THE ROLE OF PASTEURELLAE IN THE RESPIRATORY DISEASE OF SHEEP IN FIVE STATES OF NORTHERN NIGERIA

BY

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DECLARATION

This is to certify that I carried out the work reported in this thesis at the Laboratories of the Department of Microbiology, Faculty of Science, ABU, Zaria and the National Veterinary Research Institute, Vom under the supervision of Dr. (Mrs.) L. E. Odama, Professor J. U. Umoh and Dr. A. A. Makinde.

This thesis is an original work carried out by myself and no part of it has been presented elsewhere for a degree or diploma. The work of other investigators is acknowledged and referred to in the text.

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CERTIFICATION

This thesis titled "The Role of Pasteurellae in the Respiratory Disease of sheep in Five States of Northern Nigeria" by **Moses Ode ODUGBO** meets the regulations governing the award of the degree of

DOCTOR OF PHILOSOPHY

(MICROBIOLOGY)

of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation

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DEDICATION

To Perpetua,

Understanding, Loving and Helpful Wife,

To Jesse,

My Son, Who Spurred Me on to Success

And to My

Parents

Because There couldn't

Have been Me without Them

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ABSTRACT

To isolate and determine the spectrum *of P. haemolytica (Ph)* and *P. multocida (Pm),* a total of 1335 sheep lung samples with identifiable lesions of pneumonia were evaluated over a four - year period in Plateau, Bauchi, Kaduna, Sokoto and Borno states of northern Nigeria. The samples yielded 504 (37.8%) Pasteurellae isolates and 378 (75%) and 126 (25%)of the positive samples were identified as *P. haemolytica* and *P. multocida* respectively. All but one of the *Ph* isolates were biotype A. The serotypes which were encountered in this study were *PhAl=4.0%; PhA2=27.1%; PhA6=9A%; PhA7=4.4%; PhA8=2.7%; PhA9=1.3%; ph*T10=0.2%; *PhA*11 =10.2%; and *PhUT= 1*6.0%. All the *Pm* isolates were of serogroup A. Other bacteria isolated from the lungs were *Neisseria spp., Escherichia coli,* streptococci, *Corynehacterium pyogenes* and staphylococci.

To assess the pathogenicity of the serotypes isolated, conventionally reared 5 -7 month old lambs (n=44) free from *Ph* and *Pm,* randomly alloted to 10 treatment groups, were inoculated intratracheally with the log phase of one of the 10 different serotypes (dose =2.2 - 4.0 x 10^8 cfu) per group with the 11th group as control. Clinically, there was no statistically significant relationship between the clinical scores and the serotypes at the 90% or higher confidence level.

Pathologically, the lambs inoculated with *Ph*A2 and *PhA7* serotypes showed above 50% mean consolidated lung tissue followed by serotypes *PhA* 1 and *PmA* with mean per cent lung consolidation of less than 50%. There were a statistically

significant difference in pneumonia scores between groups given *PhA2 , PhA7* and that of the other groups. There were no significant differences in the mean pneumonia scores between groups inoculated with A1, A6, A8, A9, Tl0, A11, UT, PmA and of the control. Gross lesions observed with *Ph* differed morphologically from those observed with *Pm. P. haemolytica* generally caused acute fibrinous pneumonia and *P. multocida* lesions were more of bronchopneumonia. The histological findings of necrotic lesion with "oat cells" was pathognomonic in the cases of *Ph* but rare in Pm infected animals.

Because some *Ph* serotypes (AI, A2, A7) and *the Pm* singly produced pneumonic pasteurellosis in this study and has also been reported in combination with other agents, it is suggested that these serovars should perhaps receive more recognition not only as secondary pathogens but also as primary pathogens. Cognizance should be taken of the different *Pasteurella* serovars capable of causing respiratory infections in sheep and goats when developing pneumonic pasteurellosis vaccine.

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CHAPTER 1

INTRODUCTION

With a population of over 14 million sheep and 25 million goats in Nigeria (FAO, 1995), these animals have the potential for the provision of the much needed animal protein for the Nigerian populace. Various respiratory infections, including pneumonic pasteurellosis caused by *Pasteurella haemolytica* and *Pasteurella multocida* have been a major factor militating against the achievement of this expectation.

Pneumonic pasteurellosis is one of the most common bacterial infections of small ruminants and by far the most important respiratory infection with a widespread distribution occurring in temperate, subtropical and tropical climates (Gilmour and Gilmour, 1989). The pathogenesis of pneumonic pasteurellosis (sometimes referred to as shipping fever) has been described as complex and multifactorial involving management practices, stress, multiple infectious agents and the immune response. However, the ultimate cause of severe clinical signs and death is usually a fibrinous penuemonia caused by *P. haemolytica* biotype A (now being reclassified as *Mannheimia haemolytica*) (Angen *et al.*, 1999) and *P. multocida* type A. (Gilmour and Gilmour, 1989; Alley, 1991).

Field evidence suggests that the synergism between viruses and *Pasteurella* is commonly responsible for natural outbreaks of pneumonic pasteurellosis (Gilmour and Gilmour 1989), however recent reports indicate that *P. haemolytica* and *P. multocida* cause pneumonic pasteurellosis independent of viral or other microbial agents especially when virulent growing bacteria are injected intratracheally or intrabronchially (Shoo,

1989; Debey *et al.*, 1992). Reports indicate that while pre-infection with virus increases susceptibility, such pre-infection is not a pre-requisite for pneumonia to develop and the severity of pneumonia is a function of the dose and virulence of the *P. haemolytica* or *P. multocida* and not of other infectious agents (Shoo, 1989; Confer *et al.*, 1990). The viral agents *per se* lead to mild transient respiratory problem in normal animals and several viruses have been isolated from the respiratory tracts of sheep. The viruses include ovine progressive pneumonia virus, *herpes virus-1, parainflenuza-3-virus, adenovirus*, respiratory syncytial virus, etc (Lehmkuhl *et al.*, 1985; Obi and Ibu 1990; Ibu *et al.*, 1993). Animals are known to recover from singular viral agent except where there were some unusual immune deficiency or lowering of resistance (Ames *et al.*, 1985). However, the *Pasteurella* is regarded as the final and lethal agent that almost invariably kills the animal or severely damages the respiratory system. That is why an effective vaccination for *Pasteurella* is vital in the total health programme of these animals (Gilmour and Gilmour, 1989).

The current importance of pneumonic pasteurellosis to the Nigeria's livestock industry is reflected by a recent report that *P. haemolytica* and *P. multocida* account for between 35-59% of the diseases of small ruminants diagnosed at post-mortem at most. Nigerian Veterinary Investigation centres (Akerejola *et al.*, 1979; Adekeye, 1984; Ugochukwu and Nwaneri, 1984). Although it is difficult to put accurate figures on the huge economic impact of this disease in Nigeria, pneumonic pasteurellosis of small ruminants has a profound effect on the less developed economies of the world where, for the family unit, the loss of one or two animals can be catastrophic. Economic losses

occur from deaths of animals, weight loss, treatment cost, extra labour and short supply of protein. The "lungers" or respiratory cripples that result from this disease may represent a greater financial loss than do those animals that die acutely. This calls for effective control measures to combat the disease.

Based on capsular polysaccharide, 17 serotypes of *P. haemolytica* (Biberstein *et al.*, 1960; Pegram, 1974; Fraser *et al.*, 1982; Fodor *et al.*, 1988; Younan and Fodor, 1995) and 5 serotypes of *P. multocida* (Rimler and Rhoades, 1989) are recognized and immunity is mainly type-specific, although antigens other than the capsule may also be important (Confer, 1993). *P. haemolytica* is divided into 2 biotypes comprising 17 serotypes. The A biotypes are responsible for pneumonic pasteurellosis in sheep and goats while the T biotype cause systemic disease in 6-10 month old lambs or kids.

While the significance of *P. haemolytica* and *P. multocida* as pathogens of sheep and goats in Nigeria is well established (Akerejola *et al.*, 1979; Adekeye 1984; Obi, 1997) there is a dearth of information on the specific serotypes of these organisms involved in the disease. Ojo (1977) reported the serotyping of *P. haemolytica* isolates from only goat pasteurellosis in Western Nigeria. This appears to be the only documented evidence of serotyping of these agents in Nigeria. Sheep pasteurellosis in Nigeria appears to be grossly under-reported despite the significant population of sheep. This is a major challenge because in order to make a satisfactory vaccine for these animals, it is desirable to establish which serotypes are prevalent in the country and the relative importance of each serotype as a disease-producing agent. This study focuses only on sheep pasteurellosis in Nigeria. The study further builds on the information already obtained

from that on goat pasteurellosis.

This project has the following objectives:

To isolate and determine the serotypes of *P.haemolytica* and *P. multocida* commonly associated with sheep pneumonia in Nigeria.

To determine the relative importance of each representative serotype isolated as a disease-producing agent.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This section reviews the history, taxonomy and general characteristics of *Pasteurella haemolytica* and *Pasteurella multocida,* the disease, pathogenesis, diagnosis, immunology and the prevention and control of pneumonic pasteurellosis.

2.2 Historical Perspective, Nomenclature and Taxonomy

Historically, the evolution of the names *Pasteurella haemolytica* and *Pasteurella multocida* was traced in reviews by Carter (1967), Namioka (1978) and Rehmtulla and Thomson (1981). They described the earliest attempts by Bollinger and Pasteur to identify the genus *Pasteurella* in the late 19th century and then listed numerous names ascribed to them over the years (e.g. *Micrococcus gallicidus, Bacterium bipolare multocidum, Pasteurella cholerae-gallinarum, Bacterium multocidum, Bacterium Septicaemiae haemorrhagicae, Pasteurella boviseptica, Pasteurella septica*). Newson and Cross (1932) suggested *Pasteurella haemolytica* for one species within the genus and Rosenbusch and Merchant (1939) suggested *Pasteurella multocida* for another.

Bacteria included in the genus *Pasteurella* are commensals and occasional pathogens of many species of domestic and wild animals (Carter, 1981). Until very recently the genus included the species formerly known as *Pasteurella pestis, P. pseudotuberculosis, P. enterocolitica, P. tularensis* and *P. novicida.* The first three now constitute the genus *Yersinia* and have been placed in the family Enterbacteriaceae. The two remaining species, the tularaemia agents, which clearly did not fit into the *Pasteurella*

genus have been given the generic name *Francisella.*

Historically, members of the family Pasteurellacae were classified on the basis of a limited number of phenotypic characteristics. In particular organisms were assigned to the family on the basis of their requirements for growth hemin (x) and/or nicotinamide (NAD)(V) and on their ecology (MacInnes and Borr, 1990). It was not until the 1980s that a concerted effort was made to examine the genotypic rather than the phenotypic properties of members of the family. The family Pasteurellaceae contains a large group of Gram-negative facultatively anaerobic and fermentative bacteria of the genera *Pasteurella, Haemophilus* and *Actinobacillus.* Pasteurellae and allied bacteria have been the subject of taxonomic reorganization within recent years and reclassification of these organism is currently a subject of debate. The Pasteurellae are well recognized as a heterogenous group of organism and for this reason its members are subject to nomenclatural changes (MacInnes and Borr, 1990).

During the past decade, taxonomy of these organisms have been considerably improved mainly by description of new taxa with conventional methods and by extended deoxyribonucleic acid (DNA) DNA-DNA or DNA-ribosomal ribonucleic acid (rRNA) hybridization studies revealing their phylogenetic relationships (Mutters *et al.,* 1989; Confer, 1993). Approximately, 20 different species of the genus *Pasteurella* have been identified using phenotypic and genetic analysis. Modern taxonomic approaches to classifying members of the family Pasterellaceae and other bacteria have employed a combination of phenotypic properties, chemotaxonomy, protein analysis, per cent Guanine and Cytosine in the DNA (%G + C) DNA analysis and rRNA analysis (MacInnes

and Borr, 1990). Reclassification according to genomic relatedness led to the splitting of some traditional species into several new species, some into one new species, and others transferred from one genus to another, so that traditional names are changed accordingly (Mutters *et al.*, 1989).

A complete description of the taxonomy of the genus *Pasteurella* is beyond the scope of this review. However a brief review of several key points is warranted. As described in *Bergey's Manual of Systematic Bacteriology* (Carter, 1984), the genus *Pasteurella* can be divided into six species, based on *beta*-haemolysin, growth on MacConkey's agar, indole production, urease activity, gas production from carbohydrates and acid production from lactose or mannitol. These species are *P. multocida, P. haemolytica, P. pneumotropica, P. ureae, P. aerogenes* and *P. gallinarum.* Of these species, *P. multocida* and *P. haemolytica* are the most prominent pathogens in domestic animals causing severe diseases and major economic losses in the cattle, swine, sheep, goats and poultry industry (Confer, 1993). The unofficial species of *P. gallinarum* and *P. aerogenes* are of minor significance in veterinary microbiology. The non-fermentative organism called *P. anatipestifer* does not properly belong to the genus *Pasteurella* and is now correctly placed at the present in the category *species incertae sedis* (Mutters *et al.*, 1989).

Phenotypic properties, however, only account for expression of 10-20 per cent of the genome, and molecular techniques offer more critical analysis of relatedness among species. Therefore the *Pasteurellae* have been examined using such techniques as DNA/DNA hybridization, 2-dimensional protein electrophoresis and rRNA analysis.

Such analysis have resulted in reclassification of several *Pasteurella* species into 11 species in the genus *Pasteurella sensu stricto,* which can be differentiated using certain common phenotypic properties beyond those used in *Bergey's Manual* (Mutters *et al.,* 1989). One of the major organisms affected in this classification is *P.multocida* which is no longer classified merely as a single bacterial species. Appropriate subspecies of *P. multocida* are now recognized and these tend to be relatively animal species specific. Several traditional *Pasteurella* species, including *P. haemolytica* were excluded from *Pasteurella sensu stricto,* and their classification as *Pasteurella* remain questionable especially in light of recent rRNA analysis (Dewhirst *et al.,* 1992). *P. haemolytica* can be biotyped as A or T based on colony morphology and carbohydrate fermentation. Recent studies (Knight *et al.,* 1990; Sneath and Stevens, 1990; Dewhirst *et al.,* 1992; Angen *et al.,* 1997 Angen *et al.,* 1999) based on extensive investigation using quantitative evaluation of phenotypic data, ribotyping multilocus enzyme electrophoresis, 16S rRNA sequencing and DNA-DNA hybridization has resulted in reclassification of *P. haemolytica* and the identification of a new genus, *Mannheimia.* The 17 recognized serotypes of the former *P. haemolytica* complex have now been shown to represent genetically three distinct species viz: *Mannheimia haemolytica* which now contains all but A11 former A biotypes (i.e. 1, 2, 5-9, 12-14, 16 and 17); *Mannheimia glucosida* now represent the A11 serotype while all T biotypes (3,4,10 and 15) are now reclassified as *Pasteurella trehalosi* (Angen *et al.,* 1999).

There is still considerable debate regarding the taxonomic organization of the family *Pateurellaceae.* There is no doubt that this family will look very different in the

next edition of *Bergey's Manual of Systematic Bacteriology* with many long standing members moved to other families and new members added. In future it is clear that genotypic as well as phenotypic data should be considered before assigning an organism to the family *Pasteurellaceae*. Traditional DNA-DNA hybridization techniques will probably remain the "gold standard" for assessing relatedness at the species and to a lesser extent at the genus level although more rapid techniques such as slot blotting and restriction endonuclease fingerprinting may be used to reduce some of the labour involved in this analysis. 16S and 23S rRNA sequencing will be invaluable for assessing relatedness at the genus level and higher (Angen *et al.*, 1999). We should not however lose sight of the fact that modern genotypic and phylogenetic analysis should be linked to older phenotypic analysis for as MacInnes and Borr (1990) put it "classification is for the convenience of the microbiologist not for the convenience of the microbe."

2.3 General Characteristics of *P. haemolytica* and *P. multocida*

The principal characteristics of members of the genus *Pasteurella* are: small Gram-negative rods or coccobacilli, nonmotile facultatively anaerobic, catalase and oxidase positive, fermentative (except *P. anatipestifer*) producing acid but no gas from a number of carbohydrates (Carter, 1981).

The species *P. multocida* and *P. haemolytica* are found as commensal on the mucous membranes of the upper respiratory and digestive tracts of a considerable proportion of healthy animals. Carter (1981) reviewed the literature on carrier rates of these organisms in animals. For *P. multocida* the rates ranged from 3.5 to 7% for water buffaloes and cattle to 90% for cats. *P. multocida* has a remarkably broad host range and

it would seem likely that its commensal state is similarly widespread. Ordinarily, *P. multocida* does not occur as commensal in humans, although occasional carriers have been detected among individuals such as veterinary students and laboratory workers who are exposed to animals and cultures.

P. haemolytica has been recovered from nearly as many species of animals as *P. multocida.* Carter(1981) reviewed the several reports on the occurrence of *P. haemolytica* in healthy cattle and sheep. The carrier rate ranged from 6% for the tonsils of cattle to 40% for the nasal passages of sheep. This specie has also been recovered from poultry. Just as *P. multocida* is more common in the nasopharynx of cattle, *P. haemolytica* is more prevalent as a commensal in sheep.

As pathogens of animals, five different serologically specific capsular substances of *P. multocida* occur. Five serogroups (A, B, D, E and F) are currently distinguished in the Carter system of classification (Rimler and Rhoades, 1989). Serogroup C was abolished because it was found that it did not represent a capsule group. Findings of some studies had shown a relationship between the Roberts and Carter typing systems (Carter, 1963). Roberts types I, II and V were equated with Carter's serogroups B, A and D respectively. Based upon the observation that capsules of Roberts types III and IV contain hyaluronic acid, they may be equivalent to subtypes of Carter's serogroup A (Carter, 1963). The mucoid biotype includes the type A cultures; the haemorrhagic septicaemia biotype, the B and E cultures while the porcine biotype is the type D cultures.

The occurrence of different somatic or O group antigens of *P. multocida* was clearly recognized by Namioka and Murata (1961) using an agglutinin absorption procedure and later by Heddlestone *et al.* (1972) with an agar gel precipitin test. This system recognises sixteen serotypes. The disease caused by *P. multocida* can be divided into two broad categories: those in which the organism is the primary cause, e.g. fowl cholera and epizootic haemorrhagic septicaemia and the many infectious processes in which *P. multocida* is a secondary invader i.e. secondary to a primary "breaching" agent such as a virus or a *Mycoplasma*. The latter category includes many sporadic infections such as pneumonia, sinusitis, mastitis, infections of the central nervous system, abortion and also important pneumonic infections of the cattle, sheep, goats and swine (Carter, 1981). Although there has been little study of the occurrence of serotypes in various human infections what evidence there is indicates that the mucoid (type A) and the porcine biotype (type D) are the most common causes of sporadic respiratory or internal infections such as sinusitis, pneumonia, endocarditis appendicitis various abscesses and terminal septicaemias (Carter, 1967). Types B and E and F cultures have not yet been recovered from humans. Many of the human infections would appear to be of the secondary type.

P. haemolytica cultures have been classified serologically in essentially the same manner as *P. multocida.* Subdivision of *P. haemolytica* into A and T strains was first reported by Smith (1961). These varieties possess different biochemical, cultural and serological characteristics and responsible for different clinical forms of pasteurellosis of sheep, goats and cattle (Adlam, 1989). Strains of *P.haemolytica* are designated as A or

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T biotypes according to their ability to ferment either Arabinose or Trehalose after 10 days. In the fermentation of these sugars, the majority of A biotype ferment Arabinose but not Trehalose and all T biotype ferment Trehalose. The ability of strains to ferment xylose, salicin and lactose also assists in differentiating between biotypes. Thus A strains are usually salicin negative, xylose positive and lactose positive (apart from serotype A2 strains which are usually salicin positive and negative for xylose and lactose. In terms of antibiotic susceptibility, A strains are generally more susceptible to penicillin than T strains but not all A strains are susceptible. Colonial morphology on blood agar can be used as an additional criterion to distinguish between A and T strains. At 24-hour A strains appear as evenly coloured grey colonies whereas T strains produce larger (up to 2mm diameter) colonies with large brownish centres (Adlam, 1989).

The classification into A and T strains is of academic epidemiological significance as the biotype A strains are associated with pneumonic pasteurellosis in cattle, sheep and goats, while the T strains cause systemic disease in 6-10 month - old lambs or kids (Gilmour and Gilmour, 1989). The biotypes are further divided into at least 17 serotypes on the basis of classification by an Indirect Hemagglutination procedure (Biberstein *et al*, 1960; Chengappa *et al.*, 1984; Younan and Fodor; 1995), the reactions to which depend on the differences in capsular polysaccharides between the strains. Thirteen of these serotypes belong to the A biotype and four to the T biotype. All 17 serotypes have been isolated from sheep while only serotypes 1 and 2 occur in cattle. There are variations in pathogenicity among serotypes. Biotype A comprises serotypes 1,2,5-9,11,12,14,16 17 and biotype T comprises serotypes 3, 4, 10 and 15. Untypeable

strains have been isolated from both healthy and those with pasteurellosis. Most untypeable strains are however associated with the nasopharynx of healthy animals (Quirie *et al.*, 1986). Untypeable strains probably represent unencapsulated mutants (Biberstein, 1978) and probably belong to the A biotype since they exhibit sugar fermentation patterns more typical of A strains than of T strains.

The formation of a narrow zone of haemolysis on 7% ovine or bovine blood agar is used to distinguish *P. haemolytica* from other *Pasteurella species.* Thinly poured plates facilitate the observation of this zone which in some cases can only be detected when colonies are scraped off the surface of the plate. The ability of *P. haemolytica* strains to grow on MacConkey agar (on which lactose positive strains give rise to small pink or reddish colonies), their lack of a urease and their inability to produce indole serve as additional tests to distinguish them from other *Pasteurella* species (Adlam, 1989).

P. multocida occurs singly, in pairs and occasionally as chains of filaments. In tissues, exudates and recently isolated cultures, the bacterium shows bipolar staining with Giemsa or Wright's stain. After growth under unfavourable circumstances or on repeated subculture, they tend to become pleomorphic. Old laboratory cultures tend to consist predominantly of filamentous forms. Virulent strains of *P. multocida* are usually capsulated and the capsules are usually lost upon repeated subculture (Rimler and Rhoades, 1989). Ordinary cultures on agar media may develop into either or both of two principal colony forms, mucoid and smooth. Rough colony forms are encountered infrequently. The largest colonies are composed of cells with capsules consisting in part of hyaluronic acid (Carter, 1967). The mucoid characteristic can range from cultures

whose colonies are discrete, circular, convex and have a slight mucous-like consistency to cultures whose colonies are confluent, markedly moist and viscous (watery mucoid). The mucoid colonies are found particularly among isolates from the respiratory tract of sheep, goats, cattle, swine, rabbits and humans (Heddleston and Wessman, 1975).

The type A mucoid cultures yield large moist mucoid colonies with flowing margins. The type B,D and E colonies are relatively non-mucoid and smooth. The type F exhibit characteristics typical of the latter groups. The type F has reportedly been isolated only from turkeys in the US (Rimler and Rhoades, 1987). Canine strains produce the smallest colonies. Subculture of fresh cultures often results in the appearance of several different colonial variations (Carter, 1981). All typical cultures of *P. multocida* possess a characteristic musty odour.

Colonial morphology on transparent medium observed in obliquely transmitted light with a stereomicroscope is a useful characteristic for the study of *P. multocida*. Colonies refract the light in a manner that helps distinguish variants. Except for watery mucoid colonies which appear gray, colonies consisting of capsulated cells display a yellowish-green, bluish-green or pearl-like iridescence. Colonies that consist primarily of noncapsulated cells are not iridescent and appear blue, grayish blue or gray (Rimler and Rhoades, 1989).

2.4 Aetiology, epidemiology and Prevalence of infection.

Pneumonic pasteurellosis is a major economic problem militating against the production of sheep and goats in many parts of the world including Europe (Gilmour and Gilmour, 1989), Africa (Mwangota *et al* ., 1978; Adekeye, 1984; Cameron and Bester,

1986); Asia (Zamri-Saad and Basri, 1989), USA (Frank, 1982) and New Zealand (Alley, 1991). The disease is therefore geographically widespread and occurs in temperate, subtropical and tropical climates.

Pasteurellosis of sheep is caused by *P. haemolytica* and *P. multocida*, although the relative importance of these organisms may be different from place to place. Fibrinous pleuropneumonia due to *P. haemolytica* is usually a more fulminating and potentially fatal pneumonia than that produced by *P. multocida* (Gilmour and Gilmour, 1989). There are two biotypes of *P. haemolytica* - A and T - and distinct clinical syndromes are associated with each biotype. Biotype A strains cause pneumonia in all ages of sheep and septicaemia in young lambs; biotype T strains cause a well-defined systemic disease of young adult sheep. At present, five capsular types, A,B,D, E and F of *P. multocida* are recognized (Rimler and Rhoades, 1987) and most isolates from sheep with pneumonia are capsular type A (Rimler and Rhoades 1989).

P. haemolytica and *P. multocida* are carried in the nasopharynges and tonsils of apparently healthy sheep. Lambs acquire infection soon after birth probably due to intimate contact with their dams. Gilmour and Gilmour, (1989) in a survey of 100 adult sheep heads from an abattoir in Scotland found that *P. haemolytica* was isolated from 95 of the tonsils and from 64 of the nasopharynges. Normal flocks had a lower nasal carrier rate than those undergoing outbreaks of pasteurellosis. There was a random assortment of serotypes and untypable strains in normal flocks but a narrowing of the spectrum of serotypes and a dimunition of the number of typeable strains occured during disease outbreaks (Gilmour and Gilmour, 1989). The occurrence of the different serotypes from

cases of pasteurellosis indicates that serotype A2 is most common in sheep in contrast to pneumonic pasteurellosis in cattle, where serotype A1 is most commonly isolated (Gilmour and Gilmour, 1989). Mwangota *et al.* (1978) reported that all the known serotypes at the time occured in sheep and goats in Kenya and that serotypes 3, 4, 6, 8 and 10 were more commonly associated with pneumonia. Serotype A2 was most commonly isolated from healthy flocks in USA (Frank, 1982) and from pneumonic lungs in New Zealand (Alley, 1991).

Prevalence of pneumonic pasteurellosis varies annually and from farm to farm but it is invariably one of the most commonly diagnosed infectious ruminant diseases in many parts of the world including Nigeria (Akerejola *et al.*, 1979; Adekeye, 1984; Gilmour and Gilmour, 1989).

2.5 Pathogenesis and the Molecular Aspects of Virulence of *Pasteurella.*

Isolation studies have shown *P. haemolytica* and *P. multocida* to be part of the normal upper respiratory tract (URT) microflora in healthy sheep together with *Micrococcus* species, corynebacteria, saprophytic *Neisseria*, streptococci and mycoplasma (Alley, 1991). The source of Pasteurellae that cause pneumonic pasteurellosis in an individual sheep could either be its own nasopharynx or that of another sheep. In either of the cases, the affected sheep must possess a predisposing factor which allows multiplication of large numbers of the organisms. The large rapidly-replicating population of the organism in the nasopharynx can travel to the lungs in inspired aerosol droplets. Any condition that is stressful to the sheep or impairs its health and promotes

close contact between sheep will contribute to the spread of the organisms and to the development of pneumonic pasteurellosis (Gilmour and Gilmour, 1989).

A number of different viruses that are ubiquitous in sheep have been associated with pneumonic pasteurellosis. Those most frequently involved are *parainfluenza-3 (P1-3)* virus and *adenovirus* (Lehmkuhl *et al.*, 1985; Ibu *et al.*, 1993). All of these can contribute to severe pneumonic pasteurellosis but infections caused by these viruses *per se* are usually mild or asymptomatic in healthy unstressed sheep. Some of them have been shown to cause mild pneumonia experimentally. Crowding and other management factors cause stress and promotes dissemination of these viruses among sheep. Those that become ill are highly susceptible to pneumonic pasteurellosis at the time. In all these, one or more viruses might be involved but infection with *P. haemolytica* or *P. multocida* is most often the cause of death.

The disease has been studied extensively by compromising the respiratory tract of sheep with viral agents or Mycoplasma followed at variable times with aerosol or endobronchial inoculation of *P. haemolytica/P. multocida.* (Confer *et al,* 1988; Gilmour and Gilmour, 1989). There has been controversy about the role played by these organisms in the aetiology and pathogenesis of the disease. In North America for example, the aetiology of pneumonic pasteurellosis has been considered by many workers to be multifactorial (Yates, 1982; Martin, 1984). In Europe (especially Britain) on the other hand, *Pasteurella* species and in particular *P. haemolytica* biotype A are thought to be capable of initiating pneumonia as primary agents in non-immune animals in the absence of the viruses or physical stress (Gibbs *et al.*, 1984; Shoo, 1989).

Specific biotypes and serotypes of *P. haemolytica* and P. *multocida* are associated with fibrinous pleuropneumonia in cattle,sheep and goats. The pathogenicity requirements of these Pasteurellae include colonization of mucous surface, invasion of the host tissues, survival and multiplication in the host, interference with host defense and damage to the host (Whiteley *et al.*, 1992). For these purposes the bacteria possess at least the following virulence factors: fimbriae, capsular polysaccharides (CPS), endotoxin or lipopolysaccharide (LPS) and in addition in the case of *P. haemolytica* a very potent cytotoxin called leukotoxin (Lkt) (Confer, *et al.*, 1990). Other potential virulence factors whose roles are less well documented or not well defined include: outer membrane proteins (Davies *et al.*, 1992), iron- regulated protiens (Murray *et al.*, 1992), neuraminidase (Straus *et al.*, 1993) and neutral glycoprotease (sialoglycoprotease, Abdullah *et al.*, 1992). The interactions of these virulent factors with components of the pulmonary alveolus are discussed as a model for the pathogenesis of pneumonic pasteurellosis (Confer *et al.*, 1990).

Fimbriae on *P. haemolytica* may enhance colonization of the URT. The capsule of *P. haemolytica* varies in composition among serotypes. It inhibits complement-mediated antibody killing phagocytosis in intracellular killing *Pasteurella.* The capsule enhances neutrophil direct migration and adhesion of *Pasteurella* to alveolar epithelium. Lipopolysaccharides can alter ruminant leukocyte functions by dose dependent inhibition or augmentation; it is directly toxic to ruminant endothelium; it modifies cardiopulmonary hemodynamics and elevates circulatory prostanoids and serotonin. Leukotoxin is produced by all known serotypes of *P. haemolytica* and many untypable strains.

Leukotoxin is a pore-forming cytolysin that affects ruminant leukocytes and platelets by altering their functions at low levels while causing lysis at high levels (Confer *et al.*, 1990).

The availability of hysterectomy-derived, colostrum deprived, specific pathogen-free (SPF) lambs or animals allowed the investigation of the pathogenicity of the disease in animals. Current knowledge of the molecular and pathogenic aspects of virulence associated with these factors have been made possible through SPF animal (Hart *et al.*, 1971).

2.5.1 Fimbriae (Pili)

Bacteria adhere to eukaryotic cell surfaces through capsular material or surface proteins (adhesions). Specific receptors for fimbriae are present on eukaryotic cells, and the fimbriae-receptor interaction overcomes normal repulsive forces exerted between bacteria and eukaryotic cells. Recent findings indicate that fimbraie may be important in upper respiratory tract (URT) colonization (the first major step) in production of pneumonic pasteurellosis. The fimbriae is thought to cause two major alterations that lead to colonizatin of the URT-ulterations in the mucociliary apparatus and loss of fibronectin. Loss of fibronectin which expose receptors on cells and permits binding of gram-negative bacteria appears to be a key event favouring colonization of the URT (Whiteley *et al.*, 1992).

2.5.2 Capsular polysaccharide

P. haemolytica and *P. multocida* produce a polysaccharide capsule (glycocalyx) during log growth phase. The capsule can be examined or visualized by light or electron

microscopic examination of the grown cultures. The capsule of each serotype has a different sugar composition. Studies indicate that the capsule polysaccharides were less susceptible to serum agglutination, complement-mediated serum killing and phagocytosis by neutrophils compared to experimental decapsulated organism (Brogden *et al.*, 1989). These studies indicate that the *Pasteurella* capsule may be important for adherence of the organism to alveolar and bronchiolar surfaces and attraction of neutrophils to the sites of colonization. However, capsular material may inhibit phagocytic and bacteriocidal functions of neutrophils and complement mediated serum killing. *In vivo* studies indicate that capsule may not be important in directly inducing vascular damage, atelectasis or other components of the lesions of pneumonic pasteurellosis.

2.5.3 Lipopolysaccharide (endotoxin)

Pasteurella LPS is similar to LPS produced by other gram-negative bacteria and is composed of biologically active lipid A, core oligosaccharide and an antigenic polysaccharide chain (O-antigen). It is estimated that 12-25% of the gram-negative bacteria is composed of LPS. LPS appears to be a major surface antigen on formalin-killed *Pasteurella;* however high serum antibody response to the polysaccharide chain of LPS did not correlate with resistance to experimental pneumonic pasteurellosis. Changes seen in sheep lungs after direct LPS exposure indicates a role for *P. haemolytica* LPS in stimulating leukocyte adherence and infiltration, modifying leukocyte function and causing vascular leakage through endothelial damage (Confer *et al.*, 1990).

2.5.4 Leukotoxin

All known serotypes and several untypeable strains of *P. haemolytica* isolated from ruminants produce leukotoxin (Lkt) during log growth phase (Shewen and Wilkie, 1985). In culture supernatants, native Lkt is present as a multiprotein complex with a molecular weight >700 kilodalton (KDa), and the protein that confers leukotoxic activity to this complex is 105 KDa but is readily degraded to proteins of 97 and 66 KDa. The gene coding for *P. haemolytica* Lkt has been isolated and found to share extensive homology with the gene coding for *E. coli* alpha-hemolysin (Lo *et al.,* 1985). Lkt is associated with cell degeneration in alvelolar exudate of infected ruminant lung. Ruminant leukocytes exposed to low Lkt doses have reduced phagocytic killing and oxygen radical generation, whereas higher Lkt doses cause leukocyte lysis. Non-ruminant leukocytes and other types of cells and endothelium are resistant to Lkt effects (Shewen and Wilkie, 1982). Lkt is a pore-forming cytolysin. Lkt damages the plasma membrance of ruminant neutrophils causing rapid leakage of intracellular potassium ions (K^{1}). Lkt also causes rapid cytolysis of ruminant platelets. Leukotoxin-damaged platelets could release fibrinogen and vasoactive compounds contributing to the formation of thrombin and fibrin leakage into alveolar spaces.

2.6 Clinical manifestation

2.6.1 Predisposition

Many apparently healthy sheep are carriers of *P.haemolytica* and *P. multocida* but not all carriers develop pasteurellosis. Although it has generally been accepted that predisposing factors are necessary to precipitate clinical disease, this concept has been

difficult to prove epidemiologically, particularly with regard to noninfectious environmental and management factors.

Climatic changes may be particularly important and deaths have been reported in ewes during periods of warmth, still weather, not associated with any other stressful procedure such as movement. It is believed that environmental and management factors, uncomplicated by infections, stress the sheep, but as these are part of normal flock management, little can be done to remove them and thus influence the precipitation of pasteurellosis (Gilmour and Gilmour, 1989).

A number of infectious agents have a temporal relationship with outbreaks of pasteurellosis. A longitudinal serological survey of respiratory virus infections in sheep in many countries showed a temporal relationship between outbreaks of pneumonia and the presence of antibodies to parainfluenza-3 virus (P1-3) and also to an adenovirus (Gilmour and Gilmour, 1989; Ibu *et al.*, 1993). The role of reovirus and respiratory syncytial virus infections in sheep has also been shown (Lehmkuhl *et al.*, 1985).

Sheep with pulmonary adenomatosis often die suddenly from a complicating *P. haemolytica* pneumonia (Sharp and Martin, 1983). The predisposing factor may be the dysfunction which results from the tumour. Immuno-suppression in ovine trypanosomiasis may be a factor in predisposing to *Pasteurella* infection in Southern Africa (Mackenzie *et al.*, 1975). It has been shown that the cause of tick borne fever of sheep predisposes to *P.haemolytica* pneumonia (Gilmour *et al.*, 1982).

It is also currently believed that *P. haemolytica* and *P. multocida* cause pneumonic pasteurellosis independent of viral or other microbial agents when virulent rapidly

growing Pasteurellae are injected intrabronchially or transthoracically. *Pasteurella* in the stationary phase of growth, unlike rapidly growing bacteria produces less severe pneumonia in challenge experiment (Debey *et al.*, 1992).

2.6.2 Epidemiology of flock outbreak

Sporadic outbreaks of pneumonic pasteurellosis occur in all ages of sheep throughout the year. Generally while only a small number of sheep may be affected outbreaks during late spring and early summer involve a higher morbidity and mortality. In Nigeria, incidence of the disease is highest during the cold dry harmattan and wet seasons (Adekeye, 1984). In some flocks only ewes may be affected, in others ewes and lambs and in others, lambs only.

2.6.3 Clinical signs

A typical outbreak of pneumonic pasteurellosis in adult sheep starts with sporadic deaths. Close inspection of the flock at this stage may reveal some sheep with a slight, serous, oculonasal discharge and with an occasional soft cough. Clinical cases of pneumonia then occur. Affected sheep are dull and anorectic with rectal temperatures ranging from 40.6 to 42.6°C (105-107°F). A proportion of sheep with these signs become recumbent and die within a few hours. Others develop dyspnoea, manifested by exaggerated movement of the abdominal flank muscles (abdominal "lift"). Most sheep which reach this stage die, if untreated, while a small number survive to develop chronic pneumonia and then generally remain unthrifty. Total clinical morbidity and mortality seldom exceeds 10% (Gilmour and Gilmour, 1989).

The disease in lambs depend on age. It is not common under about three weeks of age, but when encountered, is a hyper-acute, rapidly-fatal septicaemia. As the lambs get older, some affected animals may live long enough to appear very dull but quickly become recumbent, collapse and die. By three months of age, a higher proportion of cases live long enough to develop the more obvious signs of pneumonia. Enzootic pneumonia is the term that describes outbreaks of pneumonia associated with *Pasteurella.* (Alley, 1991).

2.6.4 Post-morten finding.

On opening the thorax of sheep which have died in the acute phase, 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 straw-coloured 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. Blood-stained fluid and frothy exudate can be expressed from the cut surfaces. Often there is no distinct demarcation between lesions and normal lung. In sheep which have been ill for a few days the lesions are more sharply demarcated, less extensive and a darker red; extensive adhesion are usually present between the parietal and visceral pleura. Abscesses 30 to 50mm in diameter may be present. A central necrotic core and a fibrous tissue capsule is surrounded by pale grey lung tissue (Gilmour, 1978).

In young lambs up to about two months of age, the post-mortem picture is usually one of septicaemia without pathognomonic features of pasteurellosis. Petechiae are

usually present on the liver, spleen kidneys and myocardium. Severe pleurisy and pericarditis with little lung involvement are also seen in lambs up to three months of age and seem to be associated predominantly with the A2 serotype (Gilmour, 1978).

2.6.5 Histology

On histological examination, the peracute lung lesions show alveolar necrosis with the alveolar spaces filled with fluid and bacteria. The interlobular septae are extended by oedema and the capillaries are distended with fluid and red blood cells. Fibrin deposits and an occasional lymphoid cell can be seen. There is bronchiolitis with hyperplasia and sloughing of the mucosa. However, the most characteristic feature of this pneumonia resulting from *P. haemolytica* is the presence in the lung lesions of spindle-shaped cells with intensely basophilic nuclei which have been called oat cells. These cells can be seen in large numbers, lying with their long axes parallel, surrounding areas containing necrotic alveoli. Some of these alveoli are lined with hypertrophic cells and large mononuclear cells and the capillaries present in the area are thrombosed or devoid of lining cells. In sheep that survive the acute phase, a collagen capsule infiltrated with neutrophil and lymphoid leukocytes surround the area of oat cells (Gilmour, 1978).

2.7 Diagnosis

Clinical signs in an affected group may range from occasional coughing to sudden death. At the early stage, diagnosis of pasteurellosis must be provisional and confirmation depends on necropsy. It must be emphasized that the recovery of *Pasteurella* species from the upper respiratory tract of either diseased or healthy sheep does not warrant a

diagnosis of pasteurellosis (Gilmour and Gilmour, 1989), and little is to be gained by serotyping of *P. haemolytica* recovered from nasal swabs.

Although many of the features found at necropsy are almost pathognomonic, diagnosis is not easy in per-acute cases and when necropsy findings are complicated by autolysis. Under these circumstances, it is essential that tentative diagnosis are confirmed by bacteriology. Samples should be collected from cases which have not been given antibiotic and should be taken from different sites, e.g. pleural and pericardial fluid, heart blood, lung lesions, liver, spleen and kidneys (Gilmour and Gilmour, 1989). Since *Pasteurella* may be present in the lungs of sheep in the absence of pasteurellosis, it is important to have an indication of the number of the organism in the lungs. In acute pasteurellosis, 10^6 or more colony forming units (cfu) per gram of lung are present with 10^3-10^5 cfu/g in subacute and chronic cases. Residual lesions in chronic or treated cases may contain *Pasteurella*. If the serotype of *P.heamolytica* is to be ascertained, it is essential that a number of colonies be tested since a single colony from one necropsy specimen may not represent the causal serotype. It is also possible that more than one serotype is pathogenic during an outbreak (Gilmour and Gilmour, 1989).

Both *P. haemolytica* and *P. multocida* grow readily on blood agar, forming round light greyish growth at 37°C (Carter, 1981). When they are isolated from pneumonic lung, the colonies of *Pasteurella* are usually in "pure" culture or outnumber other bacterial colonies. In some cases especially from nasal mucus samples, *Pasteurella* colonies must be selected from a mixture of other bacterial colonies. In sheep dying later (say second week) in an outbreak, bacteria such as *Neisseria* species, *Corynebacterium pyogenes*,

streptococci, *Escherichia coli, Pseudomonas* species and staphylococci are frequently isolated from these lesions in addition to *Pasteurella* species (Adekeye, 1984; Alley, 1991).

Isolations of *Pasteurella* are usually done to determine the antibiotic therapy necessary for treatment and for epidemiological studies. *Pasteurella* species have a number of biochemical properties, but certain ones are most useful for identifying the organisms. Examination of colonies on blood agar under low-powered magnification will best display their morphology, and in case of *P. haemolytica,* reveal the weak zone of *beta*-haemolysis beneath the colony. The butyraceous consistency of the colony and its Gram-staining potential are useful for differentiating *Pasteurella* colonies from those organisms with a similar colony appearance (e.g. streptococci) that sometimes appear in nasal mucus sample (Carter, 1981). A sequence to isolate and identify *Pasteurella* in large numbers of nasal mucus samples with a minimum amount of time spent on each isolate was described by Frank (1982).

Measurement of serum antibodies is not a useful diagnostic aid since many healthy animals have titres to *P. haemolytica* and the multiplicity of serotypes makes the choice of antigen in any such test difficult and of little value for routine use (Gilmour and Gilmour, 1989).

2.7.1 Differential diagnosis

In per-acute cases, the necropsy observations may be common to other condition displaying acute congestion and thus bacteriological examination is essential in differentiation. These conditions include clostridial disease, phenolic dip poisoning,

aspiration pneumonia, hypostatic pneumonia and *post mortem* autolysis (Gilmour and Gilmour, 1989). Other bacterial infection e.g.streptococci, *E. coli* and *Corynebacterium pyogenes*, may cause lung consolidation, pleurisy and abscess formation, but these infections in sheep are much less common than pasteurellosis and are readily differentiated on microbiological grounds. Acute pneumonic pasteurellosis can occur in sheep with pulmonary adenomatosis and the lesions of pasteurellosis may mask those of the tumour especially if the later are not extensive. Sub-acute and uncomplicated chronic pasteurellosis must be differentiated histologically from a typical pneumonia since gross differences are unreliable and serofibrinous pleurisy may accompany both conditions. Chlamydia, P1-3 virus, and *Dictyocaulus filaria* infections also cause chronic pneumonias and must be differentiated histologically from pneumonic pasteurellosis (Gilmour and Gilmour, 1989).

2.8 Immunogens of *Pasteurella*

2.8.1 *P.haemolytica* immunogens

P.haemolytica has numerous potential antigens. Those with the most potential for stimulating immunity include capsular polysaccharide, lipopolysaccharide (endotoxin, LPS), outer membrance proteins, fimbriae, iron-regulated proteins and secreted leukotoxin (Confer, 1993)An important approach for determining the importance of various immunogens for stimulation of immunity to *P. haemolytic* is through the vaccination of sheep, goats and cattle with purified or relatively purified antigens followed by challenge with virulent *P. haemolytica.* . The antibody responses to specific antigens are quantitated and statistically correlated with the lesion score obtained after

challenge. Thus a significant correlation between a high antibody response and resistance to challenge can be used as a predictor of the importance of an antigen in stimulating immunity.

Antibody response to *P. haemolytica* fimbriae have not been documented. Capsular polysaccharides from at least five *P. haemolytica* serotypes were purified and characterized (Adlam, 1989). The capsular polysaccharide is a virulence factor that interferes with phagocytosis and killing of *P. haemolytica* (Czuprynski *et al.*, 1991) and complement-mediated bacteriolysis (Chae *et al.*, 1990). While immunization of ruminants with *P. haemolytica* capsular polysaccharide or live or killed whole cell preparation may result in an antibody response to the capsule, studies have shown it is usually ineffective at protecting animals against *P. haemolytica* challenge (Confer *et al.*, 1989).

P. haemolytica lipopolysaccharide (LPS) has classical endotoxin function, *in vivo* and it can alter leukocyte function and is toxic to ovine endothelial cells (Confer *et al;* 1990). The intensity of the antibody responses to LPs did not correlate with resistance to experimental challenge (Confer *et al., 1986).*

The antibody responses to *P. haemolytica* outer membrane protein (OMP) have not been completely characterized. Antibody responses to protein antigens from surface extracts have been correlated with resistance (Confer *et al.*,1989). Mosier *et al.*, (1989) showed that high antibody responses to several proteins in the surface extract correlated with resistance to experimental challenge. The highest correlations were for antibody response to proteins with molecular masses of 86, 66, 49 and 31 kilodalton (KDa).

Several of those proteins have molecular masses equivalent to major OMP of *P. haemolytica* (Knight *et al.*, 1990). Vaccination with *P. haemolytica* OMP-enriched preparation induces serotypic immunity against experimental challenge.

Iron-regulated protein (IRP) of *P. haemolytica* have been described (Deneer and Potter, 1989; Lainson *et al.*, 1991). The 100 and 70 KDa proteins are located in the outer membrane of *P. haemolytica* grown *in vivo* or under iron-restricted *in vitro* conditions. Gilmour *et al.* (1991) demonstrated that sodium salicylate extract vaccines of *P. haemolytica* A2 prepared from bacteria grown under iron-restricted conditions protected sheep better than similar vaccines prepared from *P. haemolytica* grown in media containing iron. These studies suggest the IRPs are involved in the immune response and might contribute to protective immunity in sheep. Murray *et al.* (1992) observed antigenic cross-reactivities of the IRPs of different serotype suggesting strongly that the IRP vaccine may be cross-protective (Donachie, 1995).

Another antigen that has received much attention recently is *P. haemolytica* leukotoxin (Lkt). It has received much acclaim as an immunogen against pnemonic pasteurellosis. The Lkt is lytic for ruminant leukocytes (Clintenbeard *et al.*, 1989) and is therefore thought to have a major role in the pathogenesis of *P. haemolytica*- induced pneumonia. The genes for Lkt have been cloned and sequenced (Lo *et al., 1985).* Antibodies to Lkt are not *P. haemolytica* serotype specific. Vaccination of cattle, sheep or goats with live *P. haemolytica* culture supernates induced high neutralizing antibody titres to Lkt and which correlated with resistance to experimental or natural infection with *P. haemolytica* (Confer *et al.*, 1989).

2.8.2 *P. multocida* Immunogens

The etiologic role of *P. multocida* relative to *P. haemolytica* in pneumonic pasteurellosis disease process is less important. Nonetheless, *P. multocida* possesses important immunogens which are worth mentioning. Capsular polysaccharides, LPS, OMP and *P. multocida* toxin (PMT) are recognized. Purified *P. multocida* capsule is a hapten when injected in most animals. Capsules of various *P. multocida* serotypes appear to be serotype specific (Rimler and Rhoades, 1989).

P. multocida LPS has similar chemical and biological properties to the LPS of other Gram negative bacteria (Lugtenberg *et al.,* 1984). Protection afforded by immunization with LPs is somewhat species dependant. In general *P. multocida* LPS seems to be a major immunogen in birds. The role of LPS as an immunogen in mammals remain controversial. Mice, cattle and rabbit have not been readily protected against *P.multocida* infection following immunity with LPS (Rhoades and Rimler, 1989).

Recently, outer membrane proteins (OMP) of *P. multocida* have been studied as potential immunogens (Abdullahi *et al.,* 1990; Hunt *et al.*, 2000). The immunogenic role of the OMP has been best characterized in rabbits. Lu *et al.* (1988) demonstrated that rabbits mounted major antibody responses against 5 *P. multocida* OMPs. They further demonstrated that vaccination with *P. multocida* OMP protected rabbits against homologous challenge. The immunogenic role of iron-regulated *P. multocida* OMP has not been investigated.

Certain *P. multocida* isolates of the A and D serogroups produce antigenically similar protein toxins (approximately 145KDa) that are toxic for bovine embryonic lung

and vero cells *in vitro,* are lethal for rodents and birds, induce osteolysis of swine turbinate bones and produce haemorrhage and necrosis when injected into guinea pig skin (Rimler and Rhoades, 1989). Because of the necrotic effect on guinea pig skin, the toxin was called dermo-necrotic toxin; however the term *P. multocida* toxin (PMT) is now in current use. Although PMT has many characteristics of an exotoxin, it is not secreted from living intact *P. multocida* (Dali et al., 1991), but must be extracted from bacteria by sonication. PMT has been demonstrated in *P. multocida* isolates from various animal species; however, it is mainly a virulence factor in atrophic rhinitis of pigs. As an immunogen, inactivated PMT (toxoid) induces protection against a lethal effect of PMT in rats and mice (Thurston *et al., 1991)* and against experimental atrophic rhinitis in pigs (Foged *et al*., 1989). The gene for PMT has been cloned and sequenced in *E. coli* and characterized (Petersen and Foged, 1989).

2.9 Immunology

2.9.1 *P. haemolytica*: Serotyping

Subdivision of A and T strains of *P. haemolytica* into different serotypes was first achieved by Biberstein *et al.* (1960) using an indirect hemagglutination assay (IHA). Recent studies of OMP and the genome of *P. haemolytica* demonstrated marked differences in electrophoretic patterns and nucleic acid sequences between the A and T biotypes (Knight *et al.*, 1990; Dewhirst *et al.*, 1992). *P. haemolytica* biotype T has been reclassified as *P. trehalosi.*

The number of serotypes which have been identified using IHA method which relies on the differences in capsular polysaccharides between strains has now reached 17

(Younan and Fodor, 1995), with 13 of these belonging to the A biotype and 4 to the T biotype. Modifications to the IHA technique have included the use of microtitre trays (Shreeve *et al.*, 1972; Sawada *et al.*, 1982). A direct plate agglutination method was developed by Frank and Wessman (1978). Chengappa *et al* (1984) compared IHA and direct plate agglutination with counter-immune electrophoresis (CIE). The CIE was shown to be rapid and reliable when absorbed sera were used. Untypable strains have been encountered. Untypable strains probably represent encapsulated mutants and probably belong to the A biotype since they exhibit sugar fermentation patterns more typical of A strains than T strains (Biberstein, 1978; Fraser *et al.*, 1982)

2.9.2 *P. multocida*: serotyping

Many systems developed by early workers for serological typing of *P. multocida* based upon agglutination tests are no longer in use (Carter, 1967), because most capsulated isolates could not be typed. Subsequent serological typing system which have enjoyed success have been based upon methods devised to circumvent the problem of inagglutinability of capsulated strains (Carter and Rundell, 1975; Carter and Subronto 1973).

2.9.2.1 Serum protection typing: Roberts (as reviewed by Rimler and Rhoades, 1989) in 1947 developed an immunological typing system based upon passive protection of mice by serum against live challenge organism. Four types (I, II, III, IV) were recognized among 37 cultures but it was considered that seven or eight types could exist. Type V was distinguished later by Hudson in 1954 (as reviewed by Rimler and Rhoades 1989).

Typing by the Roberts system is still sometimes used. Problems particular to Roberts system is that routine typing tests are expensive and isolates must be virulent for mice.

2.9.2.2 Capsule serotyping: The system most commonly used for specific capsule antigen typing is based upon passive hemagglutination of erythrocytes sensitized by capsule antigen. Five serotypes A B D E & F are currently distinguished in the Carter system (Rimler and Rhoades, 1989). Serogroups C was abolished because it was felt that it did not represent a capsule group (Carter, 1963). A relationship between Roberts and Carter typing systems has been described. Roberts types I II and V were equated with Carter's serogroups B, A and D respectively. Based upon the observations that capsules of Roberts types III and IV contain hyaluronic acid, they may be equivalent to subtypes of Carter's serogroup A.

2.9.2.3 Non-serological and Rapid serological tests: Due to the difficulties and time involved with passive hemagglutination tests, several serological and non-serogical tests have been devised in an attempt to facilitate capsule typing. The presence of hyaluronic acid in the capsule is largely specific to serogroup A strains (Carter and Rundell, 1975). Therefore, Carter and Rundell (1975) developed a simple test in which serogroup A strains are recognized by depolymerization of the capsule after growth in proximity to a hyaluronidase-producing strain of *Staphylococcus aureus*.

The basis of the test for the presumptive recognition of serogroup D isolates is the characteristic floccular reaction with acriflavine (Carter and Subronto, 1973). Some of the newly recognized serogroup F strains react similarly.

Counter-immunoelectrophoresis (CIE) was developed as a rapid serological method for recognition of serogroups B D and E (Chengappa *et al.*, 1986). A coagglutination test was developed for recognition of serogroups B and E (Rimler, 1978).

.2.9.2.4 Somatic serotying: Two systems have been commonly used for somatic antigen typing of *P. multocida*: the Namioka system based upon tube agglutination tests (Namioka, 1978) and the Heddleston system based upon gel diffusion precipitin tests (Heddleston *et al.*, 1972). Eleven serotypes (1-11) were recognized by the Namioka system and 16 serotypes (1-16) were recognized by the Heddleston system.

The Namioka system utilizes rabbit antisera. The antigens consist of HCl-treated bacterial suspension. The HCl treatment is to remove capsular material which inhibits the agglutination reaction. The system has a problem of isolates becoming autoagglutinable following HCl treatment and therefore cannot be typed.

The Heddleston gel diffusion precipitin tests utilizes antisera made in chickens. The chickens are preferred to other laboratory animals because they respond better to the type specific antigens after a short course of immunization with a bacterin. The heat-stable antigens used for the test are extracted from formalinized-saline suspension of the bacteria.

Comparisons between Namioka and Heddleston serotyping systems have been made (Brogden and Packer, 1979). They observed that serotypes determined by one system generally did not correlate with serotypes determined by the other system.

2.9.2.5 Problems with *P. multocida* serotyping: Fresh isolates of capsulated *P. multocida* are usually inagglutinable in antiserum directed against the specific

homologous capsule antigen. Serogroup B and E isolates can be agglutinated with high-titre capsule antiserum after repeated laboratory passage, but inagglutinablity persist with serogroups A, D and F isolates. The inagglutinability of serogroup A is due to the presence of hyaluronic acid in the capsule (Rimler and Rhoades, 1989).

2.9.3 Immunity to *P. haemolytica*

One or more immune system could be potentially involved in resistance to *P. haemolytica* (Confer *et al.*, 1988). First, is the cell-mediated immunity involving T-lymphocytes which can be directly toxic to the bacteria or enhance intracellular killing of the bacteria through lymphokine-induced macrophage activation. But because *P. haemolytica* primarily multiplies and exists extracellularly, this mechanism may not be of major importance in resistance to pneumonic pasteurellosis.

Second, is the IgA-induced inhibition of bacterial multiplication on mucosal surfaces. This is a potential immune mechanism for defence against *P. haemolytica* but the role of local immunity in pasteurellosis is not well understood (Confer *et al.*, 1988).

Third, is opsonization of *P. haemolytica* by specific antibodies mainly serum IgM and IgG which leak into alveoli because of bacterial-induced vascular permeability with subsequent phagocytosis by alveolar macrophages or neutrophilis. Phagocytosis is possible when the ratio of bacteria to leukocytes was low (<100 bacteria/leukocyte). However large number of opsonized bacteria (>100/cell) cause leukocyte death through the action of leukotoxin.

Fourth is the complement-dependent killing of *P. haemolytica* which has been demonstrated with cattle serum by MacDonald *et al.*(1983). Nasal secretions were not bactericidal.

The fifth potential immune mechanism involves neutralization of the leukototin of *P. haemolytica* by antibodies in serum or broncho-alveaolar secretions (Cho *et al.*, 1984). Immunologic neutralization of leukotoxin activity could enhance pulmonary resistance by protecting alveolar macrophages and infiltrating neutrophils so that opsonization and killing of *P. haemolytica* can occur.

The goals of many investigators have been to determine the immune mechanism that can effectively enhance resistance to *P. haemolytica*, determine antigens responsible for stimulating immunity, and develop an efficacious *P. haemolytica* vaccine.

The (IHA) test (Biberstein *et al.*, 1960) which detects serotype-specific antibodies directed against the capsule was widely used in early studies. A fluctuating range of relatively low (¼to 1/16) serum antibody titres to a range of serotypes is detected over a period of time in normal flocks. These are probably, the response to carriage of *P. haemolytica* in the upper respiratory tract and to intermittent, mild challenge of the lower respiratory tract. Low titres may also be due to cross-reactive antibodies as has been detected in sera of SPF lambs which had no experience of *P. haemolytica* (Gilmour and Gilmour, 1989). An ELISA using sodium salicylate extract (SSE) antigens was up to 200 times more sensitive but less specific than IHA test (Burrells *et al.*, 1983). Donachie *et al.*(1983) developed a serotype-specific ELISA using phenol/water extracts of *P. haemolytica* as antigen. The use of SPF lambs has allowed a better understanding of

immunity engendered in animals. Antibodies to antigenic components of *P. haemolytica* were sought by immunoblotting using sera from recovered animals. When immunoblot antigens prepared from *P. haemolytica* cells grown in conventional media were compared with cells recovered from pleural fluid of SPF lambs dying from pneumonic pasteurellosis (grown *in vivo*) and examined with sera from recovered animals, the antigenic profiles were significantly different. The *in vivo* grown cells expressed at least three antigens in addition to those seen in laboratory grown cells. The intensity of the antibody reaction to the *in vivo* antigens suggest that these are major antigens. (Gilmour and Gilmour, 1989).

Further evidence for the important role of humoral immunity is derived from the results of passive protection experiments in which SPF lambs were protected against experimental A2 pneumonic pasteurellosis by transfer of sera from naturally or artificially immunized sheep (Jones *et al.*, 1989). Immune sera gave 90% or greater protection.

2.9.4 Models for Assessment of Challenge and Immunity studies.

This models have evolved mainly from work carried out in cattle which also has been applicable to small ruminants (Confer *et al.*, 1988). Three basic experimental models (with variations among laboratories) are commonly used to assess immunity. The most common method involves nasal, intratracheal or aerosol exposure of the animal to P1-3 virus, followed 3 to 5 days later by aerosol, intrabronchial or intratracheal exposure to *P. haemolytica/P. multocida*. The result resembles naturally acquired pneumonic pasteurellosis but the aerosol procedure is labour-intensive and time-consuming (Davies *et al.*, 1981).

The second model involves direct instillation of *P. haemolytica/multocida* intratracheally or intrabronchially without prior exposure to a virus. This model use somewhat natural routes of exposure (Debey *et al.*,1992; Zamri-Saad *et al.*, 1996). The extent of resistance is quatified by assessing clinical responses such as rectal temperature, respiratory rate and by estimating the per cent of the lung with lesions of pneumonia or the number of infected lung lobes.

The third model involves direct injection of the *Pasteurella* into both caudal lung lobes through the thoracic wall. The system has a disadvantage in that it by- passes the upper airways and therefore does not simulate natural exposure but is usually quickly and readily accomplished (Confer *et al.*,1988). Vaccination protocols and vaccines can be assessed by use of any of these models.

2.10 Control and Prevention

2.10.1 Therapy

Numerous reports on antimicrobial agents describe sensitivity and resistance of *P. multocida* and *P. haemolytica in vitro* as well as their effects on the organisms and animals *in vivo*. While *P. multocida* may be sensitive to a variety of antimicrobial agents, treatment is rarely effective possibly because treatment results in endotoxin release from killed bacteria (Rimler & Rhoades, 1989). It is important therefore to begin antibiotics or sulphonamide treatment as soon as possible since the disease pneumonic pasteurellosis can advance within 1 - 2 days to a stage where treatment is less effective or ineffective. Ideally antibiotic sensitivity of the *Pasteurella* isolate should be determined before initiation of treatment. Lung isolates collected at necropsy would be the first choice for

determining antibiotic therapy. In an outbreak, *Pasteurella* species should be isolated from the first cases to determine the antibiotics of choice but treatment should be started with an antibiotic that is being used successfully in the local area at the time (Gilmour & Gilmour, 1989; Shoo, 1990).

The majority of strains of *P. haemolytica* from sheep are sensitive to penicillin and ampicillin and all are sensitive to oxytetracycline. The efficacy of a long-acting oxytetracycline was investigated by Gilmour *et al.* (1982) in lambs. Lambs were infected first with PI-3 virus and then with *P. haemolytica* A2. Clinical signs of pneumonic pasteurellosis were present in all lambs 24 h after last exposure. Treatment of 10 lambs at that time completely abolished clinical signs within 24 h and this effect lasted for six days, but 2 treated lambs died on day 7 post-infection. However, 7 of the 10 untreated lambs died during the seven days after infection. Another 10 lambs were given oxytetracycline at the same dose rate 24h before *P. haemolytica* challenge. Clinical signs of pasteurellosis were delayed until four days post-infection, after which time five lambs died. This suggests that deaths occurred after the blood concentration of the antibiotic had waned. The recurrence of infection presumably followed a persistent but low level of *P. haemolytica* infection since oxytetracycline is bacteriostatic and not bacteriocidal. Pasteurellosis occurs sporadically and vaccination is unlikely to be of value during an outbreak, thus the use of oxytetracycline under similar circumstance could be advantageous. However, under farm conditions, delay in treatment may make this less effective than under experimental conditions.

2.10.2. Vaccination

Since pneumonic pasteurellosis is sporadic, it seems unlikely that any long-lasting flock resistance develops as a result of disease within the flock. Many of the predisposing causes are part of the normal environment or management of flocks and some of those of infectious nature e.g. P1-3 virus infection are themselves of less importance. Thus control would best be achieved by vaccination (Brogden *et al.*, 1998).

In the early 1970s, commercial vaccines against pneumonic pasteurellosis of sheep generally contained formalin-killed cells and a narrow range of serotypes. The ability to produce pneumonic pasteurellosis in SPF lambs enabled novel vaccines to be tested. Because of the broad spectrum of serotypes now identified to be involved in pneumonic pasteurellosis, any vaccine preparation must taken multiplicity of *P. haemolytica* into account. Currently, available *P. haemolytica* biologicals are modified live vaccines, bacterins, bacterial surface extracts and culture supernates that contain leukotoxin (Gilmour and Gilmour, 1989; Confer, 1993). Biologicals available for *P. multocida* in small and large ruminants are also bacterins and vaccine usually in combination with *P. haemolytica*. Gilmour and Gilmour (1989) produced a multivalent vaccine against commonly occurring serotype which for biotype A were 1, 2, 6, 7 and 9. Initially Freund's type adjuvants and later antigens absorbed into alhydrogel were used but these oil-based vaccines caused unacceptable reactions.

Vaccines against *P. haemolytica* serotype A2 continues to be poorly immunogenic although A2 heat-killed organism with appropriated adjuvant have shown some effectiveness (Gilmour & Gilmour, 1989). Crude and purified cytotoxin (leukotoxin)

have been tested for immunogenicity and it was found to give 86% immunity (Sutherland *et al.*, 1989). In combination with sodium salicylate extract (SSF) of surface antigens of *P. haemolytica*, the vaccine gave 98% compared to the SSE alone which gave 47% protection. This suggests that cytotoxin may be an important immunogen. The lambs vaccinated with cytotoxin developed cytotoxin neutralizing and bactericidal antibodies, the titres of which in individual lambs correlated with their combined clinical and necropsy scores. these findings hold out considerable hope for further improvements in vaccine against pneumonic pasteurellosis. Subunit vaccines containing surface extracts such as iron-regulated proteins (Gilmour *et al.*, 1991) and outer membrane proteins (Morton *et al.*, 1995) are currently undergoing trials with promising laboratory results.

Since pneumonic pasteurellosis is a sporadic disease, it is necessary to institute a programme of vaccination which gives protection throughout the year to all ages of sheep. This is best achieved by vaccinating breeding sheep twice at tupping time and a third time about three weeks before lambing with an annual booster (Gilmour & Gilmour, 1989). This schedule will ensure that colostral antibodies are available. The prevalence of pasteurellosis in lambs in vaccinated flocks suggest that colostral immunity does not persist for longer than 4 - 5 weeks after birth. Active immunization of young lambs with doses of *Pasteurella* vaccine should be initiated around this time. Gilmour *et al.* (1980) have shown that the presence of colostrally-derived antibodies did not interfere with the antibody response to vaccination at 10 or 18 days of age. The duration of immunity to pneumonic pasteurellosis is not known. To ascertain this, it is necessary to carry out tests for functional immunity using sera of vaccinated conventionally-reared sheep.

The effect of vaccination with PI-3 virus or a combination of PI-3 virus-*Pasteurella* vaccine has been tested (Gilmour & Gilmour, 1989). However, since there appear to be many other predisposing causes, vaccination with PI- 3 alone would seldom reduce the risk of pasteurellosis.

2.11 Studies in Nigeria

A general review of ovine and caprine diseases in Nigeria ranked Pasteurellae isolation next to enteric infections in prominence (Akerejola *et al.*, 1979) with pneumonic pasteurellosis described as enzootic and most prevalent during the wet season (Obi, 1997).

In western Nigeria there has been several reports and isolation of Pasteurellae from normal and diseased sheep and goats. In an analysis of common diseases of livestock in Ibadan during the period 1978 - 1982, Ugochukwu and Nwaneri (1984) observed that pasteurellosis as recorded during the period were 8.47% in bovine, 58.59% in caprine, 31.71% in ovine and 1.21% in porcine. An earlier review of microbial diseases of ruminants in southern Nigeria also revealed pasteurellosis as more prevalent than pleuro-pneumonia in cases of ovine and caprine pneumonia (Ojo, 1971, 1976) with the highest incidence during the rainy and harmattan seasons. Falade *et al.* (1984) also noted that pneumonia ranked next to enteric infection and was largely due to *P. haemolytica* and *P. multocida* isolations.

In northern Nigeria, Pasteurellae has also been shown to undermine efforts at small ruminant development. Akerejola (1980) noted a 12% incidence rate of pneumonic pasteurellosis in sheep with the pneumonia ranking second to helminthiasis in Kaduna region of northern Nigeria. Similarly, *P. haemolytica* and *P. multocida* were also isolated

in large number from ovine and caprine pneumonic lungs with over 52% of the lungs positive for Pasteurellae in the same region (Adekeye, 1984). A seven-year study and analysis of the prevalence of livestock disease in eastern Nigeria by Onyekwodiri and Shoyinka (1984) also recorded pneumonia due to Pasteurellae in sheep and goats but less so in cattle.

The role of other complementary agents like PI-3 virus and adenovirus (Taylor *et al.*, 1975; Ibu *et al.*, 1993) in the caprine or ovine respiratory disease complex is also documented. Obi and Ibu (1990) using indirect ELISA techniques detected PI-3 virus in 45.5% of pneumonic lung tissues of goats taken in some parts of northern and southern Nigeria. Goats were further infected with a local PI-3 virus to follow the clinical response. The virus precipitated a mild respiratory disease in goats in the absence of stress and other infectious agents. It is generally believed that the viruses act in concert with the Pasteurellae in field conditions to cause severe injury to small ruminants (Gilmour & Gilmour, 1989).

CHAPTER 3

MATERIALS AND METHODS

3.1 ISOLATION AND IDENTIFICATION OF *PASTEURELLA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* FROM PNEUMONIC LUNGS OF SHEEP

3.1.1 Study Population

Over a four-year period (1991 - 1994), a total of 1335 pneumonic lung specimens of sheep were investigated for the isolation of *P. haemolytica* and *P. multocida*. They were collected from Plateau, Bauchi, Kaduna, Sokoto and Borno states of the northern region of Nigeria.

3.1.2 Collection of Samples

Specimens of sheep lungs with identifiable lesions of pneumonia such as consolidation were cut into sterile containers. The consolidated lung specimens were red to dark - red in colour (Valero *et al.*, 1992). Majority of the samples were collected during the wet rainy and cold harmattan periods of the year. All specimens were conveyed in a cold ice-pack container to the laboratory for immediate processing.

3.1.3 Cultural and Biochemical Identification

The outer surface of the pneumonic lung samples were first sterilized by searing with heated spatula before sterile cotton-tipped swabs were used to collect affected exudate from within the specimens, which were cultured overnight on blood agar at 37 ^{0}C, as described by Frank (1982). The resulting colonies were examined for *Pasteurella*-like morphology, colour, feature and haemolysis. The colonies were tested for Gram

reaction or Gram staining potential with 3% KOH. Possible *Pasteurella* colonies was inoculated into triple sugar iron (TSI) agar slants and seeded into a broth of 2% tryptone in 0.85% NaCl for indole determination. After 18 - 24 h, the TSI slants were observed for the typical reactions caused by *Pasteurella* species (TSI = yellow slant and butt and no gas formation). The tryptone cultures of possible *Pasteurella* was inoculated into a tube of Lead acetate agar for H_2S detection and onto a MacConkey agar after which the remaining tryptone broth was tested for indole production..

Isolates were then tested for urease, catalase, and oxidase. Suspected *P. haemolytica* colonies were also tested for Arabinose and Trehalose sugar fermentations (Adlam, 1989). Isolates were considered to be *P. multocida* if they had typical colonial morphology (moderate sized, round, gray and musty odor), with a Gram negative staining potential, had acid/acid or acid/no change reaction on TSI slant; were H_2S, catalase, oxidase and indole positive, urease negative and did not grow on MacConkey agar.

Isolates that had typical colonial morphology - small round and gray *beta*-hemolytic or sometimes non-hemolytic , had a Gram negative staining potential, had a typical reaction on the TSI slant (same as *P. multocida*), were H_2S, catalase, and oxidase positive, were indole and urease negative, and grew on MacConkey's agar were considered to be *P. haemolytica.*

The confirmed isolates of *haemolytica* and *P. multocida* were stored at -20°C and -70°C in cooked meat medium and in brain heart infusion broth (Difco) containing 15% glycerol (Sigma) respectively.

3.2 SEROTYPING OF THE LOCAL *PASTEURELLA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* ISOLATES

3.2.1 Typing Antisera

Antisera to serotypes 1-16 of *P. haemolytica* and *P. multocida* serogroups A-E were obtained from Wellcome Research Laboratories (Beckenham England) in the lyophilized form.

3.2.2 Rapid plate Agglutination (RPA) for Typing P. haemolytica.

The Rapid Plate Agglutination (RPA) procedure (Frank and Wessman, 1978) was used for the serotyping of *P. haemolytica* isolates. The lyophilized sera were first reconstituted in 4.0ml of saline containing 1:10,000 thiomersal . A drop (approx. 10µl) of antiserum was placed on a clean glass surface. A small amount of discrete *P. haemolytica* colony from blood agar was picked with an inoculating wire loop and mixed with the serum. A positive reaction in the form of clumping and clearing occurred as the mixture was stirred with the loop. Negative reactions remained turbid. Reactions were run at room temperature.

3.2.3 Hyaluronic Acid Test for Typing of *Pasteurella multocida*

.The test was conducted on blood agar containing 5% Ox blood (Oxoid) in 50mm x 12mm Petri-type dishes with tight fitting lids. Each isolate examined was inoculated transversely across the whole plate so as to provide lines of growth approximately 3mm to 5mm apart. After this inoculation, a hyaluronidase producing strain of *P. multocida* type B (NVRI, Vom) was streaked heavily at right angles to the first *Pasteurella* streak lines. The inoculated plates were incubated at 37°C and observed periodically up to 24

h. The hyaluronidase effect when present was always evident at 24 h. The effect was demonstrated as a reduction in size of the *Pasteurella* colonies in the region close to the *P. multocida* type B strain.

3.3 EXPERIMENTAL TRANSMISSION STUDIES IN CONVENTIONALLY REARED LAMBS USING LOCALLY ISOLATED SEROTYPES OF *PASTEURELLA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA*

3.3.1 Experimental Animals

Forty four healthy lambs were bought from Alkaleri, a local market in Bauchi State, Nigeria. The lambs were aged between 5 and 7 months and had average weight of 12.5kg. Upon arrival the lambs were housed in straw-covered concrete floors in a covered building built of cement with cross ventilation. They were maintained on fresh grass or hay and feed - concentrate, salt licks and fresh water *ad libitum*. They were conditioned to their new environment for at least 4 weeks during which they were screened for blood and. intestinal parasites. They were examined and found to be free of clinical signs of pneumonia. Nasal swabs were taken immediately and every three days for at least four weeks to ensure the lambs were free of *P. haemolytica* and *P. multocida*. Blood samples were taken to determine the antibody levels of *P. haemolytica* and *P. multocida* by IHA test. Only lambs with negative serum titre to the Pasteurellae and the absence of the organisms from the nasopharyngeal swabs were used for this study. Vital signs (temperature, pulse rate and respiratory rates-TPR) were monitored daily to observe whether they remained afebrile and free of clinical signs consistent with pneumonia. .

They were vaccinated with heterologous tissue culture rinderpest vaccine (TCRV) to protect against Peste des petits Ruminants (PPR) and the animals were treated with

.Terramycin LA (Pfizer Cedex - France) and IVOMEC MST (AFVET Merck and Co. Inc. Whitehouse Station NJ USA).

At the end of the period of conditioning, the sheep were randomly assigned into 10 treatment groups of four sheep per group and an equal number acting as control. Each treatment group was housed separately to avoid cross-infection. The treatment groups were: (1) infected with PhAI; (2) infected with PhA2; (3) infected with PhA6; (4) infected with PhA7; (5) infected with PhA8; (6) infected with PhA9; (7) infected with PhT10; (8) infected with PhA11; (9) infected with PhUT; (10) infected with Pmtype A; (11) inoculated with sterile meat infusion broth - control.

3.3.2 Determination of Infective dose

In order to decide on the dose of inoculum suitable for the main experiment, a pilot experiment was carried out with 40 mice. The mice were randomly divided into four groups of 10. Each group received one of four different doses of Ph or Pm representative isolate intranasally, with the aid of a 24G cannula. The different doses were viz: 4.0 x 10^6, 4.0 x 10^7, 4.0 x 10^8, 4.0 x 10^9. Four days after inoculation, all the mice were killed with chloroform. The severity of pneumonia was assessed by gross observation of the lungs. The dose capable of producing pneumonia in approximately 30% of mice at 4 days was regarded suitable for the main challenge experiment (Rushton, 1978; Jian *et al.*, 1991)

3.3.3 Preparation of Inoculum

The suspension of *P. haemolytica* and *P. multocida* used for inoculation of the lambs were prepared from the lyophilized organisms isolated and serotyped from the

pneumonic lungs of sheep in this study. Inoculum was prepared for *P. haemolytica* A1, A2, A6, A7, A8, A9, T10, A11, UT and *P. multocida* type A.

Each of the confirmed and lyophilized serotype was reconstituted in 5ml meat infusion broth and incubated for 2 h. The 2 h - culture was then passaged intraperitoneally in mice and the heart and lung sub-cultured onto 5% ox blood agar (Oxoid). They were re-identified as *P. haemolytica* and *P. multocida* by colony morphology, Gram staining and biochemical reactions (Frank, 1982). The isolates were re-confirmed as *P. haemolytica* serotype or *P. multocida* type A using the rapid indirect haemagglutination (RIHA) test (Fraser *et al.*, 1983).

A discrete colony of *Pasteurella* serotype was picked with a wire loop from an overnight blood agar culture and suspended in 15 ml meat infusion broth. After a 6 h incubation at 37°C, the viability and the approximate number of the 6 h log phase of the organism per dose were determined by a quantitative plate count (Miles and Misra, 1938). The inoculum was 4.0 x 10^8 cfu of the serotype in 7ml volume of meat infusion broth (Purdy *et al.*, 1996; 1977; Zamri - Saad *et al.*, 1996).

The experimental mode of inoculation was by direct injection intra-tracheally (Confer *et al.*, 1988). Before inoculation, the hair around the throat region of each lamb was scraped and intratracheal inoculation made with a 21G x 1½ inch needle inserted between the tracheal rings 5 cm below the larynx: This caused the lamb to cough indicating deposition of the inoculum into the respiratory passage. This procedure was repeated for the control lambs too in which 7 ml of sterile meat infusion broth was used as the inoculum.

3.3.4 Post - Inoculation Assessment

After challenge exposure, all lambs were observed for 7 days post-inoculation during which they were fed grass/hay and feed-concentrate and water given *ad libitum*. All lambs were necropsied at death or slaughter. Survivors were killed on day 7. The degree of response of the animals to infection was measured for each group and compared using the following parameters:

3.3.4.1 Clinical Observations

This consisted of monitoring daily rectal temperature, pulse and respiratory rates, lung sounds, nasal discharge, coughing, appetite and attitudes of the animals. To avoid any subjective influence, the identities of the groups were unknown to the clinician and a score for the degree of illness or presence or absence of some of the basic clinical signs was awarded daily to each lamb. According to the severity of illness, lambs were awarded graded score of 0 - 6 points (Table1) (Jones *et al*., 1989).

3.2.4.2 Serological Studies

Blood samples (3ml each) were collected from all the animals prior to infection and at necropsy. Sera were harvested, stored at -20°C and later used to determine antibody to *P. haemolytica* and *P. multocida* by IHA (Fraser *et al*., 1983).

3.3.4.3 Gross Pathology

Lambs were euthanized using an overdose of barbiturate and exsanguinated on the 7th day post inoculation or when they had reached a terminal state. The thoracic cavity of each lamb was exposed and the trachea, and all lungs were removed intact. Although necropsy examination was concentrated primarily on the respiratory organs, gross

characteristics of the liver, lymph nodes and spleen were also noted. The lungs were examined grossly and the percent of involvement was determined by measuring the lesion, by visual observation and by palpation. The proportion of the lung surface area which showed consolidation was measured (Length x Width x Thickness) with calipers by a veterinarian unaware of the group to which the lamb belonged. The extent of pneumonia was evaluated on the basis of the volume of consolidated lung tissue. The volume of the consolidated lungs was expressed as a percent of the total volume of the lungs (Table 2) (Jones *et al.*, 1989).

3.3.4.4 Histopathology

Portions of affected and normal lungs were fixed in 10% formol saline for histologic examination. Sections were paraffin-embedded, cut at 5 to 6μm and stained with hematoxylin or eosin (Jian *et al.*, 1991). They were examined by light microscopy.

3.3.4.5 Microbiology

During the period of observation post inoculation, bilateral nasal swabs were taken for bacterial culture collected on day 0, 4 and 7 for non-fatal cases. At necropsy, the following materials were obtained for bacterial culture; pleura, pericardial and peritoneal fluids, liver, kidney, spleen, lymph nodes and any tissue showing gross abnormality.

Viable counts from 10% (w/v) lung tissue suspensions were made on 5% Ox blood agar. The major lobes from each lamb were cultured for bacteria and viable counts of *P. haemolytica* or *P. multocida* were made from "lesion" and "non-lesion" pools. Bacterial cultures were performed in an attempt to re-isolate the infective inoculum and to determine if other bacteria were present.

3.3.5 Scoring System

A post-inoculation assessment of the lambs was carried out using a combined scoring system (a modification of Jones *et al.*, 1989), based on three components viz: clinical parameters, a measurement of lung lesions at necropsy and an index of organisms recovered from standard sites. This allowed for comparative studies of the infective rates of the serotypes

3.3.5.1 Clinical Score

The animals were examined clinically from days 1 to 6 inclusive and necropsied on day 7. According to the severity of illness, lambs were awarded graded score of 0 - 6 points as shown in Table 1.

Table 1: Evaluation of Clinical Signs

Sign		Score*
Pyrexia (rectal temp > 40°C)		1
Abnormal respiration (Dyspnea)		1
Dullness or Lethargy		1
Cough		0.5
Nasal Discharge		0.5
Off-Feed:	hay or grass only	0.5
	hay and grains	or 1.0
Recumbent, unable to rise or death		Plus 1.0

*Thus on any day, a lamb could score between 0 - a maximum of 6 points.

All lambs that died prematurely were scored a maximum score of 6 points for each day remaining in the experiment. A cumulative score was obtained for each lamb before the average daily scores for each lamb and group were calculated.

3.3.5.2 Pneumonia Score

The mean volume of consolidated lung tissue on dorsal and ventral surfaces was assessed by measurement. Scores of 0 represented normal and higher scores were assigned for progressively severe changes as shown below (Table 2). The volume expressed as a percent of the total lung volume was halved to give the pneumonia score per lamb. Animals which died rapidly with or without development or consolidated lung tissue scored maximum if isolations of *P. haemolytica* or *P. multocida* indicated lung infection and a generalized septicemia (one or more of heart blood, spleen and liver positive) (Table 2)

Table 2: Evaluation of Lung Lesions

Observation	Score**
*No visible Lesion	0
*Minimal focal lesions in any Single lobe	1
Consolidation, abscessation and/or	
adhesions involving < 25% of the lung	2
*As 2 involving 25 - 50% of the lung	3
*As 3 involving 51 - 75% of the lung	4
*As 4 involving > 75% of the lung	5
* additional point for pleural effusion or	
fibrinous adhesion to the thorax or pericardium	Plus 1

**The maximum score for each lung was 5; the maximum score per lamb was 10.

3.3.5.3 P. haemolytica/P. multocida Isolation Index

Lung tissue samples taken from the four lobes of each lamb were pooled, titrated using ten - fold serial dilution and cultured and the counts of viable *P. haemolytica* or *P. multocida* estimated (Miles and Misra, 1938). Representative colonies were identified by standard bacteriological (Frank, 1982) and serological (Fraser *et al.*, 1983) methods. The mean $\log_{10}$ titre of the pool was taken as the isolation index figure for each lamb (Maximum point per lamb = 1; maximum point per group = 4).

The group mean scores were then computed from the total scores of the individual lambs and compared.

3.3.6 Statistical Analysis

All data were reported as means.. Multiple range test or the Fisher's procedure was used to compare mean values between groups.

CHAPTER 4

4. RESULTS

4.1 PASTEURELLAE ISOLATES FROM PNEUMONIC LUNGS OF SHEEP

In 504 (37.8%) of 1335 samples investigated, *P. haemolytica* and *P. multocida* were isolated either alone or with other bacteria. The Pasteurellae were recovered in relatively pure aerobic culture from 370 (27.7%) of the samples. The Pasteurellae samples yielded 378 (75%) *P. haemolytica* and 126 (25%) *P. multocida*. The isolation studies within each state shows that 235/400 (58.8%) of the samples from Plateau, 124/400 (31.0%) from Bauchi, 107/400 (26.8%) from Kaduna, 22/72 (30.6%) from Sokoto and 16/63 (25.4%) from Borno, were positve for Pasteurellae (Table 3). Plate 1 shows a typical *P. multocida* colony on blood agar while Plate 2 shows *P. haemolytica* on blood agar.

Other bacteria that were occasionally isolated in combination with the Pasteurellae were *Neisseria* species, *Corynebacterium pyogenes*, streptococci, *Escherichia coli* and staphylococci (Table 4)

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Table 3: *Pasteurella haemolytica* and *Pasteurella multocida* isolates from pneumonic lungs of sheep from five Northern States in Nigeria

States	No. of Lung Sample	No. and % of Pasteurellae	*Pasteurella haemolytica* Isolates	*Pasteurella multocida* Isolates
Plateau	400	235(58.8%)	177	58
Bauchi	400	124(31.0%)	101	23
Kaduna	400	107(26.8%)	76	31
Borno	63	16(25.4%)	5	11
Sokoto	72	22(30.6%)	19	3
Total	1335	504(37.8%)	378	126

Table 4: Other aerobic bacteria isolated from pneumonic lungs of sheep from five States in Nigeria.

Bacteria	No. and % isolates
Neisseria Sp.	27 (2.0%)
Corynebacterium pyogenes	7 (0.5%)
Streptococci	11 (0.8%)
Escherichia coli	20 (1.5%)
Staphylococci	320 (24.0%)

Plate 1: *Pasteurella multocida* on blood agar: large gray mucoid colonies.

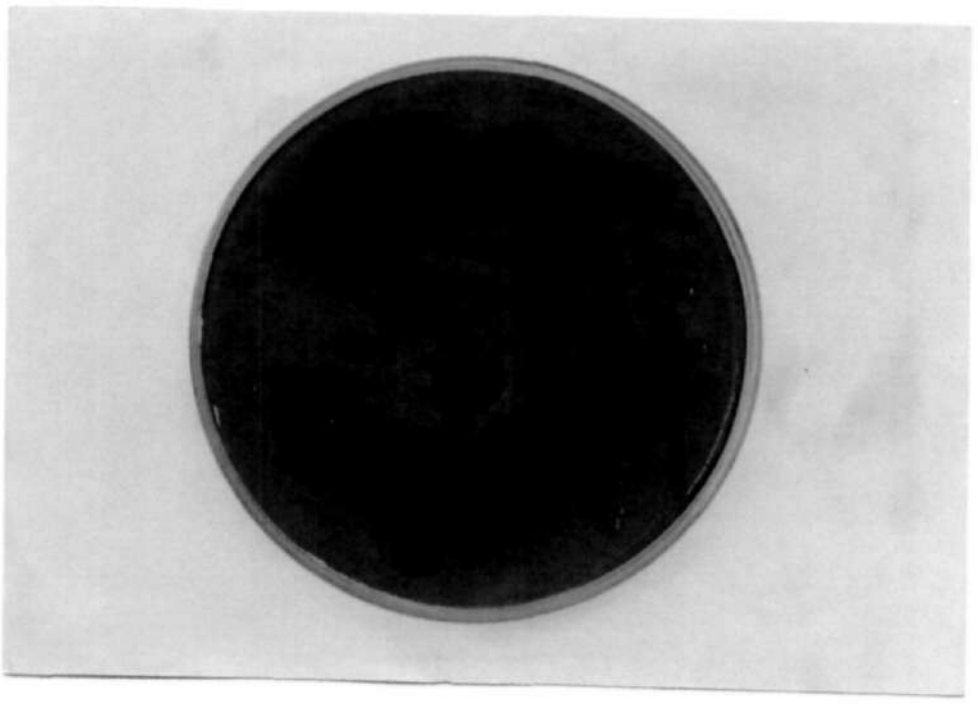
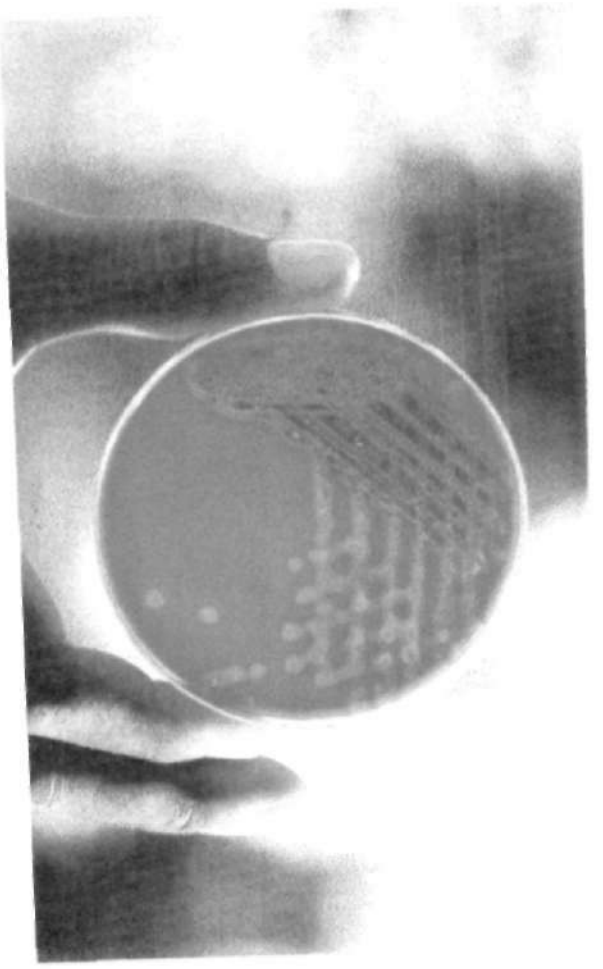


Plate 2: *Pasteurella haemolytica* on blood agar: gray colonies with haemolysis around colonies.



4.2 SEROTYPING OF *PASTEURELLA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* ISOLATES

Table 5 shows a distribution of the varieties of serotypes of *P. haemolytica* and *P. multocida* isolated in this study. The isolates belonged to either *P. haemolytica* (*Ph*) A1, A2, A6, A7, A8, A9, T10, A11 or *P. multocida* (Pm) type A. The *P. haemolytica* isolates yielded 266 typeable and 72 untypeable (UT) serotypes divided between 16 serotypes (serotype 17 not included).

In descending order of frequency of isolation, the relative frequencies of the serotypes of *P. haemolytica* (Ph) and *P. multocida* isolated in this study were: *Ph*A2(27.1%) *Pm* type A(25.1), *Ph*A11(10.2%), *Ph*A6(9.1%), *Ph*A7(4.4%), *Ph*A1(4.0%), *Ph*A8(2.7%), *Ph*A9(1.3%) and *Ph*T10(0.2%). Up to 16.0% of the isolates were untypeable Ph (UT). Fifty three (53) of the Pasteurellae isolates were however lost before the commencement of typing.

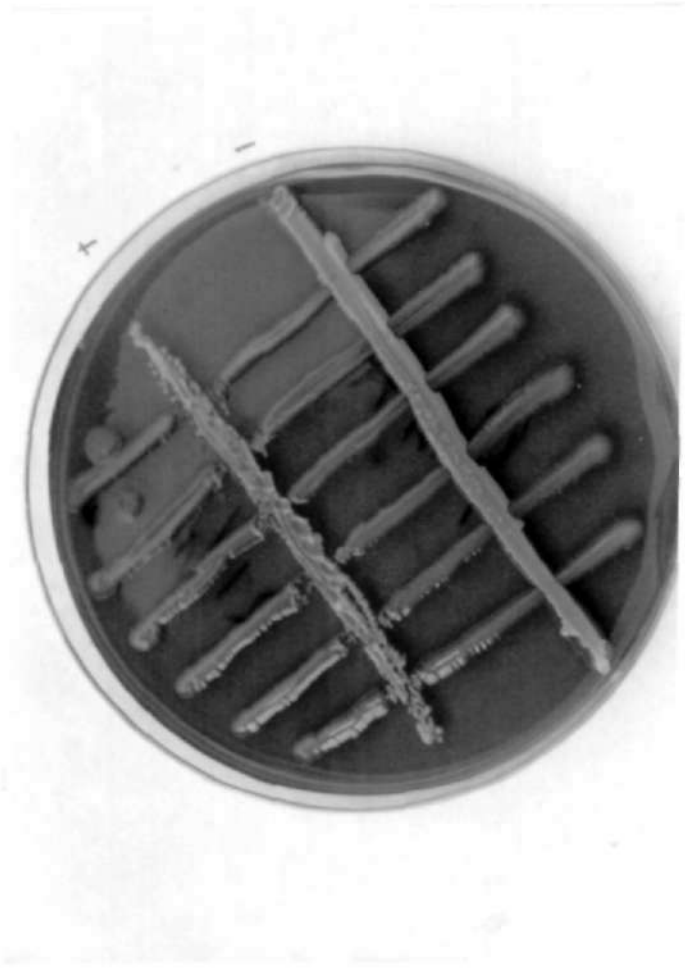
Plate 3 shows a positive hyaluronidase test for typing of *P. multocida* type A. The hyaluronidase effect with type A cultures was manifested as a diminution in the size of the *Pasteurella* colonies in the region close to the *P. multocida* type B streak.

Table 5: Distribution of Serotypes of *P. haemolytica* (Ph) and *P. multocida* (Pm) isolated from pneumonic lungs of sheep from Slaughter houses of five States in Northern Nigeria.

States Visited	No. of Lung Samples	No. of Isolates	Serotypes										
			PhA1	PhA2	PhA6	PhA7	PhA8	PhA9	PhT10	PhA11	PhUT*	PmA	L**
Plateau	400	235	7	47	17	8	5	3	1	20	37	46	44
Bauchi	400	124	3	34	12	7	5	1	-	16	19	22	5
Kaduna	400	107	4	30	9	4	2	1	-	10	12	31	4
Borno	63	16	-	5	-	-	-	-	-	-	-	11	-
Sokoto	72	22	4	6	3	1	-	1	-	-	4	3	-
Total	1335	504	18	122	41	20	12	6	1	46	72	113	53

Ph UT* = Untypable Isolates. L** = Isolates lost.

Plate 3:Hyaluronidase test: for typing of *Pasteurella multocida* type A. The hyaluronidase effect was manifested as a diminution in the size of the *Pasteurella multocida* type A growth in the region adjacent to the *P. multocida* type B streak



4.3 PATHOGENICITY STUDY OF THE *PASTEURELLA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* SEROTYPES IN LAMBS

Within the first 48 h all the mice were showing clinical signs of variable degrees of depression, loss of appetite, roughened coat and were huddled together. These signs decreased in severity and at 96 h were present in only a few mice. At necropsy on the 4th day, macroscopic lesions were more evident in lungs of mice given 4.0 x 10^8 and 4.0 x 10^9 cfu. They consisted of very small irregular red areas in the anterior portions of the lungs. The 4.0 x 10^8 dose which produced pneumonia in approximately 30% of the mice was regarded suitable for the main infection test (Table 6).

4.3.1 Group 1 (Infected with PhA1)

No mortality was recorded on or before the 7th day post-inoculation (dpi). Prominent clinical signs and symptoms recorded by 3/4 of the lambs were pyrexia (rectal temperature $\geq$ 40°C), serous nasal discharge, coughing and 2/4 of the lambs showed dyspnea , lack of appetite and were also lethargic but soon returned to clinical normalcy after the 4th dpi. All four surviving lambs were euthanized on the 7th dpi. The mean group clinical score was 8.5 (± 8.3 SE) (Table 7).At necropsy, 3/4 lambs had typical pneumonic lesions of less than 25%.

Plate 4 shows a typical macroscopic appearance of pneumonic lesions or consolidated lungs of one of the lambs after infection with *P. haemolytica* serotype A1. The lambs had circumscribed areas of pneumonia concentrated on the dorsal surfaces of the lobes. The group mean pneumonic (lesion) score was 3.5 (± 1.0 SE) (Table 8)

Pathologically, the consolidated areas were well delineated and appeared on the dorsal region of the lungs. The lungs of one lamb were free of lesions.

The pathological examination of the lung lesions showed hyperaemia, congestion and capillary haemorrhage. The alveoli contained clusters of neutrophils but the predominating cell type filling the alveoli was mononuclear, frequently appearing to be pyknotic. The cells had a characteristically elongated shape with a spindle-shaped or wavy nucleus giving rise to the appellation "oat cell". Clusters of these cells formed whorls in alveoli and appeared to stream between adjacent spaces (Plate 6).

4.3.2 Group 2 (Infected with Ph A2)

At the 4th day post inoculation (dpi) 2/4 of the lambs had died following inoculation of the lambs with Ph A2. The infection followed an acute course. About 24 hours post inoculation, pyrexia, dyspnea, inappetence, coughing and profuse nasal discharge were evident. The lambs became lethargic and were in sternal recumbency with eventual death on the 4th dpi.

In two other lambs pyrexia, serous nasal discharges and coughing were evident but they soon returned to clinical normalcy within the 4th dpi. The mean group clinical scores was 18.8 (± 16.8 SE) (Table 7).

The survivors at 7th dpi and the fatal lambs were necropsied. At necropsy the 2 fatal lambs showed extensive pneumonic lesions (> 75% consolidation with adhesions) and pleurisy. The lesions were distributed anteriorly involving a considerable portion of the apical and lateral lobes. Affected tissue was liver-like in consistency, intensely red to slight dark greenish and its cut surface exuded fluid on pressure. An important finding

was that the entire thoracic cavity appeared to be filled with coagulated fibrin which coated the heart thickly. The lungs were enlarged and edematous with sharply demarcated areas of consolidation involving essentially the apical and cardiac lobes with adhesion of the left lung to the thorax. The presence of irregular greenish-brown areas of necrosis in the consolidated portions of the lungs was also observed. About 25 - 50% lung consolidation was observed in the surviving lambs that were necropsied on the 7th dpi. The group mean pneumonic score was 5.8 (± 3.0 SE) (Table 8). Plate 5 shows macroscopic appearance of pneumonic lesion in a typical PhA2 challenged lambs.

Pathologically, the most obvious lesion common to all members of the group were areas of fibrin and edema fluid accumulation of pink color in distented and edematous interlobular septa and within the alveoli (Plate 6), mingled with masses of Gram negative coccobacilli. The lung vasculature was intensely hyperaemic with extensive irregular areas of abnormality in which the central alveoli and vasculature have undergone necrosis, while peripherally, the alveoli were packed with intensely basophilic spindle-shaped leucocytes or "oat cells" which form whorles in alveoli or appear to stream from one alveolus to another. The pleurisy was characterized by a copious fibrinous exudate, often incorporating masses of coccobacilli, presumably *P. haemolytica*.

Microbiologically, *P. haemolytica* was recovered from the pneumonic lungs pleural exudates and heart blood of all affected lambs (Table 8).

4.3.3 Group 3 (Infected with PhA6)

No mortality was recorded following inoculation of the lambs with PhA6. However 2/4 of the lambs showed pyrexia on the 2nd dpi but soon returned to clinical

normalcy on the 3rd dpi. No other clinical signs were observed. The group mean clinical score was 0.5 (± 0.6 SE) (Table 7).

At necropsy on the 7th dpi of the four lambs, no gross pulmonary lesions were seen but focal lesions were observed in 2/4 lambs and they were limited to the apical and cardiac lobe. The group mean pneumonic score was 0.5 (± 0.6 SE) (Table 8).

Pathologically, the picture seen from lesions of pneumonia were similar to those seen in typical cases of animals inoculated with PhA2.

4.3.4 Group 4 (Infected with PhA7)

Mortality was recorded in 2/4 of the lambs on the 4th dpi, with the infection following an acute course. At 24 hours post inoculation, 3/4 of the lambs began to manifest with clinical signs of pyrexia (temp ≥ 40°C). The 2/4 of the lambs which eventually died had serous nasal discharge, cough, dyspnea, inappetence, lethargy and recumbency. Less obvious clinical signs such as nasal discharge and dullness, but not recumbency were observed in two other lambs necropsied on the 7th dpi. The group mean clinical score was 18.4 (± 15.0 SE) (Table 7).

The lesions varied in degree but not in type among the animals. The apical, cardiac and anterior portions of the diaphragmatic lobes were generally consolidated. At necropsy massive pleural effusion was seen in 2/4 of the lambs which died on the 4th dpi. Extensive pneumonic lung lesions of 80 - 100% consolidation were observed in these two lambs. In the other 2 lambs, pleural changes were present but the underlying pneumonic lesions were less extensive and these lambs showed between 25 and 50% lung consolidation. The group mean pneumonic score was 5.8 (± 3.0 SE) (Table 8). Plate 7

shows a representative lung lesion from a lamb inoculated with PhA7. Generally, the lesions affected the anterior-ventral parts of the lungs. Survivors showed mild to moderate lesions of the lungs at necropsy typical of pneumonic pasteurellosis. The affected parts appeared firm.

White coloration of the liver with haemorrhage was seen. Petechiae was also seen when the thorax was opened. The spleens were swollen with smooth edges. Adhesion of the left lung to the thorax was observed in 2/4 of the lambs. Generally the pathology was similar to that of Ph A2 treated lambs.

Histologically, 2/4 of the lambs with the most extensive gross lesions had a necrotizing broncho-pneumonia and 'oat cells' rather than neutrophils were the predominant cell in the inflammatory infiltrate. Hyperaemia, desquamation of alveolar epithelial cells was a dominant feature in the other animals. The histopathology picture was similar to Ph A2 treated animals (Plate 6).

Microbiologically, *P. haemolytica* was present in large numbers in both pneumonic and congested lobes of the lungs (Table 9)

4.3.5 Group 5 (Infected with PhA8)

There were no deaths recorded and there were no apparent clinical signs or symptoms in this group of lambs treated with PhA8. Group mean clinical score = 0.

At necropsy the lungs were free of any form of consolidation. The group pneumonic score was = 0.

4.3.6 Group 6 (Infected with PhA9)

Although no mortality was recorded after treatment of the lambs with PhA9, 2/4 of the lambs showed severe clinical signs of pyrexia, serous nasal discharge, cough, dyspnea and lethargy. Inappetence was also observed among these lambs but resumed feeding on the 3rd dpi. The other 2 lambs exhibited mild clinical signs of nasal discharge and dullness but soon recovered to clinical normalcy. The group mean clinical score was 6.1 (± 9.3 SE) (Table 7).

At necropsy 2 lambs showed 25-50% lung consolidation while focal lung lesion was seen in one lamb. The group pneumonic score was 1.3 (± 2.0 SE) (Table 8). .

Pathologically, in lambs with visible pneumonic lesions, the histological changes were similar to those seen in other lambs of other groups with established *P. haemolytica* infection i.e. a necrotizing bronchopneumonia with oat cell infiltrate in the surrounding lung parenchyma (Plate 6).

4.3.7 Group 7 (infected with PhT10)

With the PhT10 treated lambs, no mortality, no apparent clinical signs or symptoms were recorded. Group clinical score was= 0.

No lesions were observed at necropsy Group mean pneumonic score was = 0. *P. haemolytica* was not isolated from the lungs.

4.3.8 Group 8 (Infected with PhA11)

No clinical symptoms were recorded in this group. Group mean clinical score was = 0. At necropsy only one lamb showed focal lesions typical of pneumonia, with Ph A11

isolated in the lung lesions of this lamb. The mean pneumonia score was 0.3 (± 0.5 SE) (Table 8)

The pathological picture of the lamb with pneumonic lesion was not different from the typical pneumonic pasteurellosis seen in the field.

4.3.9 Group 9 (Infected with Ph UT)

No apparent or overt clinical signs were recorded in this group. Group mean clinical score was = 0

No form of pneumonic consolidation or congestion was observed at necropsy, and there were no presence of bacteria seen. Group mean pneumonia score = 0.

4.3.10 Group 10 (Infected with Pm type A)

Although none of the lambs infected with *P. multocida* type A (Pmtype A) showed pyrexia (temp > 40°C), subacute infection was observed on the 3rd dpi in one of the lambs which showed clinical signs of inappetence, nasal discharge (no dyspnea), lethargy and recumbency resulting in the death of the animal on the 6th dpi. Two other lambs showed less obvious clinical signs of serous nasal discharge, coughing and inappetence which were transicent. The other one lamb failed to show any overt clinical signs. Group mean clinical score was = 13.9 (± 13.4 SE) (Table 7).

At necropsy the three lambs which showed various degree of clinical signs had swollen and congested lungs with little fibrin exudation typical of bronchopneumonia. Group pneumonia score was 2.3 (± 2.6 SE) (Table 8)

The pathological lesions could be divided into two types - subacute and chronic. The subacute lesions were in the form of severe bronchopneumonia with moderate

congestion of the interalveolar septa and blood vessels or capillaries. Most alveoli and bronchioles were filled with a mixture of neutrophils and macrophages, whereas some other alveoli were filled with edema fluid. The chronic lesions were typical of pneumonic pasteurellosis. The lesions consisted of bronchopneumonia with mainly macrophages and some little fibrin in the alveoli. Bacteria were also readily apparent in the interstitial tissue of the alveolar septa or spaces adjacent to the affected blood vessels. Plate 8 shows a typical microscopic lesion seen in a PmA - infected lung tissue.

4.3.11 Group 11 (Control)

The control group of lambs did not present with any clinical signs after inoculation with sterile meat infusion broth. Clinical score was = 0.

At necropsy there were no lesions seen. Pneumonic score was = 0. Bacteria were not present in the lung tissue. Plate 9 is a typical macroscopic appearance of the lung of sham-inoculated control lamb. Plate 10 shows a typical microscopic apperance of a sham -inoculated sheep lung.

In conclusion, Figure 1 shows the mean percentage of consolidated lung tissue of the lambs inoculated with the various local serotypes of *P. haemolytica* and *P. multocida* type A. From the bar chart it is evident that lambs inoculated with *P. haemolytica* serotypes A2 and A7 and *P. multocida* type A had the highest mean consolidated lesions. There were significant ($P< 0.05$) differences in mean percent of lung lesions between groups of serotypes A2, A7 and that of control and other groups. There were no significant differences between the mean pneumonic scores of the A1, A6, A8, A9, T10,

A11 UT PmA and that of the control (Fisher or LSD procedure). The group mean combined disease score (Table10) showed PhA2 and PhA7 to have the highest scores.

4.3.12 Bacteriological Finding

Generally, very heavy growth of the *Pasteurella* was always obtained on blood agar from consolidated lung tissue of the lambs. Much smaller numbers of viable bacteria (*P. haemolytica* or *P. multocida*) were often but not always present in non-consolidated lung tissue, pleural exudate, heart blood, liver, spleen and kidney. In lambs which did not die but showed lesions at slaughter, infection was always present but was confined to the thorax. In the absence of lesions no bacteria could be found. Isolates consisted of pure cultures of *P. haemolytica* or *P. multocida* having the characteristic of the strain inoculated.

The number of viable bacteria per gram was also determined. Table 9 shows the results of the organisms obtained at death or slaughter. It is apparent that in the fatally infected animals a high number were present in the lesion between 10 to 100 times the number of organism inoculated. The least demonstrable degree of multiplication occurred in the animal taking the longest time to die. In the non-fatally infected sheep, the numbers of organism associated with the lesions were less than those inoculated.

4.3.13 Serological Finding

The sera of all the animals pre- and post-infection were found to be negative for antibodies to *P. haemolytica* and *P. multocida* by the IHA test.

Table 6: Number of mice with lung lesions after inoculation with various doses of *P. haemolytica* (Ph) and *P. multocida* (Pm)

Group	Inocula	Dose	Total No. of mice	No. of mice with lung lesions
1	Ph	4.0×10^6	10	0
2	Ph	4.0×10^7	10	1
3	Ph	4.0×10^8	10	3*
4	Ph	4.0×10^9	10	3
5	Pm	4.0×10^6	10	1
6	Pm	4.0×10^7	10	2
7	Pm	4.0×10^8	10	5*
8	Pm	4.0×10^9	10	6

*The dose (4.0×10^8) capable of producing pneumonia in at least 30% of mice was chosen for the main experiment in lambs.

Table 7: Group mean clinical scores of the lambs infected with the various local Serotypes of *P. haemolytica* (Ph) and *P. multocida* (Pm)

Group	1	2	3	4	5	6	7	8	9	10	11
Infective Serotype	PhA1	PhA2	PhA6	PhA7	PhA8	PhA9	PhT10	PhA11	PhUT	PmA	Control
No. Of Lambs	4	4	4	4	4	4	4	4	4	4	4
Group mean (36) Clinical Scores(±SE)	8.5 ±8.3	18.8 ±16.8	0.5 ±0.6	18.4 ±15.0	0	6.1 ±9.3	0	0	0	13.9 ±13.4	0

Figure in Parenthesis indicates maximum possible Score.

Table 8: Pneumonia score of consolidated lung tissue of the lambs infected with the various local serotypes of *P. haemolytica (Ph) and P. multocida(P m)*

Group	1	2	3	4	5	6	7	8	9	10	11
Infective Serotype	PhA1	PhA2	PhA6	PhA7	PhA8	PhA9	PhT10	PhA11	PhUT	PmA	Control
No. Of Lambs	4	4	4	4	4	4	4	4	4	4	4
Group Mean Lesion (10) Score(± SE)	3.5 ±1.0	5.8 ±3.0	0.5 ±0.6	5.8 ±3.0	0	1.3 ±2.0	0	0.3 ±0.5	0	2.3 ±2.6	0

Figure in Parenthesis indicates maximum possible Score.

Table 9: Number of *P. haemolytica* (Ph) and *P. multocida* (Pm) isolates recovered at necropsy from the nasal mucosa and lungs in the lambs following infection with the various serotypes

Group	Serotypes	Nasal mucosa	Lungs	CFU/g* from lungs
1	PhA1	2/4	4/4	1.2×10^7
2	PhA2	3/4	4/4	1.6×10^{10}
3	PhA6	0/4	0/4	0
4	PhA7	3/4	4/4	3.8×10^9
5	PhA8	0/4	0/4	0
6	PhA9	2/4	2/4	2.6×10^6
7	PhT10	0/4	0/4	0
8	PhA11	0/4	1/4	1.3×10^2
9	PhUT	0/4	0/4	0
10	PmA	1/4	2/4	1.7×10^4
11	Control	0/4	0/4	0

*The Pasteurellae titres were calculated from 10g (10% w/v) of lung tissue taken consolidated/congested/normal portion blended in 90ml sterile nutrient broth (diluent) and serially diluted.

The numerator is the number of lambs from which Ph/Pm isolates were recovered; the denominator is the total number of lambs.

CFU = Colony Forming Units.

Table 10: Group Mean Disease Score

Group	Infective Serotype	No. of Lambs	No. dying or killed in extremis	Group mean clinical score	Group mean lesion score	No. with Ph/Pm in lungs	Group Mean Combine Score*(±SE)
1	PhA1	4	-	8.5	3.5	4	16.0 ±1.9
2	PhA2	4	2	18.8	5.8**	4	28.6 ±8.0
3	PhA6	4	-	0.5	0.5	0	1.0 ±0.3
4	PhA7	4	2	18.4	5.8**	4	28.2 ±7.8
5	PhA8	4	-	0	0	0	0
6	PhA9	4	-	6.1	1.3	2	9.4 ±2.6
7	PhT10	4	-	0	0	0	0
8	PhA11	4	-	0	0.3	1	1.3 ±0.5
9	PhUT	4	-	0	0	0	0
10	PmA	4	1	13.9	2.3	2	18.2 ±6.8
11	Control	4	-	0	0	0	0

*Disease Score = The mean sum of the clinical findings, consolidated lung lesions and Ph or Pm Isolation Index Scores.

**There were significant ($P < 0.005$) differences in mean lesion scores between the PhA2, PhA7 groups and the other 9 groups

Figure 1 Group Mean Percent Consolidated lung tissue of lambs infected with the various Local Serotypes of P. haemolytica (Ph) and P. multocida (Pm)

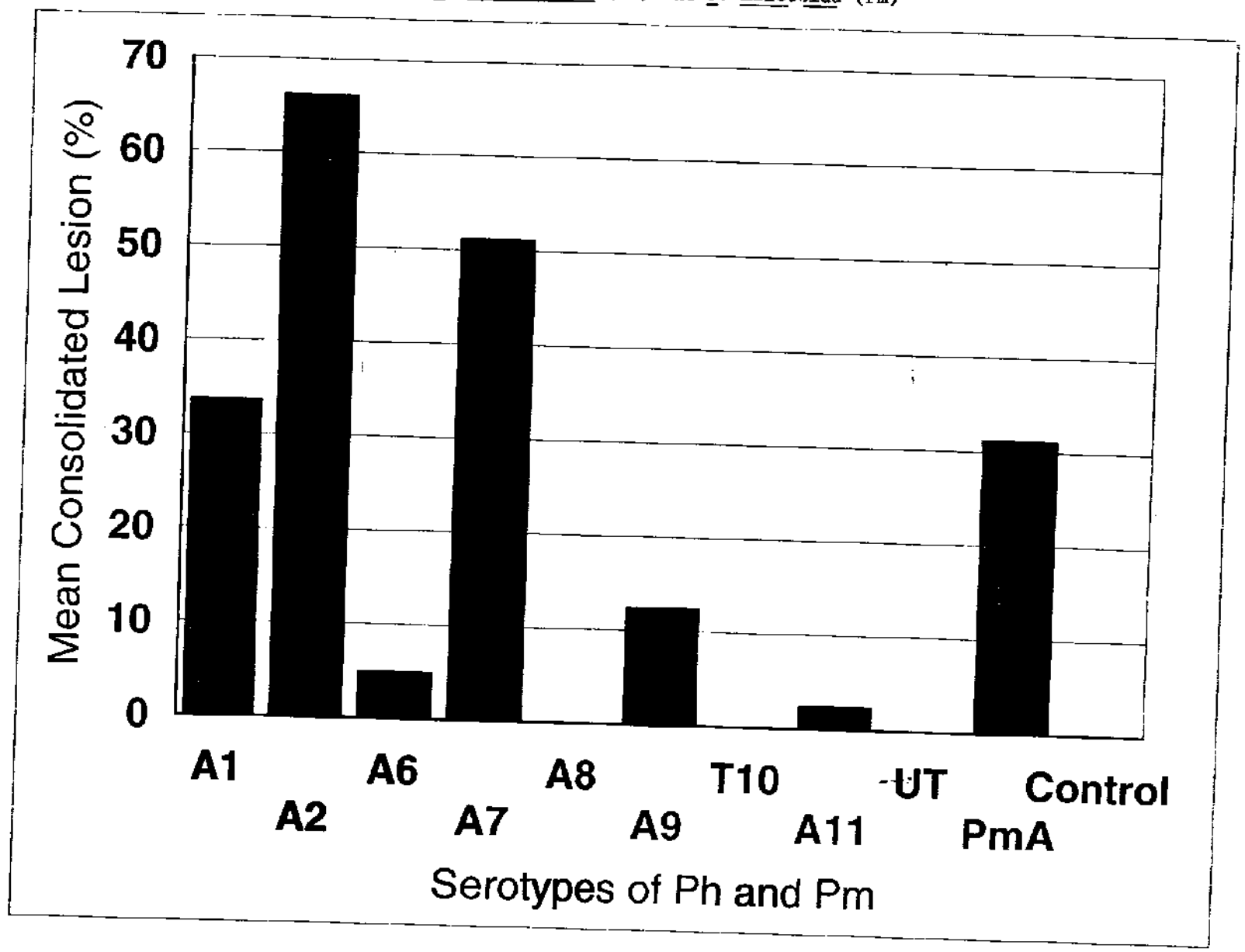


Plate 4: Representative lung lesion from a lamb in the *Pasteurella haemolytica* serotype A1 infected group



Plate 5 Representative lung lesion from a lamb in the *Pasteurella haemolytica* serotype A2 infected group

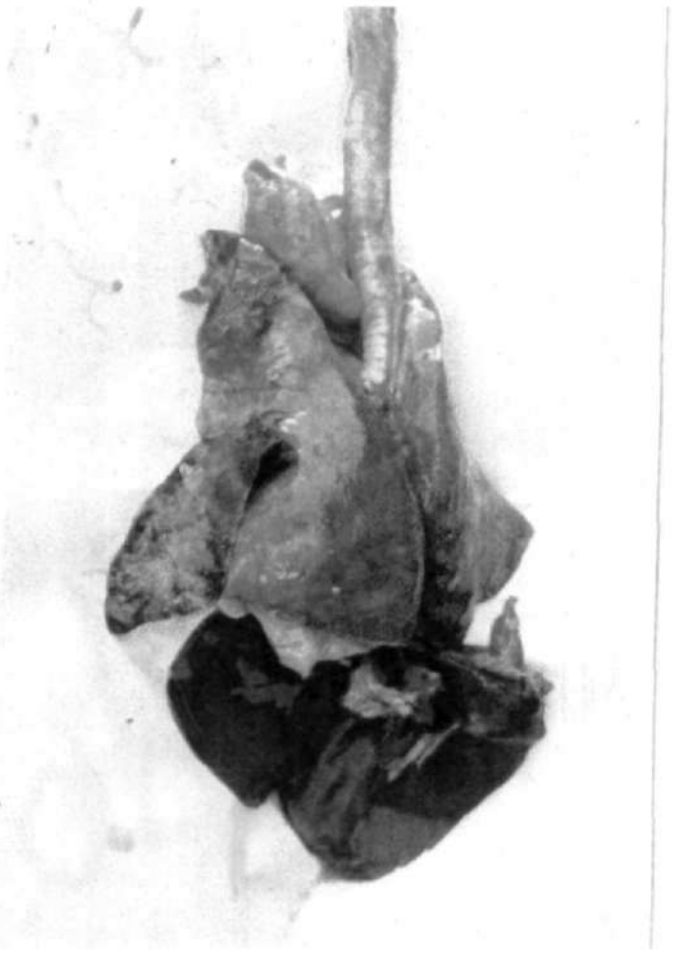


Plate 6: Pneumonic pasteurellosis. The characteristic wavy or spindle-shaped nucleus of the "oat cell" and the collective streaming effect seen in the microscopic lung lesion was typical of *Pasteurella haemolytica* infection. H & E x 100

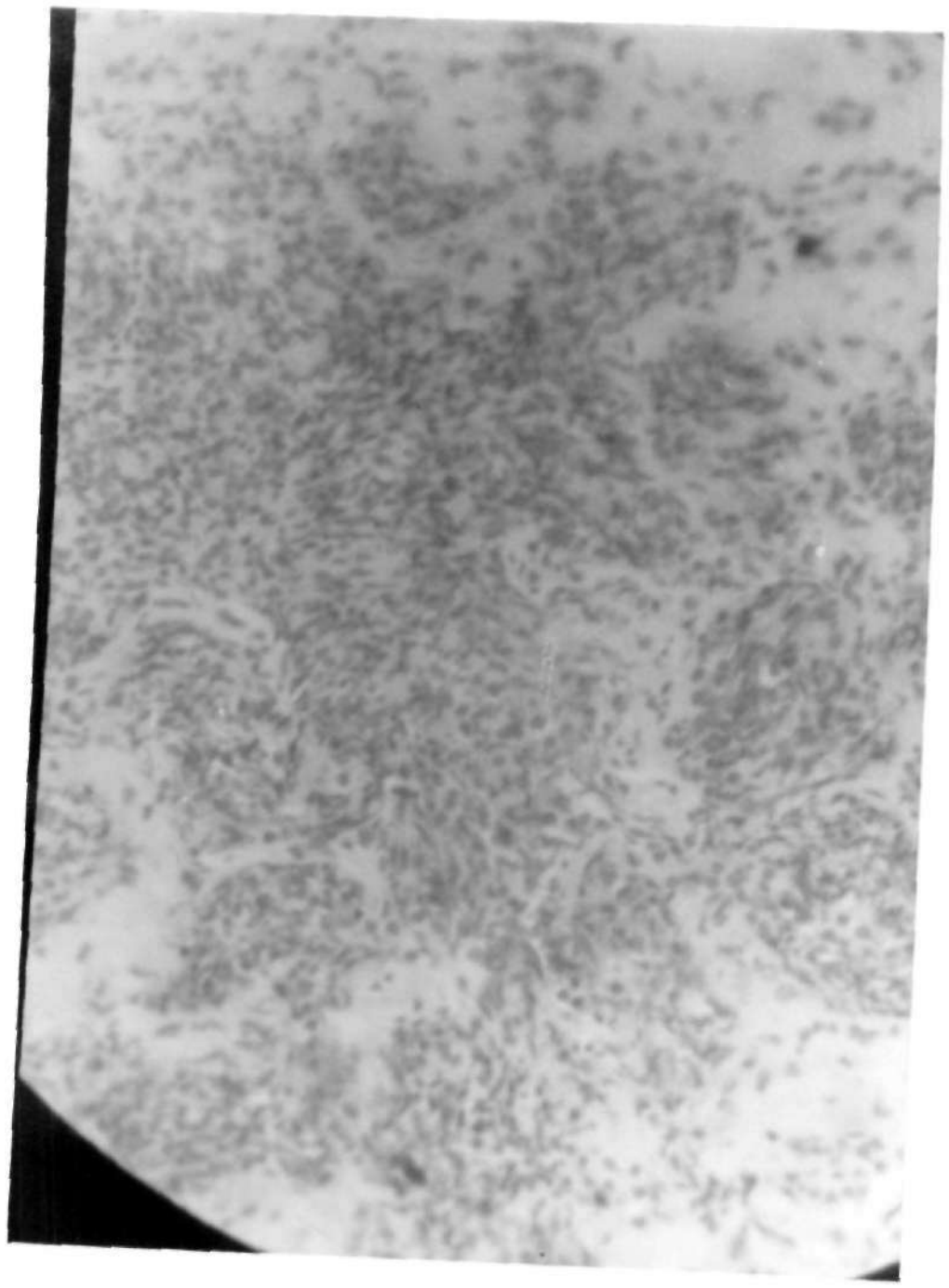


Plate 7: Representative lung lesion from a lamb in the *Pasteurella haemolytica* serotype A7 inoculated group



Plate 8: Pneumonic pasteurellosis: lung from lamb inoculated with *Pasteurella multocida* A showing primary cell types involved to be neutrophils and macrophages. H & E.x 100

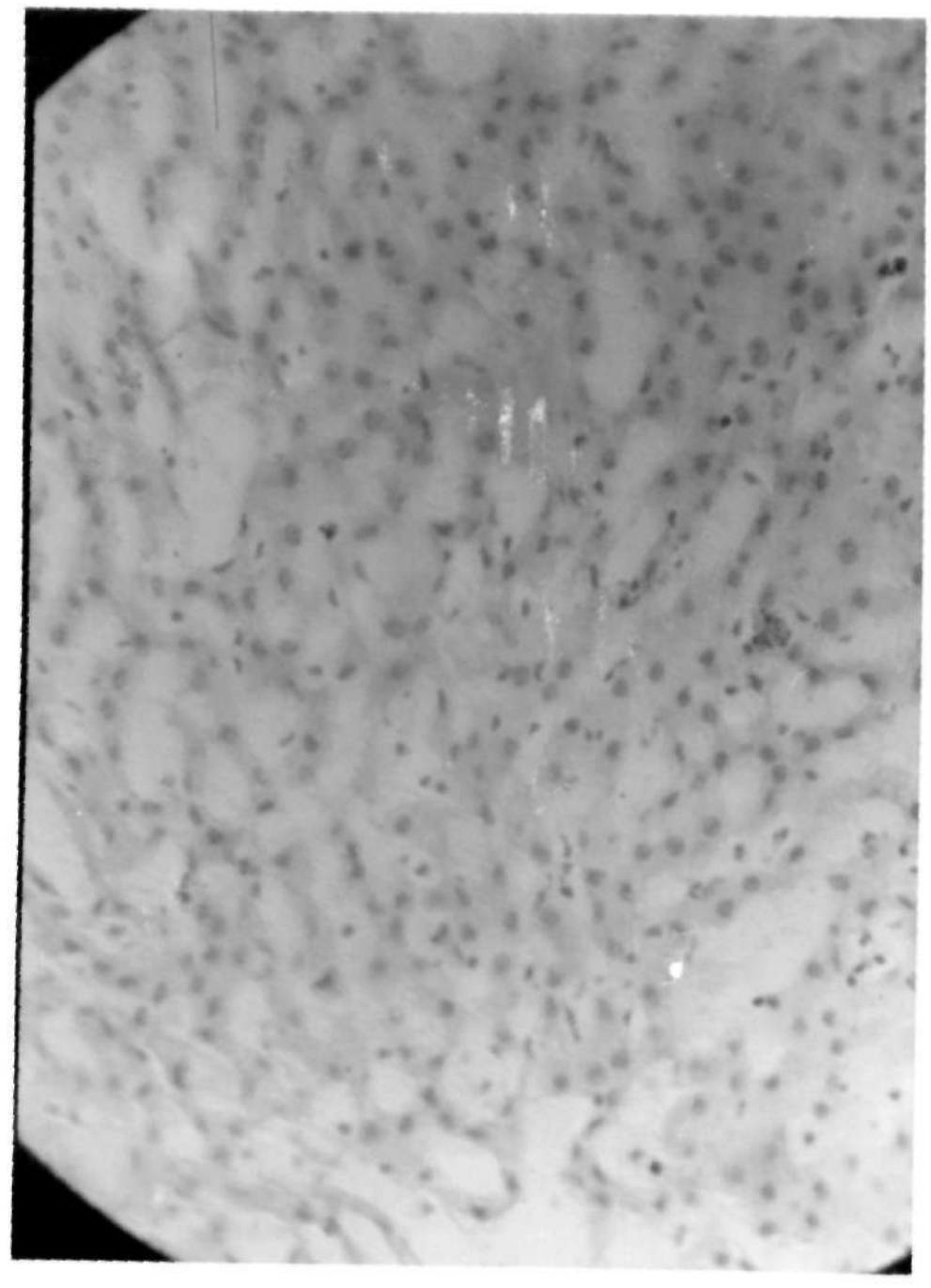
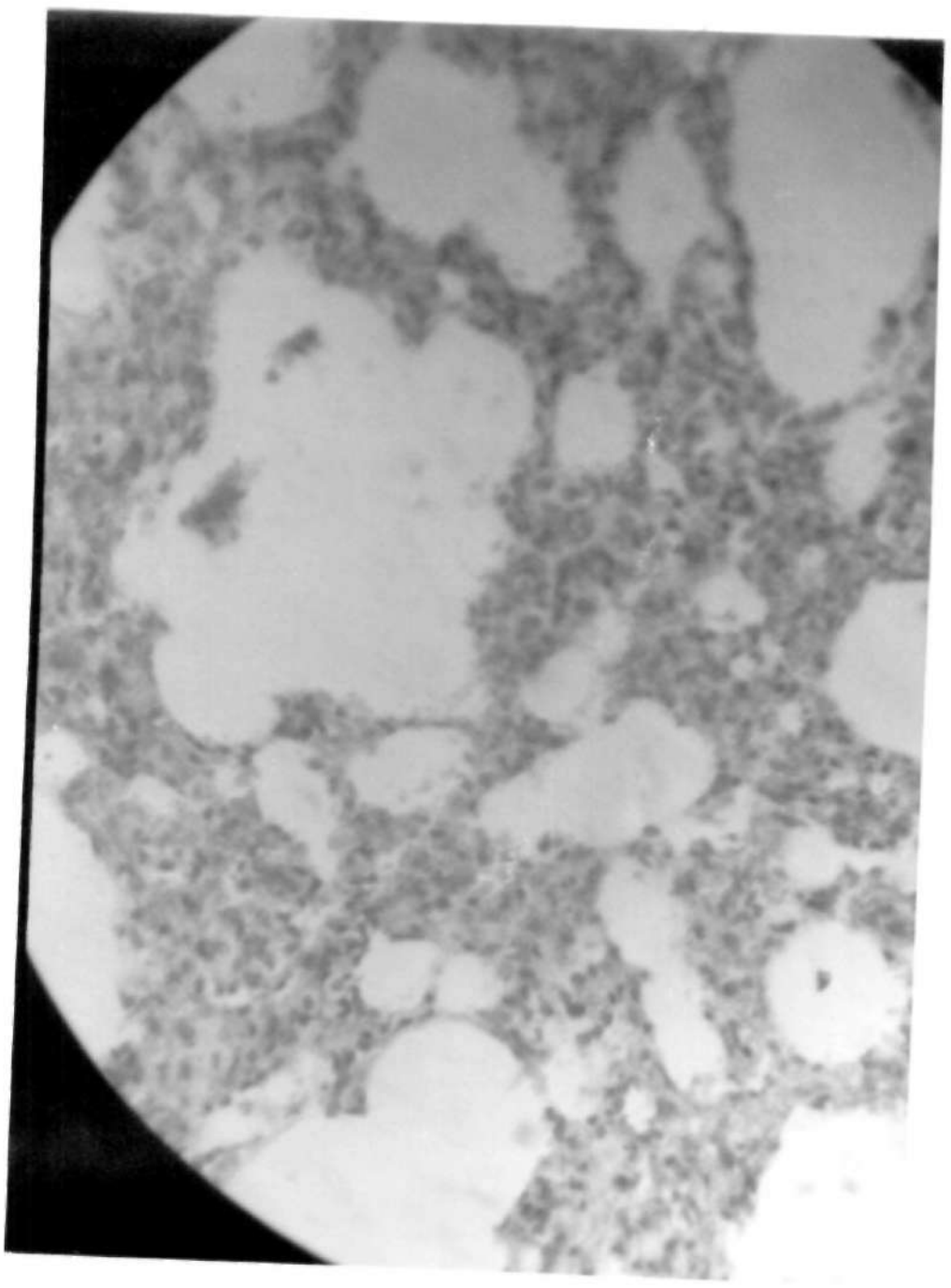


Plate 9: Macroscopic lung appearance of a lamb in the control group.



Plate 10: Microscopic lung appearance of a lamb in a typical control group. H & E x 100



CHAPTER 5

DISCUSSION AND CONCLUSION

In addition to existing information on pasteurellosis of small ruminants in Nigeria, this study has confirmed the isolation of *P. haemolytica* and *P. multocida*. Overall, *P. haemolytica* (378) was more associated with penuemonic lungs than *P. multocida* (126) confirming the widely held view that *P. haemolytica* plays a more important role in pneumonic pasteurellosis of small ruminants (Gilmour and Gilmour, 1989).

The implication of this and other similar studies in various parts of the country is that pneumonic pasteurellosis is an enzootic disease to be taken more seriously than the attention it is currently receiving. Since many cases of pasteurellosis are likely to be undiagnosed or unreported the accurate prevalence of the disease or its precise economic cost to the sheep and goat industries cannot be calculated. However, it is invariably the most common infectious disease of sheep and goats diagnosed at postmortem at most Nigerian veterinary investigation centres (Ojo 1971, 1977; Akerejola 1980; Adekeye, 1984; Onyekwodiri and Shoyinka, 1984; Ugochukwu and Nwaneri, 1984). Economic losses have resulted from death of animals or treatment cost. In most cases the animals have remained chronically unthrifty with great financial loss from frequent treatment cost.

.Other bacteria less frequently isolated were *Neisseria* species, *Corynebacterium pyogenes*, streptococci and *Escherichia coli*. They were often isolated as mixed growths with the Pasteurellae. Along with staphylococci, these group of bacteria often obscured and confused diagnosis of pasteurellosis. The isolation of these bacteria were also reported by Alley (1975) and Adekeye (1984) but less frequently than Pasteurellae. The

bacteria are often assumed to be opportunistic co-infections or may be secondary pathogens to Pasteurellae especially in long-standing pneumonic forms of the disease. The relatively less frequent manner in which they were isolated in this study suggest they may have an insignificant role in the development of ovine pneumonia, although no causal relationship of these agents was established in this study. Alley (1991), observed that in sheep dying later in an outbreak of pneumonic pasteurellosis, these bacteria were frequently isolated from the lung lesions in addition to the Pasteurellae. The role of these bacteria, though uncertain may yet be another variable in this seemingly complex disease that merits a separate investigation. However, Biberstein *et al.* (1967), had carried out a transmission study with these bacteria abounding in the environment of the sheep, and it was apparent that they failed to produce lesions in the respiratory tract nor was there any significant temperature rise elicited by these agents.

Serotyping of the isolates were carried out by the use of Rapid Plate Agglutination (RPA) (Frank & Wessman, 1978) for *P. haemolytica* and the hylauronidase test (Carter and Rundell, 1975) for *P. multocida* isolates. These two tests were found to be simple, convenient and less involved for routine use in diagnostic laboratories and recommended for large scale sample assay. Perhaps in the past, only a limited number of complete serotyping studies have been done on *P. haemolytica* and *P. multocida* isolates in Nigeria owing to the time and effort involved in the indirect haemagglutination (IHA) procedure (Sawada *et al.*, 1982). Many research workers had overlooked relatively simple typing procedures such as the RPA and hyaluronidase tests. Frank (1982) and Chengappa *et al.*, (1984) observed that simple RPA test yielded essentially the same result as the IHA

procedure for serotyping of *P. haemolytica*. Carter and Rundell (1975) noted that *P. multocida* type A strains possess large capsules composed in large part by hyaluronic acid and the presence of the acid in the capsule is largely specific to serogroup A strains.

Serotyping of the isolates was considered important because of the need to construct an effective vaccine for *Pasteurella*. With the exception of one documented serotyping study in goats in South-west Nigeria (Ojo, 1977), there appears to be a dearth of information on the specific serotypes of *P. haemolytica* and *P. multocida* in Nigeria. Ojo (1977), identified six serotypes of *P. haemolytica* viz: A2, A6, A7, A8, T10 and A11. In this present study, all these serotypes were encountered in addition to *P. haemolytica* A1 and A9 and *P. multocida* type A.

Only 16 serotypes of *P. haemolytica* were examined during this investigation. *P. haemolytica* serotype A17 which was recently added (Younan and Fodor, 1995) was not investigated for because its antiserum was not available. Of the 16 serotypes examined from the field isolates only serotypes A1, A2, A6, A7, A8, A9, T10 and A11 were represented and untypeable (UT) strains of *P. haemolytica* were also isolated. A reclassification of *P. haemolytica* has been proposed recently. The 17 recognized serotypes of the former *P. haemolytica* complex have been shown to represent three genetically distinct species. Serotypes 3, 4, 10 and 15 represent *Pasteurella trehalosi* (Sneath and Steven, 1990), serotype 11 represent *Mannheimia glucosida* while the remaining serotypes (most *P. haemolytica* biotype A) belong to *Mannheimia haemolytica* (Angen *et al.*, 1999).

Of the 5 recognized *P. multocida* serogroups, all the isolates of *P. multocida* in this study yielded *P. multocida* serogroup A. The relative frequencies of all the serotypes isolated in this study indicate that *Ph* A2 (27.1%) and *P. multocida* type A (15.1%) represented the most frequently isolated and the most numerous double combination serotypes from all the five states studied.

Untypeable *P. haemolytica* strains (UT) were also encountered in this study. Some of the so-called UT strains may belong to *P. haemolytica* A17, since isolates were not tested against *P. haemolytica* A17. This could possibly reduce the number of non-typeable isolates obtained in this study. The UT strains may represent mutants of *P. haemolytica* which have lost their ability to produce capsular polysaccharides (Gilmour and Gilmour, 1989). The isolation of the UT strains is not surprising as Donachie *et al.*, (1984) observed that 90% of *P. haemolytica* strains from cattle, sheep and goats could be grouped into recognized serotypes with the remaining UT accounting for about 10%. UT strains have been isolated from both healthy sheep and those with pasteurellosis (Gilmour and Gilmour 1989).

All the serotypes of P. *haemolytica* were of biotype A except for an isolation of T10 of *P. haemolytica*. The isolation of predominantly A biotype from pneumonic lungs further confirms that biotype A strains are associated with pneumonia of sheep, while the T biotype strains are associated with the septicemic form of pasteurellosis. The presence of an isolate of biotype T (T10) in pneumonic lung is therefore not clear but it has been reported that all serotypes of *P. haemolytica* have at various times been isolated from normal and diseased respiratory tracts of sheep (Gilmour and Gilmour, 1989).

Of the 5 serogroups of *P. multocida* (A B D E and F), only serogroup A was reported in this study. Frank (1989), and Rimler and Rhoades (1989) observed that serogroup A was the most commonly isolated serogroup associated with pneumonia in ruminants. While *P. multocida* type D may also play an important role in pneumonia of ruminants, it is often known to be of most importance in pigs (Chanter and Rutter, 1989). Serogroups B and E are important causes of septicemia in cattle (Carter and de Alwis, 1989) while serogroup F causes disease in turkeys (Rimler and Rhoades, 1989). *P. haemolytica* serotypes 3, 4, 5, 12, 13, 14, 15 and 16 were not represented in this study.

The multiplicity of serotypes of *P. haemolytica*, involved in ovine pasteurellosis is quite evident from this study, and poses a serious problem for the formulation of effective vaccines. The evidence that immunity is type-specific has been confirmed by a number of workers (Gilmour and Gilmour, 1989; Chandrasekeran *et al.*, 1991; Donachie, 1995), although there is evidence of some degree of cross-protection among serotypes (Confer, 1993). The necessity of including all the serotypes that prevail in a particular country, region or area, in a bacterin is therefore quite evident.

The data presented in this investigation suggest the relevance of some specific serotypes of *P. haemolytica* and *P. multocida* as important pathogens of small ruminants in Nigeria. The experimental transmission studies produced in some cases no overt clinical signs and in others severe signs and death. Even in the absence of death lung lesions developed in some cases and could be scored at post-mortem. Lesions at necropsy ranged from no lesion to severe lesions and in some cases adhesions and pleurisy in addition to the exudation.

The infective dose (4.0 x 10^8 cfu) of each serotype used in this study was sufficient to induce respiratory tract disease. This dosage was employed in the log phase of growth after a 6 - h broth culture of the organisms. Similar workers advocated the use of 10^8 -10^{10} cfu of bacteria as inoculum in the absence of any virus predisposing agent and that severe pneumonic pasteurellosis could be induced if a log phase growth of the *Pasteurella* was used as the inoculum (Slocombe *et al.*, 1984; Hilwig *et al.*, 1985; Zamri-Saad *et al.*, 1996).

Animals exposed to serotypes PhA2 and PhA7 appeared to suffer a more severe illness resulting in the deaths of some of the lambs. Less severe but important illness were evident in the lambs inoculated with serotypes A1, A9 and PmA. Animals challenged with these serotypes developed outward signs of illness: Pyrexia, dyspnea, inappetence and lethargy were the clinical parameters showing the most obvious changes between groups. Within each group however, subtle differences were observed clinically and pathologically among the animals infected. For example, only 3/4 lambs, 3/4 lambs, 3/4 lambs, 2/4 lambs and 3/4 lambs showed overt susceptibility to infectious exposure to PhA1, A2, A7, A9 and Pm type A respectively. The reason for lack of a 100% success rate of infection in these groups is not known but could be speculated as due to a number of factors such as predisposing factor (presence or absence of other infectious agents) (Gilmour and Gilmour, 1989) or error during the intratracheal method of administration of the inoculum (Vestweber *et al.*, 1990). Supportive experimental and field evidence however abound to affirm the pathogenicity of serotypes PhA1, A2, A7, A9 and Pm type A in ruminants as shown in this study (Gilmour and Gilmour, 1989; Gourlay *et al.*, 1989;

Zamri-Saad *et al*., 1996). Clinical and pathological signs of lethargy, inappetence, depression, pyrexia, dyspnea, lung consolidation, pleuritis and adhesion were similar to those seen in lambs in field cases of *Pasteurella* pneumonia (Gilmour and Gilmour, 1989; Alley, 1991). Rectal temperatures greater than 40°C were consistent with previous reports and *Pasteurella* isolation were made from classical lung lesions.

Although each of the serotypes appears to be potential lamb pathogen, subtle differences have been observed amongst them which may explain the importance of certain serotypes in lamb pneumonia. As revealed in this study, only 5 of the 10 serotypes isolated could cause pneumonia in the experimental lambs.

One of the potential causes of failure of the reproduction of pneumonic pasteurellosis in experimental animals has been the presence of protective immunity or antibodies to the *Pasteurella* organism (Gilmour and Gilmour, 1989). Before infection, no antibodies to *P. haemolytica* or *P. multocida* were however detectable in the serum of any of the lambs used in this study. Also all the animals were negative for *Pasteurella* species when tested repeatedly by bilateral nasopharyngeal swabbing over a period of 4 weeks before the experimental infection.

While some workers have argued that *P. haemolytica* or *P. multocida* do cause disease only as secondary pathogens following pre-disposition of the animals by other agents such as viruses, there is substantial evidence to support the thesis that *P. haemolytica* or *P. multocida* are primary causes of respiratory diseases in small ruminants. As a secondary pathogen therefore, the success of a challenge or immunity study will depend very much on the presence of the predisposing agents. The infectious agents

known to have a temporal relationship with outbreaks of pasteurellosis include parainfluenza-3 virus (PI - 3 virus), adenovirus, reovirus, chlamydia and mycoplasma (Gilmour & Gilmour, 1989; Brogden *et al.*, 1998). The presence of these agents was not investigated in this study. However, other workers in Nigeria have established the presence of some of these agents in sheep and goats (Ojo, 1977; Obi, 1984; Obi and Ibu, 1990; Ibu *et al.*, 1993). Experiment using *Pasteurella* in combination with these agents isolated in Nigeria may be necessary in the future to define the exact role of these agents in the disease complex.

Thus, many experimenters have used multiple agents in different combinations, with variable degree of success in reproducing the disease (Confer *et al.*, 1988; Gilmour and Gilmour, 1989). The success, although moderate, has reinforced the concept of the secondary role of *Pasteurella* in pneumonic pasteurellosis. In the 1980s however, it became increasingly clear that *P. haemolytica* or *P. multocida* was capable of causing the disease as a primary pathogen, provided that two conditions were fulfilled. First, the animals had to be susceptible and secondly the organism in the logarithmic growth phase had to be administered to the trachea or lungs in numbers greater than 10^8 cfu (Shoo, 1989; Zamri-Saad *et al.*, 1996). It is generally recognized that the clinical and pathological features of the disease are due to *Pasteurella* species (Gilmour and Gilmour, 1989). There has been controversy about the role played by these so-called predisposing agents in the etiology and pathogenesis of the disease. In North America, for example, the etiology of the disease has been considered by many workers to be multifactorial (Confer *et al.*, 1988; Shoo, 1989). The viruses are thought to initiate respiratory tract

lesions in the presence of physical stress in non-immune which then enable *Pasteurella* species to induce pneumonia (Gilmour and Gilmour, 1989). In Britain, on the other hand, *Pasteurella* species and in particular *P. haemolytica* biotype A are believed to be capable of initiating pneumonia as primary agents in non-immune animals in the absence of the viruses or physical stress (Donachie, 1996). In this experiment, it cannot be said with certainty if pneumonia was induced in the presence or absence of any of the viruses as their presence was not assayed for. However, the animals had no prior experimental exposure to any virus.

Another source of failure to induce experimental pneumonia by a pathogenic strain of *P. haemolytica* or *P. multocida* may be error in the intratracheal inoculation of the organism. Vestweber *et al.* (1990), demonstrated that during intratracheal administration of *Pasteurella*, if coughing by the animal occurred during inoculation, it helped to disseminate the inoculum in more quadrants of the lung field. The coughing during deposition of the bacteria intratracheally probably disseminated the bacteria through a major portion of the lungs resulting in severe clinical symptoms and consolidation of the lungs.

In the experimental groups infected with Ph A6, A8, T10, A11 and untypeable (UT) serotypes, either insignificant clinical and lung lesion or no signs at all were observed. The control animals showed no clinical signs or lesions. In all these cases or groups, pneumonia was not observed even on the 7th dpi and in most cases the bacteria were not isolated from the lungs, except for one case of Ph A11 - treated lamb which recorded a focal lesion and a low titre of Ph A11 count.

Although Ph A11 currently re-classified as *Mannheimia glucosida* (Angen *et al.*, 1999) was the third most frequently isolated serotype in this study, its pathogenicity in the conventionally reared lambs used in this study was insignificant. Speculation for its relative poor pathogenicity could be that while all *P. haemolytica* (Ph) serotypes appear to be potential pathogens, subtle differences in growth rates may indicate selective disadvantage for certain strains of Ph for disease production. Slower growth rates and consequent lower leukotoxin production *in vivo* might allow host defenses to combat an infection before the organism increased to a threshold level that could cause disease. Supportive evidence for this is provided by the studies of Frank and Smith (1983) and Gilmour *et al.* (1986).

The infective *Pasteurella* serotype could be found at sites of diseased sheep-pleural and pericardial fluid, heart blood, lung lesions, liver, spleen and kidneys. In the groups where pneumonic consolidation of the lungs occurred, the infective serotypes were isolated from the lung lesions at the titre range of 10^2 - 10^{10} cfu/g. To further confirm diagnosis of pasteurellosis in field outbreaks, Gilmour (1978) observed that since *P. haemolytica* or *P. multocida* could be present in the lungs of sheep in the absence of pasteurellosis, it was important to have an indication of the number of the organism in the lungs. In acute pasteurellosis, 10^6 or more cfu per g of lung were present with 10^3 - 10^5 cfu per gram in subacute and chronic cases. Residual lesions in chronic or treated cases may not contain *P. haemolytica* or *P. multocida.*

There is yet no evidence that serology is useful either on an individual or flock basis in the diagnosis of the disease and the presence of *P. haemolytica* or *P. multocida*

in nasal swab is of no significance (Gilmour and Gilmour, 1989). Many apparently healthy sheep have antibodies to a range of serotypes of *P. haemolytica* and *P. multocida*. A diagnosis is therefore best made on necropsy when confirmation of the presence of pneumonic pasteurellosis is obtained by finding the acute inflammatory changes in the thorax and the lesions in the lungs. Gram negative coccobacilli may be seen in smear from exudate and the cut surfaces of lung lesions but the isolation in culture of *P. haemolytica* or *P. multocida* in large numbers is required for the confirmation of the presence of the disease. Further information of the diagnosis can be got from histopathological examination when the finding of lesions with oat cells is pathognomonic in the cases of *P. haemolytica*. In acute case in this study Pasteurellae were isolated from lungs, livers, spleens and kidneys. Both in acute and in sub-acute pneumonia of up to 6 or 7 days duration, the numbers of bacteria was in the region of 10^{-5} - 10^{10} cfu/g of lung in most of the lesions. Martin (1984) reported that as sheep may have IHA titres to several serotypes, IHA is of no value in establishing which serotype is the cause of an outbreak, and no definite correlation exist between antibody titres and protection in sheep.

It is apparent from this study that not all of the Pasteurellae serotypes isolated from the field could precipitate pneumonic pasteurellosis at the same rate. *P. haemolytica* serotypes A1, A2, A7 and A9 and *P. multocida* type A should be considered important pathogens and it would appear that they can cause the disease as primary pathogens in non-immune experimental sheep, especially when log growth phase cultures containing at least 10^8 cfu was administered to sheep intratracheally. It is also the view of other

workers that virulent Pasteurellae, can by themselves initiate respiratory disease in the field without the presence of any primary incitants (Panciera & Corstvet, 1984).

While description of lesions from natural cases of pneumonic pasteurellosis may differ among authors, there seems to be a consensus as to some common basic findings. Difference in micro-organisms which contributed to the pneumonia, the severity of the disease and the stage of which the lesions were examined could account for the varied observations and descriptions. Animals which died before the 7th dpi frequently showed heavy and deeply congested lung with dark red areas of consolidation especially anteriorly and pleural fluid and layers of fibrin covering the affected lobes. The histological findings in the acute cases from *P. haemolytica* varied only in degree and not type. Generally severe pneumonia was present. *P. multocida* produced lesions of limited extent and different histopathological pattern from *P. haemolytica*. The striking difference between lesions induced by *P. multocida* and those induced by *P. haemolytica* inoculated intratracheally corroborates the work of Ames *et al*. (1985) in ruminants. With *P. multocida*, there was a suppurative exudative component with minimal to no necrosis. In this study deposition of fibrin was observed in *P. haemolytica* and not *P. multocida* infected lambs but oat cell type macrophages were rarely seen in the *P. multocida* - infected animals. The lack of an oat cell macrophages in *P. multocida* - infected animals was presumably because *P. multocida*, unlike *P. haemolytica* does not produce a leukotoxin (Confer, 1993). Several studies have shown that in the lungs, *P. haemolytica* produces a cytotoxin that lyses alveolar macrophages, neutrophils (WBC) and monocytes. This inactivates the lungs primary line of defence and allows the organism to colonize

and grow almost uninhibited. The result is the severe fibrinous pneumonia (Whiteley *et al.*, 1992; Confer, 1993).

Cognizance must be given to the different serovars (especially Ph A1, A2, A7, A9 and Pm type A) capable of inducing severe respiratory infections in sheep when developing vaccines. Reports of *Pasteurella* vaccine failure in many countries (Gilmour and Gilmour, 1989) may have been due in part to lack of a multivalent serotype in the product.

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