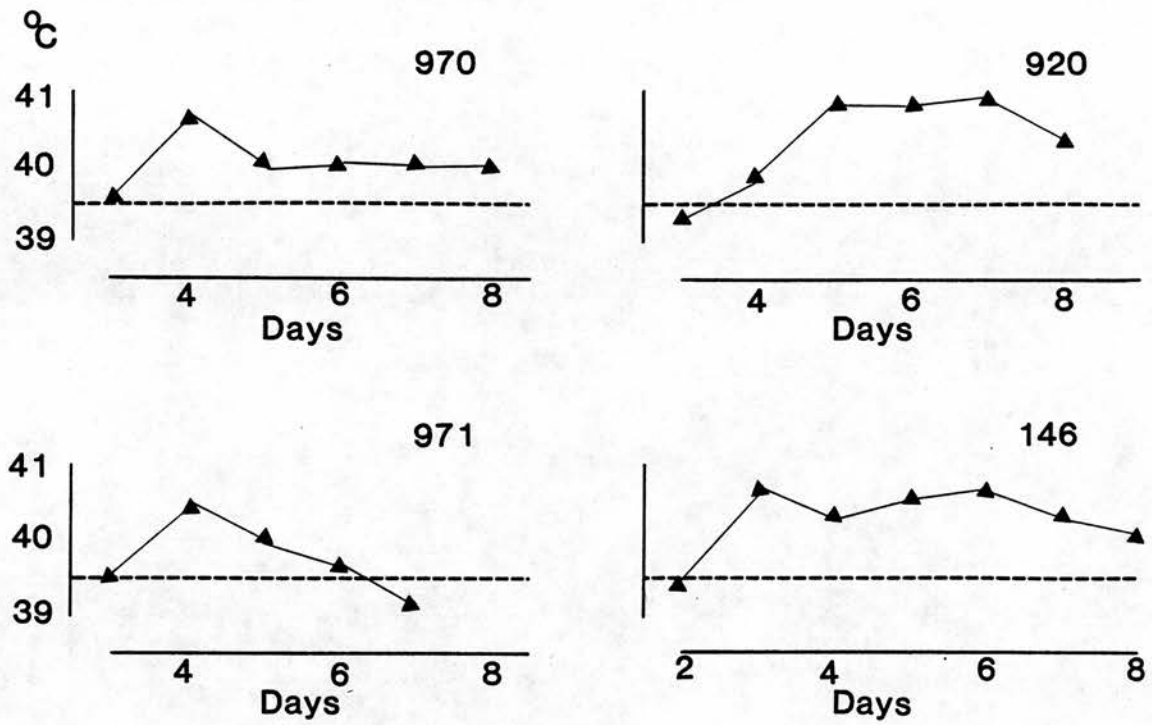
Results.

Clinical observations : Four of the 6 infected sheep showed a rise in temperature over 40°C. Pyrexia (>40.5°C) lasted between 1 - 3 days (Fig. 14). No rise in temperature occurred in any of the control animals. The principal clinical signs in infected sheep were of a respiratory character. Within 48 hours of initial infection all 6 animals developed an intermittent cough which lasted for 7 days and 4 had a nasal discharge. Two animals (970, 920) in particular reacted severely and were obviously weak and dull. On oral examination these two animals showed very severe lesions on both sides of the pharynx. Three further animals developed moderate to severe lesions while one (121) showed only a mild tonsillar reaction which appeared as enlargement and swelling of the tonsils with a mucoid exudate. Healing processes were evident by day 14 after infection.

Pathology : The two most severely affected animals (970, 920) were killed on day 8 of the experiment and post-mortem changes were recorded. Gross changes were present in the pharyngeal-tonsillar area and surrounding lymph nodes. One animal (970) had severe ulcer formation of the surrounding tissues (Fig. 15). The second animal (920) had a severe inflammatory reaction with ulceration of the tonsils accompanied by petechial haemorrhage which was evident on the outer surface of the tonsils. In both animals, lymph nodes surrounding or close to the tonsillar area were enlarged and oedematous and some showed haemorrhagic foci when incised.

Fig. 14 : Temperature responses of 4 individual sheep injected with iron and then given tonsillar infections with a mixed T serotype culture of P. haemolytica. The dotted line is the mean temperature of control sheep.

Exp. 5


°C
970
41
40
39
4
6
8
Days
920
4
6
8
Days
971
41
40
39
4
6
8
Days
146
2
4
6
8
Days

In both animals most internal organs were moderately congested but no gross lesions specific for P. haemolytica were detected.

Histological examination of tissue sections from tonsils revealed a severe purulent inflammation which extended from the crypt epithelial cells to the lymphoid and sub-epithelial tissues (Fig. 16). The inflammatory exudate was composed mainly of neutrophils and clumps of bacteria. Tonsillar crypts revealed masses of debris composed of dead and degenerated cells with colonies of microorganisms. Sections of liver showed a centrilobular pattern of fatty change indicative of degeneration. Foci of inflammatory cells were aggregated around blood vessels and portal tracts and were composed mainly of neutrophils and lymphocytes (Fig. 17). Lymph node sections revealed evidence of an active response to antigen in the form of germinal centre formation and presence of plasma cells. In the subcapsular spaces infiltrating neutrophils and macrophages were present together with oedematous fluid (Fig. 18). There were no specific microscopic changes in the spleen apart from a few neutrophils around blood vessels. The lungs showed oedema and thickening of interlobular septa with only a few neutrophils and macrophages infiltrating the walls of the alveolar capillaries (Fig. 19). Using the Prussian blue reaction iron was demonstrated inside the macrophages and reticulo-endothelial phagocytes of liver and spleen (Fig. 20a, b). No significant histological changes were seen in the remaining organs sampled.

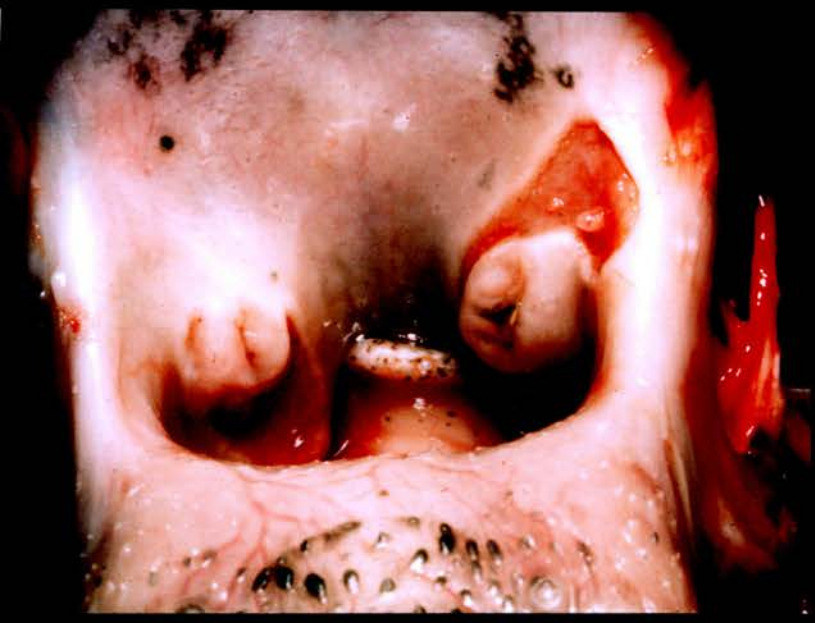
Fig. 15 : Tonsil : Severe tonsillar and peritonsillar ulcers produced after experimental abrasion and repeated infection of the tonsils of sheep given daily iron injections.

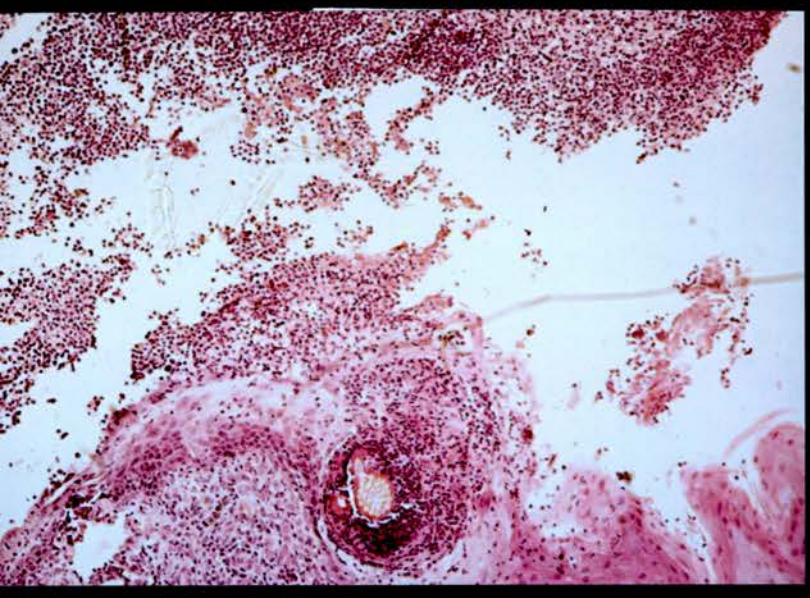
Fig. 16 : Tonsil : Necrosis of crypt epithelial cells with involvement of blood vessel. Inflammatory cells and epithelial debris are present in the crypt lumen.

H & E X250

Fig. 17 : Liver : accumulation of inflammatory cells, mainly macrophages, lymphocytes and few neutrophils around blood vessels and portal triad.

H & E X400





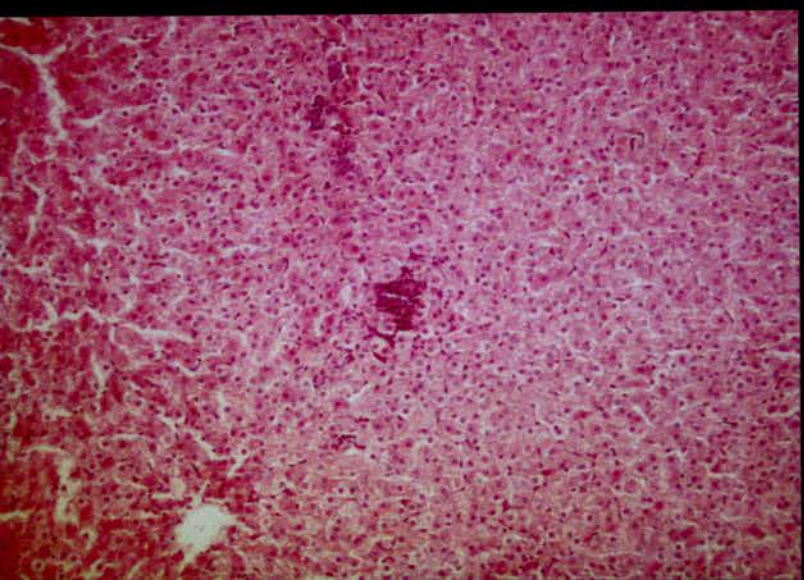
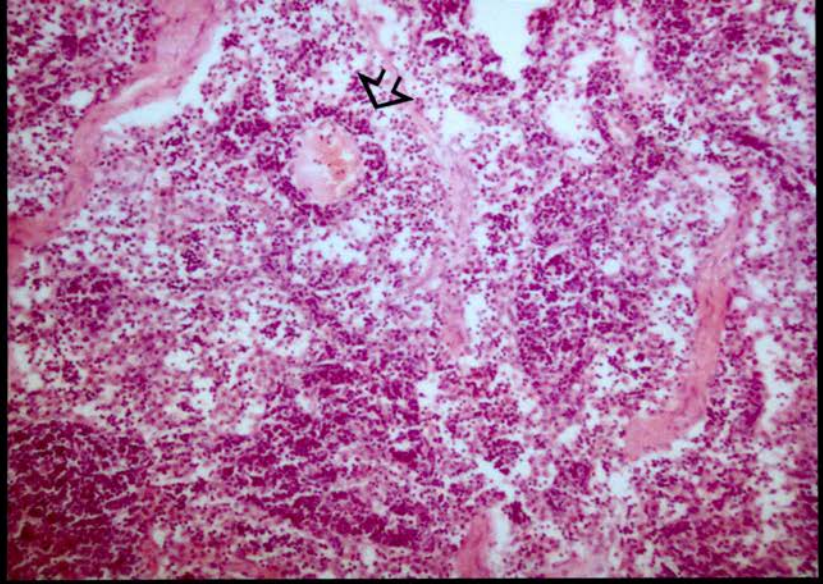


Fig. 18 : Peripharyngeal lymph node : inflammation of parenchyma and necrosis of blood vessels (arrow) with oedema in the subcapsular spaces and formation of germinal centres.

H & E X544

Fig. 19 : Lung : pneumonia produced following tonsillar infection with mixed T serotype cultures of P. haemolytica. Thickening of interalveolar septa by oedema and cellular infiltration.

H & E X544



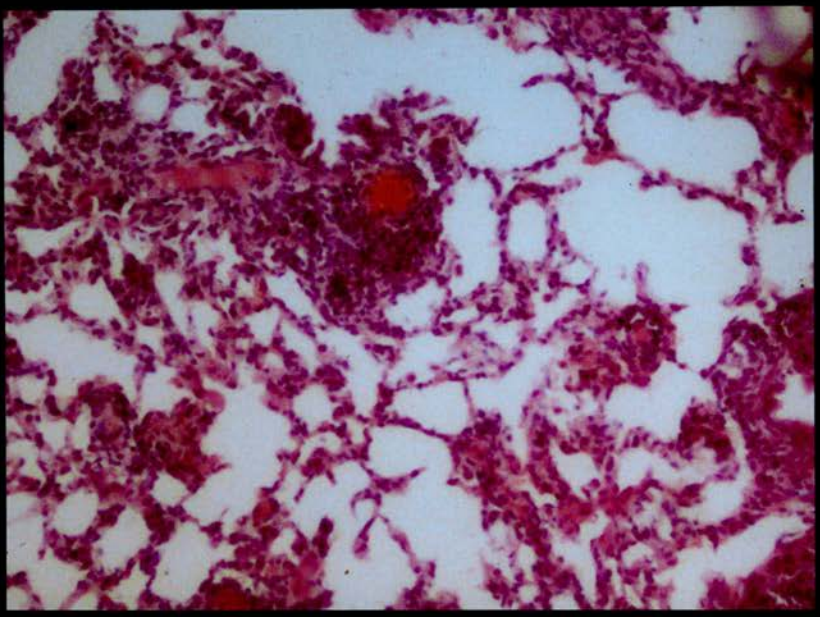


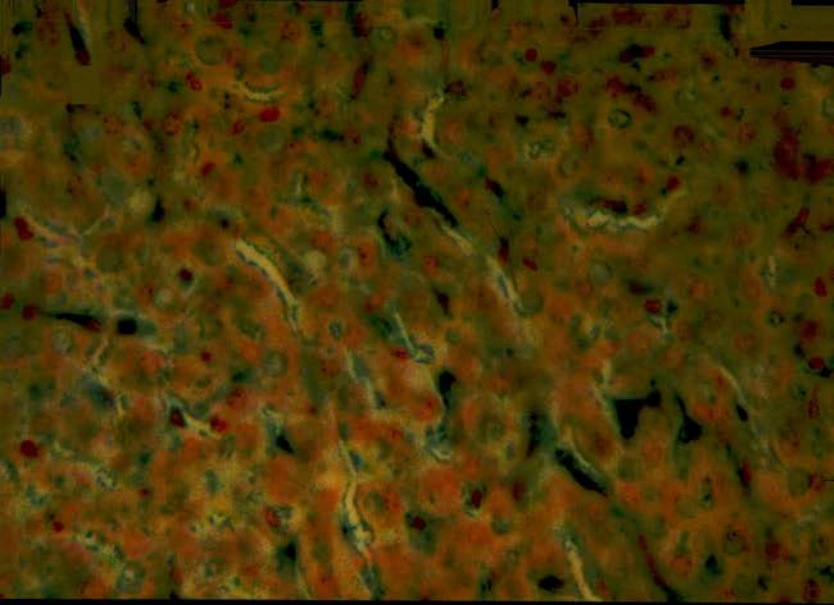
Fig. 20 : Iron in tissues of sheep killed 8 days after experimental trial for reproduction of systemic pasteurellosis.

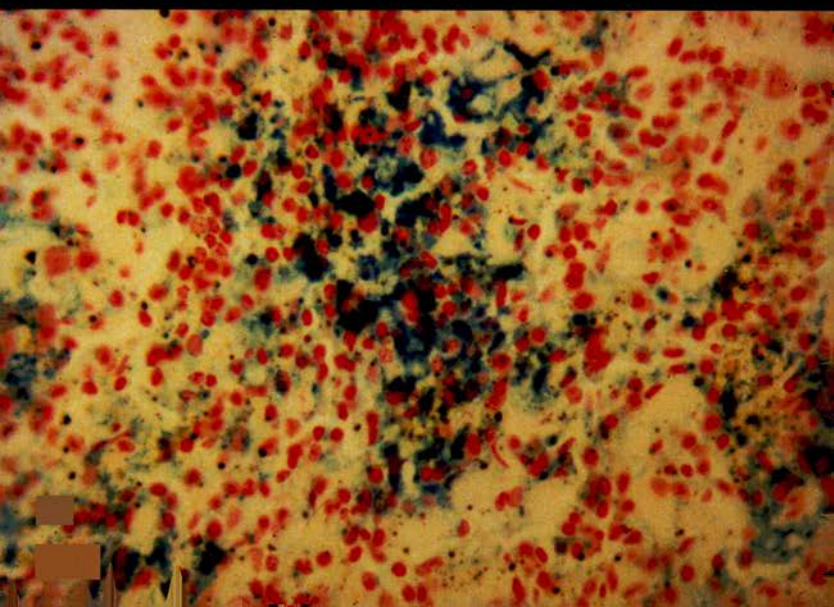
a) : Liver section showing iron engulfed by reticuloendothelial macrophages in the sinusoids. Prussian blue reaction

X 1360

b) : Spleen section showing iron in macrophages. Prussian blue reaction

X 1360.





Bacteriology : All three T serotypes were represented in the isolations made from the group before infection (Table 24). Seven days after the experiment had commenced the isolation rate from the group had more than doubled and on days 14 and 28 there was indication of transmission of infection to control sheep.

P. haemolytica T serotypes isolated from the internal tissue samples of the 2 animals killed 8 days after infection are indicated in Table 25.* All 3 serotypes were isolated from the spleen and tonsils of both animals while only T4 serotype was isolated from the lungs of animal 920 and T3 and T4 from animal 970.

Serology : No significant rise in serum IHA antibody titres against any of the three T serotypes was noted in any animal (Table 26).

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* See page 133

Table 24. Isolation of T serotypes of P. haemolytica from tonsillar swabs taken before and after infection of young adult sheep tonsils with a mixed T serotype culture. (Experiment 5).

Animal No.	Serotypes isolated											
	Before infection			Days after infection								
				7			14			28		
971			10	3		10	3	4	10		4	10
160	3	4		3	4	10	3			3		
970				3	4	10		D			D	
146	3			3		10	3					10
920			10	3	4	10		D			D	
121			10			10	3		10			
166_C				3		10		4	10		4	10
902_C							3					
956_C	3			3			3		10	3		10

C = control

D = dead

Table 26. IHA test results for serum samples collected before and after infection of the tonsils with a mixed T serotype culture of P. haemolytica.* (Experiment 5)

Animal No.	IHA titre						
	Before infection		Days after infection				
			14		28		
	T3	T10	T3	T4	T3	T4	T10
971	16	16	16	8	8	8	4
160	-**	-	8	8	32	16	-
970	32	16	D	D	D	D	D
146	-	8	16	16	8	-	-
920	8	8	D	D	D	D	D
121	8	8	16	32	16	32	8
166_C	-	16	16	32	32	32	16
902_C	8	16	-	8	32	16	-
956_C	-	8	-	8	8	8	-

* All serum samples taken before infection and at 7, 14 and 28 days after infection were tested against all three T serotypes. Those results not shown were negative (titre $\leqslant 4$)

** IHA titre $\leqslant 4$

C Control

D Dead

Experiment 6 : Tonsillar infection of lambs with a mixed T serotype culture of P. haemolytica after iron injection.

The experiment in young adult sheep established that administration of iron could exacerbate a P. haemolytica infection of the tonsils. Particularly severe local infections with some dissemination was achieved and the clinical response was obvious, although none of the animals died as a result of infection. It was considered possible, however, that lethal infections might be established in younger animals treated in the same fashion. Accordingly an experiment was undertaken to test this hypothesis and to make a direct comparison of the effect of iron injection on the progress of infection.

Experimental design : Finnish Landrace and Dorset lambs, 7 - 10 weeks old, were used. The lambs were kept with their dams in pens in an open sided building and were randomly separated into 3 groups. In each of two groups (A and B) of 8 lambs, 6 were used for experimental infection and 2 were uninfected controls, while in a third group (C) of 9 lambs, 6 were infected and 3 were controls.

In group A, lambs received a single tonsillar infection with a mixed T serotype culture. In group B, animals received an initial tonsillar infection and 24 hr later the tonsils were abraded and reinfected. Further infections without abrasion

were performed on each of the following 3 days. In group C, animals were treated as those in group B but in addition FAC was injected daily immediately before infection of tonsils. Control animals received a single daily injection of FAC. The number of viable individual T serotypes involved in the daily mixed cultures are presented in Table 27.

Results.

Clinical observations : In group A, no signs of clinical illness or rise in temperature were detected during the first 7 days after infection. However, in 2 of the 8 infected lambs an increase in temperature was recorded between days 8 and 11 and in one of them the elevation in temperature was accompanied by marked enlargement of both tonsils, pharyngeal congestion and intermittent coughing. The symptoms subsided after 2 days. It should be noted that one control animal also experienced a one-day rise in temperature 8 days after the beginning of the experiment. In group B, a small rise in body temperature occurred in three lambs (1768, 1734, 1759) 2 days after infection but lasted for 3 days only. All infected animals developed tonsillar lesions on one or both sides of the pharynx and the lesions were most pronounced and bilateral in lambs with increased temperatures. In four lambs coughing and moderate dyspnoea of 6 - 7 days duration were recorded. By the 12th day after initial infection all lambs were clinically improved and tonsillar healing had commenced.

Table 27. Number of viable T serotypes of P. haemolytica used to inoculate lamb tonsils.

Serotype	Number of viable organisms (Log 10^{10}) per ml of culture used on Day				
	1	2	3	4	5
T3	0.9	0.4	0.5	2.0	4.5
T4	0.2	0.2	0.4	3.8	1.8
T10	0.2	0.3	0.3	7.3	1.5

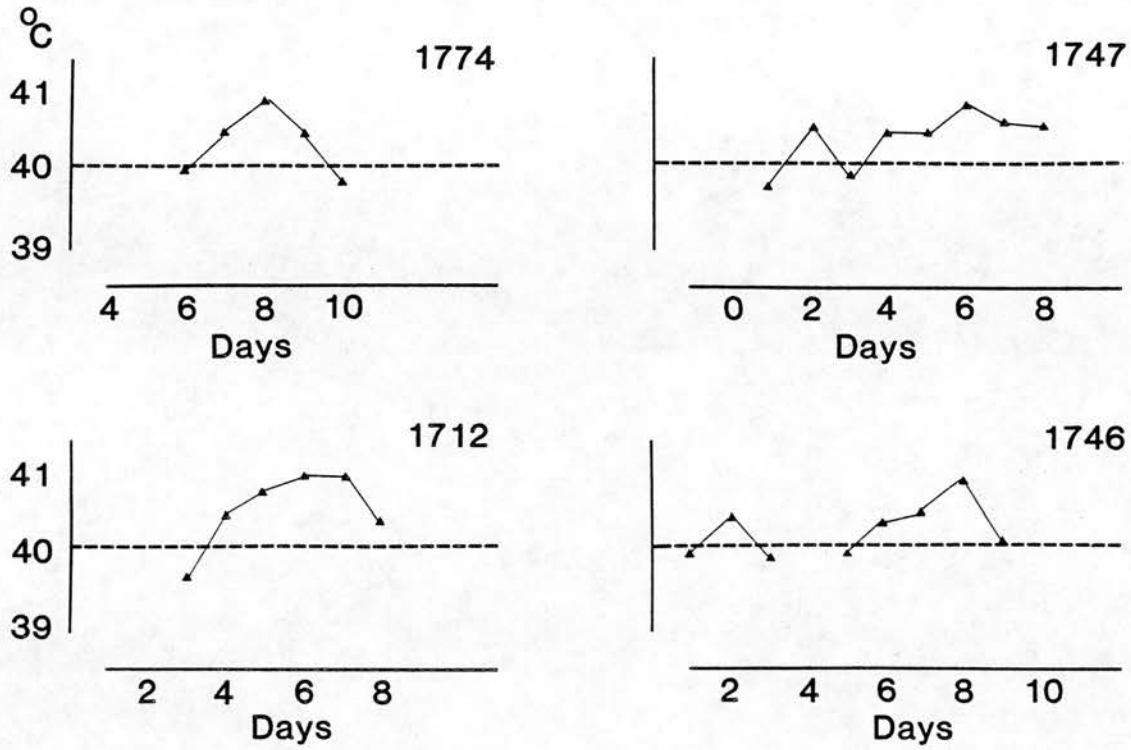
Table 29. Serotypes of P. haemolytica isolated from various organs of lambs in group C. (Experiment 6)

Lamb No.	Organs							
	Heart	Lung	Liver	Spleen	P.L.N.*	Kidney	Adrenal	Tonsil
1745 (died)	T4	T4	T4	T3, T4	T3, T4	T3, T4	T4	T4
1712 (killed)	-	T10	-	T10	T10	-	-	T3, T10
1747 (killed)	-	T10	-	T10	-	-	-	T4, T10

* Peripharyngeal lymph node.

Fig. 21 : Temperature responses of 4 individual lambs injected with iron and then given tonsillar infection with a mixed T serotype culture of P. haemolytica. The dotted line is the mean temperature of control lambs.

Exp. 6 group C


°C
41
40
39
1774
4 6 8 10
Days
1747
0 2 4 6 8
Days
1712
41
40
39
2 4 6 8
Days
1746
2 4 6 8 10
Days

The most severe clinical signs occurred in infected lambs of Group C, one of which died about 8 hours after the second infection. Four of the 5 remaining lambs developed pyrexia ($>40.5^{\circ}C$) (Fig. 21) of variable duration and were obviously dyspnoeic and dull, particularly between days 3 and 7 after initial infection. Over this period severe tonsillar swelling and ulceration were present and persisted for approximately 2 weeks after infection. About this time clinical recovery and healing of lesions commenced. One of the six infected lambs exhibited only mild clinical signs and little pathological change in the pharynx. None of the control lambs in groups B and C were clinically affected during the experiment.

Pathology : In addition to the lamb which died the two most severely affected lambs from group B and from group C were killed 8 days after the experiment began. Post-mortem examinations were carried out on all 5 lambs.

In lamb 1754 of group C which died during the experiment there was marked inflammation of the tonsils and pharyngeal mucosa, together with pronounced congestion of internal organs and widespread petechial haemorrhages on serosal and mucosal surfaces. Histologically all internal organs revealed vascular congestion and haemorrhagic inflammation. In the liver, the portal areas were infiltrated by a variety of cell types with macrophages and lymphocytes predominating. In the parenchyma foci of hepatic

cell necrosis were found. Apart from haemorrhage no distinct pathological changes were detected in the lungs.

Both lambs of group C killed on day 8 (1712 and 1747) had erosions and ulcers in the tonsillar region and in one (1747) there was abscess formation (Fig. 22). In both, peripharyngeal lymph nodes were swollen and congested and their cut surfaces were oedematous and haemorrhagic. In the lungs areas of consolidation were present in the dependent parts of all lobes. In one lamb (1947) there was limited shallow ulceration of the mucosa of the upper oesophagus with a surrounding zone of haemorrhagic inflammation. In the other lamb (1712) several necrotic ulcerative lesions about 2 - 3 mm diameter were present in the wall of the abomasum with an associated inflammatory reaction. Other than congestion no specific gross pathological features were seen in other internal organs.

In the sacrificed lambs of group B tonsillar and pharyngeal lesions were present but much less prominent than those of group C lambs. Focal pneumonia affected the dorsal surface of the apical and cardiac lobes of one lamb (1768). The only other remarkable feature was congestion of the intestine in lamb 1734.

Histologically, samples taken from animals 1712 and 1747 of group C exhibited severe inflammation and necrosis of the tonsils and adjacent lymph nodes. A varying degree of suppuration and

destruction of tonsillar parenchyma was evident as was destruction of tonsillar crypt epithelium (Fig. 23). The inflammatory reaction and necrosis extended into the subepithelial tissue. In regional lymph nodes necrosis, haemorrhage and inflammatory cell infiltration were common (Fig. 24). A cellular infiltration, consisting mainly of lymphocytes and macrophages but with few neutrophils occurred in and around the hepatic portal triads (Fig. 25). The oesophageal ulcer in lamb 1747 was being infiltrated by inflammatory cells, mainly neutrophils and macrophages which were present in the lamina propria and around the oesophageal gland (Fig. 26). Exudative inflammatory changes with infiltrating neutrophils and mononuclear cells also affected the abomasal lesions detected in lamb 1712 (Fig. 27). Lung sections from both animals revealed severe exudative reactions involving the bronchioles and alveolar spaces (Fig. 28). Alveolar vasculitis affecting the small blood vessels in the interseptal tissue was a distinct pathological feature (Fig. 29). Fibrin was present in the pulmonary alveoli and in the pleural and subpleural surfaces together with neutrophils and cellular debris.

In the lambs of group B the only significant changes detected were suppurative inflammation of the tonsils and adjacent lymph nodes and ulceration of tonsillar epithelium. Lung sections from lamb 1768 revealed only mild inflammatory changes of the interalveolar septa.

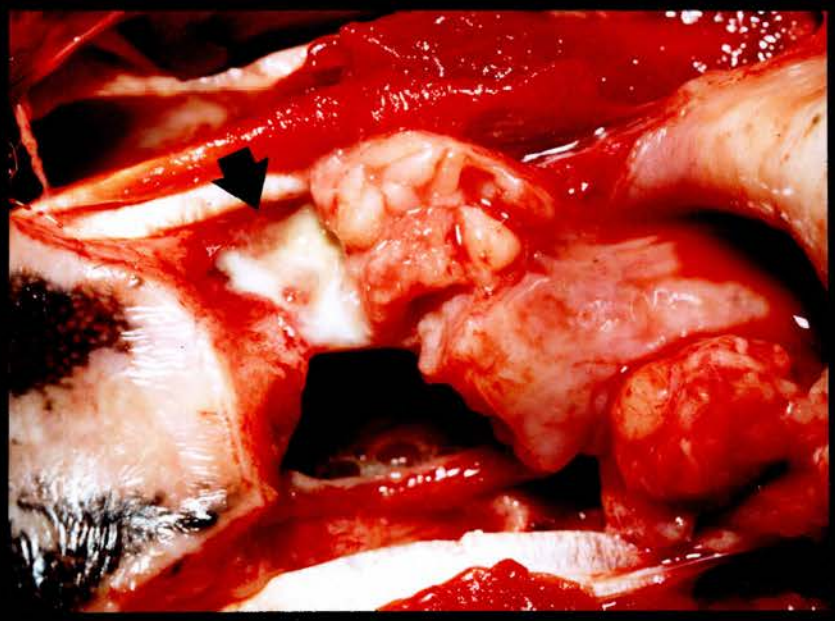
Fig. 22 : Oral cavity of lamb No. 1747 showing an opened abscess in the tonsil (arrow) after repeated topical infection with a mixed culture of T serotypes of P. haemolytica and daily iron treatment intravenously.

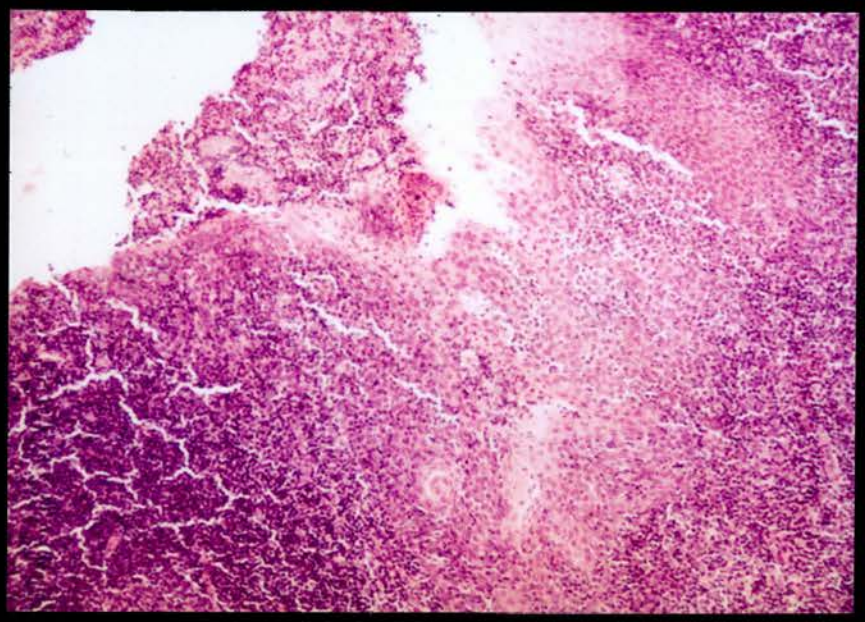
Fig. 23 : Tonsil : extension of inflammation in the crypt epithelium and subepithelium after topical infection with mixed T serotype cultures of P. haemolytica.

H & E X340

Fig. 24 : Peripharyngeal lymph node : necrosis of parenchymal cells and blood vessels with extensive cellular infiltration, mainly of neutrophils and macrophages.

H & E X544





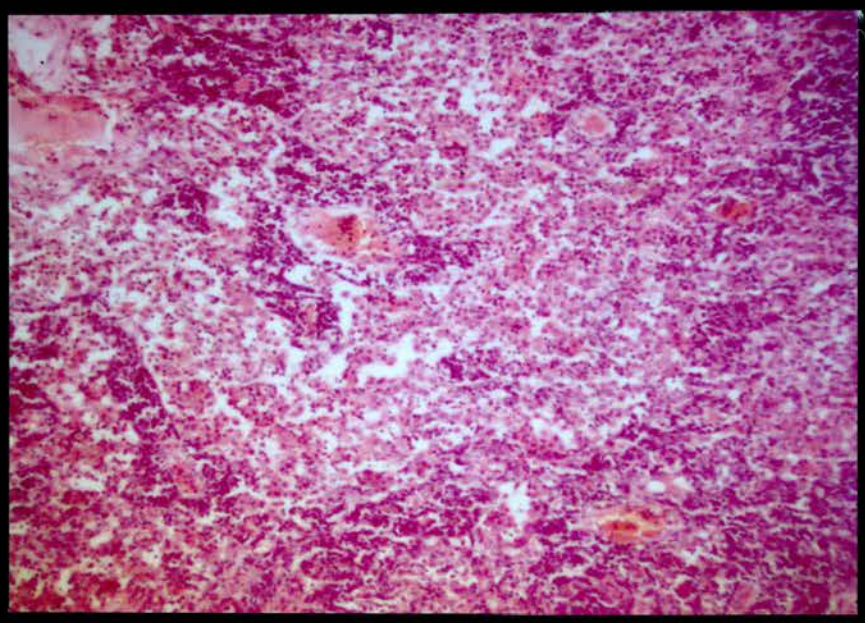


Fig. 25 : Liver : focal area of cellular infiltration in liver parenchyma composed mainly of macrophages, lymphocytes and few neutrophils.

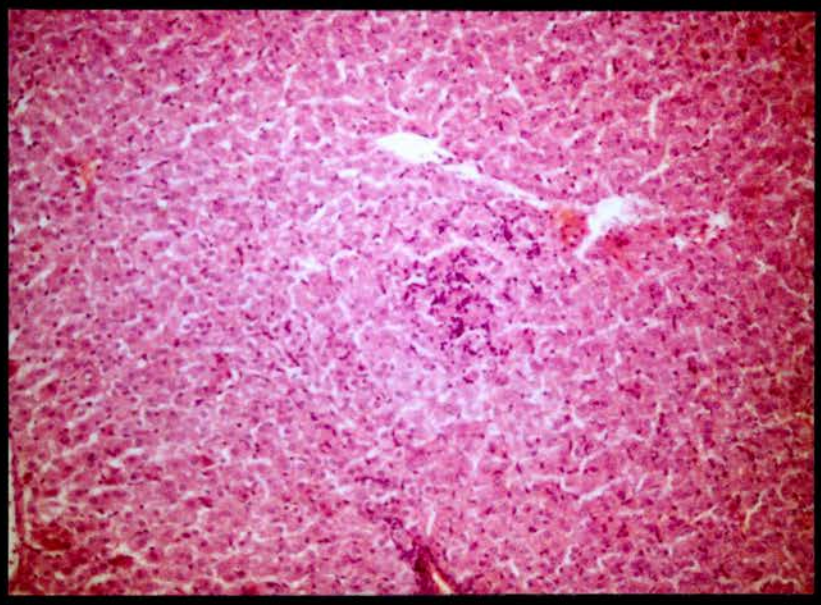
H & E X340

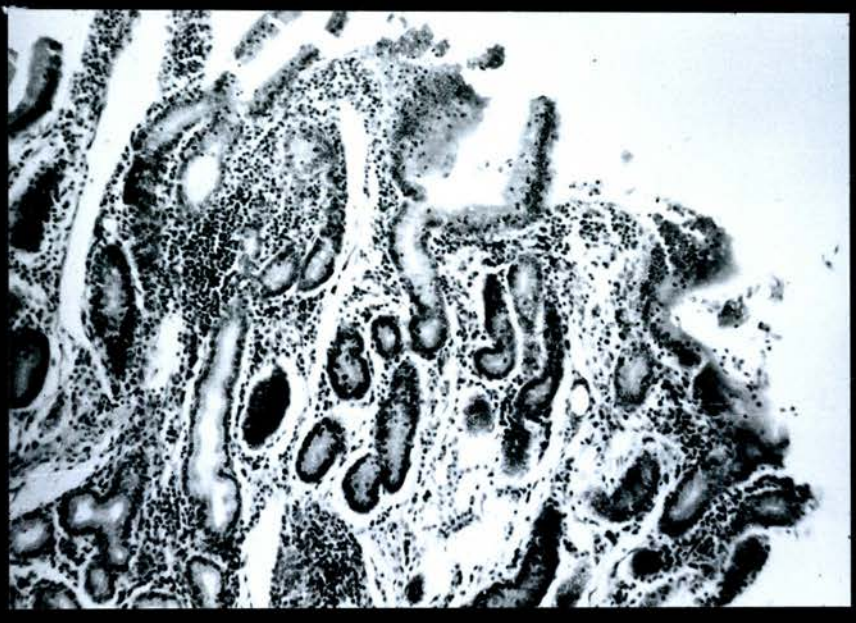
Fig. 26 : Oesophagus : necrosis of epithelial mucosa with extention of inflammation between the tubuloacinar glands. Inflammatory cells are mainly composed of macrophages, lymphocytes, neutrophils and a few eosinophils.

H & E X544

Fig. 27 : Abomasum : necrosis of the villi with a marked inflammatory reaction extending to the submucosa.

H & E X340





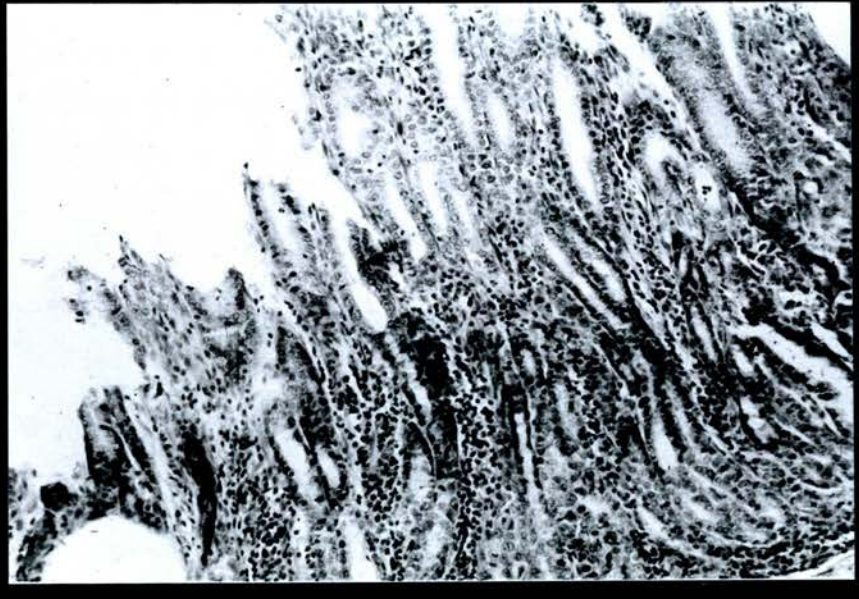
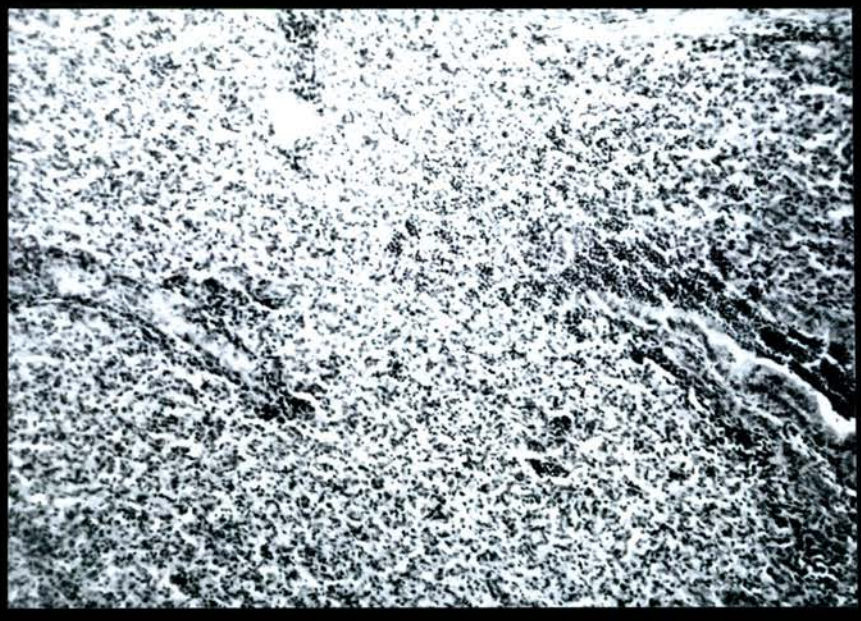


Fig. 28 : Lung : lobar pneumonia after repeated tonsillar infection and iron treatment. Note inflammatory exudate in the bronchioles and alveoli.

H & E X544

Fig. 29 : Lung : necrosis of alveolar capillaries with infiltration of the wall by inflammatory cells

H & E X 1360



Bacteriology : The individual T serotypes isolated from the tonsillar area of lambs of all 3 groups before and after infection are listed in Table 28*. In group A (single infection) no isolations were made on day 7, but the majority of swabs taken on days 14 and 21 were positive. In groups B and C (repeated infections) P. haemolytica was readily isolated on each of the 3 sampling days.

T serotypes of P. haemolytica were isolated from the tonsils, but not from any internal organ of the 2 group B lambs. In contrast isolations were made from tonsils and several organs of lambs from group C (Table 29)**. In the case of the lamb which died (1745) the bacterium was recovered from every organ sampled implying a wide-spread dissemination of infection. However, P. haemolytica was recovered only from the lungs, spleen and pharyngeal lymph node of the lambs which were killed and in each the serotype isolated was T10.

Serology : No significant IHA serological response was detected against any of the T serotypes inoculated on to the tonsils.

Discussion.

In this chapter the work undertaken with mice was designed to establish that prior administration of iron would render mice more susceptible to P. haemolytica. That this was indeed the case was evident from the substantial reduction in LD_{50} which

* see page 162

** see page 149

Table 28. Isolation of T serotypes of P. haemolytica from tonsillar swabs taken before and after infection of lambs tonsils with a mixed T serotype culture. (Experiment 6).

Animal No.	infected	Serotypes isolated			
		before infection	Days after infection		
			7	14	21
Group A					
1749	yes	3			3 4
1706	yes			3 4	4
1753	yes	3 10		4	10
1751	yes				4
1779	yes	10		4	10
1727	yes			3 4	
1729	no	10		3 10	10
1750	no			10	4 10
Group B					
1719	yes	3	3 10	3 4	3 4
1762	yes	10	3 4 10	3 4 10	4
1760	yes	10	10	10	10
1759	yes	10	3 10	3 4	3 10
1768	yes	3	3 4 10	K	K
1734	yes	3	3 4 10	K	K
1758	no		3	3 4	4
1757	no		3 4 10	4 10	3 4 10

Table 28 continued.

Animal No.	infected	Serotype isolated											
		before infection	Days after infection										
			7			14				21			
Group C													
1774	yes	3			3		10	3	4	10	3		
1756	yes	3			3	4	10		4	10	3		10
1746	yes			10	3		10	3	4			4	10
1712	yes				3	4	10		K			K	
1747	yes			10	3	4	10		K			K	
1754	yes					D			D			D	
1775	no	4		10			10		4	10	3		10
1755	no	4					10		4	10		4	
1793	no				3						3		

K : killed

D : died

was achieved (Table 21). In itself this result was not unexpected as iron is well recognized as an infection-promoting substance (Weinberg, 1978) and the mechanism by which it exerts this effect is at least partially understood (Holbein, 1981). However, so far as the author is aware, there have been no previous accounts of the use of iron to enhance the infectivity of P. haemolytica. The sole method hitherto available has been the i.p. inoculation of bacteria suspended in hog gastric mucin, the technique adopted by Smith (1958) and employed by others who have used the mouse as a model for P. haemolytica infections (Evans and Wells, 1979).

Although extensive use has been made of mucin for establishing experimental infection with various microorganisms, information on its mode of action is scanty and incomplete (Calver, Kenny and Lavergne, 1976). Speculations have ranged from the provision of a protective surface coating for bacteria (Olitzki, 1948) to the interaction of several factors, including viscosity, rather than the effect of one alone (Smith, Harris-Smith and Stanley, 1952). However, during analysis by spectrophotometry and atomic absorption of different lots of 5% hog gastric mucin, Calver et al. (1976) established that iron, at fairly high concentrations, is a component of mucin. Furthermore, they proved that the ability of mucin to promote infection was decreased to a large extent by a specific iron chelating agent which has been shown to remove iron from transferrin and to deplete iron stores in tissues (Moeschlin, 1962).

Thus it would appear that iron and mucin act in a similar fashion in promoting bacterial pathogenicity. Assuming that the mucin used in the mouse experiments 2 and 3 contained 100 μg of iron per ml (the mean value determined by Calver et al. (1976)) the amount of iron available in the injected mucin would have been 40 μg/mouse. This figure quite closely approximates the 60 μg of aqueous iron injected i.v. to each 30g mouse. That mucin and iron effected a similar reduction in LD_{50} of P. haemolytica (Table 22) can be taken as evidence in support of the view that virulence enhancement by mucin is attributable to its iron content. Calver and colleagues (1976) demonstrated that, when mice were pretreated with iron compounds in amounts equivalent to the iron in mucin before infection with Neisseria meningitidis, mortality was comparable to that of mice given the organism in mucin. However, in the experiments with P. haemolytica combination of iron pretreatment and injection of organisms in mucin reduced the LD_{50} between 50 and 100-fold (Table 22) which is considerably more than might be expected by doubling the amount of iron injected. This suggests an interactive rather than a simple additive effect and implies that mucin acts in ways additional to the effect of its contained iron.

Before proceeding to study the course of P. haemolytica infection in iron-treated sheep it was necessary to establish the pattern of iron excretion from the circulation as no previous data existed for sheep. The dose of iron used was simply scaled

up from that employed successfully in mice. Figure 13a,b, which illustrates the excretion pattern, very closely resembles curves obtained in guinea pigs given similar doses of iron by Bullen, Gushnie and Rogers (1967). Although serum iron concentration fell rapidly during the first 6 hours after injection it was still, even at 6 hours, at least twice that of normal sheep. During this period therefore the iron-binding capacity of serum would have been exceeded and excess iron would have been available in a free ionic state to microorganisms in tissues and probably to those on mucosal surfaces.

The consistently observed lip-licking which immediately followed iron injection was attributed to the rapid distribution of iron from circulation to tissues and secretions including saliva. The absence of other symptoms implied no adverse physiological effect and indeed Bullen et al. (1967) detected no more than a mild transient trembling in guinea pigs given 20 mg iron/kg. i.e. 10 times the concentration administered to sheep.

By 24 hours after injection serum iron concentrations had returned to normal values. The pattern of excretion was repeated when a further dose of iron was administered without untoward effect on the animal. Thus it was concluded that daily injections could safely be given over a 5 day period.

The first attempt to induce T type disease in iron-treated sheep (experiment 5) was successful in reproducing tonsillar-pharyngeal lesions similar in type, though not in extent or severity, to those seen in the natural disease. In addition, systemic spread of one or more of the T serotypes was demonstrated. Despite this, none of the animals died or developed severe clinical illness. These findings suggested that for induction of severe or fatal disease additional factors which either altered host resistance or favoured the growth and multiplication of the bacteria were required. Experimentally, alteration of host resistance could be achieved by immunosuppressive agents, by concurrent infection with other organisms or by phagocytic blockade. However, as any of these approaches would have introduced an additional experimental parameter, it was decided to repeat the experiment in sheep of a younger age group which might prove more susceptible to experimental infection.

Although the outcome of experiment 6 (group C) demonstrated the greater pathogenicity of P. haemolytica in iron-treated lambs, in only one of the 6 animals was the outcome fatal. In this lamb the infection had undoubtedly gone through a bacteraemic phase as 2 of the infecting serotypes were recovered in profusion from all organs tested (Table 29). In two further lambs the severity of local lesions and limited spread of at least one serotype to spleen and lung implied that systemic disease had almost been achieved. However, the histopathological picture

in internal organs (Figs. 25, 28) was indicative of a contained rather than a progressive infection. Thus, even using an experimental model weighted in favour of the organism, reproduction of the natural disease proved elusive.

Conclusions :

(1) Iron increases the pathogenicity for mice of T serotypes of P. haemolytica.

(2) Iron injection of sheep increases the severity of experimental tonsillar infection with T serotypes of P. haemolytica.

(3) In iron-treated lambs, dissemination of experimental infection from tonsils can occur in some cases.

CHAPTER 4.

PRODUCTION AND EVALUATION OF SEROTYPE SPECIFIC ANTISERA AGAINST P. HAEMOLYTICA.

Introduction

In the two preceding chapters it was shown that tonsillar infection with T serotypes of P. haemolytica could be established experimentally. The essentially mild local reaction induced by simple infection could be rendered more severe by causing superficial damage to the pharyngeal and tonsillar epithelium and by temporarily increasing the concentration of ionic iron in host tissues. While it was recognized that a true experimental reproduction of natural systemic pasteurellosis had not been achieved it was considered that useful information could be obtained by more detailed investigation of the bacteria in situ. Study of the distribution and inter-relationships of the various serotypes in key tissues of normal and diseased animals, both naturally and experimentally infected, could yield information relevant to the epidemiology and pathogenesis of ovine pasteurellosis.

Opportunity was also available to study tonsillar tissue from cases of natural systemic pasteurellosis and various tissues from sheep experimentally infected with selected A and T serotypes of P. haemolytica. The former material was received from the

Veterinary Investigation Centre at St. Boswells as tissue prefixed in absolute alcohol. The tissues from animals experimentally infected with A serotypes were provided by Dr. N.J.L. Gilmour and his colleagues in Moredun Research Institute, as was material from sheep experimentally infected by i.v. injection of serotype T4 in agar. The author gratefully acknowledges these donations of tissues which permitted extension of his study of pathogenesis at tissue level.

However, certainly in many normal sheep and in the majority of experimental infections more than one T serotype was likely to be present in the tissues to be examined. Confident identification of individual serotypes in tissue sections requires serotype-specific antisera of a potency sufficient for immunofluorescence or similar procedures. Preliminary trials with available rabbit antisera used in the routine serotyping of P. haemolytica by indirect haemagglutination indicated that they were unsuitable for identifying serotypes in tissues and attempts were therefore made to produce antisera capable of doing this.

Antisera were produced against serotypes A1, A2, A6, T3, T4 and T10 which were those most appropriate to the work being undertaken for this thesis. The production and evaluation of these antisera are the subject of this chapter.

Materials and methods

Immunization procedures : Antisera were produced in New Zealand White rabbits aged 7 - 9 months, 5-week old Leghorn type chickens and 3 - 4 week old Swiss albino mice.

(a) with live microorganisms : Each of the 6 serotypes was given i.v. to 2 rabbits and 3 chickens and subcutaneously (s.c.) to 20 - 25 mice. For rabbits and chickens the volume of inoculum was 0.3 ml, 0.5 ml and 0.5 ml on days 0, 7 and 14 respectively and for mice the corresponding volumes were 0.2 ml, 0.2 ml and 0.3 ml. The number of microorganisms in the inoculated suspensions, determined by the method of Miles and Misra (1938), varied with the serotype and day of growth but had a mean of 6.9×10^7/ml with a range of 10^6 to 6×10^9/ml.

Rabbits, mice and chickens were bled 10 days after the last inoculation while for mice, 0.3 ml of freshly harvested Landschütz mouse ascites tumour cells were injected i.p. on day 17. When the mice were killed 7 to 10 days later blood and ascitic fluid (Stuart and Elhassan, 1969) were collected.

(b) with SSE : Aqueous SSE was blended with equal volumes of FCA or FIA. The SSE in FCA was injected intramuscularly to rabbits (1.0 ml) and s.c. to chickens (0.2 ml) and to mice (0.1 ml). Ten days later antigen in FIA was given by the same routes and in the same volumes. A third injection of aqueous

suspension of SSE was similarly administered on day 20. Rabbits and chickens were bled 10 days after the last injection. In the case of mice, 5 from each serotype group were killed and blood collected from the heart and pooled for each group.

(c) with SSE and live microorganisms : Ten days after completion of the SSE schedule rabbits, chickens and surviving mice were given live microorganisms of the appropriate serotypes by the i.v. (rabbits, chickens) or s.c. (mice) route in volumes of 1.0 ml, 0.2 ml and 0.1 ml respectively. The mean inoculum count per ml was 9.5×10^8 with a range of 10^6 to 7.5×10^9. All animals were bled 7 - 10 days later.

Bacterial antigens and cell bodies : In addition to whole bacterial cells and SSE use was made of three other preparations derived by treatment of whole bacterial cells and of the respective "stripped" cell bodies. These materials were kindly donated by Mr. W. Donachie of the Institute's Microbiology Department. The procedures involved removal of surface components by the following procedures:-

(i) phenolwater extraction (PWE) (Westphal, Luderitz and Bister, 1952).

(ii) extraction by Ethylenediaminetetra acetic acid (EDTA) (Zollinger, Kasper, Veltri and Artenstein, 1972).

(iii) extraction in saline : an overnight blood agar culture was washed off with 2.5% saline and the resulting suspension homogenized with a blender (MSE)* for 5 min. Cells were removed by centrifugation and the supernatant precipitated with 5 volumes of cold (4°C) acetone. After two further washes in acetone the precipitate was suspended in 1 ml distilled water.

Treatment with 2-Mercaptoethanol (2-ME) : To demonstrate their sulphydryl sensitivity rabbit antisera were treated with one-tenth volume of 1M 2-ME for 30 min. at 37°C in a microtitre plate (Schlueberberg, 1965; Reid, Gibbs, Burrells and Doherty, 1972). Comparative IHA tests were done on treated and untreated sera.

Gel filtration : This procedure was undertaken for the author by Mr. A.M. Dawson of the Institutes' Physical Chemistry Department. Two ml aliquots of rabbit anti-A1 and of chicken anti-A6 each resulting from stimulation with live microorganisms, were fractionated on a column of Sephadex G200 using 0.1M Tris-HCl buffer pH 8 containing 1.0M NaCl and 0.02% sodium azide. The flow rate was maintained at 280 mm using an ISCO Model UA5 monitor. Fractions (6.1 ml) were collected and submitted to the IHA test.

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* MSE Scientific Instruments, England.

Serology : Sera were assayed for specificity and potency by the following tests: IHA, ID, CIE, IIF and IPO, details of which are given in general materials and methods (p.38).

Results

Only a few of the mouse serum and ascitic fluid samples were positive in IHA, ID and CIE tests. As reactions were weak and there were cross-reactions with heterologous serotypes, mouse material was not studied further.

In both chickens and rabbits repeated inoculation of live microorganisms consistently generated higher titres of IHA antibodies than did injection of SSE (Table 30). A poor response to SSE alone was generally improved by a subsequent single i.v. administration of live microorganisms although not in the case of chickens with serotypes A2 and T4 or rabbits with serotype T10. However, 2 rabbits given this immunizing regime became ill soon after administration of live microorganisms (T3 or T4). One died within 18 hours and the other was destroyed shortly afterwards.

In gel diffusion tests supernatant fluids from 24 hour broth cultures of P. haemolytica did not result in precipitin lines when tested against homologous antisera either by ID or CIE but SSE proved a satisfactory antigen for both tests. In these tests 48 of 54 (89%) chicken sera, and 17 of 36 (47%) rabbit sera were positive (Table 30). Notable was the failure of

Table 30. Serum IHA antibody titres to homologous serotypes of P. haemolytica in chickens and rabbits inoculated with live microorganisms, injected with sodium salicylate extract (SSE) or given both treatments.

P. haemolytica serotype	Chickens ♀ immunized with			Rabbits ♀ immunized with		
	live microorganisms	SSE	SSE + live microorganisms	live microorganisms	SSE	SSE + live microorganisms
A1	512**	16*	1024*	2048**	32*	512*
	1024**	64**	1024*	4096**	32*	2048**
	2048**	256**	512*			
A2	512	8*	16*	1024	< 2	32*
	512	4*	16*	1024	4	1024*
	1024	8*	16*			
A6	2048**	256	256*	1024	16	128*
	1024**	128*	256*	4096	16	64*
	2048**	64*	256*			

Table 30 continued.

P. haemolytica serotype	Chickens ∅ immunized with: live microorganisms	SSE	SSE + live microorganisms	Rabbits ∅ immunized with: live microorganisms	SSE	SSE + live microorganisms
T3	4096**	4096*	4096*	2048	4096*	<2*
	128	64*	1024*	1024	128*	D
	256	64*	4096*			
T4	128**	16*	16*	8192	<2	2048*
	4096**	32**	64**	8192	8	D
	4096**	64*	64**			
T10	2048*	1024*	4096*	2048	4*	16*
	2048**	128**	256**	1024	8*	8
	4096**	1024*	4096*			

A single asterisk indicates those sera positive in gel diffusion tests and a double asterisk those precipitating only the homologous antigen.

∅ : Highest preinoculation titre = 4

D : died

precipitin production by 3 chickens receiving only live A2 microorganisms and by 2 birds given T3 microorganisms, whereas their counterparts injected with SSE plus live microorganisms did respond. The single serotype to which rabbits receiving only live microorganisms responded was A1 whereas responses were evoked by SSE to A1, T3 and T10 and to all serotypes by treatment with SSE plus live microorganisms. In neither species did precipitin response correlate with IHA titre.

Of the 48 precipitin-positive chicken antisera 16 produced well defined single lines and 3 a double line against their homologous serotypes only and were considered serotype specific whereas the remainder showed cross-reactivity with one or more heterologous serotypes (Fig. 30). Only 3 of the rabbit antisera were serotype specific and all were anti-A1. Side by side examination by ID of individual antisera considered specific for the same serotype verified their identity (Fig. 31). In this way sera were assembled with individual specificity for serotypes A1, A6, T3, T4 and T10. No chicken or rabbit produced a serum with specificity restricted to A2 and attempts to remove cross-reactivity by absorption with heterologous serotypes also eliminated reactivity to serotype A2.

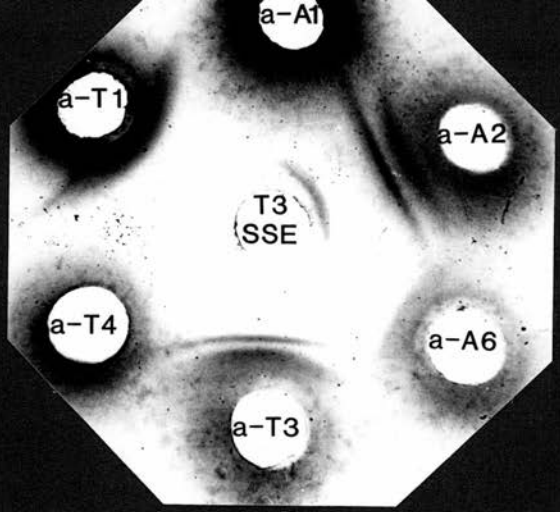
The chicken and rabbit antisera judged to be serotype specific by ID were tested by indirect IF against slide preparations of the full range of recognized serotypes and one untypable strain. The

Fig. 30 : Precipitin reactions between P. haemolytica SSE antigens and chicken antisera to individual serotypes.

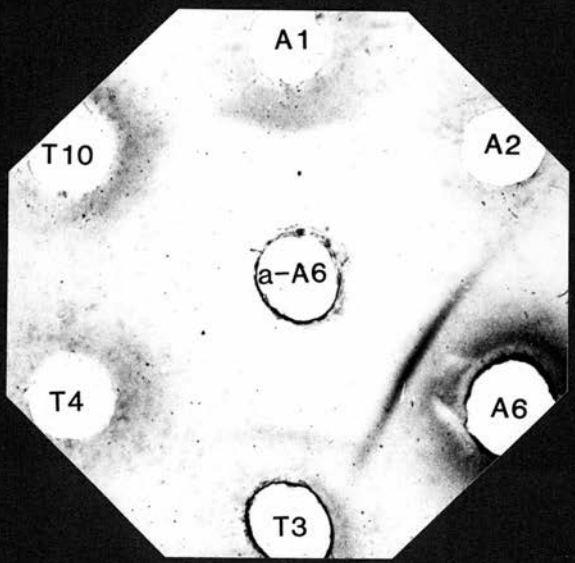
a) Only antisera to A1 and T4 fail to cross-react with T3 antigen in the centre well.

b) Antiserum to A6 reacts predominantly with homologous antigen.

c) Only antiserum to A6 reacts with A6 antigen.


a-A1
a-T1
a-A2
T3
SSE
a-T4
a-A6
a-T3

a


A1
T10
A2
a-A6
T4
A6
T3

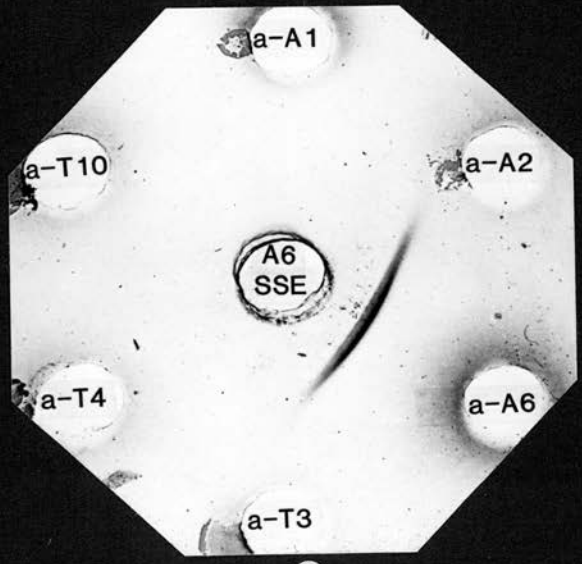

a-A1
a-T10
a-A2
A6
SSE
a-T4
a-A6
a-T3

Fig. 31 : Identity of serological reactions.

a) : Between individual homologous chicken antisera and SSE antigen of P. haemolytica serotype A6.

b) : Between individual homologous chicken antisera and SSE antigen of P. haemolytica serotype T4.

A6
SSE

T4
SSE

majority of antisera cross-reacted with a number of heterologous serotypes but cross-reactions were effectively resolved by partial dilution of the primary antisera (Table 31). No specific fluorescence resulted when conjugated reagents were applied directly to dried bacterial suspensions or to preparations initially treated with preinoculation chicken or rabbit sera. Specific fluorescence was blocked when slides exposed to serotype specific antisera were treated with unconjugated species anti-globulin before application of the respective conjugated reagent.

Tests were also conducted to establish the pH optimum of the IIF test with P. haemolytica, using the procedures described by Riser, Noone and Poulton (1976) in their work on Klebsiella species. Working over a pH spectrum from 5 to 11 it was found that satisfactory homologous reactions were obtained within a pH range of 7 to 9 (Fig. 32). Above pH9 some heterologous reactions were weakened, others became more marked. As a routine therefore IIF tests were conducted at pH 7.4.

In all cases specific fluorescence was confined to the outer part of the bacterial cells (Fig. 33).

With the IPO test recognition of microorganisms in smears was readily achieved but cross-reactions with heterologous serotypes occurred even to titres as high as the homologous serotype. The test was therefore considered not as reliable as

Table 31. Cross reactions in indirect immunofluorescence tests of antisera judged serotype-specific by immunodiffusion and dilutions needed to resolve cross-reactivity.

Specificity by immunodiffusion	Immunofluorescence		
	homologous titre	cross-reactivity detected with	dilution to remove cross-reactivity
A1	1 : 640	A7, A9	1 : 20
A6	1 : 1280	A7, A9, A12	1 : 40
T3	1 : 160	-	
T4	1 : 640	A6, A7, A11, A13, A14	1 : 80
T10	1 : 1280	A6, A7, A13, T3, T4	1 : 80

Fig. 32 : The effect of pH of antibody-antigen reaction on immunofluorescence of homologous and heterologous serotypes of P. haemolytica using

(a) anti-A1 serum

(b) anti-T10 serum

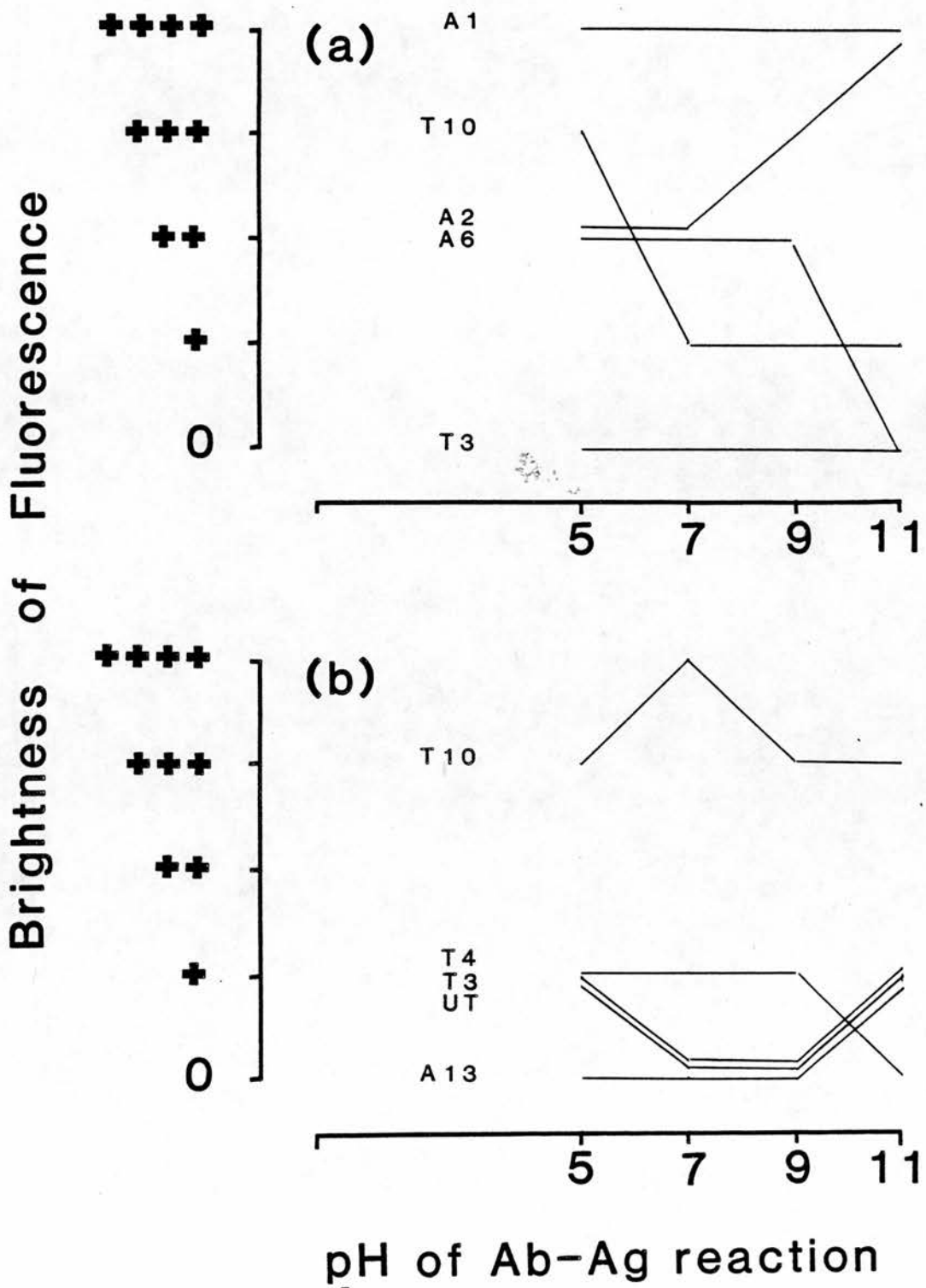

Brightness of Fluorescence
++++
+++
++
+
0
(a)
A1
T10
A2
A6
T3
5
7
9
11
(b)
T10
T4
T3
UT
A13
5
7
9
11
pH of Ab-Ag reaction

Fig. 33 : P. haemolytica serotype A6, revealed by indirect immunofluorescence.
A drop of broth culture, dried on a microscope slide was treated with homologous chicken antiserum and then with FITC conjugated rabbit anti-chicken globulin.

IIF stain X 1800

Fig. 35 : Indirect immunofluorescence staining of P. haemolytica in tissue. Serotype A1 in pulmonary alveoli.

IIF stain X 1125

IIF for the purpose of discrimination between individual serotypes of P. haemolytica and no further evaluation was carried out.

Because FCA contains Mycobacterium butyricum it was thought necessary to know what serological response to this microorganism might be produced in animals immunized with SSE in this adjuvant. In the sera of two chickens inoculated once with 0.1 ml FCA alone antibody titres of 1/256 to M. butyricum were detected in IHA and IIF tests with this microorganism. In similar tests using P. haemolytica there was no cross-reaction by IHA but in IIF titres of 1/16 and 1/32 were obtained against A1 and A6 respectively. When antisera to the 6 selected P. haemolytica serotypes were subjected to IHA and IIF tests with M. butyricum there was little evidence of cross-reaction. Trivial titres ($<1/16$) were obtained from 6 of 12 rabbit antisera and 15/18 chicken antisera. The 6 other rabbit antisera were negative while three chicken antisera had titres of 1/32, 1/64 and 1/512 respectively.

To demonstrate that most of the activity of antisera specific for individual serotypes of P. haemolytica related to surface antigens a number of absorption tests were carried out with representative anti-A1, and anti-T4 sera.

Aliquots of each serum were absorbed once with an equal volume of a 1/20 suspension in PBS of cell bodies from which surface components had been removed by one of the 4 methods

previously described (p.172). Further aliquots of each serum were absorbed with the material removed from the surfaces of the bacterial cells by SSE and by PWE.

Absorption with cell bodies prepared by treating whole cells with saline, PWE or SSE caused no more than a two-fold reduction in serum IIF titres whereas an eight-fold drop in titre resulted from absorption with cell bodies prepared by the EDTA method. In contrast, serum titres fell from 1024 to 8 and 16 respectively after absorption with SSE or PWE. This finding implied that most of the antibody activity was directed to surface antigens. Supportive evidence was obtained by carrying out IIF tests on bacterial cells from which surface components had been removed. The following are typical of the results obtained.

Whole cells	IIF titres obtained with cells prepared by			
	SSE	EDTA	PWE	Saline
1024	64	128	32	128

The immunoglobulin class specificity of rabbit antibody detected by the IHA test was investigated using 2-Mercaptoethanol. Antisera specific for each of the 6 serotypes tested before and after 2-ME treatment gave the results shown in Table 32. The very substantial reduction in titres obtained indicated a major role for IgM in this test. This was substantiated for both chicken and rabbit antisera by the results of gel filtration studies. Fig. 34a and b illustrate the elution profiles and Table 33 records the IHA titres of individual unconcentrated fractions.

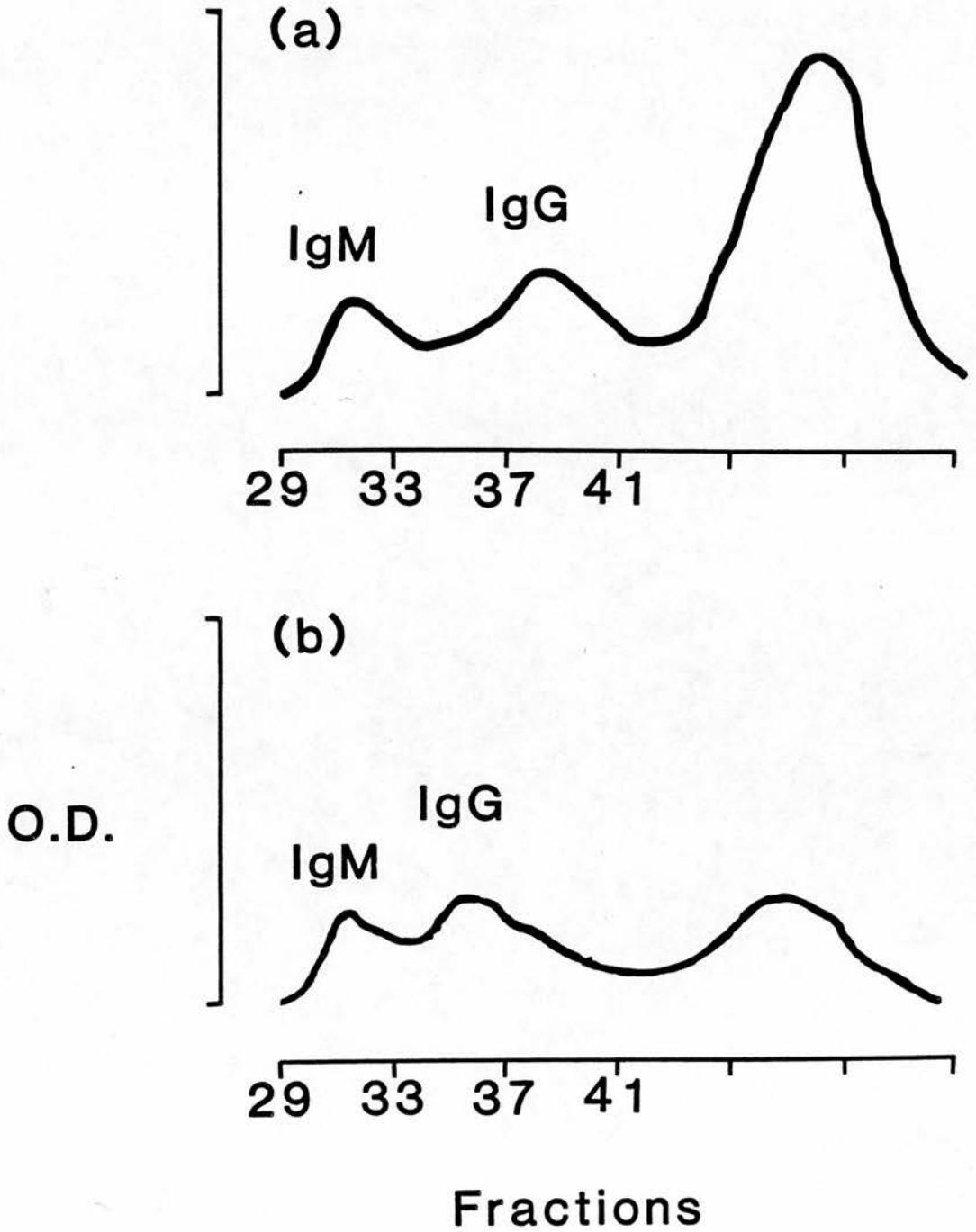
Table 32 : IHA activity of rabbit antisera before and after treatment with 2-Mercaptoethanol.

Test	Antisera against serotypes									
	A1			A2		A6	T3	T4	T10	
	1	2	†	2	1	2	1	1	1	2
Before	256	1024	32	512	512	512	0	1024	8	16
After	16	128	0	0	16	32	0	16	0	0

Table 33 : IHA activity of IgG and IgM containing fractions of chicken and rabbit.

Antisera	IgM Fractions				IgG Fractions						
	30	31	32	33	34	35	36	37	38	39	40
Rabbit	16	32	16	-	-	-	-	4	4	4	4
Chicken	64	128	64	16	16	8	8	8	8	4	-

Fig. 34 : Sephadex G200 elution profiles of (a) rabbit and (b) chicken antisera to P. haemolytica.


(a)
IgM
IgG
29 33 37 41
(b)
O.D.
IgG
IgM
29 33 37 41
Fractions

When antisera which had been rendered serotype-specific by absorption were used for IIF on tissue sections the bacterial cells were clearly delineated (Fig. 35)* the use of these antisera to study the distribution of P. haemolytica in various tissues is described in the following chapter.

Discussion.

Immunofluorescent techniques have been successfully exploited for improvement of bacterial serotyping e.g. with Klebsiella species (Riser, Noone and Poulton, 1976; Riser, Noone and Bonnet, 1976), Streptococci (Watson, Kunz and Moellering, 1975) and members of the family Bacteroidaceae (Griffin, 1970). They have also been valuable for the detection in tissues of various bacteria including members of the genus Pasteurella e.g. P. tularensis (White and McGavran, 1965), P. multocida (Sulong and Maheswaran, 1976) and P. haemolytica, serotype A1 (Pass and Thomson, 1971). Therefore it was considered likely that IF would be a useful tool with which to study the distribution in infected tissues of various serotypes of P. haemolytica, provided sera of suitable specificity could be obtained.

Serotyping of P. haemolytica isolated from domestic ruminants by IHA uses antisera produced in rabbits by repeated i.v. inoculation of live or inactivated bacteria (Biberstein et al., 1960; Biberstein, 1978). High titres of IHA antibody are generally produced and though serotype cross-reactions do occur

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* See page 187

in the IHA test (Biberstein, 1965) they can be avoided by dilution of the serum. However, even with that degree of dilution the serotyping sera available to the author failed to discriminate clearly between individual serotypes of each biotype when subjected to IIF.

This preliminary observation was substantiated by the finding in the experiments reported here that of the 6 serotypes (A1, A2, A6, T3, T4 and T10) used for immunization of rabbits only A1 live microorganisms induced precipitating antibody, although each of the 6 serotypes evoked a satisfactory IHA response in recipient rabbits (Table 30). On the other hand, precipitin production to A2, A6 and T4 was achieved when live microorganisms were given to rabbits which, though failing to respond to SSE, probably had been primed by that antigen. As assessed by ID the only serotype specific rabbit antisera obtained were from rabbits immunized with A1, but the high dilution needed to achieve specificity in IIF limited their usefulness for this work.

Chickens make a vigorous antibody response to a variety of antigens (Schmidt and Wolfe, 1953; Furesz, 1960; Wang and Dunne, 1972; Frerichs and Frerichs, 1973) and this ability was evident in their precipitin production to P. haemolytica after each mode of stimulation (Table 30). However, chickens produced only IHA antibody after immunization with live microorganisms of A2 serotype. It was particularly unfortunate that in this series

of experiments no antiserum to A2 was produced as this is the serotype most commonly associated with pneumonic pasteurellosis of sheep.

The general failure of mice to produce antibodies against P. haemolytica serotypes was surprising in view of the relative ease with which protective immunity can be demonstrated in this species (Evans, 1979), at least for certain serotypes. However, few studies have been made of the serological response of mice to vaccination or infection and thus no general conclusion can be drawn at the present.

Fractionation of representative rabbit and chicken antisera indicated that antibody activity was present in both IgM and IgG Fig. 34, and Table 33). Because of its large molecular structure and multiple antibody binding sites IgM is particularly efficient in agglutinating reactions (Greenbury, Moore and Nunn, 1963) and this efficiency was revealed in the dramatic fall in IHA titre which resulted from treatment of serum with 2-ME (Table 32). Residual titres were probably due to IgG antibody. Conversely, precipitin reactions are mainly mediated by IgG antibodies and thus the lack of correlation between IHA titres and the results of ID tests (Table 30) is understandable.

In their IIF studies with Klebsiella species Riser, Noone and Poulton (1976) demonstrated that the pH of the initial anti-

body-antigen reaction had an important bearing on the degree of discrimination that could be achieved in the fluorescence test between homologous and heterologous capsular types of Klebsiella. The greatest differences were detected after the initial reaction had been conducted at pH 9.0. This was not the case with P. haemolytica. While there was always good discrimination at pH 7.0, the distinction narrowed at higher and lower pH values for some individual serotypes (Fig. 32). The reasons for these changes have not been established but they may result from alteration in the balance of ionic charges on antigenic determinants at the surface of the bacterial cell which in turn affects the binding of antibody. That this occurred only with certain serotypes implies differences in chemical structure of surface components. Too little is known of the surface chemistry of P. haemolytica to take this discussion further. Indeed the only available information is a brief account of a general analysis of extracellular substance derived from what was probably serotype A1 (Wetzel and Collier, 1974). This account reported the presence of protein (40%) and carbohydrate (8 - 27%) with galactose being identified as the major sugar. Because of the importance of surface antigens in relation to protective immunity and serotyping there is clear need for research in this area.

Evidence that the antibodies in the serotype-specific antisera were directed against surface antigens came from absorption studies and from the pattern of bacterial fluorescence. In the former case

only surface derived material (SSE and PWE) caused a marked reduction in IIF titre and bacteria were invariably delineated by surface staining (Fig. 33). In all immunofluorescence systems it is crucial that specificity be established by appropriate controls. These were applied in the tests carried out on bacterial smears before the antisera were judged suitable for screening infected tissues.

On the basis of the experimental results obtained a strong case can be made for using chickens to raise serotyping antisera for P. haemolytica. Though chickens can be naturally infected with P. multocida they are not natural hosts for P. haemolytica and can withstand high doses of live microorganisms. Most birds immunized with live microorganisms produced high titres of IHA antibody and the majority gave precipitin reactions only with the homologous serotype. Only with A2 was the response less than wished for, a point which serves to emphasise the probable uniqueness of serotype A2 in terms of surface antigens.

Conclusions

(1) For the production of serotype-specific antisera against P. haemolytica chickens are better than rabbits or mice.

(2) For the same purpose live microorganisms stimulate a better antibody response than do derived antigens.

(3) Serotype-specific antibodies produced in chickens immunized with live microorganisms are directed against surface antigens of P. haemolytica.

(4) In the IIF test the pH of the initial antibody-antigen reaction affects the extent of cross-reactivity obtained with certain heterologous serotypes.

(5) The IIF test affords a means for detecting and differentiating between various P. haemolytica serotypes in tissue sections.

CHAPTER 5.

THE RECOGNITION AND TISSUE DISTRIBUTION OF INDIVIDUAL SEROTYPES OF P. HAEMOLYTICA.

Introduction

A variety of methods has been used to study mechanisms of infection by various microorganisms and to follow the course of disease processes in host tissue which resulted from infection. A reliable technique for the detection in tissue of individual types of the same bacterial species is necessary for pathogenicity studies. Immunological methods, particularly immunofluorescence have been used to locate microorganisms in tissues or smears. Bacteria that have been detected and identified in these ways include Streptococci (Watson et al., 1975), Streptococcus suis (Williams, Lawson and Rowland, 1973), Escherichia coli (Thomason, Cherry, Davis and Pomales-Lebron, 1961; Drees and Waxler, 1970; Haddad and Gyles, 1978), Klebsiella pneumoniae (Murcia and Rubin, 1979), Klebsiella species (Riser, Noone and Poulton, 1976; Riser, Noone and Bonnet, 1976), Aeromonas (Kloeckl and Rouf, 1977), Vibrio cholerae (Schrank and Verwey, 1976), Bacteroides fragilis subspecies fragilis (Abshire, Lombard and Dowell, 1977), Bacteroides melaninogenicus (Lambe, 1974), Bacteroides, Fusobacterium, Sphaerophorus (Griffin, 1970), Bordetella pertussis (Eldering, Eveland and Kendrick, 1962), Salmonella typhosa (Thomason, Cherry and Moody, 1957), Salmonella serotypes (Caldwell,

Stulberg and Peterson, 1966), Brucella abortus (Thomas and McCausland, 1980), Mycobacterium avium, M. johnei, M. bovis (Gilmour, 1971), P. multocida (Sulong and Maheswaran, 1976), P. tularensis (White and McGavran, 1965) and P. haemolytica (Pass and Thomson, 1971). Most of these investigations concerned recognition of one or more bacterial species and antisera specific for each species proved adequate. However, in some cases (Riser, Noone and Poulton, 1976; Griffin, 1970; Caldwell et al., 1966) distinction between subtypes (serotypes) of the same species required the use of suitably cross-absorbed antisera. The successful outcome of these studies encouraged a similar approach to investigation of natural and experimental infection with T serotypes of P. haemolytica and to examination of lung tissue from sheep experimentally infected with A serotypes. It was considered that the information gained could contribute to knowledge of the pathogenesis of T-type disease and to A-serotype pneumonia.

It is, of course, possible to follow the establishment and spread of bacterial infection by isolation of the microorganism coupled with determination of viable counts per unit weight of tissue but these procedures provide no insight into the pattern of distribution of invading bacteria within an organ or tissue. It is in this respect that immunofluorescent techniques are particularly valuable. Because T-serotypes of P. haemolytica colonize the tonsils of conventional sheep from an early age

(Chapter 1) there was need for antisera capable of identifying individual serotypes in tissues. The preparation and evaluation of these reagents and of antisera specific for serotypes A1 and A6 have been described in Chapter 4. This chapter presents and discusses results obtained by using those sera to examine tissues obtained from both naturally and experimentally infected sheep.

Materials and methods

For the study of pneumonic pasteurellosis samples were obtained from SPF and conventional lambs used by colleagues in the course of their own experimental work. Four methods of inducing the disease were used:

(a) Four conventional 3-week old lambs were exposed to an aerosol of P. haemolytica serotype A1 using equipment specifically designed and built for that purpose (Smith, 1975). The aerosol was generated by a Wright's nebuliser,* from a concentrated broth-grown culture containing 1.5×10^{11} microorganisms/ml. The aerosol was measured using a multistage liquid impinger (May, 1966) and found to contain 5.6×10^{5} microorganism/litre air. The lambs were killed immediately after 15 min. exposure and tissue samples taken from the lungs (Fig. 36), trachea, bronchi and left tonsil.

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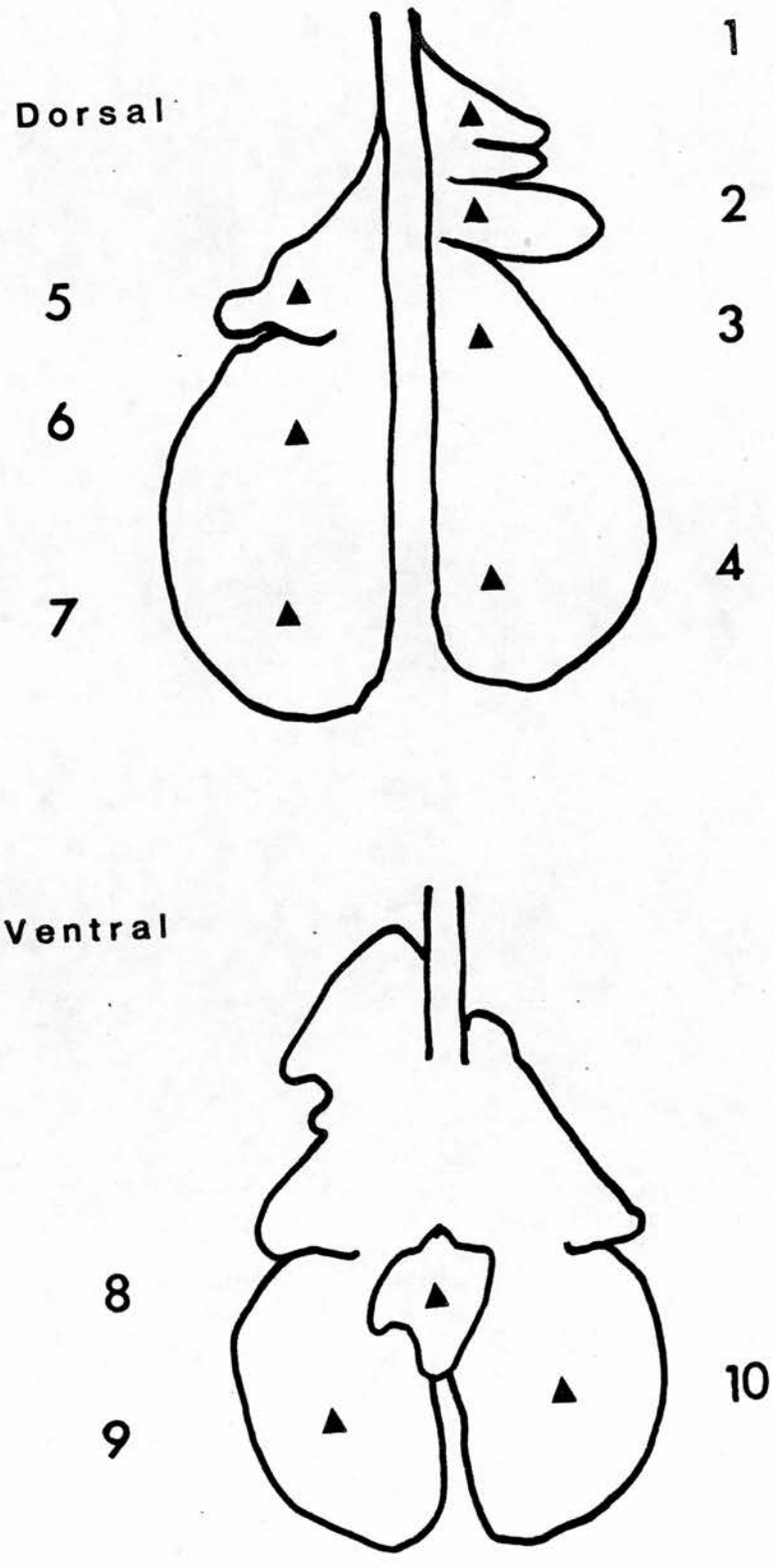
* Aerosol Products, Colchester, Ltd., England.

Fig. 36 : Lung diagram; sites sampled.


Dorsal
1
2
3
4
5
6
7
Ventral
8
9
10

(b) Infection of SPF lambs with PI3 virus and P. haemolytica : lambs were inoculated intratracheally and intranasally with PI3 virus followed at intervals of 3 - 7 days by exposure for 15 min. to an aerosol of P. haemolytica biotype A serotypes 1, 2 or 6 (Sharp et al., 1978).

(c) Infection of SPF lambs with P. haemolytica incorporated in agar : bacteria were added to a just-molten suspension of agar which, after solidifying, was converted to a suspension of fine particles and injected i.v. (N.J.L. Gilmour, personal communication).

(d) Infection of conventional lambs by IVAA method : lambs were injected i.v. with the agar and then exposed for 15 min. to an aerosol of bacteria (Gilmour et al., 1982a, b).

For procedure (a) the author collaborated with Mr. J. Fraser of the Institute's Microbiology Department but was not involved in procedures (b), (c) and (d) although he was personally responsible for collecting specimens at post-mortem examinations being done.

For the study of systemic pasteurellosis samples were obtained from lambs or adult sheep used in attempts at reproduction of the disease detailed at Chapters 2 and 3 of this thesis. In addition, lungs and internal organs of conventional sheep infected i.v. with serotype T4 in agar were provided by Dr. N.J.L. Gilmour.

For both the A and the T serotype studies, tissues were obtained from animals which died or were killed in the course of experiments. These tissues were fixed in absolute alcohol and processed following the method of Saint-Marie (1961). Tissue sections of 4 - 5 µm thickness were screened by the IIF test to study the distribution of P. haemolytica.

Results

I. Observations on the distribution of A serotypes.

Following direct exposure of 3-week old lambs by aerosol, P. haemolytica was isolated from all samples except the tonsil and viable counts on 10 lung portions from each lamb yielded very similar numbers of microorganisms (means ± SD of 5.6 ± 0.4, 5.4 ± 0.6, 4.8 ± 0.6, 4.6 ± 0.6, all of 10^6/g). IIF tests on lung tissue adjacent to blocks taken for viable counts revealed bacteria in all slides examined. The microorganisms were present within alveoli and some were attached to alveolar walls (Fig. 35). Bacteria were also demonstrated in the trachea and bronchi of two individual lambs but not in any tonsil.

Examination of tissues taken from animals infected by different methods with A serotypes of P. haemolytica indicated considerable colonization of lung tissues in all cases (Table 34). Irrespective of the mode of infection bacteria were also demonstrated in the nares, trachea and retropharyngeal lymph node of some animals, but only after i.v. injection of microorganisms in agar was P. haemolytica detected in tonsils.

Table 34. Demonstration of P. haemolytica A serotypes by IIF on tissues of experimentally infected animals.

Mode of infection *	Serotype used	Animals Status	Animals No.	Day of sampling	No. of animals positive for P. haemolytica in Nares	Trachea	Lungs ** A	Lungs ** C	Lungs ** D	Tonsil	RPLN ***
1	A1	SPF	10	7	2	-	10	6	10	-	3
1	A6	SPF	14	7	3	4	14	8	13	-	2
1	A2	SPF	2	1	2	-	2	2	2	-	-
			6	7	-	1	4	-	2	-	4
2	A1	CONV ø	7	3	2	1	7	3	7	-	1
3	A1	SPF	5	1	-	2	4	3	5	2	3
	A2	SPF	4	1	1	2	3	3	4	1	2

* 1 : PI3 + P. haemolytica

2 : IVAA

3 : P. haemolytica in agar i.v.

** A : apical

C : cardiac

D : diaphragmatic

*** RPLN : retropharyngeal lymph node.

ø Conventional.

In considering the distribution of bacteria in lung attention was paid to the mode of lodgement in the tissue, the relative number of microorganisms and whether the bacteria were present singly or in clumps. All 3 serotypes administered by aerosol after PI3 infection had a similar pattern of distribution. The bacteria were present on or near the lining epithelium of air passages and were seldom detected in inter-alveolar septa. The majority of microorganisms occurred singly and in small numbers with clumps being seen only in frankly pneumonic areas. When aerosol infection immediately followed an i.v. injection of agar (IVAA) bacteria were found both in the air passages and alveoli, often beside apparently inflamed blood vessels and in large numbers, usually clumped together. After administration of P. haemolytica in agar by the i.v. route a diffuse distribution of bacteria in blood vessels and lung parenchyma was the major feature and microorganisms were rarely detected in air passages. Clumps of bacteria were intermingled with agar and few single microorganisms were seen.

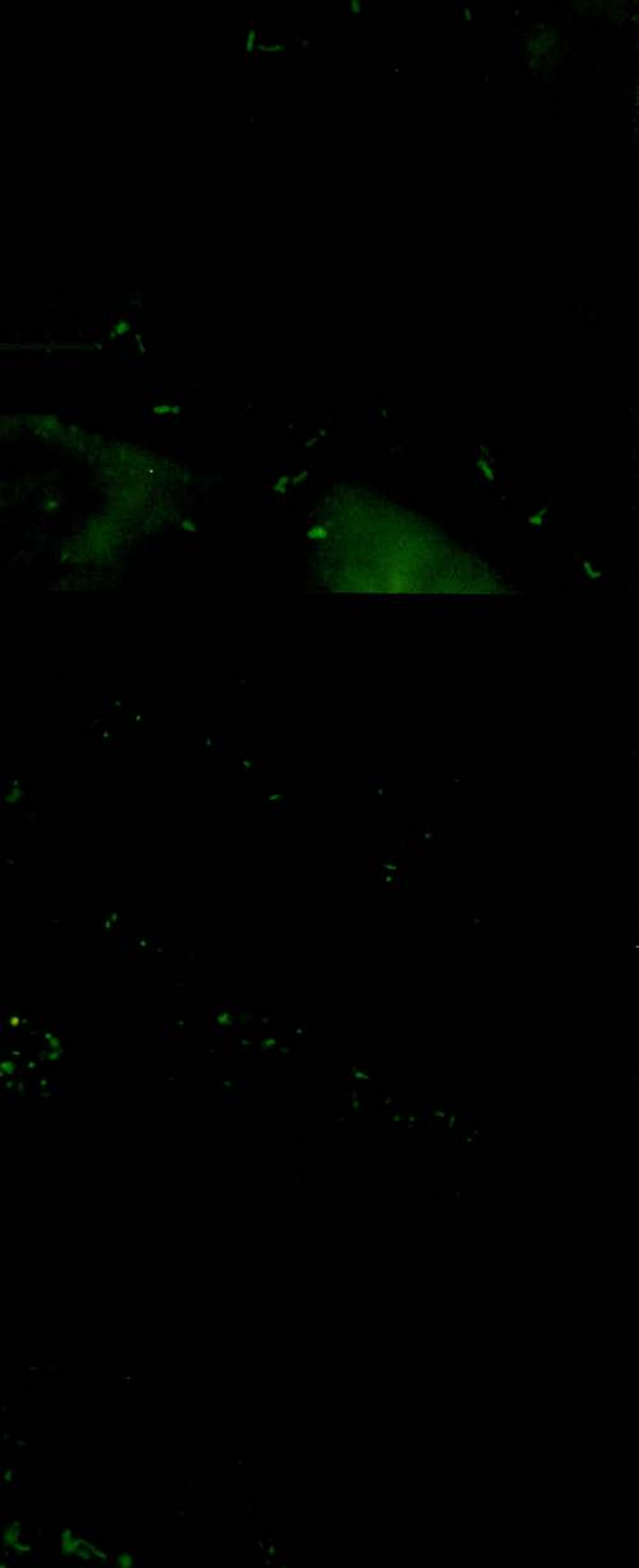
II Observations on the distribution of T serotypes

In tonsils taken from 10 healthy adult sheep all 3 serotypes were demonstrated in 6 cases, while 2 revealed only a single serotype and two were negative. The bacteria were distributed mainly as single microorganisms in the loose connective tissue surrounding the tonsil and on or near the surface epithelium (Fig. 37). Bacterial bodies were not detected within the tonsillar

Fig. 37 : Tonsil from normal sheep : P. haemolytica serotype T10 on the surface epithelium.
IIF stain X 1650

Fig. 38 : Tonsil of diseased sheep : P. haemolytica serotype T10 located deeply within tonsillar crypts in areas of inflammation and necrosis.
IIF stain X 1125

Fig. 39 : Tonsil of diseased sheep : P. haemolytica serotype T10 in the lymphoid parenchyma.
IIF stain X 1125



crypts. The pattern of bacterial distribution was common to all 3 serotypes.

The tonsils of 4 sheep which died of natural T type pasteurellosis were studied. In three cases evidence of mixed infection with 2 or 3 serotypes was demonstrated by IIF although only a single serotype was isolated in the fourth animal only T10 was detected. In each case bacteria were located deep within the tonsillar crypts in areas of inflammation together with inflammatory cells and tissue debris (Fig. 38). Penetration into the lymphoid tissue was also evident (Fig. 39). Occasional faintly-staining, disintegrating bacteria were seen within cells suggesting that phagocytic digestion might be occurring.

The majority of extracellularly located bacterial cells were bacillary in form.

The results obtained from the study of experimentally infected animals are summarized in Table 35. Irrespective of the age of animal or mode of infection P. haemolytica was easily detected in all tonsil samples examined. In all but one of the samples obtained after infection with a mixed T serotype culture each of the three T serotypes was present. Their pattern of distribution did not differ significantly from that observed in naturally infected tissue i.e. they were present within the crypts and subepithelial tissue of the tonsils (Figs. 38, 39). However,

Table 35 : Demonstration of individual T serotypes of *P. haemolytica* by IIF in the tonsils of lambs and adult sheep after experimental infection with a mixed T serotype culture and of adult sheep given T4 in agar i.v.

Experiment No. *	Animals			No. samples positive for		
	Age	No. infected	No. sampled	T3	T4	T10
1	3-7 w	6	1	1	-	1
2	8-11 w	6	2	1	1	2
3	8-11 w	6	3	3	3	2
4	7-9 m	6	2	2	2	2
5	7-9 m	6	2	2	2	2
6	adult	4	4	2+	4	3+

* 1 single infection of intact tonsils (Experiment 1, Chapter 2)

2 repeated infection of intact tonsils (Group B, Experiment 6, Chapter 3).

3 repeated infection of intact tonsils after iron treatment (Group C, Experiment 6, Chapter 3).

4 repeated infection of abraded tonsils (Experiment 4, Chapter 2)

5 repeated infection of abraded tonsils after iron treatment (Experiment 5, Chapter 3).

6 T4 only in agar given i.v.

w = week

m = month

\+ Few in number and confined to surrounding connective tissue and epithelial surface.

they were often detected in clumps perhaps because of the weight of experimental infection applied directly to the tonsils. Fluorescing but indistinctly shaped particles were seen within macrophages and epithelial cells and may have been remnants of bacterial cells.

In contrast, in the tonsils of adult sheep given T4 in agar, only that serotype was seen within the tonsil parenchyma, though small numbers of each of the 3 serotypes were present at the tonsillar surface.

Scrutiny of internal organs for P. haemolytica by IIF was undertaken in lambs treated with iron (group C of experiment 6, Chapter 3). Table 36 presents the results of these observations which show that dissemination of all three T serotypes occurred in each of the 3 animals examined though there was variation in the number of serotypes demonstrable in any one tissue. In lamb 1747 serotype T10 was detected within the oesophageal ulcer (Fig. 26, p.157) found at post-mortem examination but no evidence of infection was seen in the abomasal lesions of lamb 1712 (Fig. 27, p. 157). As expected serotype T4 was demonstrable within the major vascular organs of the 4 adult sheep injected i.v. with this serotype (Table 37).

In individual organs P. haemolytica was distributed in a focal fashion near vascular areas, usually as clumps of micro-

Table 36 : Individual T serotypes detected by IIF in tissues of lambs after tonsillar infection with a mixed T serotype culture.

Lamb No.	Organs																				
	heart			lung			spleen			liver			kidney			adrenal			P.L.N.*		
1754 (died)	3	4		3	4		3	4	10	3	4		3	4		3	4		3	4	10
1712 (killed)		-		3		10	3	4	10		4	10			10			10	3	4	10
1747 (killed)		-				10		4	10	3	4	10	3		10	3		10	3		10

* PLN : Peritonsillar lymph node.

Table 37 : Detection of homologous serotype by IIF in internal organs of 4 adult sheep given T4 in agar by i.v. injection.

Organ	Sheep No.			
	1	2	3	4
Lung	+	+	+	+
Liver	ND*	+	+	+
Spleen	ND	+	+	+
Kidney	ND	-	+	-
RPLN**	+	-	+	-

* ND = not done.

**RPLN = Retropharyngeal lymph node.

organisms e.g. within sinusoids and periportal areas of the liver (Fig. 40), around arterioles or larger follicles of the spleen and around congested interalveolar capillaries of the lung (Fig. 41). Bacteria were also detected in the subcapsular spaces of the peritonsillar lymph node and in the interstitium of the renal cortex.

In the internal organs of sheep given T4 in agar by i.v. injection the microorganism was particularly evident in blood vessels occluded with agar (Fig. 42).

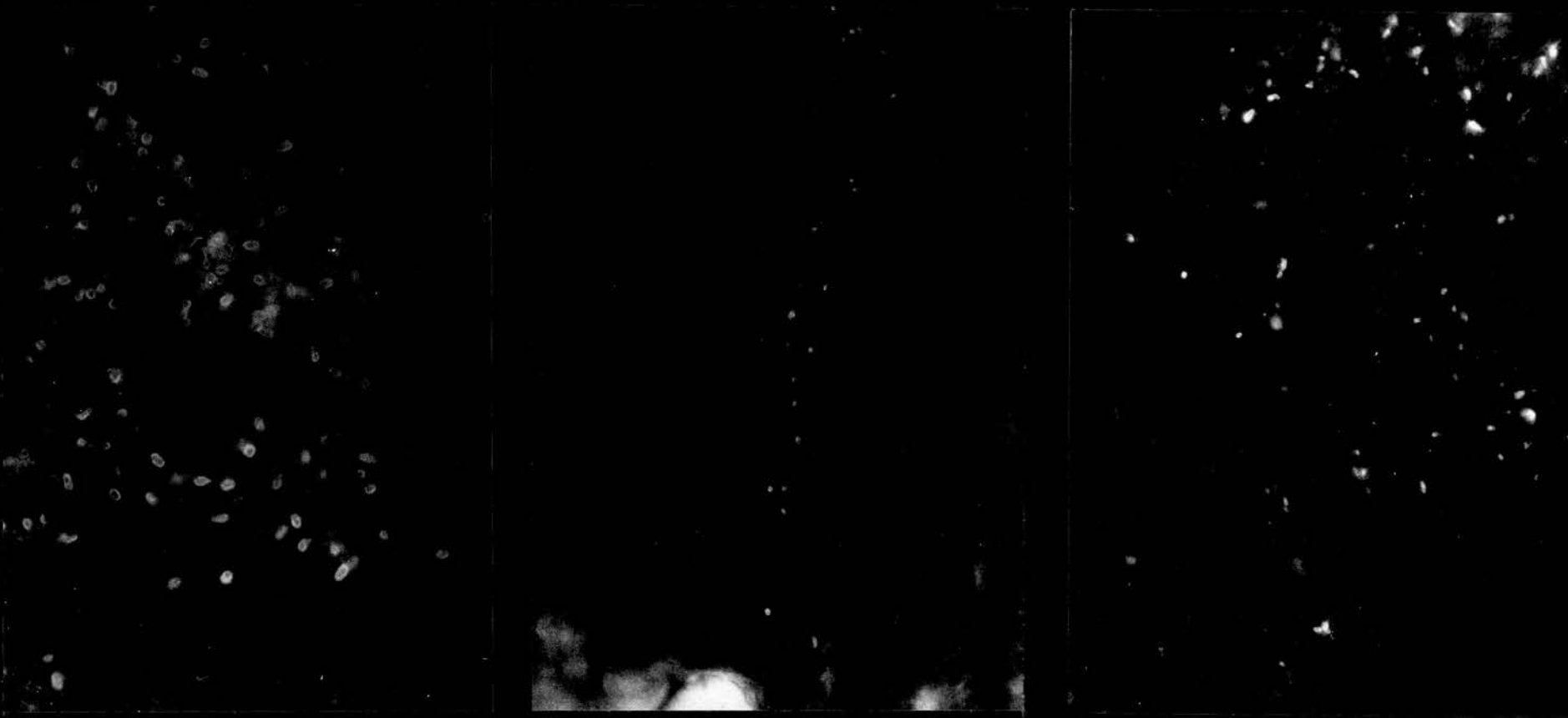
Discussion

Pathogenesis studies on P. haemolytica have relied mainly on the use of bacterial counts from selected organs and on histological examination of gram stained tissue sections. In the usual histological preparation, many bacteria are difficult or impossible to identify, because of their poor staining and confusion with tissue fragments or cellular organelles. For this and other reasons the IIF test was chosen to study tissue sections and to detect P. haemolytica in situ. Apart from its successful use with other bacteria fluorescent microscopy has proved an efficient means of detecting and recognizing P. haemolytica serotype A1 in tissue (Pass and Thomson, 1971; Newman, Corstvet and Panciera, 1982).

Fig. 40 : Liver of experimentally infected sheep with mixed T serotypes of P. haemolytica : presence of serotype T4 shown in periportal area and within sinusoids.
IIF stain X 1125

Fig. 41 : Lung of experimentally infected sheep with mixed T serotypes of P. haemolytica : presence of serotype T10 shown around congested blood vessel.
IIF stain X 1650

Fig. 42 : Spleen of experimentally infected sheep given T4 serotype in agar by intravenous injection. Bacteria in occluded blood vessel with agar.
IIF stain X 1800



The results of the immunofluorescence studies on P. haemolytica reported in this chapter concur with those of the above mentioned authors in regard to the efficacy of the procedure with A1. In addition, they have confirmed its general applicability with 4 other serotypes. In the author's experience the choice of tissue preservative did not appear to be critical as the various serotypes of P. haemolytica retained their antigenicity and fluoresced with equal intensity whether fixed in absolute alcohol or 10% formol saline. Thus for future pathogenesis studies it would be preferable to use formalin fixative so as to preserve tissue integrity as far as possible.

Each of the methods used to establish pneumonic pasteurellosis with A serotypes resulted in extensive pulmonary colonization (Table 33). However, critical comparison between the methods was not possible because of variation in sampling times and other factors over which the author had no direct control. Past experience at Moredun has established that aerosol infection with P. haemolytica alone seldom results in pneumonia, even in SPF lambs and it is considered that some predisposing factor is required. One such factor is a preceding respiratory viral infection which impairs bacterial clearance from the lungs (Davis et al., 1977; Sharp et al., 1978) and which prevents intracellular killing of bacteria by alveolar macrophages (Jakab and Green, 1972). Persistence of considerable bacterial infection was certainly evident in lung tissue obtained 7 days

after P. haemolytica infection of PI3 virus-inoculated lambs (Table 33) and the bacteria were still attached to alveolar epithelium or free in extracellular locations.

Whatever predisposing or precipitating factors may be involved in initiating disease there was no doubt that aerosol infection alone resulted in uniform deposition of bacteria in each lobe of the lung in all 4 lambs. This was evident from the consistency in the number of microorganisms recovered from the different areas of the lung (p. 205) and the similar patterns of bacterial distributions revealed by IIF (Fig. 35). In contrast, microorganisms in agar, given i.v., failed to reach the alveolar spaces and were largely retained within particles of agar. Thus, although pulmonary infection was established the pathogenesis of subsequent disease would differ from that associated with normal respiratory infection. For this reason the model is not an ideal one for studies on pneumonic pasteurellosis.

The case for tonsillar involvement as a portal of entry for T serotypes of P. haemolytica in ovine systemic pasteurellosis was put forward by Dyson et al. (1981) on the basis of observations made on animals dying as a result of natural field disease. The fluorescence microscopy studies on tonsils of normal animals and those of naturally or experimentally infected sheep support this speculation. In normal sheep bacteria were absent from the tonsillar crypts, a site in which they were readily detected in

diseased or experimentally infected animals, and only in cases of disease was P. haemolytica found to have breached the epithelial barrier of the tonsil. This finding is similar to that of Williams et al. (1973), who, working with S. suis, demonstrated that the microorganism was confined to the tonsillar crypts after infection. Likewise, Drees and Waxler (1970) while investigating colibacillosis in piglets, identified E. coli in the crypts of the pharyngeal tonsils.

As the tonsils are virtually in continual contact with inhaled or ingested microorganisms which they normally arrest and destroy they have long been suggested as a portal of entry for pathogens including the tubercle bacillus (Griffith, 1907) and M. johnei in calves (Payne and Rankin, 1961). Other workers have taken the broader view that the tonsils, together with the small intestine and lung are the major sources of entry for pathogenic bacteria (Payne, Sansom, Garner, Thomson and Miles, 1960; Payne and Derbyshire, 1963). The former group of workers showed that radioactive particles 1 - 5 μm in diameter (P. haemolytica is 0.5 - 2.0 μm or more) may pass the tonsillar barrier. The latter group demonstrated that infection of the pharyngeal lymph nodes resulted from infected macrophages originating from the tonsillar crypts and suggested that the tonsils are continually "sampling" antigens which come in contact with crypt epithelium. However, there is no information on the frequency with which bacteria, commensal or pathogenic, may pass

the tonsillar barrier in the normal animal. Williams et al. (1973) illustrated that crypt epithelial cells are capable of phagocytosis of bacteria, as previously demonstrated for non-infectious material applied especially to the palatine tonsils of piglets (Williams and Rowland, 1972).

Whatever the mechanism, P. haemolytica applied to the tonsils experimentally were able to reach internal organs in sufficient numbers to allow their easy isolation (Tables 25 and 29) and detection by IIF (Figs. 40 and 41). In some cases T serotypes were demonstrable by IIF but failed to grow on standard culture media. It is probable that these were either dead or very weak microorganisms which had not yet been phagocytosed.

Conclusions

In this study it has been shown by cultural isolations and histology (Chapter 3) and by fluorescence microscopy that oral infection of sheep can lead to the establishment of persistent T serotype colonization of the tonsils. Under certain experimental conditions (abrasions, repeated infection, iron excess) the bacteria can multiply and invade the tonsillar crypt epithelium. From there they can pass to the draining lymph node and be disseminated systemically. The tonsils may therefore be regarded as portals of entry for this bacterium.

CHAPTER 6

GENERAL DISCUSSION

The main objective of the work presented in this thesis was to study some aspects of the pathogenesis of ovine pasteurellosis. The disease, both in its pneumonic and systemic forms, continues to be a cause of flock and sporadic losses.

Epidemiological investigations have been hampered by the wide range of serotypes of P. haemolytica, by the commensal nature of the organism, by the general unpredictability of disease outbreaks, by the lack of a serological response to some serotypes, (notably A2 the most common isolate from cases of pneumonic pasteurellosis in Britain) and by the general lack of premonitory clinical signs in systemic T-type pasteurellosis. Better understanding of the disease syndromes, their pathogenesis and the means of controlling the disease have depended therefore on development of experimental models.

In recent years a reliable and reproducible method for experimental induction of pneumonic pasteurellosis has been developed using sequential infection with PI3 virus and P. haemolytica (Sharp et al., 1978) but the procedure requires SPF lambs and is therefore very expensive. An alternative strategy is the use of the IVAA method (Gilmour et al., 1982a, b) for

which conventional animals are suitable. These procedures have been exploited with success in testing the efficacy of vaccines against experimental challenge with A serotypes (Wells, 1981) and some pathogenesis studies have also been carried out (Rushton, Sharp, Gilmour and Thompson, 1979). However, much still remains to be learned about the initiation and progress of the pneumonic disease. Recently published evidence (Rimsay, Coyle-Dennis, Lauerman and Squire, 1981; Himmel, Yates, Lauerman and Squire, 1982; Kaehler, Markham, Muscoplat and Johnson, 1980; Markham and Wilkie, 1980) points to the probable importance of a component of P. haemolytica A1 which is toxic for bovine pulmonary macrophages and mononuclear leucocytes. Experimental studies at Moredun have confirmed that P. haemolytica exerts a similar selective toxic effect on ovine pulmonary phagocytes (A.D. Sutherland, personal communication). Inhibition of phagocytic function could explain the ability of P. haemolytica to establish itself within the lung, and to initiate pneumonic changes.

The major contribution of pathogenesis studies with A serotypes reported in this thesis, has been in determining the distribution of the organism after experimental infection. It has been demonstrated that IIF is a suitable tool for making such observations provided antisera of adequate serotype-specificity are available. While this is not so crucial in the case of SPF lambs exposed to infection with a single defined serotype it is important for work on tissues derived from

conventional lambs which are likely to harbour a commensal infection in the upper respiratory tract (Shreeve and Thompson, 1970; Gilmour et al., 1974). The results obtained confirmed that the IVAA procedure results in a pattern of bacterial distribution comparable to that which followed sequential infection of SPF lambs with PI3 virus and P. haemolytica. This finding reinforces the view that the IVAA method is an acceptable alternative for induction of pneumonic pasteurellosis in conventional lambs for certain types of study e.g. assessment of immunity resulting from vaccination. However, it is too artificial to be a suitable model for pathogenesis studies.

While little is known of the pathogenesis of either form of pasteurellosis it is the systemic form of the disease which has been less amenable to study because it has been difficult to reproduce experimentally. Many methods have been tried, as indicated in the Literature Review (p.29) but while most have proved fatal none has succeeded in producing the clinical and pathological syndrome of the natural disease. For this reason an alternative approach was adopted which centred on the postulate that the tonsil is a primary focus of infection (Dyson et al., 1981). It was known that the majority of adult sheep harbour T serotypes in the pharynx and a survey of growing lambs (Chapter 1) demonstrated that tonsillar colonization, which commences at 3 weeks, is well established in 9 week old lambs (Fig. 1). Association between T serotypes of P. haemolytica and the tonsil

thus develops at an early age and is persistent. The nature of this association and the possible involvement of attachment factors has not been investigated but would merit study.

As all previous attempts to reproduce disease had depended upon parenteral administration of bacteria it was decided to use only topical application of live organisms for the series of experiments that were planned. Also it was considered that a mixed culture of the 3 known T serotypes would provide maximum opportunity for initiation of active infection. While the number of bacteria in the infecting inocula was always determined the number actually applied to the tonsils could only be estimated because of losses due to salivation and swallowing. Nonetheless the lodgement and persistence of the applied bacteria was evident for up to 3 weeks after infection (e.g. Table 13).

The experiments described in Chapter 2 established that application of T serotypes to intact or abraded tonsils promoted a local inflammatory reaction. However, the lack of systemic spread of P. haemolytica indicated that the microorganism failed to breach the tonsillar barrier either because of the efficiency of the host defence system or the relatively avirulent character of the serotypes employed. The strains used for preparing the cultures were derived from natural systemic infections (Table 2), had gone through a minimal number of culture passages in the laboratory and were capable of causing fatal infection of mice.

However, P. haemolytica is a rather delicate bacterium, preferring a chemically defined medium for optimal growth (Wessman, 1966; Biberstein, 1978) and even limited laboratory culture may be associated with some loss of virulence. It is well recognized that microorganisms can adjust their metabolism or biochemical composition in response to changing nutritional or physical environmental conditions (Ellwood and Tempest, 1972; Macdonald and Adams, 1971; Neidhart, 1963; Robinson and Tempest, 1973). Two observations, not reported in the thesis, suggest that culture-associated changes can occur with P. haemolytica; microorganisms grown in NB supplemented with 1% galactose had a more brightly fluorescing surface than those grown in conventional P. haemolytica medium; a similar difference was noted between cultured P. haemolytica and bacteria in tissues of infected animals. While these preliminary observations require confirmation and extension they hint that culture conditions affect surface components, perhaps including virulence factors. An analogous phenomenon has been reported for animal-passaged Bacteroides fragilis which proved significantly more able to resist phagocytosis and intracellular killing by neutrophils than those cultured in-vitro (Simon, Klempner, Kasper and Gorbach, 1982). As surface components of P. haemolytica have proved of considerable importance in regard to vaccine development (Gilmour et al., 1979) further study of factors which can influence surface composition is recommended. Such study should also encompass attempts to grow microorganisms in vivo

using suitable culture chambers. Such an approach, used to culture certain strains of E. coli within the rabbit peritoneal cavity, has revealed several differences between bacteria grown in vivo and in vitro (Finn, Arbuthnott and Dougan, 1982).

That iron has a virulence-enhancing effect on P. haemolytica infections in mice and sheep emerges from the experiments reported in Chapter 3. Iron is required for the growth of nearly all bacteria (Lankford, 1973; Neilands, 1973) but to acquire their essential iron pathogens must compete with the host's iron-binding and storage proteins usually by production of siderophores (Weinberge, 1978). Whether P. haemolytica can generate siderophore is not known but it is tempting to speculate that its haemolytic property reflects a demand for iron. Frank haemolysis is not a feature of ovine pasteurellosis but multiple haemorrhages are a common and consistent pathological finding in acute systemic disease.

As well as a direct influence on bacterial virulence iron may also indirectly depress the host defence system by interfering with availability of copper. In vitro experiments have indicated that deficiency of the latter element impairs defence mechanisms (Boyne and Arthur, 1981; Jones and Suttle, 1981) and in-vivo studies have demonstrated a relationship between copper depletion and increased susceptibility to experimental infection of rats with Salmonella typhimurium (Newberne, Hunt and Young, 1968) and

of mice with P. haemolytica (Jones and Suttle, 1982). Moreover, the incidence of mortality at 8 weeks from all causes was significantly greater in groups of lambs with low plasma copper levels than in those of normal copper status (Suttle, personal communication). Grazing animals ingest much of their dietary iron in soil and soils rich in iron inhibit uptake of copper (Suttle, Abrahams and Thornton, 1981). As soil ingestion is maximal during autumn, the time of peak incidence of systemic pasteurellosis, it is conceivable that limited copper availability so impairs host defence that commensal T serotypes can penetrate the tonsil and initiate the fatal disease process. This hypothesis could be tested by tonsillar infection of lambs with experimental copper deficiency. Also in outbreaks of natural disease it would be useful to determine plasma copper levels in surviving animals and to assess iron concentration of soil.

As a model, tonsillar infection has definite limitations. Fairly extreme measures, viz. repeated injection of iron, multiple infection of the tonsil and abrasion of the pharyngeal surface in the tonsillar area, were needed to induce true systemic infection and associated clinical disease. Even so, disease never occurred in all members of a treated group. Thus, for routine experimental purposes, the model has serious shortcomings, but did provide a source of material for investigation of pathogenesis. The main outcome of these studies has been to demonstrate that the tonsils can indeed be a focus of initial infection, as

speculated by Dyson et al. (1981). However, the fact that not all animals developed systemic disease after experimental infection reinforces the concept of predisposing or triggering factors which have yet to be identified.

As a means of establishing P. haemolytica infection in mice administration of bacteria in a suspension of hog gastric mucin, introduced by Smith (1958), has been very successful. By means of the mouse model useful information has been obtained on serotype pathogenicity and on vaccine efficacy (Evans, 1979). The mode of action of mucin has never been clearly defined but it may be in part due to its iron content (Calver et al., 1976). However, in a study comparing iron and mucin as virulence-enhancers of Y. enterocolitica Smith et al. (1980) noted significant differences in effect. Mucin allowed the growth of the microorganism on the surface of livers and spleens of mice without associated internal lesions. On the other hand, extensive hepatic and splenic lesions developed in mice infected i.p. with microorganisms suspended in 10% iron dextran.

A major problem in working with hog gastric mucin is batch-to-batch variation in composition (Calver et al., 1976). This problem does not exist with chemically defined preparations such as aqueous solutions of iron compounds which are also easier to administer with quantitative accuracy. It would be valuable therefore to consider using iron as an alternative to mucin in

mouse studies and to assess the relative efficacies of different iron formulations.

At present, serological classification (serotyping) of P. haemolytica depends solely on the IHA test which, according to Biberstein et al. (1960), recognizes specific diffusible surface components of individual serotypes. The practice developed of labelling "untypable" any isolate which failed to react with known typing antisera, but the recent discovery of new serotypes — A13 and A14 (Pegram et al., 1979) and T15 (Fraser et al., 1982) — emphasises the need to reserve judgement on isolates which do not react with existing typing sera. Only if routine immunization of rabbits with these "unrecognized" strains fails to produce specific antibody should they be regarded as "untypable". On the basis of results presented in Chapter 4 a case can also be made for attempting to produce typing antisera in chickens as well as in rabbits when investigating unrecognized isolates.

The relatively high percentage of untypable isolates, all apparently biotype A, recovered from the tonsils of lambs in the early weeks of life (Fig. 1) merits attention. These isolates did not persist in any numbers beyond 6 weeks and their loss was associated with an increase in T serotypes. The question arises whether biotype and serotype specificities are stable characteristics in vivo. It may be relevant that in the course of repeated subculture on blood agar of a serotype A2 isolate there appeared

new IHA negative isolates (J. Fraser, personal communication). Biberstein *et al*. (1970) also reported that after 10 years' storage of typable cultures of *P. haemolytica* there emerged rough "untypable" (unrecognized) variants, although no change was noted in the serological specificity of the typable cultures. In Kenya, Mwangota *et al*. (1978) detected both A and T biotypes within serotypes 3, 4, 10, 6 and 12.

Taken together, these various observations suggest that it would be useful to investigate biotype and serotype stability *in vivo*. One approach to this problem could be by infection and monitoring of SPF lambs over a period of several weeks. The findings could have important implications both for the classification of *P. haemolytica* and for the epidemiology of the diseases of sheep with which it is so closely associated.

The experimental results obtained in the course of studies described in this thesis have provided new information which adds to the general body of knowledge concerning the commensal and pathogenic associations between *P. haemolytica* and sheep. An experimental method of reproducing a syndrome resembling the natural systemic disease caused by T serotypes has been developed and is suitable for pathogenesis studies. From observations made on tissues derived from experimentally infected sheep and from some which died of natural field disease it has been concluded that tonsillar penetration is an important initiating event in the

disease process. Before a complete account of the pathogenic mechanisms can be offered there is need for further work and several lines of investigation have been suggested. Such investigations are justified because of the economic importance of ovine pasteurellosis in this and other sheep rearing countries.

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Appendix 1. Reagents.

Coomassie blue stain :

Coomassie brilliant blue R*	50.0 g
Ethanol 96%	4.5 L
Glacial acetic acid	1.0 L
Distilled water	4.5 L

Filter before use.

Phosphate buffered saline pH 7·4 (10 x) :

KCl	10.0 g
NaCl	400.0 g
$Na_2 HPO_4$	57.5 g
$KH_2 PO_4$	10.0 g
Distilled water	5.0 L

Filter through filter paper.

Barbital Ca-lactate buffer, pH 8·6 :

Sodium barbital (sodium 5,5-diethylbarbiturate)	105.1 g
Barbital (5,5-diethylbarbituric acid)	16.6 g
Calcium lactate	15.2 g
Distilled water	10.0 L

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* Edward Gurr Ltd., London.

Appendix 1 contd.

Briton-Robinson Universal buffer :

Citric acid	6.008 g
$KH_2 PO_4$	3.893 g
$H_3 BO_3$	1.768 g
diethylbarbituric acid	2.266 g
Distilled water	1.000 L

100 ml of this mixture is titrated with X ml of 0.2 N (4g/100 ml distilled water) Na OH to give the required pH ($18^{\circ}C$).

Formol buffered saline :

Phosphate buffered saline pH 7.4	1.0 L
neutral formalin	3.0 ml

Appendix 2: Isolations of P. haemolytica serotypes from the extracted tonsils of lambs between birth and 7 weeks of age.

Lamb No	Age range			Isolate
	≤1d*	1-7d	1-7w**	
1		3d		-
2	+			-
3	+			-
4	+			-
5	+			*U
6	+			-
7		3d		-
8		3d		-
9	+			-
10	+			U
11	+			-
12	+			-
13	+			-
14		4d		-
15	+			-
16	+			-
17	+			A11
18	+			-
19		1d		A11
20	+			-
21		2d		U
22		1d		A11
23	+			-
24		1d		U,A11
25		1d		-
26		2d		A11
27	+			U
28	+			-
29		1d		U
30		1d		-
31	+			-
32		1d		-
33			2w	A11
34			2w	A11
35	+			U
36	+			U

Lamb No				Isolate
	≤1d*	1-7d	1-7w**	
37	+			U
38		3d		U
39	+			-
40			6w	A11
41			6w	U,A7
42			6w	A11,T10
43			6w	U,'A11
44		1d		U
45	+			U
46	+			-
47		2d		-
48		2d		-
49		1d		-
50		2d		-
51		3d		-
52		3d		U
53		1d		-
54		3d		U
55		2d		-
56		2d		-
57		4d		-
58		4d		-
59		2d		-
60			>1w	A11
61		4d		A2
62		2d		-
63		1d		-

*d : Day

**w : Week

*U : Untypable

Appendix 3: Isolations of P. haemolytica serotypes* from the tonsils of 28 ewes swabbed on 6 occasions over a 12 week period

Ewe No.	Swabbing No.					
	1	2	3	4	5	6
1	-	10	-	-	4,10	-
2	3	3,7	3,10	-	-	-
3	-	-	-	-	-	-
4	3	-	3	-	3,10	-
5	3,10	10	10	5	-	-
6	10	-	-	10	3,4,7,10	3
7	10	-	4,10	-	-	-
8	-	-	10	-	-	-
9	3,5	-	*ND	-	3,10,11	3,10,11
10	-	-	..	-	-	-
11	10	-	ND	-	-	-
12	4	-	-	-	-	-
13	10	..	4,10	-	-	-
14	-	ND	ND	10	-	-
15	-	-	10	2,3	-	-
16	3,11	3,10	ND	-	3	3,4
17	3	2,3	2,3	-	-	-
18	11	-	-	-	3,10	10
19	3,11	2	2,9	2,3	-	3,10
20	10	-	-	-	-	-
21	-	3	2,3	-	-	-
22	-	ND	10	-	-	-
23	3,4	-	3,10	-	-	-
24	1,3,7	-	3,4	U**	8	-
25	10	10	10	-	2	-
26	-	-	-	-	-	-
27	10	-	9,10	3,10	-	3,10
28	10	3,10	3,10	-	3,10	-

*Biotype designations omitted: 3,4 and 10 are type T, others type A.

**U : Untypable

*ND : Not done

Appendix 4: Isolation of P. haemolytica serotypes* from the tonsils of 37 lambs swabbed on 6 occasions between birth and 12 weeks of age

Ewe No	Lamb No	Swabbing No. 1	2	3	4	5	6
1	1	U**	3	-	U	3	-
	2	1,6,11	-	-	-	-	-
2	3	-	-	7	-	3	-
	4	-	-	U	-	-	3,4,10
3	5	-	10	3,10	-	-	-
4	6	-	-	-	-	4	-
5	7	-	-	-	10	-	-
	8	-	-	-	3,7	3,7	4
6	9	-	9	-	-	-	-
7	10	U	-	-	-	10	-
8	11	U	-	-	3	3	3,4
9	12	1	1,9	U	2	-	3,4
	13	U	-	-	-	10	3,10
	14	5	-	6,7	11	-	-
11	15	U	-	-	-	-	-
	16	-	-	10	-	-	-
	17	1	-	-	-	3	4
15	18	-	-	2	-	-	-
	19	-	-	10	-	4,10	10
16	20	-	-	-	7	-	3,4
17	21	U	U	-	7	-	-
18	22	-	-	U	-	10	3,4
19	23	1,11	-	-	-	10	4
20	24	-	-	-	7	-	-
	25	-	-	-	-	10	4,10
21	26	U	-	-	-	-	-
22	27	-	-	-	-	3	10
23	28	-	U	10	10	-	-
	29	-	-	2,10	10	-	-
24	30	U	-	U	-	-	-
	31	-	U	-	10	4	3,4
	32	-	-	-	11	3,8,11	3,4,11
26	33	1,11	-	-	-	-	3,4
	34	-	-	-	-	8	3,8
27	35	-	-	-	-	10	10
	36	-	-	10	10	10	3,10
	37	-	10	-	10	10	3,4,10

*Biotype designations omitted : 3, 4 and 10 are type T, others type A

**U : Untypable