PASTEURELLA MULTOCIDA IN INDIGENOUS CHICKENS AND DUCKS IN KENYA: - A study of carrier status, susceptibility, molecular diagnosis and pathogenesis

By

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A thesis submitted in fulfillment for the degree of Doctor of Philosophy in the Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my beloved wife Winifred Wothaya; children: Caroline Gathoni, Emmaculate Wambui, Alexander Gichohi, and Grace Kagure for their support and encouragement; and my parents Jeremiah Gichohi Gitata and Teresiah Gathoni Gichohi who inspired every member in the family to strive for the highest goals in the education system.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

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ABSTRACT

Scavenging indigenous birds constitute over 70 % of poultry in Kenya and contributing 71 % of the total eggs and poultry meat produced in the country. *Pasteurella multocida* causes fowl cholera, a disease of economic importance in commercial bird production. Its importance in village indigenous chickens and ducks is unknown. In Kenya there is very little documentation on *P. multocida* and fowl cholera, although these are reported in other African countries. Little is known about the carrier status, age susceptibility, disease severity, cross-infection, molecular diagnosis, and pathogenesis of *P. multocida* in farmed and traded birds and the effect of stress (simulated by immunosuppression in these studies) on *P.multocida* infections.

For the *P. multocida* carrier status, a total of 162 scavenging indigenous chickens and 54 ducks were examined. The birds were from smallholder farms, trading centres and slaughterhouses in Kiambu, Machakos, and Nairobi districts; the slaughterhouses receive birds from rural districts in various parts of the country. The samples collected were oropharyngeal and cloacal swabs. Isolation was done by inoculation onto blood agar, and characterized using staining and biochemical tests. All the three documented subspecies of *P.multocida* were recovered at varying rates. Chickens gave recoveries of 6.2 % or 10/162 (3 *P.m. multocida*, 2 *P.m. septica* and 5 *P.m. gallicida*) and duck had 25.9 % or 14/54 (4 *P.m. multocida*, 3 *P.m. septica* and 7 *P.m. gallicida*) *P.multocida* isolates. Carrier birds with *P.m. multocida* originated from Kiambu (4/7), Kisii (2/7) and Nairobi (1/7); those with *P.m.septica* originated from Machakos (2/5), Nairobi (2/5) and Kiambu (1/5); while those with *P.m. gallicida* were from Makueni (3/12), Machakos (2/12),

Kiambu (3/12) and Nairobi (4/12) districts. This study documents the first report of *P.multocida* carrier status in local scavenging chickens and ducks.

Age susceptibility to *P.multocida* was evaluated in duplicate in two separate experiments, one for chickens and the other for ducks. Each experiment consisted of 10 experimental birds and 5 control birds, for respective ages: 4,8,12 and 16 weeks. The experimental chickens and ducks in each age group were infected intratracheally with 1.2 –2.0 x 10^8 CFU of *P. multocida* organisms, strain NCTC 10322^T, and monitored for total number and severity of clinical signs, and weight changes over a period of 14 days post inoculation (p.i). Control birds were inoculated with brain heart infusion (BHI) broth. The clinical signs observed in sick birds in all the age groups, that is, 4,8,12, and 16 week-old birds were anorexia, fever, ataxia, depression, ruffled feathers, cyanosis, nervous tics, nasal discharges, sneezing, mouth discharges, dyspnoea, spontaneous tracheal rales, diarrhoea and head scratching. A total of 173, 272, 372 and 187 clinical signs were shown by 4,8,12 and 16-week-old chickens, respectively. Of these, the severe signs were 28 for the 4week-old, 59 for the 8week-old, 65 for the 12week-old and 24 for the 16week-old chickens. The infected birds lost weight by 14.1, 20, 25.7 and 166.9 grams for 4, 8, 12 and 16week-old chickens, respectively as compared to respective control bird. Overall, the 12week-old local chickens expressed many and severe clinical signs more times than those of other age groups, consequently this was considered the most susceptible age.

The observed clinical signs for ducks were the same as chicken except there were no cyanosis, and head scratching but there were additional eye discharges. A total of 117, 188, 80 and 83 clinical signs were shown by 4, 8, 12 and 16week-old ducks, respectively.

Of these, the severe signs were 23 for the 4week-old, 49 for the 8week-old, 18 for the 12week-old and 22 for the 16week-old ducks. The infected ducks lost weight by 7.5, 12.3, 28.5 and 28.8 grams for 4, 8, 12 and 16 week-old ducks, respectively as compared with the respective control birds. These weight changes were associated with *P.multocida* infection, although less severe in older ducks as compared to older chickens. Overall, the 8week-old local ducks expressed many and severe clinical signs more times than those of other age groups and consequently this age group was considered the most susceptible age. The most susceptible ages for chickens and ducks (12 and 8 weeks, respectively) were the ones used for subsequent experiments on clinical sign monitoring, cross-infection and pathogenesis studies.

Comparison was done with respect to severity of the disease (clinical signs), in normal, non-immunosuppressed (NIS), and immunosuppressed (IS) chickens and ducks. Immunosuppression was done by injecting the respective birds with 4.0 mg dexamethasone per kg body weight daily, for 6 days prior to inoculation. Chickens and ducks of the previously determined susceptible ages, were infected intra-tracheally with $1.1 - 2.5 \times 10^8$ organisms of *P.m. multocida* type strain; and monitored for clinical sign expressions for 14 days post inoculation. The main clinical signs expressed by NIS chickens were: fever, depression, nasal discharges, sneezing, ruffled feathers, dyspnoea, ataxia, diarrhoea, nervous tics, spontaneous tracheal rales, mucous discharges from mouth, cyanosis, head scratching and anorexia. Immunosuppressed chickens had similar signs although there were no rales, diarrhoea and cyanosis but nervous tics were many compared to NIS chickens. A total of 372 clinical signs were observed in NIS as compared to 179 in IS chickens.

The NIS and IS ducks had signs similar to those for chickens except nervous tics, head scratching and cyanosis. Ducks showed eye discharges, a sign that was not observed in chickens. A total of 188 clinical signs were observed in NIS ducks as compared to 133 for IS ducks. The clinical signs occurred singly or in various combinations per bird per observation time. Overall, fewer signs were observed in IS birds compared to NIS birds in both chicken and ducks. Thus, immunosuppression appeared to modify and subsequently decrease expression of clinical signs. New signs such as ataxia, nervous tics and head scratching have been revealed in this study and need to be considered in cases of suspected fowl cholera.

P.multocida cross infections between the chickens and ducks were done in two experiments: - (1) from chickens to ducks and (2) from ducks to chickens. Birds were used at the respective susceptible ages. In the first experiment, six 12week old chickens were inoculated intra-tracheally with 1.6×10^8 CFU of *P.multocida* organisms and, six hours later, mixed with 15 eight week-old sentinel ducks. Of the 15 ducks, five were swabbed regularly, on daily basis while the other 10 were sacrificed for post-mortem and histological examination at specified days: 1, 3, 5, 7 and 10 after mixing them with infected chickens. Of the 5 ducks that were swabbed severally for 14 days, 2 had *Pasteurella* organisms recovered from them by day 1, 4 at day 2, and all had the organism on day 6 after mixing the birds. Most of the birds developed clinical signs and lesions typical of intermediate to chronic fowl cholera. *P.multocida* organisms were recovered from these birds and also from the experimentally infected chickens at the end of the study.

In the 2nd experiment, 6 eight week-old ducks were intra-tracheally infected with 1.8 x 10^8 C.F.U. of *P.multocida* organisms and mixed with sentinel chickens 6 hours later. Four of the 5 chickens swabbed severally had the *P.multocida* on day 1 and all had the organisms on day 2, after mixing. Typical signs and lesions similar to those observed in the first respective experiment were recorded and *P.multocida* organisms recovered at the end of the experiment.

These two experiments showed that the *Pasteurella* organisms could easily be transmitted across the two types of birds, simply by mixing the birds, and that chickens acquired *P.multocida* infections more quickly than the ducks. The possibility of contact transmission occurring at farm level, market places and during scavenging, may account for the maintenance and propagation of the *P.multocida* organisms at village level.

A *P.multocida* species - specific RNA probe was developed for use in the fluorescent *in situ* hybridization (FISH) test to effectively detect and locate *P. multocida* organisms in culture and tissues. The probe was an oligonucleotide, pmhyb449, which targets the bacterial 16S rRNA. The probe was evaluated using whole-cell hybridization on bacterial cultures and tissues. Twenty two reference strains of different bacteria, formalin-fixed and paraffin embedded pig and chicken lungs were used for the evaluation. The probe differentiated *P.multocida* from other bacterial species of the families *Pasteurellaceae*, *Enterobacteriacae* and also from divergent species of the order *Cytophagales*. Biovar 2 strains of *Pasteurella avium* and *Pasteurella canis*, could not be differentiated using this probe since their 16S rRNAs have high similarity to that of *P.multocida*. In chicken and pig lung tissues, *P.multocida* cells were detected singly, in pairs, as microcolonies and as massive colonies within air capillaries septa and lumen, parabronchial septa, and blood

vessels wall and lumen. The results showed that a fluorescent *in situ* hybridization technique offers a fast method for specific detection of *P.multocida* in histological formalin-fixed paraffin-embedded tissues. This particular FISH test was replicable and is recommended as another test for diagnosis and as a tool in pathogenesis studies of fowl cholera and other *P.multocida* infections.

The pathogenesis experiments were done on chickens and ducks, separately, using the established susceptible ages. Both experiments comprised two groups of NIS and IS birds with respective controls. The immmunosuppression was done using dexamethasone as given above. All the groups of birds, except controls, were infected intra-tracheally with 1.2 - 1.9 x 10^8 CFU of *P. multocida* organisms. Control birds were inoculated with BHI as before. The birds were then sacrificed, two at a time, over a period of 14 days, at specified times, namely: 0 (immediately after inoculation), 1, 3, 6, 12, 24 hours, and days 2, 3, 5, 7, 10, and 14, post infection. Post mortem examination was done on each animal; gross pathological lesions in selected 24 organs (lung, trachea, conjunctiva, airsacs, spleen, liver, heart, thymus, harderian gland, pancreas, brain, sciatic nerve, kidney, testis/ovary, adrenal gland, bursa of Fabricius, proventriculus, duodenum, midgut, caecal tonsils, large intestine, pruning gland, comb or head skin and pectoral muscles) were noted. The selected organs were aseptically removed, immediately swabbed for conventional bacterial isolation and then formalin fixed for histopathology, and tested with FISH for the presence of *P.multocida* organism. A total of 95.8% of NIS and 91.7% of IS chicken organs had gross lesions, while all NIS organs and 83.3% of the IS chicken organs had microscopic lesions.

Lesions were numerous in the first week for both groups of chickens. The common lesions observed were pneumonia, airsacculitis, fibrino-suppurative exudate, congestion, haemorrhages, serositis, hepatitis, splenitis, peri-and-myocarditis, nephritis, sinusitis and peritonitis. They were many and severe between 1 hr and 2 days p.i for NIS chickens and 3 hrs and 24 hrs for IS birds. The picture was similar for histological lesions. On FISH test, all organs of NIS birds had *P.multocida* signals while 87.5% of IS organs had the signal. The expression of FISH signals correlated closely with lesions up to 7 days p.i, and closely with *P.multocida* re-isolation up to the 2nd day p.i. Thereafter the FISH test had more positive cases than the other methods.

The trend in the duck pathogenesis experiment was similar to that of the chickens but lesions were more severe than those in chicken organs. In both NIS and IS birds FISH signals were observed in all organs. FISH signals were in more organs at 1hr to 3rd day for NIS, and 1st hour to 2nd day for IS duck organs. *P.multocida* recovery from selected organs followed a similar trend as in chickens. As observed in previous experiments, immunosuppression was found to modify the lesion expression as well as the ease of detecting the *P.multocida* through both the FISH test and cultural method.

In conclusion, the study has established that a *P.multocida* carrier status exists in ducks and chickens, susceptible age groups; were 12 weeks and 8 weeks for chickens and ducks, respectively; typical signs and newly observed signs were seen, and cross infection of *P. multocida* occurs between village chickens and ducks. Further, a molecular diagnostic stool (probe pmhyb 449) has been developed: pathogenesis of *P.multocida* in chickens and ducks mapped out, and immunosuppression has been shown to modify *P.multocida* infections.

CHAPTER 1

1.0 INTRODUCTION

There are 15 billion poultry in the world and domestic chickens, ducks, turkeys and geese are the common species (Anon., 1996). Kenya has a poultry population of over 29 million, of which over 21 million are scavenging indigenous chickens (Njue, 2003). The rest are commercial chickens, ducks, turkeys, and geese. The indigenous chickens and ducks are descendants of random crossings between the local and exotic strains of birds (Gueye, 1998), hereby referred to as the indigenous birds. The average flock size is 7 to 14 birds per household (MLD, 1989; Mbugua, 1990a; Gueye, 1998). The scavenging chickens contribute 71 % of the total egg and poultry meat produced in Kenya (Mbugua, 1990 b) and impact significantly to the rural trade and the welfare of the rural smallholder family (MLD, 1989). Inspite of this, the birds are poorly housed, and scavenge on green feeds, seeds, insects and other residues from the environment, and are supplemented at times from household wastes (MLD, 1989; Mbugua, 1990a). Free-range is the most common poultry-raising method in developing countries (Minga *et al.*, 1989; Nyaga *et al.*, 2002). This management system predisposes the birds to various hazards, such that, the survival rate to maturity is estimated at 20-30 % of the hatched chicks and ducklings (MLD, 1989). Any remedial measure that may reduce this loss would greatly enhance the economic well being of these households. Conditions that lead to these losses include; diseases, inadequate feeds, poor housing, and predators; diseases being the most important constraint (MLD, 1989; Gueye, 1998; Aini, 1999a,b).

Bacterial infections of the respiratory system of birds include *Pasteurella* spp., *Haemophilus* spp., *Actinobacillus* spp., *Bordetella* spp., *Gallibacterium* spp. *Riemella* spp. *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli*, *Mannheimia* spp., *Ornithobacterium spp.*, and *Mycoplasma* spp. All these produce similar clinical and pathological lesions (upper respiratory tract inflammations, pneumonia, airsacculitis, polyserositis, and septicaemia) in birds. They can infect alone or in combinations (Glisson, 1998, and 2003; Bojesen, *et al.*, 2003). There is a need therefore to differentiate these causative agents in clinical infection in chickens, ducks and other birds.

Pasteurella multocida, comprising *Pasteurella multocida* subspecies ***multocida***, *Pasteurella multocida* subspecies *septica*, and *Pasteurella multocida* subspecies *gallicida* (Mutters *et al.*, 1985b) infects many food animals and is, an opportunistic human pathogen (Bisgaard, 1993; Frederiksen, 1993). It is the causative agent of fowl cholera in poultry (Rhoades and Rimler, 1989; Christensen and Bisgaard, 2000), a disease of economic importance in commercial production whose importance in rural indigenous chickens and ducks is yet to be determined. Fowl cholera occurs as peracute, acute and chronic infection (Christensen and Bisgaard, 2000; Glisson, *et al.*, 2003). Flocks with fowl cholera can be carriers of *P. multocida* (Carpenter *et al.*, 1989); and the organisms have been demonstrated in healthy scavenging poultry in farms in Tanzania (Muhairwa *et al.*, 2001b) and Denmark (Muhairwa *et al.*,2000).

In Kenya there is very little documentation on *P. multocida*, since the case report in a grey parrot by Miringa (1975). Suspected cases of fowl cholera have been reported in other African countries such as Nigeria (Oladele and Raji, 1997), Tanzania (Muhairwa *et al.*, 2001b) and Zimbabwe (Kelly *et al.*, 1994). Therefore recovery of *Pasteurella* species

from birds would directly confirm the occurrence of the organisms in Kenyan indigenous chickens and ducks. Little is known about the occurrence of healthy-looking carriers in farmed and traded indigenous birds in Kenya.

Despite considerable research on fowl cholera in domestic and wild birds, the source of new infection in a flock is still uncertain (Petersen *et al.*, 2001a,b). Furthermore little is known about the prevalence of healthy carriers and *Pasteurella* serovars involved in individual outbreaks of fowl cholera (Muhairwa *et al.*, 2000). In the Kenya indigenous chickens and ducks: - the status of *P. multocida* in terms of prevalence of healthy-appearing carriers, inter-species transmission at village level and disease susceptibility, clinical signs and pathogenesis is unknown.

Fowl cholera caused by *P. multocida* is reported to be a disease of adult and grower birds (Heddleston and Watko, 1965; Salami, 1989; Christensen *et al.*, 1998). However the most susceptible age group and the ensuring clinical picture for the scavenging chickens and ducks has not been determined and there is therefore a need to establish this.

Indigenous chickens and ducks interact at village level while scavenging. There is a possibility that *P. multocida* is shared across the species. To evaluate this, contact cross-transmission studies were under-taken to give an insight on possible ways of maintaining *P. multocida* at village level.

Detection and phenotypic characterization of *P. multocida* depends on the ability to cultivate and purify the bacteria in the laboratory (Christensen and Bisgaard, 2000). The fact that *P. multocida* infections do not show pathognomonic signs or lesions, makes it necessary to develop a specific technique that can easily detect the presence of these

organisms in culture and tissues of infected birds. *In situ* hybridization (ISH) using a specific fluorescent-labeled probe (McNicol and Farquharson, 1997; Brown, 1998) was chosen for these studies because fluorescent labeled probes, increase spatial resolution (Poulsen *et al.*, 1994; Moter and Göbel, 2000). Flourescent *in situ* hybridization (FISH) has been used to visualize various bacteria such as: *E. coli* (Poulsen *et al.*, 1994), *Salmonella typhimurium* (Licht *et al.*, 1996), *Salmonella* serovars (Nordentoft *et al.*, 1997), *Actinobacillus pleuropneumoniae* (Fussing *et al.*, 1998; Jensen *et al.*, 1999), *Lawsonia intracellularis* (Boye *et al.*, 1998a), *Brachyspira (Serpulina) pilosicoli* (Jensen *et al.*, 2000), *Streptococcus suis* (Boye *et al.*, 2000), bacteria in blood cultures (Jansen *et al.*, 2000; Kempf *et al.*, 2000), and in clinical samples (Krimmer *et al.*, 1999) and in counting of bacteria (Christensen *et al.*, 1999).

On invasion of the airways *P. multocida* infects the respiratory system and spreads to various tissues in the body of the fowl (Matsumoto *et al.*, 1991). The precise location in the organs and tissues of the indigenous chicken and ducks during the course of this disease has not been well documented. It is necessary to evaluate this, by documenting the pertinent pathological lesions, bacterial isolation using cultural methods and the FISH test at specified periods post-infection in order to understand its pathogenicity in various sentinel indigenous birds.

Predisposing factors may increase severity of outbreaks of *P.multocida* infections (Rimler *et al.*, 1998). Examples of these are stressful and immuno-suppressive conditions that are prevalent under village rearing set-ups, namely: exposure to infectious bursal disease, mycotoxins, subclinical infections and infestations, malnutrition, and excessive heat during drought conditions (Aini, 1999a,b). In traded birds fowl cholera can be

precipitated by varied stress factors (Minga *et al.*, 1989; Blackall *et al.*, 1995). The transportation of the indigenous chickens from far districts to the main market in Nairobi and their subsequent holding in congested and crowded cages and coops at various marketing points, and the rough handling of these birds before slaughter, poor feeding and housing can stress the birds and easily precipitate the flare-up of *P. multocida* multiplication and onset of fowl cholera at the marketing points. It would be essential to determine whether stress has an effect on the distribution of *P. multocida* in organs and tissues of scavenging chicken and ducks to give different clinical and pathological lesions? In view of all the above reported literature, it was hypothesized that *Pasteurella multocida* is present and causes severe disease in all ages of normal indigenous chickens and ducks in Kenya. The objectives below were derived and experiments designed and carried out to test the hypothesis.

1.1 The **Objectives** of this study were to:

1. Assess the occurrence of carrier status of *P. multocida* among farmed and traded (market and slaughter) indigenous chickens and ducks.
2. Evaluate the age susceptibility of indigenous chickens and ducks to *P. multocida* infection.
3. Document the clinical signs of fowl cholera in birds (chickens and ducks) experimentally infected with *P. multocida* and which were immunosuppressed or non-immunosuppressed.
4. Undertake cross - transmission studies of *P. multocida* between indigenous chickens and ducks.

5. Develop a fluorescent *in situ* hybridization (FISH) test as a diagnostic tool for *P. multocida*.
6. Study the pathogenesis of *P. multocida* in experimentally infected normal and immuno-suppressed indigenous chickens and ducks of specified susceptible age groups.

1.2 Justification

Considering that indigenous chickens and ducks comprise the highest number of poultry in Kenya, and that with very little input from the owners they contribute significantly to the socio-economic welfare of the village communities, any efforts towards increasing production of these birds, in form of egg or meat, will help towards poverty alleviation. Diseases are the main constraints to poultry production. One of the diseases, whose occurrence in indigenous chickens and ducks is not well documented and which produces high morbidity and mortality in commercial birds is fowl cholera, caused by *P. multocida*. Establishing the carrier status, age susceptibility, clinical signs, diagnosis and pathogenesis of *P. multocida* in chickens and ducks, as well as checking for cross infections between these birds, will go a long way to provide data usable in working out control measures for this disease.

CHAPTER 2

2.0 GENERAL LITERATURE REVIEW

2.1 Background information

In the tropical countries poultry contributes significantly to the economic welfare of the smallholder family (MLD, 1989; Gueye, 1998). In Kenya, scavenging local village chickens and ducks are kept under free-range or backyard systems of management and are widely distributed in rural households in small flocks (MLD, 1989; Mbugua, 1990a,b). The local chickens on free-range management are adapted to scavenging for food where they obtain nutrients for growth, production and reproduction. Ducks are reared together with indigenous chickens and co-host some important diseases; although ducks are commonly believed to be less susceptible to major poultry diseases compared to chickens. The marketing of local ducks and scavenging local village chickens starts with farm gate sales after which the birds are transported to urban markets, a process that could easily facilitate the spread of various poultry diseases (MLD, 1989).

2.2 Biology of *Pasteurella* organisms: *Pasteurella multocida*

The family *Pasteurellaceae* was first effectively published by Pohl in 1979 (IJSB, 1981); it accommodates most of the species in the genera *Actinobacillus*, *Haemophillus*, and *Pasteurella* (Trevisan, 1887). Genus *Pasteurella sensu stricto* includes at least 11 species, but only 7 species have been associated with avian hosts as follows: *P.multocida* (*P.multocida* subspecies *multocida*, *septica*, and *gallicida*), *P.gallinarum*, *P.avium*, *P.volantium*, *P. anatis*, *P. langaa*, and *P.* sp. *A* (Mutters *et al.*, 1985a and 1989; Dewhirst

et al., 1993, Christensen and Bisgaard, 2000). *P. multocida* subspecies *multocida* is the commonest cause of fowl cholera while the other two subspecies of *P. multocida* cause fowl cholera – like disease (Hirsh *et al.*, 1990). *P. multocida* subspecies *gallicida* is mainly associated with web – footed birds (Bisgaard, 1995; Christensen and Bisgaard, 2000).

Pasteurella species are identified by colonial and cellular morphology, Gram stain, and reactions in biochemical and other tests (Mutters *et al.*, 1989; Rhoades and Rimler, 1990; Bergey's manual, 1994). After 24 hours of incubation on blood agar or dextrose starch agar (DSA), colonies are circular, 1 – 3 mm in diameter, smooth, transparent or grayish, glistening, and butyrous. Significant variation in colonial morphology is observed even within the same species. ß-haemolysis does not occur, but a greenish discolouration may occur. Yellowish colonies or large, watery, mucoid and rough colonies may be seen with some strains. Twenty four hour capsulated colonies observed on DSA using a dissection microscope are iridescent while non-capsulated ones are non-iridescent (Henry, 1933; Bond *et al.*, 1970; Christesen and Bisgaard, 2003; Glisson *et al.*, 2003).

Pasteurella multocida cells are rod shaped, 0.2 – 0.4 x 0.6 - 2.5 µm in size and occur singly or occasionally in pairs or short chains. The bacteria are Gram negative. *P. multocida* organisms usually show bipolar staining with Giemsa or Wright's stain in tissues or exudates. Capsules can be demonstrated using an indirect India-ink method (Rimler and Rhoades, 1989; Rimler *et al.*, 1998; Glisson *et al.*, 2003).

Evaluation of reactions in differential media is made after 2 days of incubation at 37 ° C and again 5 days of incubation at room temperature (21 ° C), in case of delayed

reactions. The carbohydrate broth medium used for identification is phenol red broth base containing 1 % of the carbohydrate substrate (Rimler *et al.*, 1998). *P. multocida* ferments fructose, galactose, glucose, mannitol, without gas production. Inositol, inulin, salicin, maltose, dextrin, and rhamnose are not fermented. It reduces nitrate but does not produce urease, arginine dihydrolase or galactosidase. Indole and oxidase are almost always produced. Blood haemolysis and growth on MacConkey's agar do not occur (Table 1) (Mutters *et al.*, 1989; Rimler and Rhoades, 1989; Rimler *et al.*, 1998; Glisson *et al.*, 2003).

Table 1:Biochemical reactions of *Pasteurella* organisms

Reagents	*Pasteurella spp.*														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	N	+	+	N	+	w	w	+	+	+	+	+	+
Indole	+	D	+	(+)	+	-	-	-	-	D	-	W	-	-	-
Dulcitol	D	d	N	d	-	N	-	-	w	-	-	-	-	N	N
Nitrate	+	+	N	+	+	N	+	+	+	+	+	+	+	N	N
Urease	-	-	N	-	-	N	-	-	-	+	-	-	-	N	-
Glucose/A	+	+	N	+	+	N	+	+	+	+	+	+	+	N	N
Glucose/G	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Fructose	+	+	N	+	+	N	+	+	+	+	+	+	+	N	N
Galactose	+	+	N	+	+	N	+	+	+	+	+	+	(+)	N	N
Lactose	-	-	N	-	-	N	dw	(+)	(+)	-	-	-	-	N	N
Maltose	-	-	+	-	-	N	+	-	-	+	+	-	-	N	d
Mannitol	+	+	-	(+)	-	N	+	+	+	-	-	-	-	N	d
Mannose	+	+	N	+	+	N	N	+	+	+	+	+	(+)	N	N
Salicin	-	-	N	-	-	N	N	-	-	-	-	-	-	N	N
Sucrose	+	+	N	+	+	N	N	+	+	+	+	+	-	N	N
Trehalose	-	d/+	+	d	d	N	+	-	+	+	+	+	+	N	+
Xylose	+	+	N	d	(-)	N	d	-	+	-	d	-	d	N	N
Rhamnose	(-)	(-)	N	(-)	N	N	-	-	N	-	-	-	N	N	N
Arabinose	d	-	N	-	-	N	-	-	-	-	-	-	-	N	+
Dulcitol	(+)	-	+	-	-	-	-	-	-	-	-	-	-	N	-
Sorbitol	+	/+	-	/+	-	N	d	-	-	-	(-)	-	-	N	-
Haemolysis	-	-	N	-	-	N	-	-	-	+	-	-	-	N	-
H_2S	+	+	N	+	-	N	N	N	N	N	(+)	N	N	N	N
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/dehydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O/decarboxyl	+	+	N	+	N	N		-	-	-	-	-	-	N	-
Bipolar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	-	-	N	-	-	N	+	+	/d	-	-	-	-	N	d
V-factor Req	-	*	-	-	-	-	+	-	-	-	-	-	+	-	+

Legends:
Pasteurella spp.
1-*P.m.gallicida*; 2- *P.m.multocida*; 3- *Pasteurella* B; 4- *P.m.septica*; 5- *P.canis* type 1; 6-*P.canis* type 2; 7- *P.*volantium; 8- *P.langaa*; 9- *P. anatis*; 10- *P. dagmatis*; 11- *P. gallinarum*; 12- *P. stomatis*; 13- *P. avium* Type 1; 14- *P. avium* Type 2; 15- *Pasteurella* A.; w- weak reaction; D - Deviating strains; ( ) – Delayed reaction; N – Not done; d - + and –ve reported; O/decarboxyl- ornithine decarboxylase; ONPG- ortho-nitrophenol; Glucose/A-Glucose acid production; Glucose/G-Glucose gas production; H_2S-Hydrogen sulphide production; A/dehydrolase-Arginine dehydrolase; H_2S - Hydrogen sulphide.
Sources: Mutters *et al.*, 1989; Rimler and Rhoades, 1989; Rimler *et al.*, 1998; Bergey's manual, 1994, and Christensen and Bisgaard, 2000.

2.2.1 Sero-types

Five capsular sero-types (A, B, D, E, and F) have been reported in *P. multocida*. Sero-types A, D, and F produce capsules containing mucopoplysaccharides, and presumptive identification of these sero-types can be made using specific mucopolysaccharidases and their respective antisera in a disk-diffusion test. Sero-type E has not been diagnosed in avian hosts. Of the 4 sero-types of *P. multocida* that have been isolated from avian species, sero-type A strains are the major cause of fowl cholera (Rimler and Rhoades, 1987; Rimler *et al.*, 1998). Sixteen somatic sero-types may occur among *P. multocida* organisms. Of these, somatic sero-types 1, 3, and 4 are commonly isolated (Rimler and Rhoades, 1989; Rimler *et al.*, 1998). Capsular and somatic antigens sero-typing are useful for detection and identification of this bacterium, although limited by insufficient information to distinguish different strains of the same sero-type (Amonsin *et al.*, 2002).

Serologic tests are commonly used for antigenic characterization of *P. multocida* organisms. A gel - diffusion precipitin test (GDPT) is used for sero-typing based on differences in somatic antigens. Frequently, antigens from a single strain react with more than one type of anti-serum. An indirect (passive) haemagglutination test is normally used for specific sero-grouping. It is based on differences in capsular antigens (Glisson *et al.*, 2003). Serologic tests are, however, not routinely used to detect infections by *Pasteurella* species in poultry (Rimler *et al.*, 1998).

2.3 Culture of *Pasteurella multocida*

2.3.1 General media

Dextrose starch agar (DSA), blood agar (BA), or trypticase soy agar are recommended for primary isolation of *P. multocida*. Isolation is improved by supplementing these media with 5 % heat - inactivated serum. Colonies appear as previously described (Rimler *et al.*, 1998).

2. 3.2 Selective media

Selective enrichment media for *P. multocida* are prepared by addition of anti-microbial substances to the recommended growth agar or broth (Rimler and Rhoades, 1989). Avril *et al* (1990) made such a medium for isolation of *P. multocida* from pigs and contaminated animal sources. Moore *et al.* (1994) developed enrichment media for isolating *P. multocida* from avian and environmental samples by addition of gentamycin (0.75 µg/ml), potassium tellurite (2.5µg/ml), and amphotericin B (5.0µg/ml) to brain heart infusion agar and broth. Muhairwa *et al.* (2001a,b) used selective media to investigate *P. multocida* carrier birds, by addition of bacitracin (5000U/l), polymyxin B (20µg/l), and gentamycin (30µg/l) to blood agar. Lee *et al.* (2000) developed a selective medium with polymyxin B, crystal violet, thallous acetate, bacitracin and cycloheximide to detect *P. multocida* from the alimentary tract of chickens after experimental oral infection.

2.3.3 Use of mice for selective isolation of *P. multocida*

Mouse inoculation may improve recovery rate from field specimens. Virulent strains of *P.multocida* can be recovered from specimens (Baldrias *et al.*, 1988) dominated by other flora by subcutaneous (Rimler and Rhoades, 1989) or intra-peritoneal (Muhairwa *et*

al., 2001a,b) inoculation of mice. After death (Rimler and Rhoades, 1989) or 48 hours (mice are sacrificed) (Muhairwa *et al.*, 2001a,b) cultures are made from the liver, spleen and heart (Rimler and Rhoades, 1989) or from spleen only (Muhairwa *et al.*, 2001a,b), by usual bacteriological methods. This way the false negative culture results that occur when many *P.multocida* bacteria die in transportation or are overgrown by other bacterial flora (nasal and contaminants) in cultures are minimized. However, mouse inoculation is expensive and therefore, not practical for extensive epidemiological research or routine diagnostic testing. In the latter, selective media can be used (Rimler and Rhoades, 1989).

2.4 Virulence factors

No single virulence factor has alone been associated with the virulence of *P. multocida*. Many factors are involved (Christensen and Bisgaard, 2000). These include the capsule, endotoxin, outer membrane proteins, iron binding systems, heat shock proteins, neuraminidase production, and antibody cleaving enzymes. Production of RTX (repeats in toxin: related toxins that share structural features consisting of tandemly arranged repeats of a nine-amino-acid sequence) family of cytolytic toxins that are of major importance in the pathogenesis of some members of the family *Pasteurellaceae* has not been observed in *P. multocida* (Christensen and Bisgaard, 2000).

2.5 Transmission of *P.multocida*

Different routes of *P. multocida* administration may cause differences in severity of the fowl cholera in birds (Derieux, 1983; Pehlivanoglu *et al.*, 1999). On intravenous administration of *P. multocida* to the pekin ducks, there was more mortality than when

the bacteria were given through intranasal, intra-ocular, oral, and subcutaneous routes (Pehlivanoglu *et al.*, 1999) while oral route was found better than stick-wing for the quails (Derieux, 1983).

2.5.1 Contact transmission

Transmission through contact has been shown between dogs (Loubinoux *et al.*, 1999) from ewe to lambs (Jaworski *et al.*, 1998) and between turkey flocks (Christiansen *et al.*, 1992). Pathogenicity of *P. multocida* is however, increased by bird-to-bird passage of over a short period of time (Matsumoto and Strain, 1993).

2.6 Epidemiology of *P.multocida*

Pasteurella multocida has been isolated in a wide range of hosts worldwide among terrestrial, as well as aquatic species of mammals and birds (Christensen and Bisgaard, 2000). The mechanisms of colonization, survival, multiplication, invasion and pathogenic action of *P. multocida* are incompletely understood (Bisgaard, 1993). The natural reservoir of *Pasteurella* seems to be the mucosal membranes of the respiratory, genital and intestinal tract of mammals and birds where they exist as opportunistic pathogens. However, certain virulent strains of *P. multocida* occur as obligate parasites (Christensen and Bisgaard, 2003). In animals, the respiratory tract is reported as the frequent source of *P. multocida* isolates (Bisgaard, 1993). Exchange of *P. multocida* subspecies *multocida* between wild birds and domestic poultry has also been reported (Faddoul *et al.*, 1967; Christensen *et al.*, 1998; Petersen *et al.*, 2001b). This presents a risk to domestic birds especially those under industrial production. In Tanzania, *P. multocida* was isolated from

0.7% of scavenging chickens, 7 % of ducks, 4 % of dogs and 51 % of cats investigated (Muhairwa *et al.*, 2001b).

A possibility of dogs, cats and pigs acting as reservoirs for *P. multocida* virulent for poultry has been suggested (Muhairwa *et al.*, 2001b; Glisson *et al.*, 2003). However, there was no genotypic similarity of *P. multocida* isolates from chickens, ducks, dogs and cats in Tanzania (Muhairwa *et al.*, 2001a), hence a need for further study on potential spread between animal groups (Christensen and Bisgaard, 2003). Infected birds remain carriers of the organism for a long time and may spread *P. multocida* to healthy flocks (Carpenter *et al.*, 1989; Muhairwa *et al.*, 2000). Spread of infection through aerosol, equipments and insects seems to be of less importance (Simensen and Olson, 1980; Glisson *et al.*, 2003). Under natural conditions, mortality may range from only a few percent to close to 100 % (Glisson *et al.*, 2003). The molecular ribotyping of *P. multocida* from fowl cholera outbreaks in turkeys (Blackall *et al.*, 1999), ducks (Muhairwa *et al.*, 2001a), pigs (Bowles *et al.*, 2000), and dogs (Loubinoux *et al.*, 1999) have been clonal, with different clones involved in different outbreaks.

Pasteurella multocida may be transferred from domestic animals, including healthy looking pets, to result in serious disease in man (Christensen and Bisgaard, 20003).

2.6.1 *P. multocida* carrier status

Potential of cats being carriers of *P. multocida* has been reported (Baldrias *et al.*, 1988; Korbel *et al.*, 1992; van Sambeek *et al.*, 1995). Christensen *et al.* (1998) have reported a single clone to be involved in fowl cholera outbreaks among several avian species including semi-confined commercial poultry. In Tanzania, *P. multocida* subspecies were isolated from village chickens and ducks, as well as cats and dogs

(Muhariwa *et al.*, 2001b). Isolation of *P. multocida* from apparently healthy poultry has often been associated with previous outbreaks of fowl cholera in the flocks (Curtis and Ollerhead, 1981; Carpenter *et al.*, 1989).

The importance of wild birds as a reservoir of *P. multocida* was demonstrated experimentally by Petersen *et al.* (2001b), however, the potential of spread of this organism between different animal groups require further study (Christensen and Bisgaard, 2003).

2.7 Fowl cholera

2.7.1 General remarks

Fowl cholera is an acute fatal septicaemic infection of birds, caused by *P. multocida,* with high morbidity and mortality (Faddoul *et al.*, 1967; Glisson *et* al., 2003). Chronic fowl cholera may follow the septicaemic stage, particularly when the disease is by organisms of low virulence (Rimler *et al.*, 1998). Fowl cholera occurs as a primary disease that does not require predisposing factors (Glisson, 1998), although predisposing factors may increase severity of outbreaks. Birds that have survived outbreaks of the disease normally remain carriers of the organism (Rimler *et al.*, 1998; Muhairwa *et al.*, 2000). Most species of birds are susceptible to infection with *P. multocida;* the organism has been isolated from more than 100 different varieties of wild and domestic birds (Faddoul *et al.*, 1967; Heddleston *et al.*, 1972; Curtis and Ollerhead, 1981; Botzler, 1991) and in commercial duck farms (Takahashi *et al.*, 1996) where it occurs as sporadic or epizootic outbreaks. It may affect birds of any age, but it rarely occurs in commercially raised poultry of less than 8 weeks of age (Rimler *et al.*, 1998).

Susceptibility of the domestic fowl to fowl cholera is in the following order turkeys, pheasants, and Japanese quail as the most susceptible species, followed by web – footed birds (Botzler, 1991), while chickens are considered relatively resistant (Glisson *et al.*, 2003) compared with turkeys. Mature chickens (over 16 weeks) are reported to be more susceptible than young chickens (Heddleston and Watko, 1965; Christensen *et al.*, 1998). Caponization of tom turkeys did not significantly have a difference in the susceptibility of these birds to *P. multocida* (Friedlander *et al.*, 1992).

2.7.2 Pathogenesis

Under natural conditions, *P. multocida* organisms infect birds via the mucous membranes of the upper air passages (Matsumoto *et al.*, 1991). After oral infection, survival of *P. multocida* through the gastrointestinal tract appears limited (Iliev *et al.*, 1964) but Muhairwa *et al.* (2000 and 2001b) have demonstrated carrier birds using cloacal swabs. *P. multocida* may also enter through cutaneous lesions (Christensen and Bisgaard, 2000). Following the initial natural infection through airways, the *P. multocida* spreads to the lungs and multiplies there before entering the blood stream. In the blood stream, it multiplies rapidly and localizes in the liver and spleen, where multiplication occurs resulting in massive bacteraemia. Death is presumed to be due to the effects of endotoxin (Christensen and Bisgaard, 2000).

2.7.3 Clinical disease and its course

Sudden death may be the first indicator of fowl cholera in chickens. Typical clinical signs are depression, diarrhoea, ruffled feathers, increased respiratory rate, and cyanosis (Rimler *et al.*, 1998). Experimental disease produced by swabbing of the palatine cleft, may cause mortality of 90-100% in mature chickens within 24 – 48 hours, depending on

the strain of *P. multocida* used, but most spontaneous contact infections cause 10 – 20 % mortality of adult birds within 2 weeks (Glisson *et al.*, 2003).

2.7.4 Gross lesions

Acute fowl cholera commonly expresses lesions of passive hyperemia, swollen liver and spleen, fibrino-purulent air-sacculitis, and increased pericardial and peritoneal fluids (Rhoades, 1964; Hunter and Wobeser, 1980; Rimler *et al.*, 1998; Aye *et al.*, 2000). Birds with chronic fowl cholera may show swellings in joints, wattles, sternal bursae and exudates in the conjunctiva and nasal turbinates (Christensen and Bisgaard, 2000). Chickens often develop localized pneumonic lesions prior to death but turkeys show severe necrotic pneumonia and 100 % mortality after 48 hours of infection (Petersen *et al.*, 2001b).

2.7.5 Histopathological lesions

The standard histological lesions occur as fibrino-purulent inflammation of the lungs and airsacs characterized by heterophilic infiltrations, fibrin deposition, oedema, congested blood vessels and vasculitis (Rimler *et al.*, 1998). In some cases pyogranulomatous inflammatory reaction is present in various tissues, with giant cells, macrophages and lymphocytes surrounding heterophils and cell debris (Aye *et al.*, 2000; Petersen *et al.*, 2001b). There is extensive damage in the lungs, sinuses, airsacs, and the liver (Glisson *et al.*, 2003).

2.8 Fluorescent *in situ* hybridization (FISH) test

In situ hybridization (ISH) was developed in 1969 (John *et al.*, 1969; Pardue and Gall, 1969) as a technique for the localization of specific nucleic acid sequences within

individual cells either in tissue sections or in whole cell preparation (Giovannoni *et al.*, 1988; McNicol and Farquharson, 1997). Giovannoni *et al.* (1988) introduced ISH for the first time in bacteriology when they used radioactively labeled rRNA-oligonucleotide probes for the microscopic detection of bacteria.

A nucleic acid probe is a single-stranded fragment of DNA or RNA that has been labeled so that it can be detected following hybridization reaction. The nucleic acid probe sequence is complimentary to that of the segment of interest and is prepared such that it hybridizes only to the unique region on a genome and specifically detects the causative agent (Tenover, 1988; Jackwood and Jackwood, 1998). Specificity of probe binding is influenced by the composition of the probe where G-C bonds are stronger than A-T (U), the temperature, the salt concentration and the content of formamide, a denaturing agent, in the hybridization mixture, and the post-hybridization washes. These are calculated to minimum non-specific binding while leaving specific hybrids intact for detection (McNicol and Farquharson, 1997). FISH method allows the cell morphology, abundance and spatial distribution to be analyzed *in situ* (Delong *et al.*, 1989; Amann *et al.*, 1991 and 1995; Poulsen *et al.*, 1994), especially in diagnostic application such as identification of gene expression by detection of mRNA, diagnosis of infections, cell cycle or apoptosis, and in interphase cytogenetics in the investigation of fetal abnormalities and tumors (McNicol and Farquharson, 1997). Thus, FISH is a major tool for the identification of infectious agents in tissues using unique ribosomal RNA sequences (Montone, 1994). However, few studies have used ISH in the detection of bacterial infection (McNicol and Farquharson, 1997).

The FISH test is an important method for the detection of nucleic acid target sequences within cell and tissue specimens (Brown, 1998). The FISH technique allows simultaneous identification, enumeration and localization of individual microbial cells, in all fields of microbiology. FISH can be used to detect both the cultured and uncultured or yet-to-be cultured microorganisms. This method combines the precision of molecular genetics with the visual information from microscopy to permit visualization and identification of individual microbial cells within their natural microhabitat or diseased tissues (Moter and Göbel, 2000). Compared to the radioactive probes, fluorescent probes are safer, have better resolution without additional detection steps, and can be labeled with dyes of different emission wavelength target (Moter and Göbel, 2000). The sensitivity and speed offered by FISH has made it a powerful tool for phylogenetic, ecologic, diagnostic, and environmental studies in microbiology (Amann *et al.*, 1990a,b). To allow the detection of the signal, the probe is conjugated with rhodamine; fluorescein-derivatives [fluorescein-isothiocyanate (FITC), 5 - (-6-) carboxyfluorescein-N-hydroxysuccimide-ester (Fluox)], rhodamine-derivatives {(tetramethyl-rhodamine-isothiocyanate) (TRITC)}, Texas red, digoxigenin and cyanine dyes (CY^3 and CY^5). The latter ones have been shown to be superior to classical dyes because they have brighter staining and are very stable to photo bleaching (Wescendorf and Brelje, 1992). Previously, radioactive labeling was the standard procedure. But with the development of fluorescent labels (Delong *et al.*, 1989), radioactive labels were steadily overtaken by non-isotopic dyes (Moter and Göbel, 2000). Delong *et al.* (1989) first used fluorescently labeled oligonucleotides for the detection of single microbial cells.

In microbiology the target for FISH is 16S rRNA. This is because of genetic stability of its dormain structure with conserved and variable regions, its high copy number (Woese, 1987), and the fact that genus-specific and species-specific probe can be designed according to the region of rRNA targeted (Amann *et al.*, 1995). Other targets like 23S rRNA, 18S rRNA and mRNA can also be detected by FISH (Amann *et al.*, 1991 and 1995; Moter and Göbel, 2000). When FISH is carried out on tissue sections, pretreatment procedures have been applied to increase accessibility of the probe to the target and to reduce non-specific binding. For paraffin sections, de-waxing with xylene prior to the hybridization step is mandatory (Boye *et al.*, 1998a,b and 2000). When compared to microbiological culture or *in vitro* amplification of nucleic acids, FISH is fast, cheap and easy to carry out, and valuable for the detection of slow-growing, fastidious or un-culturable bacteria (Moter and Göbel, 2000). It can be developed quickly, allow a high spatial resolution and are sensitive. Despite of this, FISH detects only medium to high copy nucleic acid targets. Target amplification strategies, such as, direct or indirect *in situ* polymerase chain reaction (IS-PCR) and self – sustained sequence replication, have enabled increased sensitivity in detecting low-copy nucleic acids in formalin–fixed, paraffin-embedded tissue specimens. Using FISH technique, single infected cells can be identified in a section (Jackwood and Jackwood, 1998) and can localize an organism and provide information about the area in which it exists (Brown, 1998). FISH test has not been used to detect *P. multocida* cells in culture or tissues previously from indigenous chickens and ducks.

CHAPTER 3

3.0 EXPERIMENT 1: CARRIER STATUS OF *PASTEURELLA MULTOCIDA* IN FARMED AND TRADED HEALTHY-LOOKING SCAVENGING INDIGENOUS CHICKENS AND DUCKS IN KENYA

3.1 Introduction

The three subspecies of *P. multocida* infect many animal species and humans (Bisgaard, 1993; Frederiksen, 1993). *P.multocida* and fowl cholera have been reported in poultry in various countries in Africa such as Nigeria (Salami *et al.*, 1989; Oladele and Raji, 1997; Oladele *et al.*, 1999), Zimbabwe (Kelly *et al.*, 1994; Mohan *et al.*, 2000), and Tanzania (Muhairwa *et al.*, 2001b). Occasionally healthy waterfowls that carried *P. multocida* in the nasal or oral cavity have been reported (Hunter and Wobeser, 1980) while wild birds are documented to be reservoir for *P. multocida* organisms (Faddoul *et al.*, 1967; Snipes *et al.*, 1990). Furthermore, the reservoirs for *P. multocida* as a source for the bacterium for susceptible turkeys are unknown (Snipes *et al.*, 1988). Wild birds especially migratory ones, whether clinically infected or as healthy carriers may be the source of infection for poultry flocks (Gooderham, 1999). Rats are also a reservoir for *P. multocida,* while the bacterium has been demonstrated in poultry flocks (chickens, turkeys and ducks) that have had no history of the disease (Gooderham, 1999). It is reported that, just as for other diseases, feed consumption and productivity of indigenous chickens (such as egg production, fertility and hatchability) can be affected by respiratory diseases (Negesse, 1993), especially fowl cholera (Oladele *et al.*, 1999) it can cause heavy losses (drop in egg production and mortalities) in commercial poultry enterprises

(Carpenter *et al.*, 1989; Morris *et al.*, 1989; Rhoades and Rimler, 1989; Barnum, 1990). In Kenya there is very little documentation on the occurrence *P. multocida* in chickens and ducks since the case report in a grey parrot by Miringa (1975). Therefore recovery of *Pasteurella* species from indigenous chickens and ducks would directly confirm the occurrence of these organisms in farmed and traded indigenous birds in Kenya.

The objective of this study was therefore to determine the carrier status of *P. multocida* in farmed and traded indigenous chickens and ducks in Kenya.

3.2 Materials and methods

3.2.1 Birds from farms

Indigenous chickens in Kenya were sampled from 4 farms in Embakasi division, Nairobi; one farm from Githunguri division, Kiambu district; and 3 farms from Athi river division, Machakos district. Ducks were sampled from 3 farms in Embakasi and one farm from Dagoretti division, Nairobi; and a farm each from Kikuyu and Githunguri divisions, Kiambu district. In all, 88 indigenous chickens and 47 ducks were sampled from the 14 farms. The chicken flock sizes ranged from 20 to 74 birds per farm. Six to 35 birds were sampled from each chicken farm (Appendix 1). In the six duck farms, the bird population ranged from 18 to 85 birds per farm and 6 to 12 birds were sampled from each farm during this study (Fig. 1; Appendix 1).

FIG.1. A FLOCK OF FARMED INDIGENOUS CHICKENS AND DUCKS AT KATANI

3.2.2 Traded and slaughter birds

A total of 45 birds were sampled from 2 slaughterhouses in Nairobi. Of these, 32 birds were sampled during slaughter at Kariokor slaughterhouse. These had originated from Kitui (12), Bomet (10), Kericho (6), and Uasin Gishu (4) rural districts. The other 13 birds were sampled during slaughter at Burma-Maziwa slaughterhouse. They had originated from Makueni rural district. Live birds were sampled in the laboratory or the site of purchase at Jogoo road (Fig.2), Kariokor and Kasarani open-air markets, and Nairobi South and Westlands shopping centres. At the time of purchase or sampling, the bird's district of origin was recorded from the traders. In all, 29 chickens and 7 ducks were sampled from the open-air markets and centres in Nairobi. Of the 29 chickens, 9 sampled from the Jogoo road market originated from Bomet (4), Kisii (3), and Makueni (2) rural district; seven sampled from Kariokor market originated from Kitui (1), Makueni (2), Mwingi (2) and Uasin Gishu (2) rural districts; eleven sampled from Nairobi South centre originated from Machakos (8) and Makueni (3); while the remaining 2 chickens sampled at Westlands centre originated from Mwingi district. The seven ducks were sampled from Kasarani open-air market and originated from Nairobi (Fig.2; Appendices 2 and 3).

FIG.2. INDIGENOUS CHICKENS ON OPEN BASKETS AT JOGOO ROAD MARKET, NAIROBI

3.2.3 Sampling procedure

Each of the one hundred sixty two indigenous chickens and 54 ducks was swabbed on the oro-pharynx and cloaca separately using sterile cotton-tipped applicator swabs. A total of 432 swabs were collected. The swabs were placed in 2 ml of sterile physiological saline and transported in a cool box to the laboratory for screening for *Pasteurella* spp.

3.2.4 Isolation and identification of bacterial isolates

Each swab was thoroughly vortexed, streaked on blood agar plates (BA, Oxoid Ltd., CM55, Basingstoke, Hempshire, England) that were then incubated aerobically at 37 ° C for 24 hours, for initial culture. The remaining 0.5 ml of the contents of the swab was inoculated into *Pasteurella* – free 21day-old Balb C mice by intra-peritoneal route as described by Muhairwa *et al.* (2001b) to improve the bacterial recovery rate from these samples. Prior to this use, 5 of the mice from the colony were sacrificed, screened, and certified free of *Pasteurella* spp. Inoculated mice were observed for 48 hours after which the surviving mice were sacrificed. The mice were then dissected and the livers and spleens aseptically removed, and macerated. The macerated material was streaked onto blood agar and incubated aerobically at 37 ° C for 24 hours. The bacteria were identified according to the criteria given in the 9th edition of the Bergey's manual (1994).

Bacterial colonies from initial swabs and mice organs morphologically resembling those of *P. multocida* (Rimler *et al.*, 1998) were sub-cultured on BA and McConkey agar. *Pasteurella* organisms recovered from cloacal and oropharyngeal swabs of the same bird, whether directly on BA or via mouse inoculation were regarded as one isolate.

The *P. multocida* subspecies were differentiated following the procedures described elsewhere (Dorsey, 1963; Bisgaard and Mutters, 1986). Pure colonies were inoculated

into bijou bottles (with Durham tubes to detect gas formation) containing glucose broth, arabinose, dulcitol, fructose, galactose, maltose, mannitol, mannose, salicin, sorbitol, trehalose, xylose, tryptose and nitrate broth, and urea agar, incubated aerobically at 37 ° C overnight. An overnight tryptose culture was tested for indole production. Nitrate reduction and motility tests were performed. The biochemical reactions of the isolates were compared with those of *P. multocida*, NCTC 10322 ^{T} (T = type strain; NCTC = national collection of type cultures, London, United Kingdom; kindly donated by Professor Magne Bisgaard of the Royal Veterinary and Agricultural University, Denmark).

3.3 Results

In a period of 14 months (August 2001 to September 2002) a total of 432 oropharyngeal and cloacal swab samples from 216 indigenous birds (162 chickens and 54 ducks) were screened for *Pasteurella* species on both culture and mouse passage. They yielded 24 *Pasteurella* species and many other aerobic bacterial isolates. The organisms were isolated from both the cloacal and oropharyngeal swabs.

Table 2 shows the biochemical characteristics of the *Pasteurella* species isolated in this study and those of *P. multocida* type culture NCTC 10322^{T}. All the twenty four *Pasteurella* species isolates fermented glucose (without gas formation), fructose, galactose, mannitol, mannose, and did not ferment salicin, dulcitol or maltose. They showed no haemolysis on blood agar. They were urea negative and did not grow on MacConkey agar. All reduced nitrate and produced indole. Their colonies were oxidase and catalase positive. These are features that are characteristic of *P.multocida*.

Differentiation into subspecies was determined using the reactions on trehalose, xylose, sorbitol and arabinose.

Seven isolates characterized as *P. multocida multocida* fermented xylose, sorbitol, trehalose (weakly) but not arabinose while 12 isolates that fermented xylose and sorbitol (weakly) but not trehalose and arabinose were characterized as *P. multocida gallicida* and 5 isolates which fermented trehalose, xylose, arabinose (weakly) but not sorbitol were characterized as *P. multocida septica*.

Table 3 and Appendices 1 to 3 show the different *Pasteurella* species recovered from apparently healthy birds from various localities. Of the 216 birds sampled, 24 (11.11%) yielded *Pasteurella* organisms; thus 10 (4.63%) from chickens and 14 (6.48%) were from the ducks. The proportion of infected ducks is significantly higher than that of infected chickens ($P< 0.05$). At the same time, the proportion of infected traded birds, 13/81 (16%) was also significantly higher than that of farmed birds, 10/135 (8.1%) ($P< 0.05$). Of the 10 chicken isolates, five *P. multocida gallicida* isolates were from Machakos (2), and Makueni (3) districts; two P. *multocida septica* isolates were from Machakos district and three *P. multocida multocida* from Kiambu (1) and Kisii (2) districts. Of the 14 duck isolates, seven *P. multocida gallicida* were from Kiambu (3) and Nairobi (4); four *P. multocida multocida* from Kiambu (3) and Nairobi (1); and three P. *multocida septica* were from Nairobi (2) and Kiambu (1).

Table 2: Biochemical reactions used to differentiate the *P. multocida* isolates and their comparison with the type strain

Bacterial Code	NCTC10322^T	PGM 1	PGM 2	PGM 3	PGM 4
Nitrate	+	+	+	+	+
Indole	+	+	+	+	+
Glucose	+	+	+	+	+
Urea	-	-	-	-	-
Mannose	+	+	+	+	+
Fructose	+	+	+	+	+
Salicin	-	-	-	-	-
Maltose	-	-	-	-	-
Galactose	+	+	+	+	+
Trehalose	+/-	+	-	+/-	-
Xylose	+	+	+	+	+
Sorbitol	+	-	+/-	+	+/-
Arabinose	-	+/-	+	-	-
Dulcitol	-	-	-	-	-
Growth in McConkey	-	-	-	-	-
Haemolysis	-	-	-	-	-
Motility	-	-	-	-	-
Identification	*P.m.multocida*	*P.m.septica*	*P.m.gallicida*	*P.m.multocida*	*P.m.gallicida*
Number of isolates		5	7	7	5

Legends:

+ positive; - negative; +/- weak positive

T = type strain

NCTC = national collection of type cultures, London, United Kingdom

PGM 1, 2, 3, 4 – Paul G. Mbuthia isolate 1, 2, 3 and 4.

Table 3: Showing total *P. multocida* subspecies isolated from farm and traded indigenous birds by samples sourced from different localities in Kenya

Sample source	Total birds sampled	District Source	No. Birds	*Pasteurella* species isolated		
A.Chickens				**Isolates**		**Subspecies of *Pasteurella***
				Number	**%[c]**	
Farm birds	88	Kiambu	35	1	2.9	*P.m.multocida*
		Others	53	0	0	None
Slaughter birds	45	Makueni	13	1	7.7	*P.m.gallicida*
		Others	32	0	0	None
Market Birds	29	Kisii	3	2	66.7	*P.m.multocida*
		Machakos	8	2	25.0	*P.m.gallicida*
				2	25.0	*P.m.septica*
		Makueni	7	2	2.9	*P.m.gallicida*
		Others	11	0	0	None
Subtotal	**162**			**10**	**6.2[a]**	
B. Ducks						
Farm Birds	47	Nairobi	33	1	3.0	*P.m.multocida*
				2	6.0	*P.m.septica*
		Kiambu	14	3	21.5	*P.m.multocida*
				3	21.5	*P.m.gallicida*
				1	7.2	*P.m.septica*
Market Birds	7	Nairobi	7	4	57.1	*P.m.gallicida*
Subtotal	**54**			**14**	**25.9[b]**	
Total	**216**			**24**	**11.1**	

C. Total recovered *Pasteurella multocida* subspecies

Subspecies	**Number of isolates**	**%[c]**	**Chickens**	**Ducks**
P.m.multocida	7	29.0	3 (30%)	4 (29%)
p.m.gallicida	12	50.0	5 (50%)	7 (50%)
p.m.septica	5	21.0	2 (20%)	3 (21%)
Total	**24**	**100.0**	**10 (42%)**	**14 (58%)**

Legends: *P.m.multocida – Pasteurella multocida multocida*; *P.m.gallicida - Pasteurella multocida gallicida*; *P.m.septica - Pasteurella multocida septica*; %[c] -- percentage isolates; (X^2 calculated [(a,b)] = 16, is significantly higher than tabulated X^2 =3.84 ($P< 0.05$)

3.4 Discussion

Systematic investigation on the occurrence of *P. multocida* subspecies has previously been carried out on healthy farm chickens but not on traded (slaughter and live market) indigenous birds (Curtis and Ollerhead, 1981; Muhairwa *et al.*, 2000 and 2001b), waterfowls, and wild birds (Faddoul *et al.*, 1967; Hunter and Wobeser, 1980; Gooderham, 1999). Such a study had not been carried out in Kenya.

Based on the classification of Mutters *et al.* (1985b) and as biochemically characterized by Dorsey (1963), three subspecies of *P. multocida* were demonstrated in this study. The *Pasteurella* isolates emanated from birds coming from different districts of Kenya, namely; Kiambu, Kisii, Machakos, Makueni and Nairobi. In this study, the majority of *P.multocida* isolates were *P.m.gallicida* (12/24), followed by *P.m.multocida* (7/24), and *P.m.septica* (5/24). Although previous studies have shown that healthy carriers of *P. multocida* are associated with outbreaks of fowl cholera (Curtis and Ollerhead, 1981), this could not be demonstrated in this study. Traded birds are transported to Nairobi using public transport and other types of vehicles in open crates, coops or cages, inside or on top of vehicles to reach the slaughterhouses and market centres, conditions that can easily precipitate the multiplication of *P. multocida* in the carrier birds (Blackall *et al.*, 1995). In addition to transportation the birds are held in crowded cages at various marketing points (Fig. 2), fed and housed in coops that are never cleaned or disinfected, environment that can easily be a vehicle for various pathogens including *P. multocida.* This can expose market birds to *P. multocida*, its multiplication, leading to outbreak of fowl cholera at the marketing points. Although, *P. multocida* was isolated from both farmed and traded birds, it is not surprising that the

proportion of infected traded (market and slaughter) birds (16%) was higher than that of farmed (8.2%) ones. Infected birds can easily disseminate *P. multocida* along the transport route, and at trading points through their discharges and excretions, hence, pose as potential reservoir to commercial poultry. Besides other uses, the indigenous chickens can be used as gifts in many African countries (Whyte, 2002) including Kenya. Some of these gift birds are slaughtered at the home of destination while others become part of the farm's breeding stock and may be potential carriers of. *P. multocida.* This may pose a big risk to farms with commercial flocks when they come in contact with carrier birds.

Unlike Muhairwa *et al.* (2001b) who isolated *P. multocida* in 0.7% (2 out of 330) of the birds examined, in this study, it was isolated at the rate of 4.63% (10 out of 216) from the indigenous chickens. Njue *et al.* (2002) in their study of poultry problems among indigenous chickens in Kangundo division of Machakos district did not isolate any *P. multocida.* However, in this study four isolates are reported in healthy birds from the same division. The carrier rate for *P.multocida* appears to be low. It is possible therefore that there were no birds with overt fowl cholera disease during the study by Njue *et al.* (2002) albeit there being subclinical infections in birds. From 54 duck samples, 25.9% (14 out of 54) had *P. multocida* organisms isolated with half of them being *P.m.gallicida.* The duck samples had more *P. multocida* isolates than the chickens as reported elsewhere (Muhairwa *et al.*, 2001b) although the duck samples were fewer than those of chickens. This may imply that, the duck is an important reservoir of *P. multocida* in the field.

Muhairwa *et al.* (2001b) reported only *P. multocida multocida* from chickens. In this study all the three subspecies of *P. multocida* (*P. multocida multocida*, *P. multocida gallicida*, and *P. multocida septica*) were isolated from healthy farm and traded (market

and slaughter) birds. The organisms were recovered from both the oropharyngeal and cloacal swabs, but more frequently from the oropharyngeal than the cloacal region, as reported by others (Lee *et al.*, 2000).

The last documented case of fowl cholera and *P. multocida* in Kenya was in a grey parrot (Miringa, 1975). The current study documents the isolation of *P. multocida* from indigenous chickens and ducks raised under the village free-range management in Kenya indicating potential danger of contracting fowl cholera for the commercial chicken industry.

CHAPTER 4

4.0 EXPERIMENT 2: AGE SUSCEPTIBILTY OF INDIGENOUS CHICKENS TO *P. MULTOCIDA*

4.1 Introduction

In Kenya, poultry population is 29 million of which over 70% are indigenous local chickens (Anonymous, 2000; Njue, 2003). One of the constraints to poultry production is fowl cholera, a disease caused by *P. multocida* (Mutters *et al.*, 1985a,b) and which is reported to be more common in late stage of growers and adult birds (Rhoades and Rimler, 1989; Gooderham, 1999).

The degree of susceptibility to *P. multocida* varies among different types of birds and different age groups within a type (Rhoades and Rimler, 1989). However, all species of birds are susceptible (Gooderham, 1999) to *P. multocida* infections. *P. multocida* carriers are present in indigenous chickens in Kenya as has been previously reported in this study and in Tanzania (Muhairwa *et al.*, 2001 b). Fowl cholera has in the past been diagnosed in mature chickens of 30-35 weeks of age (Salami *et al.*, 1989) and in commercial turkeys aged 7-16 weeks old (Morris *et al.*, 1989). The age group of indigenous chickens most susceptible to *P.multocida* infection has not been determined yet.

The aim of this study was therefore to establish the most susceptible age group among the indigenous chickens to *P. multocida* in Kenya.

4.2 Materials and methods

4.2.1 Experimental chickens

Indigenous chicks hatched from incubated eggs at Kabete and day old chicks bought from farms with indigenous flocks were brooded and reared in an isolation house, away from other birds. They were fed on commercial chick and grower's feed (Unga Limited, Kenya) and given water *ad libitum*, up to the required experimental age of 4, 8, 12, and 16 weeks. A total of one hundred and twenty indigenous chickens comprising of 30 birds of each stated age group were used in this study. Each bird was wing tagged.

4.2.2 Bacteria used to infect chickens

Pasteurella multocida reference type strain (NCTC 10322^T) that is of low virulence was used in this study. It was maintained on Dorset egg agar, in our laboratory. To prepare innoculum, for the birds, it was spread onto blood agar (BA) plates (enriched with 5 % citrated calf blood), incubated aerobically at 37 °C, for 24 hours to check for purity. An individual colony of this culture was inoculated into brain heart infusion broth and incubated aerobically at 37 °C, for 24 hours. The concentration of bacteria in this broth was determined by the colony forming unit plate method. Briefly, a ten fold serial dilution of one milliliter of the bacterial broth was made in sterile saline. A volume of twenty five microlitres from each dilution was spotted onto BA plates in duplicate. The plates were incubated for 18 hours. Colonies were counted and colony forming units per ml calculated. The broth culture was appropriately diluted to obtain the required concentration of bacteria in the innocula used.

4.2.3 Experimental procedure

Each chicken age group had 15 birds, of which 10 were infected with *P. multocida* while 5 were used as controls and were inoculated with BHI broth only. The experiments were replicated. The infected and control groups were housed separately in different rooms located in different houses away and apart from each other.

Before commencement of the experiment, all birds were screened by taking their oropharyngeal and cloaca swabs that were cultured onto BA and also inoculated intraperitoneally into mice as done in Chapter 3.2.4 of this study. All birds were shown to be negative for *P.multocida* infection. The birds were put into groups and housed in the experimental rooms 48 hours prior to inoculation. Table 4 shows the outline of the experimental design.

Table 4: The experimental design on age susceptibility in chickens (each experiment was duplicated)

Chicken age groups	Infected Birds	Control birds	Total birds
4 week-old	10	5	15
8 week-old	10	5	15
12 week-old	10	5	15
16 week-old	10	5	15
Total birds	**40**	**20**	**60**

The innoculum was prepared as described above and appropriately diluted (Petersen *et al.*, 2001b) and each bird was inoculated intratracheally with 0.5 ml culture containing 1.2 – 2.0 x 10^8 colony forming units of *P.multocida* organisms in BHI. Each of the control bird was inoculated with 0.5 ml of BHI broth. To avoid possible cross infection the daily observations started with birds in the control house before proceeding to the infected house. Biosecurity was maintained during the entire period of the study.

Clinical sign observations, temperature and weight measurements were carried out and recorded once daily, by the same person throughout the study period. In each room, of the control or infected birds, the observer took 30 minutes per day at the same time each day to observe the presenting clinical signs and carry out the measurements. Each time, initially the observations were made without disturbing the birds and later a close examination was carried out while holding each bird that is wing tagged for identification. During the latter examination, the bird's cloacal temperature was taken using a clinical thermometer (laboratory thermometer 305 mm); the reading was recorded and the thermometer disinfected with alcohol. After close examination, the bird's weight was taken using Soehnle German balance (measuring a minimum of 25 grams and a maximum of 5 kilograms) and recorded.

The birds were examined for the presence anorexia, fever, ataxia, depression, ruffled feathers, cyanosis, nervous tics, nasal and mouth discharges, sneezing, dyspnoea, spontaneous tracheal rales, diarrhoea and head scratching. Pathological lesions in poultry due to various causes are semi-quantitatively scored (Gross and Siegel, 1965; Gross and Colmano, 1967; Shivaprasad and Droual, 2002) and a similar trend was adopted for clinical signs in this study. The frequency and intensity of the clinical signs were graded in semi-quantitative manner as severe (3), moderate (2), or mild (1). Clinical signs were mild if a sign observed 1 to 3 times; moderate if the signs were 4-6 times; and severe when observed 7 or more times on an individual bird during the 30 minutes period of clinical sign observations. Mild depression was dullness alone; when it occurred together with drooping wing it was taken as moderate depression; while dosing and/or tucked head under the wings was regarded as severe depression. Ruffled feathers around the head and neck was

taken as mild; when general body was affected it was taken as moderate; while with drooping wings it was regarded as severe. Cloacal temperature below 41.5 °C was taken as normal; 41.6 – 41.8 °C as mild; 41.9 - 42.1 °C as moderate; and that of over 42.2 - 42.5 °C was taken as severe fever. Post mortem examination was done as described by Bermudez and Stewart-Brown (2003). The sacrificed birds were opened aseptically at the end of the study and post mortem examination done and swabs taken from the oro-pharynx, cloaca, lungs, liver, spleen, caecal tonsils, and pruning gland, for bacterial examination.

During the post mortem examination, gross lesions were noted and recorded.

4.2.4 Re-isolation and identification of *P. multocida*

At 24 hours post-infection, all the birds were swabbed on oro-pharynx and cloaca to confirm the establishment or absence of *P.multocida* in the infected and control birds, respectively. These swabs and those taken during the post mortem examination were used for the re-isolation of *P.multocida*. The re-isolation and eventual identification procedures for *Pasteurella* organisms were done as given in chapter 3, section 3.2.4.

4.3 Results

4.3.1 Type and total number of the observed clinical signs

The observed clinical signs were anorexia, fever, ataxia, depression, ruffled feathers, nasal discharges, sneezing, dyspnoea, spontaneous tracheal rales, and diarrhoea. Table 5 shows the summary of the clinical signs observed in the infected 4 week-old chickens. All the birds showed clinical signs intermittent throughout the study period, except 1 chicken that showed clinical signs on two days only. A total of 173 clinical signs were recorded. Of these, 55 were depression, 36 ruffled feathers, 29 fever, 22 rales, 13 sneezing, 10 diarrhoea, 4 dyspnoea, 3 nasal discharges, and 1 ataxia. Observed clinical signs were high from day 2

when they numbered 14, peaked on the third day to 17, and stayed high up to day 9 before decreasing gradually to 4 on day 14. Majority of the signs were observed between days 2 to 9. Nervous tics, mouth discharges, cyanosis and head scratching were not recorded in this age group (Appendix 4).

Table 6 shows the clinical signs observed in the 8 week-old chickens. Three birds showed clinical signs all days of the observation while others showed signs intermittently on various days. The observed clinical signs were the same as those reported for the 4 week-old chickens but these birds in addition also showed nervous tics and head scratching. A total of 272 clinical signs were recorded. Of these, 63 were depression, 61 ruffled feathers, 53 fever, 27 sneezing, 24 rales, 17 dyspnoea, 6 ataxia, 6 nasal discharges, 5 diarrhoea, 4 head scratching, 3 mouth discharges, and 3 nervous tics. The daily total clinical signs increased from 12 on day one to a peak of 30 on day six, and declined to 13 on last day of observation. Cyanosis was not observed in this age group. There were more clinical signs observed in this age group as compared with the 4 week-old chickens (Appendix 5).

Table 7 shows the clinical signs observed in the infected 12 week-old chickens. Three birds expressed clinical signs throughout the 14 days and another one bird had signs for 13 days, while the rest had intermittent clinical signs on various days of observation. The observed clinical signs were the same as those reported for the 4 week-old chickens but these birds also showed nervous tics, cyanosis and head scratching. A total of 372 clinical signs were recorded. Of these, 79 were depression, 74 ruffled feathers (Fig.3), 60 fever, 36 sneezing, 34 nasal discharges, 16 dyspnoea, 15 head scratching, 14 ataxia, 14 diarrhoea, 13 nervous tics, 8 rales, 6 mouth discharges, and 3 cyanosis. The observed clinical signs were

high, at 31 on day one, peaked to 35 on day two and decreased gradually to 12 on day 14 of observation. All types of clinical signs were recorded in this age group. There were more clinical signs observed in this age group than in the 4 and 8 week-old chickens (Appendix 6).

Table 8 shows the clinical signs observed in the infected 16 week-old chickens. One bird exhibited clinical signs for the entire period, another one showed signs on one day only, while 2 birds expressed no clinical signs at all. The observed clinical signs were the same as those reported for the 4 week-old chickens but these birds also showed nervous tics cyanosis and head scratching. A total of 187 clinical signs were recorded. Of these, 42 were depression, 38 fever, 27 ruffled feathers, 21 nervous tics, 20 sneezing, 13 dyspnoea, 9 diarrhoea, 7 head scratching, 4 ataxia, 2 mouth discharges, 2 rales, 1 nasal discharges, and 1 cyanosis. These signs peaked from 13 on day 1 to 21 on the third day, stayed high up to day 6 and declined gradually thereafter to 9 on day 14 of the observation. All clinical signs were recorded in this age group. The total clinical signs were almost equal to those of 4 week-old chickens but fewer than those of 8 and 12 week-old chickens, respectively (Appendix 7).

In all infected chicken age groups anorexia was observed 2 to 3 days post infection. Control birds of all chicken age groups did not show any clinical sign throughout the observation period.

The total daily clinical signs were significantly high among the 12 week-old, followed by the 8 week-old chickens while the 4 week-old and the 16 week-old were almost equal ($p<0.05$; Table 9). There were significant differences between observed clinical signs in various chicken age groups ($P<0.05$) except for diarrhoea. The nervous tics, cyanosis and

head scratching appeared to occur as chickens increased in age while spontaneous rales were significantly more in the younger birds ($p<0.05$). Other signs were many in the 12 and 8 week-old indigenous chickens than other ages (Table 10). Depression, ruffled feathers, sneezing, diarrhoea, mouth discharges, and nasal discharges varied significantly on replication ($p<0.05$), unlike other observed clinical signs. These are the signs that will always be observed in cases of fowl cholera. All the observed clinical signs varied significantly with days post infection ($p<0.05$) except sneezing, ataxia, and nasal discharges which were not significant ($p>0.05$). Diarrhoea varied significantly between replication and with days ($p<0.05$) p.i, unlike ataxia that significantly varied between chicken ages ($p<0.05$) but was not significantly affected by replication or days p.i ($p>0.05$). Dyspnoea, nervous tics, cyanosis and head scratching, significantly varied with chicken age groups and days p.i. ($p<0.05$). Ruffled feathers, sneezing, diarrhoea, and nasal discharges varied significantly between replications unlike other observed clinical signs ($p<0.05$). In all age groups, clinical signs were generally, significantly, observed in infected birds between days 1 to 9, except mouth discharges that were observed between days 11 to 14 p.i. ($p<0.05$), as compared to other days after infection.

Table 5: Type and total number of clinical signs observed daily in infected 4 week-old indigenous chickens

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	3	5	5	5	4	5	4	4	5	3	4	3	3	2	**55**
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Ruffled feathers	2	4	2	4	4	3	3	3	3	1	3	1	2	1	**36**
Sneezing	2	1	2	-	1	1	1	1	1	1	1	1	-	-	**13**
Ataxia	-	-	-	-	1	-	-	-	-	-	-	-	-	-	**1**
Nasal discharges	-	-	-	-	1	-	-	1	-	-	-	1	-	-	**3**
Dyspnoea	-	-	-	-	-	2	-	1	-	1	-	-	-	-	**4**
Mouth discharges	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Diarrhoea	-	-	3	1	-	1	1	1	-	1	1	1	-	-	**10**
Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Rales	2	2	2	2	3	-	2	1	2	-	1	1	3	1	**22**
Fever	1	2	3	2	2	-	4	3	3	2	3	3	1	-	**29**
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Total signs	**10**	**14**	**17**	**14**	**16**	**12**	**15**	**15**	**14**	**9**	**13**	**11**	**9**	**4**	**173**

Table 6: Type and total number of clinical signs observed daily in infected 8 week-old indigenous chickens

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	2	5	4	5	4	7	5	5	5	4	5	5	4	3	**63**
Nervous tics	-	1	-	1	-	-	-	-	-	-	-	-	1	-	**3**
Ruffled feathers	2	3	4	5	4	7	5	5	5	4	5	5	4	3	**61**
Sneezing	2	1	1	2	2	2	3	2	1	2	3	2	2	2	**27**
Ataxia	-	-	1	-	-	1	-	1	-	1	-	-	1	1	**6**
Nasal discharges	-	-	-	-	-	-	-	1	-	-	1	2	1	1	**6**
Dyspnoea	-	1	-	1	-	2	1	3	3	1	1	2	2	-	**17**
Mouth discharges	-	-	-	-	-	-	-	-	1	1	-	-	1	-	**3**
Diarrhoea	-	1	-	1	-	1	1	-	-	-	-	1	-	-	**5**
Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Rales	2	-	1	2	2	5	2	1	2	2	1	2	1	1	**24**
Fever	3	7	6	5	5	5	5	3	3	3	3	1	2	2	**53**
Head scratching	1	1	2	-	-	-	-	-	-	-	-	-	-	-	**4**
Total signs	**12**	**20**	**19**	**22**	**17**	**30**	**22**	**21**	**20**	**18**	**19**	**20**	**19**	**13**	**272**

Table 7: Type and total number of clinical signs observed daily in infected 12 week-old indigenous chickens

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	8	8	7	7	7	6	6	5	5	5	3	4	5	3	**79**
Nervous tics	-	-	-	1	1	3	2	1	1	2	2	-	-	-	**13**
Ruffled feathers	8	8	7	7	7	5	5	4	5	4	3	4	4	3	**74**
Sneezing	4	3	3	4	3	2	2	2	3	2	2	3	2	1	**36**
Ataxia	-	1	1	2	2	1	1	2	2	1	-	-	1	-	**14**
Nasal discharges	3	4	3	2	3	3	2	2	2	3	1	2	3	1	**34**
Dyspnoea	1	1	1	1	1	1	2	1	2	2	1	1	1	-	**16**
Mouth discharges	-	1	1	-	-	-	-	-	-	-	1	3	-	-	**6**
Diarrhoea	1	1	1	1	1	2	1	1	1	1	1	1	1	-	**14**
Cyanosis	-	-	-	-	-	1	1	-	1	-	-	-	-	-	**3**
Rales	-	1	1	-	-	-	1	1	-	-	1	2	1	-	**8**
Fever	5	2	3	4	5	4	5	6	6	4	4	4	4	4	**60**
Head scratching	1	5	3	4	2	-	-	-	-	-	-	-	-	-	**15**
Total signs	**31**	**35**	**31**	**33**	**32**	**28**	**28**	**25**	**28**	**24**	**19**	**24**	**22**	**12**	**372**

Table 8: Type and total number of clinical signs observed daily in infected 16 week-old indigenous chickens

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	4	4	4	4	3	3	2	3	3	2	3	3	2	2	**42**
Nervous tics	1	1	2	-	3	2	2	2	2	2	1	1	1	1	**21**
Ruffled feathers	3	3	4	2	3	1	1	2	2	1	1	2	1	1	**27**
Sneezing	1	-	3	2	3	1	2	1	-	1	1	1	2	2	**20**
Ataxia	-	-	1	-	-	1	-	-	-	-	-	-	1	1	**4**
Nervous discharges	-	1	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Dyspnoea	1	1	1	1	1	3	1	1	-	1	1	1	-	-	**13**
Mouth discharges	-	-	-	-	-	-	-	-	-	-	1	1	-	-	**2**
Diarrhoea	-	-	-	-	1	2	1	1	1	-	2	1	-	-	**9**
Cyanosis	-	-	-	-	-	1	-	-	-	-	-	-	-	-	**1**
Rales	-	-	-	-	-	1	-	-	-	1	-	-	-	-	**2**
Fever	2	3	4	3	1	1	2	5	3	5	3	2	2	2	**38**
Head scratching	1	2	2	1	1	-	-	-	-	-	-	-	-	-	**7**
Total signs	**13**	**15**	**21**	**13**	**16**	**16**	**11**	**15**	**11**	**13**	**13**	**12**	**9**	**9**	**187**

Table 9: The comparison between the total daily clinical signs observed in each indigenous chicken age group

Chicken age groups	Days post infection														Total sign
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
4 week	10	14	17	14	16	12	15	15	14	9	13	11	9	4	173[a]
8 week	12	20	19	22	17	30	22	21	20	18	19	20	19	13	272[c]
12 week	31	35	31	33	32	28	28	25	28	24	19	24	22	12	372[b]
16 week	13	15	21	13	16	16	11	15	11	13	13	12	9	9	187[a]

X^2 calculated = 100.65 ($p < 0.05$ for [b] and [c])

Table 10: Comparison between the various types of clinical signs as observed in each chicken age group

Clinical signs	Chicken age groups			
	4 week-old	**8 week-old**	**12 week-old**	**16 week-old**
Depression	55	63	79	42
Nervous tics	0	3	13	21
Ruffled feathers	36	61	74	27
Sneezing	13	27	36	20
Ataxia	1	6	14	4
Nasal discharges	3	6	34	1
Dyspnoea	4	17	16	13
Mouth discharges	0	3	6	2
Diarrhoea	10	5	14	9
Cyanosis	0	0	3	1
Rales	22	24	8	2
Fever	29	53	60	38
Head scratching	0	4	15	7
Total signs	**173[a]**	**272[b]**	**372[c]**	**187[a]**

X^2 calculated = 100.65 ($p < 0.05$ for [b] and [c])

FIG.3. DEPRESSED CHICKENS WITH RUFFLED FEATHERS AND TAIL DOWN 3 – 5 DAYS POST INFECTION WITH *P. MULTOCIDA*

4.3.2 Severity of the clinical signs

Of the 173 clinical signs from the 4 week-old chickens, 82 were mild, 63 moderate, and 28 severe (Table 11; Appendix 4); while of the 272 clinical signs from the 8 week-old chickens, 143 were mild, 70 moderate, and 59 were severe signs (Table 11; Appendix 5). Of the 372 clinical signs from the 12 week-old chickens 217 were mild, 90 moderate and 65 were severe (Table 11; Appendix 6), while of the 187 clinical signs from the 16 week-old chickens were 116 mild, 47 moderate, and 24 severe ones (Table 11; Appendix 7).

4.3.3 Weight changes between control and infected chickens in the respective age group

The control birds in all the age groups gained more weight than the infected birds. The 4 week-old control chickens gained 14.1 grams per bird more than the infected birds in this age group (Table 12, Appendix 8; $p > 0.05$), while the 8 week-old control birds gained 20.0 grams more than their infected age mates (Table 12, Appendix 9; $p > 0.05$). 12 week-old control chickens gained 25.7 grams more than the infected group (Table 12, Appendix 10; $p > 0.05$), while the 16 week-old control chicken had 166.9 grams more than the infected group (Table 12, Appendix 11; $p > 0.05$).

Table 11: Comparison between the number of clinical signs and their severity among the indigenous chicken age groups

Chicken age group	Number and severity of the clinical signs			Total clinical sign
	Mild (1)	Moderate (2)	Severe (3)	
4 week-old	82	63	28	173[a]
8 week-old	143	70	59	272[b]
12 week-old	217	90	65	372[c]
16 week-old	116	47	24	187[a]

X^2 calculated = 16.84 ($p<0.05$)

Table 12: Comparison between the average weight changes in grams per bird among the different chicken age groups

Chicken age group	Average weight gains of control chicken (grams)	Average weight gains of infected chicken (grams)	Difference in weight gains between control and infected (grams)
4 week-old	83.3	69.2	14.1[a]
8 week-old	180.5	160.5	20.0[a]
12 week-old	206.9	181.2	25.7[a]
16 week-old	246.2	79.3	166.9[a]

F value calculated = 0.2976 ($p>0.05$)

4.3.4 Gross lesions and *P.multocida* re-isolation from the experimental chickens

At the end of the study, post mortem was carried out on the sacrified chickens. The 4 week-old chickens had no visible lesions, while 3 birds in 8 week-old group had fibrin remnants on the airsacs and thickened airsacs, one bird had fibrotic lung and another had splenomegally. Five from the 12 week-old ones had remnants of fibrin and fibrosis of the lungs and airsacs, one had necrotic liver lesions, and another one had splenomegally, while 3 of the 16 week-old ones had fibrosis on the lungs and airsacs. Of these, 4 had remnants of fibrin in the airsacs.

Pasteurella multocida was isolated from 3 of the 12 week-old and 2 of the 16 week-old chickens but not from the 4 and 8 week-old chickens, respectively from oropharyngeal, lungs, spleen and the liver samples.

There were no gross pathological lesions or *P. multocida* recovered from the control birds from all the chicken age groups.

4.4 Discussion

Pasteurella multocida has a wide range of avian host susceptibility, with some species of wild birds being more susceptible than others (Faddoul *et al.*, 1967). The degree of susceptibility varies between different types of birds and age groups (Rhoades and Rimler, 1989). Using 16 and 45 week-old New Hampshire chickens and 2 different *P. multocida* sero-types, Heddleston (1962) showed that mature chickens were more susceptible than the young chickens. In another study, Heddleston and Watko (1965) using 9 and 52 week-old New Hampshire chickens and 6 and 8 week-old, Beltsville small white turkeys, reported that turkeys are more susceptible to virulent *P. multocida* than chickens, while mature chickens were more susceptible than young chickens. In both studies, the criteria used was

mortality, as the *P. multocida* strains were lethal to the chicken. No studies have been done using a less virulent strain, expressing clinical signs but no mortality as *P.multocida* strain NCTC 10322^T of this study. Neither have clinical signs, their severity or weight changes been used to evaluate susceptibility.

In this study, the 12 week-old indigenous village chickens had more clinical signs, that were more severe, and were expressed within a few days after exposure than those of the 8, 16 and 4 week-old chickens. There were marked differences between 12 week-old chickens and other ages in expression of depression, ataxia, nervous tics, nasal discharges, and head scratching ($p<0.05$); and ruffled feathers, mouth discharges, and sneezing, with other ages except with 8 week-old; while diarrhoea was significantly different with 8 week-old chicken only ($p<0.05$) (Rhoades and Rimler, 1989).

Most signs were observed in the 12 week-old birds, except nervous tics that were more in 16 week-old chickens. Rales and dyspnoea were also marginally more in the 8 week-old chickens. Some signs varied on replication while others did not.

Coughing and eye discharges were not observed and will need further evaluation. The twelve week-old chickens had more cases of birds with fever, than the other age groups. All the control birds did not show any clinical signs. These findings agree with what previous authors have reported that different age groups have variable susceptibility to *P. multocida* (Heddleston, 1962; Heddleston and Watko, 1965; Rhoades and Rimler, 1989). However, unlike Heddleston (1962) and Heddleston and Watko (1965), the 12 week-old village chickens were found to be more susceptible than younger (4 and 8 week-old) and older (16 week-old) chickens. The difference could be due to the strain of *P. multocida* used; age variation and the breed of chickens used in the different experiments. In

commercial turkeys a retrospective study showed the onset of fowl cholera to be at 89.6 days (12.8 weeks) in Georgia, 10.8 weeks in California and 16.3 weeks in Missouri, in U.S.A. (Morris *et al.*, 1989). The observations in Georgia are similar to observations in this study where the most susceptible age of chickens was found to be 12 weeks.

One of the effects of fowl cholera, like other diseases, is decrease in the feed utilisation efficiency among affected turkeys (Morris *et al.*, 1989). Older chickens sacrificed at 19 days after exposure to *P. multocida* were found to be emaciated (Heddleston and Watko, 1965). In the current study, all infected chickens had less weight gains than the control chickens of their corresponding age group, while older chickens had more weight loss than the younger ones, when sacrificed at 14 days post inoculation. There is, therefore, a possibility that all virulent strains of *P. multocida* affect the weight gains in different age groups of birds.

On the basis of these parameters, the 12 week-old village chickens, when infected with *P. multocida* NCTC 10322^{T}, appear to be more susceptible than other age groups of the grower indigenous birds. This is the first account of age susceptibility to *P. multocida* among the chicks and growers of village indigenous chickens.

CHAPTER 5

5.0 EXPERIMENT 3: AGE SUSCEPTIBILITY OF INDIGENOUS DUCKS TO *P. MULTOCIDA*

5.1 Introduction

The duck population is 71,000 in Kenya but they contribute significantly to the smallholder economy in the rural areas (Anonymous, 2000; Njue, 2003). They are reared with chickens and closely interact with them when scavenging for food (Nyaga *et al.*, 2002; Mbuthia *et al.*, 2003). Their production is limited by diseases (Aini, 1999b; Nyaga *et al.*, 2002). One such disease is fowl cholera caused by *P.multocida* (Mutters *et al.*, 1985a); it causes waterfowl mortality in various countries (Hunter and Wobeser, 1980; Aini, 1999b; Pehlivanoglu *et al.*, 1999; Amonsin *et al.*, 2002).

Pasteurella multocida healthy-looking carriers have been observed in ducks in Kenya (Chapter 3 experiment1, of this thesis) and in Tanzania (Muhairwa *et al.*, 2001b). However the age susceptibility among the indigenous grower ducks has not been documented, yet, knowledge of susceptible age group is important in disease control measures in various production systems and development of duck health programs.

The aim of this study was to establish the most susceptible age group among the indigenous ducks to *P. multocida*.

5.2 Materials and methods

5.2.1 Experimental ducks

Indigenous ducklings hatched from incubated eggs at Kabete and day old ducklings bought from farms with indigenous flocks were brooded and reared in an isolation house,

away from other birds. They were fed on commercial chick and grower's feed (Unga Limited ®, Kenya) and given water *ad libitum*, up to the required experimental age of 4, 8, 12, and 16 weeks. A total of one hundred and twenty indigenous ducks comprising of 30 birds of each stated age group were used in this study.

5.2.2 Bacteria used to infect ducks

Pasteurella multocida type strain (NCTC 10322^T) maintained on Dorset egg agar, in our laboratory that had low virulence than other *P.multocida* was used in this study. It was spread onto BA with 5 % citrated calf blood, incubated aerobically at 37 °C, for 24 hours to check for purity prior to preparation of the innoculum. An individual colony of this culture was inoculated into brain heart infusion broth, incubated aerobically at 37 °C, for 24 hours. A ten fold serial dilution of one milliliter of the bacteria in BHI was made and 25µl of each dilution spotted onto BA plates. Colonies were counted at the dilution with countable colonies and the counts translated into colony forming units per milliliter (CFU/ml).

5.2.3 Experimental procedure

Each duck age group had 15 birds, of which 10 were infected with *P. multocida* while 5 were used as controls and were inoculated with BHI broth only. The experiments are replicated. The infected and control groups were housed in different rooms located in different houses away and apart from each other. Before commencement of the experiment, all birds were screened by taking their oropharyngeal and cloaca swabs that were cultured onto BA and inoculated into mice as done in Chapter 3.2.4 of this study and shown to be negative for *P.multocida*. The birds were separated and put in the

experimental rooms 48 hours prior to inoculation. Table 13 shows the outline of the experimental design.

Table 13: The experimental design on age susceptibility in ducks

Duck age groups	Infected Birds	Control birds	Total birds
4 week-old	10	5	15
8 week-old	10	5	15
12 week-old	10	5	15
16 week-old	10	5	15
Total birds	**40**	**20**	**60**

The innoculum was prepared as described by Petersen *et al.* (2001b) and each bird was inoculated intratracheally with 0.5 ml culture containing 1.2 – 2.0 x 10^8 CFU of *P.multocida* organisms in BHI. Each of the control birds was inoculated with 0.5 ml of BHI broth at the start of the study. To avoid possible cross infection the daily observations started with birds in the control house before proceeding to the infected house. Biosecurity was maintained during the entire period of the study.

Clinical sign observations, temperature and weight measurements were carried out and recorded once daily, by the same person throughout the study period. In each room, of the control or infected birds, the observer took 30 minutes per day at the same time each day to observe the presenting clinical signs and carry out the measurements. Initially, observations were made without disturbing the birds and later a close examination was carried out while holding each bird that is wing tagged for identification. During the latter examination, the bird's cloacal temperature was taken using a clinical thermometer (laboratory thermometer 305 mm), reading recorded and thermometer disinfected with alcohol. After close examination, the bird's weight was taken using Soehnle German balance (measuring a minimum of 25 grams and a maximum of 5 kilograms) and

recorded. The birds were examined for the presence anorexia, fever, ataxia, depression, ruffled feathers, cyanosis, nervous tics, eye discharges, nasal and mouth discharges, sneezing, dyspnoea, spontaneous tracheal rales, coughing, diarrhoea and head scratching. Pathological lesions in poultry due to various causes are semi-quantitatively scored (Gross and Siegel, 1965; Gross and Colmano, 1967; Shivaprasad and Droual, 2002) and a similar trend was adopted for clinical signs in this study. The frequency and intensity of the clinical signs were graded as severe (3), moderate (2), or mild (1). A mild sign was observed 1 to 3 times; moderate sign, 4-6 times; while severe one was observed 7 or more times on an individual bird during the 30 minutes of clinical sign observations. Mild depression was dullness alone; when it occurred together with drooping wing it was taken as moderate depression; while dosing and/or tucked head under the wings was regarded as severe depression. Ruffled feathers around the head and neck was taken as mild; when general body was affected it was taken as moderate; while with drooping wings it was regarded as severe. Cloacal temperature below 41.5 °C was taken as normal; 41.6 – 41.8 °C as mild; 41.9 - 42.1 °C as moderate; and that of over 42.2 - 42.5 °C was taken as severe fever. Post mortem examination was done as described by Bermudez and Stewart-Brown (2003). The sacrificed birds were opened aseptically, at the end of the study and post mortem examination done and swabs taken from the oro-pharynx, cloaca, lungs, liver, spleen, caecal tonsils, and pruning gland, for bacterial examination. During the post mortem examination, gross lesions were noted and recorded.

5.2.4 Re-isolation and identification of *P. multocida*

At 24 hours post-infection, all the birds were swabbed on oro-pharynx and cloaca to confirm the establishment or absence of *P.multocida* in the infected and control birds,

respectively. These swabs and those taken during the post mortem examination were used for the re-isolation of *P.multocida*. The re-isolation and eventual identification procedures for *Pasteurella* organisms were done as given in chapter 3, section 3.2.4.

5.3 Results

5.3.1 Type and total number of observed clinical signs

Table 14 shows the clinical signs observed in infected 4 week-old wing tagged ducks. All the birds expressed intermittent clinical signs over the 14 days period. One duckling expressed clinical signs for 12 days while 2 ducklings expressed signs on one day only during the 14 days' period. The clinical signs observed were anorexia, fever, depression, ruffled feathers, nasal discharges, mouth discharges, sneezing, dyspnoea, spontaneous tracheal rales, and diarrhoea. A total of 117 clinical signs were recorded. Of these, 27 were fever, 22 dyspnoea, 18 depression, 18 ruffled feathers, 17 rales, 16 nasal discharges, 3 sneezing, 3 mouth discharges, and 2 diarrhoea. Observed clinical signs were many from day one when they numbered 13, peaked on the third day to 21 and stayed high up to day six at 11, declining gradually to 2 on day 14. Majority of the signs were observed between days 1 to 6. Nervous tics, cyanosis, eye discharges, coughing, head scratching, and ataxia were not observed in this age group (Appendix 12).

Table 15 shows the clinical signs observed in infected 8 week-old wing tagged ducks. The birds had intermittent clinical signs with most of them being observed in the first 8 days of observation. Two ducks expressed clinical signs for 11 days while one duck expressed signs on 2 days only during the 14 days period. The observed clinical signs were anorexia, fever, ataxia, depression, ruffled feathers, nasal discharges, sneezing, dyspnoea, coughing, eye discharges, spontaneous tracheal rales, and diarrhoea. A total of

188 clinical signs were recorded. Of these 58 were nasal discharges, 26 dyspnoea, 25 sneezing, 22 fever, 13 depression, 13 ruffled feathers, 10 mouth discharges, 8 ataxia, 7 coughing, 2 eye discharges, and 2 rales. The number of signs peaked on day one when they were 25, stayed high up to day 8 when they were 13, and declined gradually to 1 by day 14 of observation. Nervous tics, cyanosis and head scratching were not recorded in this age group. Cyanosis was difficult to assess due to the birds' black skin (Appendix 13), and examination of other body parts could have stressed the bird.

Table 16 shows the clinical signs observed on examination of infected 12 week-old wing tagged ducks. The ducks had intermittent clinical signs over the 14 days of observation. The observed clinical signs were anorexia, fever, depression, ruffled feathers, nasal discharges, mouth discharges, sneezing, coughing, dyspnoea and head scratching. A total of 80 clinical signs were recorded. Of these 32 were nasal discharges, 16 sneezing, 15 fever, 6 depression, 6 ruffled feathers, 2 mouth discharges, 1 dyspnoea, 1 coughing, and one head scratching. Observed clinical signs were high from day one when they were 8, peaked on day five to 11, and declined gradually to 2 on day 14. Majority of the sign were observed between days 1 to 6. Nervous tics, rales, ataxia, eye discharges, and diarrhoea were not observed in this age group while cyanosis was difficult to assess as in the 8 week-old ducks (Appendix 14) as birds were handled with minimum stress.

Table 17 shows the clinical signs observed in infected 16 week-old wing tagged ducks. They showed intermittent clinical signs for the 14 days period. Two ducks showed no clinical sign another 4 had one clinical sign on 1 day only, while the rest had 1 to 5 clinical signs, with an average of 1 sign per bird per day of observation. A total of 83 clinical signs were recorded. Of these 42 were nasal discharges, 15 fever, 14 sneezing, 4

depression, 4 ruffled feathers, 3 dyspnoea, and 1 mouth discharge. The number of signs peaked on day one to 8, stayed high up to day 5 when they were 7 and declined gradually thereafter to 3 on day 14. Nervous tics, ataxia, diarrhoea, rales, eye discharges, head scratching, and cyanosis were not observed (Appendix 15).

5.3.2 Summary results of the four duck age groups

The total daily clinical signs were high among the 8 week-old ducks while the 12 and 16 week-olds were almost equal. Majority of clinical signs were observed between day 1 to 6, except 8 week-old ones that were high up to day 8 post infection (Table 18). There were significant differences between the observed clinical signs expressed by various duck age groups ($p< 0.05$), except head scratching which was not significant ($p>0.05$) and most signs were shown by the 8 week-old ducks, except rales and fever that were more in the 4 week-old birds. The 4 week-old appeared marginally variable from the 12 and 16 week-old ($p<0.05$) but the 12 and 16 week-old ducks were not statistically different from each other ($p>0.05$). Eye discharges and ataxia were observed in 8 week-old only while head scratching was recorded in the 12 week-old ducks. Diarrhoea and spontaneous rales were more in the young ducks (4 and 8 week-old). Depression, ruffled feathers and dyspnoea appeared more marked in birds 8 week-old and lower. Generally, younger ducks had more clinical signs than the older birds (Table 19).

In all age groups anorexia was observed from 1 to 4 days post infection.

Dyspnoea, diarrhoea, ataxia and coughing varied significantly on replication ($p< 0.05$), while all signs varied significantly with days, p.i ($p< 0.05$), except coughing ($p>0.05$). The expression of clinical signs was severest between days 1 to 6, and varied significantly from other days, p.i ($p<0.05$) except for head scratching. Most signs were

mild between days 7 to 9 but were expressed similarly between days 10-14 and were not significantly different ($p>0.05$). Control birds of all ages did not show any clinical sign throughout the observation period.

5.3.3 Severity of the clinical signs

Of the 117 clinical signs from the 4 week-old ducks, 23 were severe, 22 moderate, and 72 mild (Table 20; Appendix 12); while of the 188 clinical signs from the 8 week-old ducks, 49 were severe, 46 moderate, and 93 mild (Table 20; Appendix 13). Of the 80 clinical signs from the 12 week-old ducks, 18 were severe, 12 moderate and 50 mild (Table 20; Appendix 14), while of the 83 clinical signs from the 16 week-old ducks, 22 were severe, 20 moderate, and 41 mild (Table 20; Appendix 15) ($p< 0.05$).

5.3.4 Weight changes between control and infected ducks in various age groups

All the age groups had increase in weights, with the control 4 week-old ducks having an average of 248.4 grams while the respective infected ducks gained 240.9 grams per bird, a difference of 7.5 grams over the 14 days period (Table 21; Appendix 16). The eight week-old control ducks gained 431.2 grams, while the respective infected ducks had 418.9 grams, a decrease of 12.3 grams (Table 21; Appendix 17). Among the 12 week-old ducks the control gained 366.3 grams while the respective infected ducks gained 338.0 grams per bird, a difference of 28.3 grams (Table 21; Appendix 18). Among the 16 week-old ducks control birds gained 119.4 grams while the respective infected ducks gained 90.6 grams, a decrease of 28.8 grams (Table 21; Appendix 19) ($p< 0.05$).

Table 14: Type and total number of clinical signs observed daily in infected 4 week-old indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	-	1	4	2	3	2	1	-	-	1	-	-	1	-	**15**
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Sneezing	1	-	-	-	1	1	-	-	-	-	-	-	-	-	**3**
Ruffled feathers	-	1	4	2	3	2	1	-	-	1	-	-	1	-	**15**
Ataxia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Nasal discharges	2	4	2	1	1	-	-	1	-	1	-	3	-	1	**16**
Dyspnoea	3	5	4	2	2	3	1	-	-	1	1	-	-	-	**22**
Mouth discharges	-	-	2	1	-	-	-	-	-	-	-	-	-	-	**3**
Diarrhoea	1	-	-	1	-	-	-	-	-	-	-	-	-	-	**2**
Cyanosis[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Eye discharges	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Rales	3	3	2	2	2	1	1	-	1	-	-	-	-	-	**15**
Coughing	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Fever	3	3	3	3	2	2	2	2	2	1	1	-	1	1	**26**
Total signs	**13**	**17**	**21**	**14**	**14**	**11**	**6**	**3**	**3**	**5**	**2**	**3**	**3**	**2**	**117**

Legend: [a] Cyanosis was difficult to observe on the duck skins

Table 15: Type and total number of clinical signs observed daily in infected 8 week-old indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	2	2	2	2	1	2	1	1	-	-	-	-	-	-	**13**
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Sneezing	3	3	3	3	2	1	2	2	1	2	1	1	1	-	**25**
Ruffled feathers	2	2	2	2	1	2	1	1	-	-	-	-	-	-	**13**
Ataxia	-	2	1	2	1	1	1	-	-	-	-	-	-	-	**8**
Nasal discharges	7	4	7	5	2	6	5	5	4	4	4	3	1	1	**58**
Dyspnoea	4	3	2	3	2	2	2	1	1	1	1	4	-	-	**26**
Mouth discharges	2	1	1	1	1	2	1	1	-	-	-	-	-	-	**10**
Diarrhoea	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Cyanosis[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Coughing	2	1	-	1	-	1	-	-	-	1	1	-	-	-	**7**
Eye discharges	-	-	-	-	1	-	1	-	-	-	-	-	-	-	**2**
Rales	-	-	-	-	-	-	-	1	1	-	-	-	-	-	**2**
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Fever	2	4	3	3	1	2	2	1	1	1	-	2	1	-	**23**
Total signs	**25**	**23**	**21**	**22**	**12**	**19**	**16**	**13**	**8**	**9**	**7**	**10**	**3**	**1**	**188**

Legend: [a] Cyanosis was difficult to observe on the duck skins

Table 16: Type and total number of clinical signs observed daily in infected 12 week-old indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	-	1	1	1	1	1	-	1	-	-	-	-	-	-	**6**
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Sneezing	1	1	2	2	2	2	1	1	1	1	1	1	-	-	**16**
Ruffled feathers	-	1	1	1	1	1	-	1	-	-	-	-	-	-	**6**
Ataxia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Nasal discharges	3	4	4	4	4	1	1	2	2	3	1	1	1	1	**32**
Dyspnoea	-	-	-	-	-	-	1	-	-	-	-	-	-	-	**1**
Mouth discharges	1	-	-	-	1	-	-	-	-	-	-	-	-	-	**2**
Diarrhoea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Cyanosis[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Eye discharges	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Rales	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Coughing	-	-	-	-	-	1	-	-	-	-	-	-	-	-	**1**
Head scratching	-	-	-	1	-	-	-	-	-	-	-	-	-	-	**1**
Fever	3	2	-	1	2	1	1	1	1	1	-	1	-	1	**15**
Total signs	**8**	**9**	**8**	**10**	**11**	**7**	**4**	**6**	**4**	**5**	**2**	**3**	**1**	**2**	**80**

Legend: [a] Cyanosis was difficult to observe on the duck skins

Table 17: Type and total number of clinical signs observed daily, in infected 16 week-old indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	-	1	1	1	1	-	-	-	-	-	-	-	-	-	**4**
Sneezing	1	2	1	1	1	-	1	1	2	1	-	1	1	1	**14**
Ruffled feathers	-	1	1	1	1	-	-	-	-	-	-	-	-	-	**4**
Ataxia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Nasal discharges	3	2	5	4	3	3	3	3	3	3	2	3	3	2	**42**
Dyspnoea	1	1	1	-	-	-	-	-	-	-	-	-	-	-	**3**
Mouth discharges	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Diarrhoea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Cyanosis[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Eye discharges	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Rales	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Coughing	-	-	-	-	-	-	-	-	-	1	-	-	-	-	**1**
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Fever	2	1	2	1	1	2	1	1	2	1	-	-	-	-	**15**
Total signs	**8**	**8**	**11**	**8**	**7**	**5**	**5**	**5**	**7**	**6**	**2**	**4**	**4**	**3**	**84**

Legend: [a] Cyanosis was difficult to observe on the duck skins

Table 18: The comparison between the total daily clinical signs observed in each indigenous duck age group

Duck age groups	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
4 week-old	13	17	21	14	14	11	6	3	3	5	2	4	3	2	**117** [b]
8 week-old	25	22	21	22	12	19	16	13	8	9	7	10	3	1	**188** [b]
12 week-old	8	9	8	10	11	7	4	6	4	5	2	3	1	2	**80** [a]
16 week-old	8	8	11	8	7	5	5	5	7	6	2	4	4	3	**83** [a]

X^2 calculated (^{a,b})= 64.67 ($p<0.05$)

Table19: Comparison between the various types of clinical signs as observed in each duck age group

Clinical signs	Duck age groups			
	4 week old	8 week old	12 week old	16 week old
Depression	15	13	6	4
Nervous tics	0	0	0	0
Sneezing	3	25	16	14
Ruffled feathers	15	13	6	4
Ataxia	0	8	0	0
Nasal discharges	16	58	32	42
Dyspnoea	22	26	1	3
Mouth discharges	3	10	2	1
Diarrhoea	2	1	0	0
Cyanosis	0	0	0	0
Coughing	0	7	1	1
Eye discharges	0	2	0	0
Rales	15	2	0	0
Head scratching	0	0	1	0
Fever	26	23	15	14
Total signs	**117** [b]	**188** [b]	**80** [a]	**83** [a]

X^2 calculated (^{a,b}) = 64.67 ($p<0.05$)

Table 20: Comparison between the number of clinical signs and their severity among the indigenous duck age groups

duck age groups	Number and severity of the clinical signs			Total clinical signs
	Mild (1)	Moderate (2)	Severe (3)	
4 week-old	72	22	23 [a]	117 [b]
8 week-old	93	46	49 [b]	188 [b]
12 week-old	50	12	18 [a]	80 [a]
16 week-old	41	20	22 [a]	83 [a]

X^2 calculated (^{a,b}) = 7.82 ($p < 0.05$)

Table 21: Comparison between the average weight gains in grams per bird among the different duck age groups

Duck age groups	Average weight gains of control ducks	Average weight gains of infected ducks	Difference in weight gain between control and infected
4 week-old	248.4	240.9	7.5 [b]
8 week-old	431.2	418.9	12.3 [b]
12 week-old	366.3	338.0	28.3 [a, b]
16 week-old	119.4	90.6	28.8 [a, b]

F-value, calculated (^{a,b}) = 634.20 ($p<0.05$)

5.3.5 Gross lesions of the experimental ducks

At the end of the study, post mortem was carried out on the ducks. Two birds had remnants of fibrin and fibrous strands, among the 4 week-old ducks. One of these had pericarditis and perihepatitis. Eight of the 8 week-old ducks had remnants of fibrin and fibrous strands, 2 had fibrosis on the lungs and another 2 of these had necrotic spots on spleen and another on the liver. Two of the 12 week-old ducks had remnants of fibrin and fibrous strands; while 4 of the 16 week-old ducks had remnants of fibrin; one of these had necrotic spots on the liver.

5.3.6 *P.multocida* re-isolated from the experimental ducks

P.multocida was isolated from 4 of the 8 week-old and 2 of the 16 week-old ducks and one each from the 4 and 12 week-old ducks, respectively.

There were no gross pathological lesions or *P. multocida* recovered from the control birds from all the age groups.

5.4 Discussion

P.multocida has a wide range of avian host susceptibility with some species of birds and different age groups being more susceptible than others (Faddoul *et al.*, 1967; Rhoades and Rimler, 1989; Gooderhan, 1999). In the present study, the age susceptibility with respect to *P.multocida* NCTC 10322^{T} was based on the number and severity of clinical signs, number with fever, and weight changes between the infected and control birds. Using 5,11,16 and 18 week-old Mallard ducks, birds older than 11 weeks were less susceptible to *P.multocida* than young ones (Hunter and Wobeser, 1980). These findings agree with the results of the current study, in which it was found that the 8week-old

ducks had more severe clinical signs, which were observed after 1 day post exposure, than older 12 and 16 week-old indigenous village ducks except for 4 week-old ducks which were less susceptible than the 8 week-old ones. There were marked differences in clinical signs shown by ducks 8 week-old and those of other ages for depression, ruffled feathers, sneezing, ataxia, nasal discharges, mouth discharges, coughing and eye discharges ($p< 0.05$). The 8 week-old birds showed more of the above signs. Signs that did not vary with replication may be indicators of fowl cholera, while those that varied on replication may indicate fowl cholera severity.

They had also more birds with fever as compared to other age groups. The 8 week-old were followed by 4 week-old ducks in susceptibility to *P.multocida*. All infected ducks had lower body weights 14 days p.i than the control ducks. This is as reported by Faddoul *et al.* (1962) who found that the infected wild birds had weights that were below normal. However the growth rate of the 12 and 16 week-old ducks was less than the 8 and 4 week-old ducks. In all, this could be due to decreased feed efficiency in all the infected ducks but more so in the older ducks. A wide variation in virulence can exist for a given sero-type (Snipes *et al.*, 1990) and possibly the strain used in this study is less virulent. Like in turkeys (Bond *et al.*, 1974), older ducks were found to be less susceptible than the 8 week-old ones.

Based on these findings, the 8 week-old village ducks were the most susceptible age for infection with *P.multocida* compared to the other age groups. This age group was used for subsequent experiments with *P.multocida*. This is the first account of age susceptibility to *P.multocida* among village indigenous ducks.

CHAPTER 6

6.0 EXPERIMENT 4: COMPARISON BETWEEN THE CLINICAL SIGNS OF FOWL CHOLERA IN IMMUNOSUPPRESSED AND IN NON-IMMUNOSUPPRESSED INDIGENOUS CHICKENS AND DUCKS

6.1 Introduction

P. multocida, the cause of fowl cholera in poultry (Rimler and Rhoades, 1989) was first reported in Kenya as a cause of avian pasteurellosis epizootics in wild waterfowls at lake Nakuru (Hudson, 1959). The organism was last isolated from a grey parrot in Kenya in 1975 (Miringa, 1975). Current studies have indicated that indigenous birds are carriers of *P.multocida,* a confirmation that *P.multocida* is still prevalent and poses a risk to healthy poultry flocks (chapter 3 of this thesis). During scavenging, indigenous birds are subjected to stressful conditions under village rearing set ups (Minga *et al.*, 1989; Aini, 1999a,b; Nyaga *et al.*, 2002). This can be immunosuppressive and lead to the flare-up of fowl cholera in carrier birds (Blackall *et al.*, 1995). The bacteria can also be spread to other in-contact animals (Muhairwa *et al.*, 2001b). Clinical signs of fowl cholera as described in current literature are not very characteristic or differential and can be confused with other upper respiratory tract infections (Glisson 2003; Glisson *et al.*, 2003). Clinical signs that more consistently indicate the presence of this disease among indigenous chickens and ducks need to be established so that farmers, animal health assistants, and veterinarians are able to recognize the disease on encounter. This is especially important since little is known about fowl cholera clinical manifestation in normal and immunosuppressed local indigenous chickens and ducks. Variations in

clinical sign manifestations may occur due to immunosuppression requiring keener attention to differentiate fowl cholera from other diseases.

The objective of this study was, therefore, to determine the clinical signs of fowl cholera in normal and immunosuppressed indigenous chickens and ducks.

6.2 Materials and methods

6.2.1 Experimental chickens and ducks

Indigenous chickens aged 12 weeks and 8 week-old local indigenous ducks, previously shown in these studies to be the most susceptible were used in this experiment. Some birds were bought at day-old from farmers while others were hatched from indigenous bird eggs purchased locally. Before commencement of the experiment, all birds were screened by taking their oropharyngeal and cloaca swabs that were cultured onto BA and inoculated into mice as done in Chapter 3.2.4 of this study and shown to be negative for *P.multocida* infection. They were reared and kept separately in deep littered rooms, and provided with feed and water *ad libitum*.

6.2.2 Bacteria used to infect birds

The bacteria used and procedures for preparing inoculum were carried out as given in chapter 4, section 4.2.2 and chapter 6, section 6.2.4.

6.2.3 Immunosuppression of the birds

Birds to be immunosuppressed were injected intramuscularly using dexamethasone (Agar Holland, 3760 AL Soest, Holland), 4mg/Kg body weight per day as done elsewhere for 6 days prior to experimental infection (Birrenkott and Wiggins, 1984; Corrier and DeLoach, 1990; Corrier *et al.*, 1991; Nakamura *et al.*, 1994).

6.2.4 Experimental procedure

A total of 70 wing-tagged birds comprising of 35 indigenous ducks and 35 indigenous chickens were used in this study. Four separate experiments were run in duplicates, that is, for immunosuppressed (IS) ducks; for non-immunosuppressed (NIS) ducks; and for immunosuppressed chickens; and for non-immunosuppressed chickens; each with respective controls. The birds were separated and put in the experimental rooms 48 hours prior to inoculation. Table 22 shows the outline of the experimental design.

Table 22: The experimental design of the comparison between the clinical signs of fowl cholera in indigenous chickens and ducks

Experimental Birds	Infected birds	Control birds		Total birds
		Normal	Immunosuppressed	
Normal chicken	10	5	0	15
Immunosuppressed chicken	10	5	5	20
Normal ducks	10	5	0	15
Immunosuppressed ducks	10	5	5	20
Total birds	**40**	**20**	**10**	**70**

Of the 35 birds, 20 were infected with *P. multocida* while 15 were used as control birds. In the immunosuppressed studies, 10 birds were immunosuppressed and infected with *P.multocida*, while the uninfected control group comprised of 5 immunosuppressed and 5 non-immunosuppressed birds that were inoculated with brain heart infusion (BHI) broth. For the non-immunosuppressed studies, 10 birds were infected with *P. multocida*, while 5 control birds were given BHI broth. Each infected bird was inoculated intratracheally with 0.5 ml culture containing $1.1 - 2.5 \times 10^8$ CFU of *P.multocida* organisms while the controls were given 0.5 ml of sterile BHI broth like the one used to grow the *Pasteurella* inoculum.

The infected and control birds were housed in different rooms away and apart from each other. To avoid possible cross infection, the daily observations started with birds in the control house before proceeding to the infected house. Biosecurity was maintained during the entire period of the study.

The birds were observed daily for any clinical signs (including temperature changes) over a period of 14 days, as described in chapter 4, section 4.2.3 and chapter 5, section 5.2.3. Postmortem and bacterial recovery from tissues of sacrificed birds were done as given in chapter 4, section 4.2.3 and 4.2.4, respectively.

6.3 Results

All infected birds expressed clinical signs of fowl cholera during the 14 days of observation. They were: fever, depression, nasal discharges, sneezing, dyspnoea, ataxia, nervous tics, rales, mouth mucoid discharges (mucoid strings between the beaks), diarrhoea, cyanosis, coughing, head scratching, and eye discharges. The signs were noted among the infected birds but not in the control birds (Tables 23-26); they occurred singly or in combinations of 2 to 5 signs per infected bird on a day of observation (Appendices 20 to 24).

6.3.1 Clinical signs observed in indigenous chickens

A total of 179 clinical signs were manifested by the IS chickens. These were: 33 depression, 30 nervous tics, 25 sneezing, 24 ruffled feathers, 20 fever, 13 nasal discharges, 12 dyspnoea, 5 head scratching, 2 mouth discharges and 1 diarrhoea. The signs were high from day 1 when they numbered 13, peaked on the fifth day to 20, and decreased gradually to 5 on day 14. Majority of the signs were observed on days 1 to 12. Cyanosis, coughing and eye discharges were not recorded for this group (Table 23).

The common combinations of clinical signs in IS chickens were 12 depression/ruffled feathers, 6 dyspnoea/nervous tics, 5 sneezing/nervous tics/ruffled feathers, and 5 depression/nervous tics/ruffled feathers. Others were 3 sneezing/nasal discharges, 2 depression/sneezing/ataxia, 2 depression sneezing/ruffled feathers, 1 depression/ nasal discharges/ataxia/mouth discharges, 1 sneezing/ataxia/nervous tics, 2 depression/nasal discharges, 1 sneezing/ataxia, 1 depression/sneezing/dyspnoea, 1 depression/ ataxia/nervous tics, and 1 depression /dyspnoea/ataxia/nervous tics (Appendix 20). Other signs were expressed as individual clinical signs per bird per day of observation.

A total of 372 clinical signs that were shown by the NIS 12 week-old chickens in the age susceptibility test in chapter 4 were used for comparison. Of these, 79 were depression, 74 ruffled feathers, 60 fever, 36 sneezing, 34 nasal discharges, 16 dyspnoea, 15 head scratching, 14 ataxia, 14 diarrhoea, 13 nervous tics, 8 spontaneous rales, 6 mouth discharges and 3 cyanosis. Clinical signs were high from day 1 when they numbered 31, peaked on the second day and decreased gradually to 12 on day 14 of the observation. The majority of the signs were observed on days 1 to 13 of the observation. Coughing was not recorded in this group (Table 24).

The common clinical sign combinations for NIS chickens were namely: 22 for depression/sneezing/nasal discharges/ruffled feathers, 9 for depression/sneezing, 8 for depression/nasal discharges, 8 for depression/ruffled feathers, 5 for sneezing/nasal discharges, 4 for depression/nasal discharges/ataxia/ruffled feathers, 3 for depression/sneezing/nasal discharges/dyspnoea/ruffled feathers, 2 for sneezing/nasal discharges/dyspnoea/ruffled feathers, 2 for depression/sneezing/nasal discharges/ ataxia

The common combinations of clinical signs in IS chickens were 12 depression/ruffled feathers, 6 dyspnoea/nervous tics, 5 sneezing/nervous tics/ruffled feathers, and 5 depression/nervous tics/ruffled feathers. Others were 3 sneezing/nasal discharges, 2 depression/sneezing/ataxia, 2 depression sneezing/ruffled feathers, 1 depression/ nasal discharges/ataxia/mouth discharges, 1 sneezing/ataxia/nervous tics, 2 depression/nasal discharges, 1 sneezing/ataxia, 1 depression/sneezing/dyspnoea, 1 depression/ ataxia/nervous tics, and 1 depression /dyspnoea/ataxia/nervous tics (Appendix 20). Other signs were expressed as individual clinical signs per bird per day of observation.

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The common clinical sign combinations for NIS chickens were namely: 22 for depression/sneezing/nasal discharges/ruffled feathers, 9 for depression/sneezing, 8 for depression/nasal discharges, 8 for depression/ruffled feathers, 5 for sneezing/nasal discharges, 4 for depression/nasal discharges/ataxia/ruffled feathers, 3 for depression/sneezing/nasal discharges/dyspnoea/ruffled feathers, 2 for sneezing/nasal discharges/dyspnoea/ruffled feathers, 2 for depression/sneezing/nasal discharges/ ataxia

/ruffled feathers, and 2 for depression/sneezing/nasal discharges/ nervous tics/ ruffled feathers. One each for the following combinations: - depression/sneezing/ dyspnoea, depression/sneezing/nasal discharges/ dyspnoea/ rales, depression/sneezing/nasal discharges/depression/ dyspnoea/ /rales, depression/sneezing/nasal discharges/rales, depression/nasal discharges/diarrhoea, sneezing/nasal discharges/nervous tics, depression/nasal discharges/ataxia/cyanosis, nervous tics/cyanosis, sneezing/rales, depression/sneezing/rales, nasal discharges/nervous tics/ dyspnoea, depression/sneezing/nasal discharges/ataxia/cyanosis, sneezing/mouth discharges/ataxia/diarrhoea, and nasal discharges/ mouth discharges/ruffled feathers were also recorded. Other clinical signs were expressed as individual signs per bird per day of observation (Appendix 21). The NIS chickens had more clinical signs with more clinical sign combinations than the IS chickens.

6.3.2 Clinical signs observed in indigenous ducks

A total of 133 clinical signs were observed in the IS ducks. Of these, 57 were nasal discharges, 34 dyspnoea, 13 sneezing, 8 fever, 5 ataxia, 5 depression, 4 ruffled feathers, 3 mouth discharges, 2 rales, and 2 eye discharges. These signs were highest from day 1 when they numbered 22, and decreased gradually to 5 on day 14 of the observation. Majority of the signs were observed on days 1 to 8. Cyanosis, coughing and nervous tics were not recorded for this group (Table 25).

The common clinical sign combinations were 10 nasal discharges/ dyspnoea, 4 sneezing/ dyspnoea/nasal discharges, and 14 sneezing/dyspnoea. Others were one each of nasal discharges/ rales, sneezing/ dyspnoea/rales/ataxia, sneezing/dyspnoea/nasal discharges/ataxia/mouth discharges, and nasal discharges/sneezing/dyspnoea (Appendix

/ruffled feathers, and 2 for depression/sneezing/nasal discharges/ nervous tics/ ruffled feathers. One each for the following combinations: - depression/sneezing/ dyspnoea, depression/sneezing/nasal discharges/ dyspnoea/ rales, depression/sneezing/nasal discharges/depression/ dyspnoea/ /rales, depression/sneezing/nasal discharges/rales, depression/nasal discharges/diarrhoea, sneezing/nasal discharges/nervous tics, depression/nasal discharges/ataxia/cyanosis, nervous tics/cyanosis, sneezing/rales, depression/sneezing/rales, nasal discharges/nervous tics/ dyspnoea, depression/sneezing/nasal discharges/ataxia/cyanosis, sneezing/mouth discharges/ataxia/diarrhoea, and nasal discharges/ mouth discharges/ruffled feathers were also recorded. Other clinical signs were expressed as individual signs per bird per day of observation (Appendix 21). The NIS chickens had more clinical signs with more clinical sign combinations than the IS chickens.

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The common clinical sign combinations were 10 nasal discharges/ dyspnoea, 4 sneezing/ dyspnoea/nasal discharges, and 14 sneezing/dyspnoea. Others were one each of nasal discharges/ rales, sneezing/ dyspnoea/rales/ataxia, sneezing/dyspnoea/nasal discharges/ataxia/mouth discharges, and nasal discharges/sneezing/dyspnoea (Appendix

22). Other clinical signs were expressed as individual sign per bird per day of observation.

A total of 188 clinical signs shown by the NIS 8 week-old ducks in the age susceptibility in chapter 5 were used for the comparison in this study. Of these, 58 were nasal discharges, 26 dyspnoea, 25 sneezing, 13 depression, 13 ruffled feathers, 10 mouth discharges, 8 ataxia, 7 coughing, 2 eye discharges, and 2 spontaneous rales. Nervous tics, cyanosis and head scratching were not recorded in this group. The signs were highest from day 1, when they numbered 25, and decreased gradually to 1 on day 14 of the observation. Majority of the clinical signs were observed on days 1 to 8 (Table 26).

The common clinical sign combinations were 8 nasal discharges/sneezing, 4 nasal discharges/dyspnoea, 3 nasal discharges/ mouth discharges, and 3 nasal discharges/ sneezing/dyspnoea. One each for the following clinical sign combinations, nasal discharges/dyspnoea/rales, nasal discharges/sneezing/ataxia, nasal discharges/dyspnoea/ataxia, and nasal discharges/mouth discharges/eye discharges were also recorded (Appendix 23). Other signs were expressed as individual signs per bird per day of observation. As observed in chickens, the NIS ducks had more clinical signs with more clinical sign combinations than the IS ducks.

6.3.3 Comparison between the type and number of clinical signs observed in immunosuppressed and non-immunosuppressed infected chickens and ducks

More clinical signs were expressed by the non-immunosuppressed birds compared with the immunosuppressed ones. Head scratching, nervous tics and cyanosis were observed in chickens but not ducks while coughing and eye discharges were noted in ducks but not in chickens (Table 27). The normal watery fluid nature of duck faeces

made the diarrhoea assessment in ducks difficult, although infected birds had foul smelling faeces compared with control birds.

Comparing the various combinations of clinical signs in ducks and chickens, there were more combinations in chickens than in ducks. The frequency of showing 4 to 5 clinical signs at a time were more in chickens than in ducks (Appendix 24).

The chickens had more cases of depression, ruffled feathers, sneezing, rales, diarrhoea and fever as compared with ducks, while ducks had more nasal discharges, dyspnoea, and mouth discharges observed as compared to the chickens. IS chickens had more nervous tics than NIS chickens; while all other observations were more in NIS than IS. Immunosuppressed ducks had more cases of dyspnoea when compared with NIS ones, but all other observations were more in NIS ducks (Table 27).

On examination of the total daily clinical signs, the IS birds had most cases up to day 9 and 8 for chickens and ducks, respectively; while NIS birds had most cases up to day 13 and 8 for chickens and ducks, respectively. Most clinical signs were expressed within the first week post inoculation and waned off in the second week. Generally the IS birds appeared to express less signs in the second weeks as compared with the NIS birds (Table 28). The control birds had no clinical signs observed throughout the experimental period of 14 days.

There were significant differences between IS and NIS chickens in the expression of fever, ruffled feathers, rales, mouth discharges, with most signs being expressed by NIS chickens ($P< 0.05$). Nervous tics were also significantly different between the IS and NIS chickens, with most cases being expressed by IS birds ($P< 0.05$). Fever, depression, ataxia, nervous tics, head scratching and mouth discharges varied significantly ($P< 0.05$)

with days, p.i, unlike other clinical signs in chickens. More of these signs were observed within 10 days, p.i. Fever, nervous tics and head scratching were significantly affected by immunosuppression ($P< 0.05$) and also significantly varied with days post infection in chickens.

There were significant differences between NIS and IS ducks in the expression of depression, fever, ruffled feathers, coughing, sneezing and mouth discharges ($P< 0.05$); with majority of signs being shown by NIS ducks. Immunosuppression with dexamethosone, suppressed these signs. Ataxia, nasal discharges, dyspnoea and eye discharges in ducks; and ataxia and nasal discharges in chickens were not significantly affected by immunosuppression ($P>0.05$) but varied significantly with days, p.i ($p< 0.05$). Depression, Ruffled feathers, sneezing, dyspnoea, ataxia, nasal discharges and mouth discharges varied significantly with days, p.i, with majority of these signs being observed within 7 days p.i, ($P< 0.05$). However, coughing, fever, rales and diarrhoea did not significantly vary with time post infection ($P> 0.05$).

Immunosuppression significantly reduced depression, ruffled feathers, fever, sneezing and mouth discharges ($P< 0.05$) but not other signs in ducks ($P> 0.05$). Nervous tics, cyanosis and head scratching were not recorded and need further studies in indigenous ducks. In chickens the immunosuppression significantly reduced depression, nasal discharges, head scratching and mouth discharges ($P < 0.05$) unlike other signs. Most signs were observed in ducks between day 1 to 8 post infection and the number observed were significantly different from other days ($P< 0.05$). The clinical signs significantly decreased between day 9 to 14; the observed numbers at this time had no daily significant differences ($P> 0.05$). Different signs peaked on different days namely:

ruffled feathers, sneezing, and mouth discharge, on day 1 p.i; nasal discharge on day 3 p.i; ataxia and dyspnoea on day 4 p.i, and rales on day 9 post infection. In chickens, most signs were observed on days 1 to 8 p.i with a peak on days 1 to 5, p.i, especially depression, ruffled feathers, rales , sneezing, dyspnoea, ataxia, nervous tics, cyanosis and head scratching. These observations changed significantly with days, p.i ($p< 0.05$).

6.3.4 Gross lesions and bacterial re-isolation from the chickens and ducks

At the end of the study period, necropsy examination was carried out and bacterial re-isolation done from various organs of each bird. There were few lesions in both ducks and chickens and those present were in form of remnants of fibrin, cloudy airsacs (fibrosis), and scar on the abdominal lobes of the lungs.

Seven IS ducks had remnants of fibrin and fibrosis; on the right side of 5 ducks and two other ducks had fibrosis on the left abdominal airsacs. Two of these ducks, had, in addition, scars on the right lungs. *P.multocida* was re-isolated from oropharynx of three of these ducks. Five NIS ducks had remnants of fibrin and fibrosis; 4 on the right side and one on the left abdominal airsac. One of these ducks had a scar on the left lung. *P.multocida* was re-isolated from oropharynx of three of these ducks.

Four IS chickens had remnants of fibrin and fibrosis; on the right side of three chickens and one on the left airsac. *P.multocida* was not re-isolated from these chickens. One chicken had a scar on the right lung. Two NIS chickens had remnants of fibrin and fibrosis on the left airsacs. *P.multocida* was re-isolated from oropharynx of one of the chickens.

Generally, the immunosuppressed birds had more fat deposits on the subcutaneous and abdominal tissues than the non-immunosuppressed ones.

Table 23: Type and total daily clinical signs observed in immunosuppressed indigenous chickens

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	4	4	3	2	3	3	3	3	2	1	1	3	-	1	**33**
Nervous tics	-	2	2	5	5	3	4	-	3	1	-	1	2	2	**30**
Ruffled feathers	3	2	2	2	3	2	2	3	1	1	1	2	-	-	**24**
Sneezing	2	3	3	2	1	1	2	1	2	2	3	-	2	1	**25**
Ataxia	-	-	-	2	3	2	1	1	1	3	-	1	-	-	**14**
Nasal discharges	-	-	1	1	-	1	1	1	1	2	1	2	1	1	**13**
Dyspnoea	2	1	1	1	1	2	-	-	2	-	1	-	1	-	**12**
Mouth discharges	-	-	1	-	-	-	-	-	-	-	-	1	-	-	**2**
Diarrhoea	-	-	-	-	-	-	-	-	-	-	-	1	-	-	**1**
Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Rales	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Fever	1	2	2	3	3	4	1	2	1	-	1	-	-	-	**20**
Head scratching	1	-	1	1	1	1	-	-	-	-	-	-	-	-	**5**
Total signs	**13**	**14**	**16**	**19**	**20**	**19**	**14**	**11**	**13**	**10**	**8**	**11**	**6**	**5**	**179**

Table 24: Type and total daily clinical signs observed in non-immunosuppressed indigenous chickens

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	8	8	7	7	7	6	6	5	5	5	3	4	5	3	**79**
Nervous tics	-	-	-	1	1	3	2	1	1	2	2	-	-	-	**13**
Ruffled feathers	8	8	7	7	7	5	5	4	5	4	3	4	4	3	**74**
Sneezing	4	3	3	4	3	2	2	2	3	2	2	3	2	1	**36**
Ataxia	-	1	1	2	2	1	1	2	2	1	-	-	1	-	**14**
Nasal discharges	3	4	3	2	3	3	2	2	2	3	1	2	3	1	**34**
Dyspnoea	1	1	1	1	1	1	2	1	2	2	1	1	1	-	**16**
Mouth discharges	-	1	1	-	-	-	-	-	-	-	1	3	-	-	**6**
Diarrhoea	1	1	1	1	1	2	1	1	1	1	1	1	1	-	**14**
Cyanosis	-	-	-	-	-	1	1	-	1	-	-	-	-	-	**3**
Rales	-	1	1	-	-	-	1	1	-	-	1	2	1	-	**8**
Fever	5	2	3	4	5	4	5	6	6	4	4	4	4	4	**60**
Head scratching	1	5	3	4	2	-	-	-	-	-	-	-	-	-	**15**
Total signs	**31**	**35**	**31**	**33**	**32**	**28**	**28**	**25**	**28**	**24**	**19**	**24**	**22**	**12**	**372**

Table 25: Type and total daily clinical signs observed in immunosuppressed indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	1	-	1	2	-	1	-	-	-	-	-	-	-	-	**5**
Ruffled feathers	1	-	1	1	-	1	-	-	-	-	-	-	-	-	**4**
Sneezing	5	1	-	2	1	1	-	1	-	1	1	-	-	-	**13**
Ataxia	4	1	-	-	-	-	-	-	-	-	-	-	-	-	**5**
Nasal discharges	2	8	3	3	3	4	4	6	4	4	5	4	3	4	**57**
Dyspnoea	5	3	4	6	1	1	2	3	2	3	3	-	-	1	**34**
Mouth discharges	2	1	-	-	-	-	-	-	-	-	-	-	-	-	**3**
Rales	-	-	-	1	1	-	-	-	-	-	-	-	-	-	**2**
Eye discharges	-	-	-	-	1	1		-	-	-	-	-	-	-	**2**
Diarrhoea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Fever	2	2	1	-	1	1	1	-	-	-	-	-	-	-	**8**
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Total signs	**22**	**16**	**10**	**15**	**8**	**10**	**7**	**10**	**6**	**8**	**9**	**4**	**3**	**5**	**133**

Table 25: Type and total daily clinical signs observed in immunosuppressed indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	1	-	1	2	-	1	-	-	-	-	-	-	-	-	5
Ruffled feathers	1	-	1	1	-	1	-	-	-	-	-	-	-	-	4
Sneezing	5	1	-	2	1	1	-	1	-	1	1	-	-	-	13
Ataxia	4	1	-	-	-	-	-	-	-	-	-	-	-	-	5
Nasal discharges	2	8	3	3	3	4	4	6	4	4	5	4	3	4	57
Dyspnoea	5	3	4	6	1	1	2	3	2	3	3	-	-	1	34
Mouth discharges	2	1	-	-	-	-	-	-	-	-	-	-	-	-	3
Rales	-	-	-	1	1	-	-	-	-	-	-	-	-	-	2
Eye discharges	-	-	-	-	1	1		-	-	-	-	-	-	-	2
Diarrhoea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Fever	2	2	1	-	1	1	1	-	-	-	-	-	-	-	8
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Total signs	**22**	**16**	**10**	**15**	**8**	**10**	**7**	**10**	**6**	**8**	**9**	**4**	**3**	**5**	**133**

Table 26: Type and total daily clinical signs observed in non-immunosuppressed indigenous ducks

Clinical signs	Days post infection														Total signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Depression	2	2	2	2	1	2	1	1	-	-	-	-	-	-	**13**
Nervous tics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Sneezing	3	3	3	3	2	1	2	2	1	2	1	1	1	-	**25**
Ruffled feathers	2	2	2	2	1	2	1	1	-	-	-	-	-	-	**13**
Ataxia	-	2	1	2	1	1	1	-	-	-	-	-	-	-	**8**
Nasal discharges	7	4	7	5	2	6	5	5	4	4	4	3	1	1	**58**
Dyspnoea	4	3	2	3	2	2	2	1	1	1	1	4	-	-	**26**
Mouth discharges	2	1	1	1	1	2	1	1	-	-	-	-	-	-	**10**
Diarrhoea	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Coughing	2	1	-	1	-	1	-	-	-	1	1	-	-	-	**7**
Eye discharges	-	-	-	-	1	-	1	-	-	-	-	-	-	-	**2**
Rales	-	-	-	-	-	-	-	1	1	-	-	-	-	-	**2**
Head scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**
Fever	2	3	3	3	1	2	2	1	1	1	-	2	1	-	**22**
Total signs	**25**	**22**	**21**	**22**	**12**	**19**	**16**	**13**	**8**	**9**	**7**	**10**	**3**	**1**	**188**

Table 27: Comparison between the type and total clinical signs expressed in immunosuppressed and non-immunosuppressed indigenous chickens and ducks

Clinical signs	Type of bird			
	Chickens		Ducks	
	IS	NIS	IS	NIS
Depression	33	79	5	13
Nervous tics	30	13	-	-
Ruffled feathers	24	74	4	13
Sneezing	25	36	13	25
Ataxia	14	14	5	8
Nasal discharges	13	34	57	58
Dyspnoea	12	16	34	26
Mouth discharges	1	6	3	10
Rales	-	8	2	2
Diarrhoea	-	14	-	1
Cyanosis	-	3	-	-
Coughing	-	-	-	7
Eye discharges	-	-	2	2
Fever	20	60	8	22
Head scratching	5	15	-	-
Total signs	**179[b]**	**372[a]**	**133[c]**	**188[d]**

Legend: - No clinical sign seen; IS –Immunosuppressed; NIS- Non-immunosuppressed.
X^2 calculated $^{(a,b \text{ and } c,d)} = 6.68$ ($p<0.05$)

Table 28: Comparison between the total daily clinical signs observed in immunosuppressed and non-immunosuppressed chicken and ducks

Type of Birds		Days post infection														Total signs
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Chickens	**IS**	13	14	16	19	20	19	14	11	13	10	8	11	6	5	**179[b]**
	NIS	31	35	31	33	32	28	28	25	28	24	19	24	22	12	**372[a]**
Ducks	**IS**	22	16	10	15	8	10	7	10	6	8	9	4	3	5	**133[c]**
	NIS	25	22	21	22	12	19	16	13	8	9	7	10	3	1	**188[d]**

Legend: IS –Immunosuppressed; NIS- Non-immunosuppressed; X^2 calculated $^{(a,b \text{ and } c,d)}$ = 6.68 ($p<0.05$)

6.4 Discussion

P. multocida may affect birds of any age causing acute septicaemic disease with high morbidity and mortality (Rhoades, 1964; Rimler *et al.*, 1998). Previously the most susceptible age for the indigenous chickens and ducks was determined, and was used in this study. NIS chickens infected with *P.multocida* expressed fever, depression, nasal discharges, sneezing, ruffled feathers, dyspnoea, diarrhoea, ataxia, nervous tics, spontaneous tracheal rales, mucous discharges from the mouth, cyanosis, head scratching and anorexia. The IS chickens showed all these signs except spontaneous tracheal rales and cyanosis. Ducks, both IS and NIS, expressed similar signs of fever, nasal discharges, dyspnoea, sneezing, ataxia, eye discharges, spontaneous tracheal rales, depression, ruffled feathers, and anorexia. Cyanosis was not possible to evaluate in ducks as most of them had dark skins and examination of other organs could stress the birds. Anorexia was qualitatively gauged using the amount of feed left after 24 hours feeding and just before addition of more feed in the feeders. Some combined clinical signs were similar between chickens and ducks, but others were different and variable. The difference between the IS and NIS was due to the total number, combinations, and period of occurrence of most clinical signs; with NIS birds showing more of these than IS birds.

Immunosuppression with dexamethosone, suppressed these signs. This raises the question of the pathogenesis of the *P.multocida*; are the signs due to presence of the bacteria; or are the mediators released from the phagocytes causing the signs? Therefore, stressful conditions that may suppress inflammatory cells will result in less clinical sign expression. Which cells phagocytise *P.multocida* and on phagocytosis what mediators do they release? If released, are mediators harmful to the tissues? Further research is

required. Nervous tics were significantly different between the IS and NIS chickens, with most cases being expressed by IS birds ($P< 0.05$). A possibility that this sign may depend on the number of *P.multocida* organisms or their toxic products, but not phagocytic effects of the phagocytes. Immunosuppression significantly reduced expression of some signs ($P< 0.05$) but not other signs in chickens and ducks ($P> 0.05$). Maybe, the significantly variable signs are the ones to look for, when evaluating the severity of *P.multocida* infections in NIS chickens and ducks, while other signs will always be present regardless of the state of the bird. The latter may show the presence of disease but not its severity in chickens. Nervous tics, cyanosis and head scratching were not recorded. Clinically it is possible to pick the clinical manifestation between day 1 to 5 p.i in chickens and ducks. Possibly these are days that it is easier to pick this clinical signs in chickens and ducks. The clinical signs observed in this study are similar to those reported by others, for intermediate to chronic fowl cholera (Rimler *et al.*, 1998; Gooderham, 1999; Christensen and Bisgaard, 2000; Glisson *et al.*, 2003) except for a few. Sero–mucous discharges from the mouth, nose and eyes are as reported by Glisson *et al* (2003) although in this study there was occasional occurrence of muco-purulent discharges on one or both nostrils. The discharges could sometimes block the nostrils of the affected birds. Watery white diarrhoea that turned mucoid with time was easily observed in chickens, as reported elsewhere (Rhoades and Rimler, 1989; Glisson *et al.*, 2003) but it was difficult to evaluate diarrhoea in ducks due to the nature of the watery faeces in normal birds, although in some infected duck cases, the faeces had foul smell and were white or greenish in colour (Rhoades and Rimler, 1989; Gooderham, 1999). Severity of depression was variable, with some birds being lethargic (birds being slow in moving

away when excited or frequently lying down), drooping their wings, dosing or having tucked heads under their wings, while others were inactive and not aware of their surroundings. Ruffled feathers were expressed in form of raised feathers around the neck, or around the neck plus whole body. In severe cases, birds showed ruffled feathers, drooped wings and dosing. In chickens, dyspnoea was in form of open mouth breathing (or frequent gaping) and increased respiratory rate, but in ducks there was additional increase and marked abdominal movements and harsh "hissing-like" sounds. Torticollis (Gooderman, 1999; Glisson, *et al.*, 2003) was not observed. However, in chickens, nervous tics were present in both IS and NIS though more prominent in IS birds. Nervous tics were not expressed in ducks. Much as the observed nervous tics could have been a sign of meningitis or meningo-encephalitis, they could have been a reaction to mild laryngitis or severe laryngitis, or an effort to clear the nostrils and the upper respiratory tract. Ataxia was observed in chickens and ducks as wobbling movements (staggering unsteady gaits); on standing the birds could shiver, wobble, or stand for a few seconds before flipping-over or sitting on their hocks. Head scratching using legs was common in chickens but not in ducks. The affected birds could briefly scratch on the areas around the ears, nostrils and eyes. Inspite of the diversity of clinical signs observed, very few lesions were observed at post mortem, after the study period, except remnants of fibrin, and fibrous strands in form of fibrotic scars on the lungs and airsacs indicated chronic process. Immunosuppressed birds appeared, qualitatively to have more fat deposits on the subcutaneous and abdominal tissues compared with the NIS birds. This was unexpected in view of the severity of the disease in this group.

Immunosuppression appears to modify and decrease expression of clinical signs while ataxia, nervous tics, and head scratching are additional signs to be considered in cases of suspected fowl cholera. Individual and combined clinical signs of fowl cholera caused by a low virulent *P.multocida* organism are hereby reported.

CHAPTER 7

7.0 EXPERIMENT 5: CONTACT CROSS TRANSMISSION OF *P. MULTOCIDA* FROM INDIGENOUS CHICKENS TO INDIGENOUS DUCKS

7.1 Introduction

Pasteurella multocida carrier birds have been observed in apparently healthy-looking indigenous chickens and ducks in Kenya (Chapter 3 of this thesis) and elsewhere (Muhairwa *et al.*, 2001a,b). There is a possibility therefore, that *P.multocida* enzootic situation exists among the indigenous birds at farm and market areas. The susceptible age group and clinical signs of *P.multocida* have been documented in this thesis (Chapters 4,5 and 6). Many small-scale farmers in peri-urban and rural areas keep chickens as well as ducks (Nyaga et al., 2002; Mbuthia *et al.*, 2003). Others keep chickens and ducks separately but the birds interact while scavenging for food. These birds are housed, watered and given minimal kitchen waste supplementation that they share together (Minga *et al.*, 1989; Nyaga *et al.*, 2002). Little is documented on contact cross transmission of *P. multocida* between indigenous chickens and ducks at farm and market places, although the fowl cholera clinical signs reported (Chapter 6 of this thesis) are similar in both type of birds.

This study was designed to determine whether there is cross transmission of *P. multocida* from infected indigenous chickens to indigenous ducks through contact.

7.2 Materials and methods

7.2.1 Experimental birds

A total of 21 wing-tagged birds comprising of 15 eight week-old indigenous ducks and 6 twelve week-old indigenous chickens were used in this study (Fig.4). The specific

FIG.4. CHICKENS TO DUCKS CONTACT CROSS TRANSMISSION FOR *PASTEURELLA MULTOCIDA*

susceptible age groups had earlier been determined (this thesis: Chapters 4, experiment 2 for chicken and chapter 5, experiment 3 for ducks). These birds were kept in a 3 metres by 3 metres room with deep litter floor, provided with growers' marsh feed from Unga limited ® (Nairobi, Kenya), and water *ad libitum*.

All birds were screened and shown to be negative for *P.multocida* prior to the study. The birds were separated and put in the experimental rooms 48 hours prior to the start of the experiment.

7.2.2 Bacteria used to infect chickens

The bacteria and inoculum were prepared as described in chapter 4 section 4.2.2. Each of the chickens was inoculated intratracheally with 0.5 ml culture containing 1.6 x 10^8 CFU of *P.multocida* organisms.

7.2.3 Post mortem examination

Post mortem examination was done as described by Bermundez and Stewart-Brown (2003) and findings noted and recorded.

7.2.4 Re-isolation and identification of *P.multocida*

Swabs for bacterial examination were taken from the oropharynx, cloaca (live birds) and also from spleen, liver, lungs and caecal tonsils from the carcasses. The swabs were placed in 2 ml of sterile physiological saline and transported in a cool box to the laboratory for culturing for *Pasteurella* spp.

7.2.5 Experimental procedure

The 15 uninfected sentinel ducks were mixed with the six *P. multocida* infected chickens six hours after infection for contact cross infection. Five ducks were swabbed daily from days 1 to 14 after mingling with the infected chickens. Each of the 5 ducks

was swabbed separately using sterile cotton-tipped applicator swabs on the oro-pharynx and cloaca. On day 1 post-infection the infected chickens were similarly swabbed on the oro-pharynx and cloaca to establish their infection with *P.multocida.* The remaining 10 ducks of the 15 ducks were randomly paired as done in Steel and Torrie (1980) and sacrificed at specified days 1, 3, 5, 7, and 10 after mixing with the infected chickens. On day 14 post-infection the remaining 5 ducks that were regularly swabbed and the infected 6 chickens were sacrificed, examined for gross lesions and swabs taken for bacterial isolation.

7.3 Results

7.3.1 Recovery of *P. multocida* from the sentinel ducks

Table 29 gives the summary of daily *P. multocida* recoveries from swabs collected from the sentinel ducks after mixing with the infected chickens. A day after mixing the sentinel ducks with the experimentally infected chickens, 2 of the 5 ducks had *P. multocida* organisms from their oro-pharynx and cloacal swabs. By the second day of contact sentinel 4 ducks had the bacteria while all the 5 ducks being swabbed had *Pasteurella* infection by the 6th day after the mixing. From one bird (No.1414) *P. multocida* was recovered from day 6 to 14 but other birds had intermittent recovery of the bacteria. Between one and 4 ducks contracted *P. multocida* infection from chicken on any one day of swabbing, while the bacterium was isolated in 5/14 (35.7%) to 9/14 (64.3%) times in each duck during the 14 days swabbing period. A total of 35 (50%) *P. multocida* isolates were recovered from a 70 samples for the whole period.

Table 29: Daily isolation of *P. multocida* organisms from the sentinel ducks mixed with the infected chickens

Duck number	Days of swabbing the ducks post infection													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1414	-	-	-	-	-	+	+	+	+	+	+	+	+	+
1425	-	+	-	-	+	+	-	-	+	-	+	+	-	+
1427	-	+	+	+	-	+	+	-	+	+	-	-	-	+
1428	+	-	+	+	-	-	+	+	-	-	+	-	-	-
1433	+	-	+	-	+	-	-	+	-	+	+	+	+	-
Total isolates	**2**	**2**	**3**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**4**	**3**	**2**	**3**

Legend:

\+ *P. multocida* organisms isolated from the bird

\- No *P. multocida* organisms isolated from the bird

7.3.2 Clinical signs, post mortem lesions and bacterial isolations from both ducks and chickens

All the experimental birds showed clinical signs as described earlier in chapter 6, experiment 4, of this thesis. Chickens were found to frequently peck on the bills of the ducks and among themselves in an attempt to remove feed stuck around the mouth and head regions. The common gross lesions observed are as described in chapters 10 and 11, of this thesis. Lesions were seen on airsacs, lungs, and peritoneum in ducks between days 1 to 5. Thereafter the lungs had scar lesions due to fibrosis on days 7, 10 and 14. *P. multocida* was also recovered in different birds on days 1, 3, 5, 7, 10 and 14 of the infected. Infected chickens swabbed on day 1 post-infection, 4 out of 6 chickens had *P. multocida* recovered from their oro-pharyngeal and cloacal swabs, while 2 out of 6 chickens had *P. multocida* isolated from their oro-pharynx on day 14. Chickens sacrificed at day 14 post-infection had fibrosis on the lungs, and remnants of inspissated fibrin on their abdominal airsacs (Appendix, 25).

7.4 Discussion

Intra-tracheal inoculation of chickens with *P.multocida* ensured that they were infected with the bacteria in a manner simulating the natural infection under scavenging conditions (Matsumoto *et al.*, 1991; Petersen *et al.*, 2001b). Having the ducks and chickens sharing the same feed and water simulated the field setups and ensured contact between the birds (Minga *et al.*, 1989; Nyaga *et al.*, 2002).

On mixing, chickens were found to peck on the bills of ducks and other chickens in an attempt to remove feed stuck around mouth and head areas. This behaviour may have facilitated fast transmission of bacteria from one bird to another (Glisson *et al.*, 2003).

P. multocida organisms were earlier (chapter 3 of this thesis) isolated in large numbers from the oro-pharyngeal swabs from farm and traded birds. This indicates that drinking water and feed could easily be contaminated with the organisms excreted via mouth and nasal discharges, which increases the chances of cross-infection. Various routes of transmission have been described, such as: contaminated water and aerosols in turkeys; intranasal, intraocular and oral routes in ducks; and through direct inoculation in turkeys (Siemesen and Olson, 1980; Pehlivanoglu *et al.*, 1999). There is no report to date on contact transmission between indigenous chickens and ducks, as given here.

The clinical signs and gross lesions observed in this study were similar to those reported by others (Hunter and Wobser, 1980; Christensen and Bisgaard, 2000; Glisson, *et al.*, 2003) as well as those observed in pathogenesis studies here in reported. This, together with the high frequency of isolation of *P. multocida* from ducks in contact with the infected chickens confirms that the organisms were successfully transmitted from chickens to ducks during this study. Similar situation may be occuring in market places and at farm level, during scavenging. Therefore disease control strategies should target both species of birds in the field. The inter-and intra-species cross-transmission may also be playing a role in the maintenance and propagation of *P.multocida* organisms at village level.

CHAPTER 8

8.0 EXPERIMENT 6: CONTACT CROSS TRANSMISSION OF *P. MULTOCIDA* FROM INDIGENOUS DUCKS TO INDIGENOUS CHICKENS

8.1 Introduction

All the three *Pasteurella multocida* subspecies have been recovered from healthy-looking indigenous chickens and ducks in Kenya (chapter 3 of this thesis) and in other countries (Muhairwa, *et al.*, 2001a,b). The susceptible age group for chickens and ducks as well as the clinical signs of *P. multocida* have been documented in this thesis (Chapters 4,5 and 6). Both the chickens and the ducks had similar clinical signs as reported elsewhere (Glisson *et al.*, 2003 and in chapter 6 of this thesis). However, in our study there were variable magnitudes in each species for the observed clinical signs. There is a need to evaluate if an enzootic situation exists among the indigenous birds. A number of smallholder farmers in rural areas of Kenya keep both ducks and chickens (Nyaga *et al.*, 2002). In Chapter 7 of this thesis *P.multocida* was shown to be successfully transmitted from infected chickens to ducks. However, little is documented on contact cross transmission of *P. multocida* from infected indigenous ducks to none infected indigenous chickens at market places and village farms.

The aim of this study was to determine whether there is cross transmission of *P.multocida* from infected indigenous ducks to susceptible indigenous chickens through contact.

8.2 Materials and Methods

8.2.1 Experimental birds

A total of 21 wing-tagged birds comprising of 15 twelve week-old indigenous chickens and 6 eight week-old indigenous ducks were used in this study. The specific susceptible age groups had earlier been determined (this thesis: Chapters 4, experiment 2 for chickens and chapter 5, experiment 3 for ducks). These birds were kept in a 3 metres by 3 metres room with deep litter floor, provided with growers' marsh feed from Unga limited ® (Nairobi, Kenya), and water *ad libitum*. All birds were screened and shown to be negative for *P.multocida* prior to the study. The birds were separated and put in the experimental rooms 48 hours prior to the start of the experiment.

8.2.2. Bacteria used to infect ducks

The bacteria and inoculum were prepared as described in chapter 4 section 4.2.2. Each of the ducks was inoculated intratracheally with 0.5 ml culture containing 1.8×10^8 CFU of *P.multocida* organisms.

8.2.3 Post mortem examination of the birds

Post mortem examination was done as described by Bermundez and Stewart- Brown (2003) and findings noted and recorded.

8.2.4 Re-isolation and identification of *P.multocida*

Swabs for bacterial examination were taken from the oropharynx, cloaca (live birds) and also from spleen, liver, lungs and caecal tonsils from the carcasses. The swabs were placed in 2 ml of sterile physiological saline and transported in a cool box to the laboratory for culturing for *Pasteurella* spp.

8.2.5 Experimental procedure

The 15 uninfected sentinel chickens were mixed with six *P. multocida* infected ducks six hours after infection for contact cross infection. Five chickens were swabbed daily from days 1 to 14 after mingling with the infected ducks. Each of the 5 chickens was swabbed separately using sterile cotton-tipped applicator swabs on the oro-pharynx and cloaca. On day 1 post-infection the infected ducks were similarly swabbed on the oro-pharynx and cloaca to establish their infection with *P.multocida.* The remaining 10 chickens of the 15 chickens were randomly paired as done in Steel and Torrie (1980) and sacrificed at specified days 1, 3, 5, 7, and 10 after mixing with the infected ducks. On day 14 post-infection the remaining 5 chickens that were regularly swabbed and the infected 6 ducks were sacrificed, examined for gross lesions and swabs taken for bacterial isolation.

8.3 Results

8.3.1 Recovery of *P. multocida* from sentinel chickens

Table 30 gives the summary of the daily *P. multocida* recoveries from swabs collected from ducks after mixing with the infected chickens. Within 24 hours of mixing the sentinel chickens with the experimentally infected ducks, 4 out of 5 chickens had *P. multocida* organisms recovered from their oro-pharynx and cloacal swabs. By the 3rd day, all the 5 chickens had *P. multocida* organisms. The bacterium was recovered from 3 to 5 birds up to day 8 following mixing of the birds, after which there was reduction to 2 infected birds out of 5 on days 9 and 10, and then rose to 4 and 3 birds on days 11 and 12, respectively. Thereafter, the bacterial recovery was low. The bacterium was isolated in

each chicken between 7 (50%) and 10 (71.4%) times in the 14 days'period. A total of 42 (60%) *P. multocida* isolates were recovered from 70 samplings for the whole period.

8.3.2 Clinical signs, post mortem lesions and bacterial isolations from chickens and ducks

Chickens were found to frequently peck on the bills of the sentinel ducks and among themselves in an attempt to remove feed stuck around the mouth. All the test birds showed clinical signs as described in chapter 6, experiment 4, and pathological lesions as described in chapters 10 and 11, of this thesis. The gross lesions, including fibrino-purulent necrotic lesions, were seen in chickens 1-3 days after mixing with the infected ducks. Thereafter, the lungs had scar lesions due to fibrosis seen up to day 7. *P. multocida* was also recovered from different chickens sacrificed on days 1, 5,7, and 14 but not on days 3, and 10.

Infected ducks were swabbed on day 1 post-infection and 5 out of the 6 ducks had *P. multocida* recovered from their oro-pharyngeal and cloacal swabs, an indication that the intratracheal inoculation was successful and that they could shed the *P. multocida* organisms. Ducks sacrificed at day 14 post-infection had fibrosis on the lungs, and remnants of inspissated fibrin on their abdominal airsacs (Appendix 26). *P. multocida* was recovered from 4 out of the 6 sacrificed ducks and all from the oro-pharynx at the end of the study.

Table 30: Daily isolation of *P. multocida* isolation from the sentinel chickens after mixing with infected ducks

Chicken number	Days chickens were swabbed post infection													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1284	+	+	+	-	-	+	+	+	-	-	+	+	-	+
1287	+	+	+	+	+	-	+	-	-	-	+	+	-	-
1288	+	+	+	+	-	+	-	+	+	-	-	-	-	-
1289	+	-	+	-	+	+	+	+	+	+	+	+	-	-
1291	-	+	+	+	+	+	+	-	-	+	+	-	-	-
Total isolates	**4**	**4**	**5**	**3**	**3**	**4**	**4**	**3**	**2**	**2**	**4**	**3**	**0**	**1**

Legend:

\+ *P. multocida* organisms isolated from the birds

\- No *P. multocida* organism isolated from the birds

8.4 DISCUSSION

In this study, indigenous ducks were infected intra-tracheally with *P.multocida*, organisms. This ensured that the ducks were infected with the bacteria, simulating cases of natural infection under scavenging conditions and as has been done by others (Matsumoto *et al.*, 1991; Takahashi *et al.*, 1996; Petersen *et al.*, 2001b). Having the ducks and chickens sharing the same feed and water simulated the field rural set ups and ensured contact between these birds (Minga *et al.*, 1989; Nyaga *et al.*, 2002).

On mixing, the birds pecked on bills of others in an attempt to remove feed stuck around mouth and head areas. This behaviour may have facilitated fast transmission of bacteria from ducks to chickens. *P. multocida* organisms were earlier (chapter 3 of this thesis) isolated in large numbers from the oro-pharyngeal swabs of ducks. This is an indication that drinking water and feed could easily be contaminated with the organisms excreted from the mouth, nasal and eye discharges. This would increase the chances of cross-infection. *P. multocida* has been transmitted through water, aerosols in turkeys; and intranasally, intraocularly, orally and intratracheally in commercial ducks and chicken (Siemesen and Oslon, 1980; Matsumoto *et al.*, 1991; Pehlivanoglu *et al.*, 1999). However contact transmission from indigenous ducks to chickens has not been determined.

The clinical signs and gross lesions in this study are similar to those in chapters 5, 6, and 11 of this thesis and as reported elsewhere (Christensen and Bisgaard, 2000; Glisson *et al.*, 2003). This, together with the frequency of isolation of *P. multocida* from chickens in contact with the infected ducks, confirmed the bacterial transmission from ducks to chickens in this study. The inter-and intra-species *P. multocida* transmission is a possible scenario that maintains the organism at village farms and market places.

CHAPTER 9

9.0 EXPERIMENT 7: DEVELOPMENT OF FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) AS A DIAGNOSTIC TOOL FOR *P.MULTOCIDA* IN TISSUES AND CULTURES

9.1 Introduction

The diagnosis of fowl cholera in the sick birds depends on the specific detection of the causative organism, *P.multocida,* and its subsequent characterization. Its detection and phenotypic characterization depend on the ability to cultivate and purify the bacteria in the laboratory (Christensen and Bisgaard, 2000). Cultivation and identification by standard bacteriological methods can be ambiguous because of V-factor requirement or non–typable strains, including cross-reaction in sero-typing and viable but non-culturable cells (Krause *et al.*, 1987).

In situ hybridization (ISH) allows precise localization of a specific segment of nucleic acid within a histologic section (Brown, 1998) or detection of specific ribosomal ribonucleic acids in morphologically intact bacterial cells (Amann *et al.*, 1995). *In situ* hybridization combines basic molecular biologic techniques and the ability to appreciate subtle histomorphologic changes (Brown, 1998). The key feature distinguishing ISH from other molecular methodologies (filter hybridization and PCR) is that the sample DNA/RNA is detected directly in the intact cell rather than being extracted from the cell before testing (Nordentoft *et al.*, 1997). With fluorescent labeled probes, *in situ* hybridization has excellent spatial resolution (Poulsen *et al.*, 1994) and has been used in

various studies (Licht *et al.*, 1996; Boye *et al.*, 2000; Christensen, *et al.*, 1999; Jansen *et al.*, 2000; Jensen *et al.*, 2000; Kempf *et al.*, 2000).

The aim of this study was to develop a culture-independent, fluorescent *in situ* hybridization (FISH) test for *P. multocida* based upon hybridization with fluorescent tagged oligonucleotide probes to bacterial rRNA and testing its diagnostic potential on a variety of *P.multocida* and other bacterial cultures; and also on chicken and pig lungs infected or injected with *P. multocida* organisms.

9.2 Materials and methods

9.2.1 Cultivation of bacteria

A total of twenty two strains of *P. multocida* and other bacterial species, isolated from normal and diseased lungs of poultry and other animals as indicated on Table 31, were used in this study for the development of this tool. These bacteria were stored at -80 °C and cultivated overnight on blood agar base (Oxoid Ltd., CM55, Basingstoke, Hampshire, England) containing 5% citrated calf blood. Single colonies were cultured in brain heart infusion broth (Oxoid), at 37 °C for 3-4 hours in a shaker incubator, and optical density (A_{600}) was measured before fixation.

9.2.2 Fixation of bacterial cells

The bacterial cell cultures were fixed in 4 % paraformaldehyde (Sigma, Chemical Co., St. Louis, Mo.) as previously described (Amann *et al.*, 1990a,b), and collected through centrifugation at 10,000 X g (10K) for five minutes; the supernatant was discarded, while the fixed cells (sedimented) were washed in phosphate–buffered saline with 0.1 % Nonidet P40 (Sigma) and re-suspended in 200 µl of 2 x storage buffer (40

mM Tris, pH 7.5; 0.2 % v/v Nonidet P40) and 200 µl of 96 % alcohol and stored at – 20 °C until use.

9.2.3 Processing of tissue sections

Lung tissues from chickens infected with *P. multocida* subsp. *multocida* strain 40605–1 (Christensen *et al.*, 1998) were used. The respective chickens were infected intra-tracheally with 10^4 colony forming units (CFU) of *P. multocida* per 0.5 ml and sacrificed 12, 24, and 48 hours post inoculation. These birds developed typical clinical signs and gross lesions of fowl cholera and *P. multocida* subsp. *multocida* was re-isolated from their spleens after post mortem examination. For comparison to other animal species, lung tissues from a healthy sacrificed post mortem pig injected with *P. multocida* subsp. *septica* strain HIM 746–6^T were kindly provided by Dr. Tim K. Jensen, SVS, Denmark. All infected chicken and pig tissues as well as controls non-infected tissues were fixed in 10 % neutral buffered formalin, processed for histology, embedded in paraffin wax, sectioned 3 to 4 µm thick and mounted on adhesive slides (Super Frost/plus slides, Menzel–Gläser, Germany) and kept at 4 °C until use.

9.2.4 Oligonucleotide probes

16S rRNA sequences obtained from GenBank and unpublished sequences were compared and emphasis was made on finding at least four mismatches separating *P. multocida* and biovars 2 of *P. canis* and *P. avium* and other members of *Pasteurellaceae* as well as from other bacterial species. Distinction of the segment was done through Pileup alignment using Wisconsin Sequence Analysis Package (GCG, Madison, USA)

(Table 31). Comparison with similar 16S rRNA secondary structures of *E. coli* predicted this region to give strong detection (Fuchs *et al.*, 1998).

The selected probe pmhyb449 (S-S-Pmul-0449-a-A-20 [Alm *et al.*, 1996]) and its complementary, non-pmhyb449 (S-*-Npmol-0449-a–S–20 [Alm *et al.*, 1996]) were labeled with rhodhamine, fluorescein or CY3 (cyanine dyes), and purified by high performance liquid chromatography (HPLC). The signals from the tagged probe were compared when tested with strains of *P. multocida*. Probe EUB338 (S–D–Bact–0338–a–A–18 [Alm *et al.*, 1996; Amann *et al.*, 1990a,b]) that is specific for the bacterial domain, and its complementary probe non–EUB338 (S–*-non–0338–a–S–18 [Alm *et al.*, 1996]) labeled with rhodamine, flourescein or CY3, was used as control.

9.2.5 *In situ* hybridization of cultured bacterial cells

Prior to hybridization, fixed bacterial cells were aclimatized at ambient (room) temperature, mixed by vortexing, and bound to 10-wells teflon–coated slides (Novakemi, AB, Enskede, Sweden) which had been coated by immersion in a 1:10, 0.1% w/v aqueous solution of poly–L–Lysine (Sigma) for 2 minutes, and air dried. One microlitre of bacterial cells were applied in each well. For control some wells had bacteria only, probe only, cells plus buffer (no probe), buffer plus probe (no cells) and empty wells. Slides were air-dried, dehydrated in serial concentrations of 50%, 80%, and 96% ethanol, 3 minutes each and air-dried again. Ten microlitres of hybridization buffer (15% formamide, 100 mM Tris, pH 7.2; 0.9 NaCl, 0.1% sodium dedocyl sulfate [SDS]) containing 5 ng probe were applied per well and hybridization was performed in a humidified moist chamber over night at 37 ° C. Slides were washed in 100 ml of hybridization buffer prewarmed to 37 °C in a coplin jar for 10 minutes, and thereafter

changed to 100 ml of prewarmed (37 °C) hybridization buffer containing 20 % formamide for 10 minutes. Finally, the slides were rinsed in 100 ml of Milliq-water prewarmed to 37 °C and air dried in the dark (Poulsen *et al.*, 1994). Some control slides with bacteria in the wells were hybridized with the complementary probe.

9.2.6 Sorting out optimal hybridzation conditions using fixed bacterial cells

Post hybridization washing with formamide was evaluated using different concentrations (15%, 20%, 25%, and 30%), in two steps, where hybridization time was varied between 5 and 30 minutes at each step. The FISH signals under different formamide concentrations were evaluated.

9.2.7 *In situ* hybridization of tissue sections

Tissue sections were deparaffinized in coplin jars by two changes of xylene and dehydrated twice with 99.9 % ethanol for 3 minutes at each step. Slides were air-dried and a circle drawn around the tissue section using a hydrophobic pen (Dako PAP pen, DAKO A/S, Glostrup, Denmark). The hybridization and wash steps were done as described for the cultured bacterial cells but with 50 µl hybridization buffer and 30 ng of probe per tissue section. The PAP–pen circle was removed from the slides with tissue sections using xylene solvent (Boye *et al.*, 1998a; 2000).

9.2.8 DAPI (4`, 6–diamidino–2–phenylindole) staining

To evaluate the proper morphology and the presence of the bacterial cells where no signal was obtained with probe pmhyb449, DAPI (Sigma) staining was performed for selected bacteria on hybridized slides. Prior to DAPI staining, cover slips were removed, slides immersed in 99.9 % ethanol for 10 minutes to remove mountant and air dried.

Bacteria were stained with 40 µg/well DAPI solution (6ng /ml) for 10 minutes. Slides were washed with 100 ml of Milliq water in a coplin jar for 10 minutes, and air-dried, prior to epi-fluorescence microscopy (Amann *et al.*, 1990a).

9.2.9 Epi-fluorescence microscopy

A cover slip was mounted on hybridized slides with application of a small amount (2 - 3 drops) of paraffin oil or Vectashield (Vector Laboratories, Inc., Burlingame, CA) oil (tissue sections) on the dry slide. Non–fluorescent oil was applied and slides were examined using Zeiss filter sets 05, 09 and 15 for visualization of DAPI, fluorescein, and Rhodamine (tetramethyl rhodamine isothiocynate) or CY3, respectively, using a Zeiss Axioplan II microscope (Oberkochen, Germany) with a 100 W mercury lamp and magnification X 1000. Filter set XF53 (Omega Optical, Brattleboro, VT.) was applied during micrograph photography with a MC 200 Zeiss camera and exposed on a Kodak Ektachrome Elite 400 film (Boye *et al.*, 1998a).

9.3 Results

9.3.1 Developed probe sequence and tagging system

A specific oligonucleotide probe, pmhyb 449, 5`- CTATTTAACAACATCCCTTC-3` (S-S-Pmul-0449-a-A-20 [Alm *et al.*, 1996]) (Tags, Copenhagen, Denmark) for specific detection of *P. multocida* was selected based on 16S rRNA sequence comparison. The probe and its complementary, non-pmhyb449, (S-*-Npmol-0449-a–S–20 [Alm *et al.*, 1996]) were labeled with different fluorescent dyes. The CY3 label was chosen for further experiments because it gave a stronger signal than fluorescein or rhodamine when

tested on strains of *P. multocida* and compared to probe EUB338 that is specific for the bacterial domain, while its complementary probe non–EUB338 labeled with either rhodamine or CY3 was used as control.

9.3.2 Specificity of the probe sequence on fixed bacterial cells

The oligonucleotide probe pmhyb449 and its complementary oligonucleotide probe non–pmhyb449 specific for *P. multocida* were initially tested with fixed bacterial strains. By use of the universal bacterial probe EUB338, all strains tested were found positive which showed that the rRNA in the fixed bacterial cells was accessible for the probe to bind and give a strong fluorescence signal. Its complementary probe non–EUB338 did not give any signal. Probe pmhyb449 was tested on strains of *P. multocida* and gave good signals (Table 30, Fig. 5A), comparable to EUB338 labeled with CY3, while its complementary probe non–pmhyb449 did not give any signals with the same bacterial strains. All other bacteria tested, except *P. avium* biovar 2 and *P. canis* biovar 2 were negative with probe pmhyb449 (Table 31). No signal was detected with controls including empty wells, bacterial cells only, probe only, cells plus buffer (no probe), and buffer plus probe (no cells), showing that there was no non–specific staining.

9.3.3 Optimal hybridization conditions with fixed bacterial strains

Ten minutes washing time at each step gave the best results without background noise. By use of 30% or 25% formamide in the second washing buffer, probe pmhyb449 resulted in weaker signal compared with 20% formamide, while the washing buffer with 15% formamide, under these stringent conditions of hybridization gave strong signal with

P. multocida but also weak signals with other *Pasteurella* spp. and non-*Pasteurella* bacteria at the hybridization temperature of 37 °C.

The optimum formamide concentration during post hybridization washing for probe pmhyb449 was found to be 15% in the first and 20% in second wash using a 10 minutes washing period at each step.

9.3.4 Ability of probe pmhyb449 to pick *P.multocida* from tissues

The ability of the probe pmhyb449 to detect *P. multocida* in tissues was tested, using chicken lungs from experimentally induced fowl cholera and normal pig lungs injected with *P. multocida.*

The specific and narrow fluorescence of probe pmhyb449 distinguished *P. multocida* bacterial cells by a red fluorescence, while lung tissue cells fluoresced greenish to brownish and red blood cells were yellow on examination after *in situ* hybridization (Fig. 5 B - I).

In fowl cholera cases, *P. multocida* was clearly demonstrated within the lung tissues. However, differences in distribution were observed (Table 33). In early cases, 12 hours post-infection, most bacteria were seen in the air capillaries (lumen and wall), bronchi, parabronchial areas (lumen, inter-parabronchial septa), perivascular (arterioles), and as masses in anatomically destroyed lung tissues. A few bacteria were also seen in the infundibulum space linings, pleura, vascular lumen and their walls (Table 33). The bacteria appeared small in size (as compared with pure culture cells), and were coccobacilli to rods in shape, and occurred singly, in pairs, in fours or in clusters of microcolonies (Fig.5 B, D, E) and as massive colonies. Twenty four hours post infection, the bacteria were commonly demonstrated in air capillaries, parabronchial lumen and as

masses of bacteria in necrotic lung tissues and air capillaries. Low numbers were illustrated in air capillary walls, blood vessels (lumen and perivascular), infundibulum and respiratory atria. Some bacteria were seen in pairs or in fours in a ring formation in phagocytic cells (macrophages or heterophils) within the parabronchial spaces, blood vessels and in aggregation of lymphoid tissues within the lungs tissues. At 48 hours, most bacteria were detected in the air capillaries, air capillary walls, infundibulum and a few on the spiral smooth muscles of the parabronchi (Table 33). Some bacteria were found in a ring formation in phagocytes (in bronchi and blood vessels) and in lymphoid aggregation areas of the lung tissues indicating either phagocytosis or presence of circulating phagocytes with bacteria. The role of these cells in pathogenesis of *P. multocida* needs further investigation.

In the pig lung, *in situ* hybridization with probe pmhyb449 clearly detected *P. multocida* among tissues and cells of the lung. The bacteria were found mainly in the alveoli, terminal bronchioles and interstitial areas and a few were observed in the bronchi, bronchioles, and terminal bronchioles (Table 33; Fig 5 G, H).

Both infected and non-infected tissues were negative when hybridized with the non–pmhyb449 probe (Fig.5 F, I). The inflammatory reaction of the lung tissues in response to the bacteria did not hinder the binding of the probe in fowl cholera. The results of this study indicated that probe pmhyb449 is suitable for detection and determination of the *in vivo* localization of *P. multocida* in tissues.

Table 31: Specificity test results of oligonucleotide probe pmhyb 449 and its complementary non-pmhyb 449 labeled with CY3 on bacteria grown in pure culture

Bacterial strain	Bacterial species	Animal Source	Hybridization signal intensity	
			Pmhyb 449	Pmhyb non–449
NCTC 10322[Ta]	*P. multocida* subsp. *multocida*	Pig	+++[b]	-ve[c]
214	*P. multocida* subsp. *multocida*	Calf	+++	-ve
40605–1	*P. multocida* subsp. *multocida*	Eider	+++	-ve
RA 12/2	*P. multocida* subsp. *multocida*	Calf	+++	-ve
NCTC 10204[T]	*P. multocida* subsp. *gallicida*	Cow	+++	-ve
77179	*P. multocida* subsp. *gallicida*	Chicken	+++	-ve
NCTC 11619[T]	*P. multocida* subsp. *septica*	Human (cat bite)	+++	-ve
5	*P. avium* biovar 2	Calf	+++	-ve
25	*P.canis* biovar 2	Calf	+++	-ve
NCTC 11188[T]	*P. gallinarum*	Chicken	-ve	-ve
ATCC 43326[T]	*P. canis.* biovar 1	Dog	-ve	-ve
F 149[T]	*P.anatis*	Duck	-ve	-ve
CCM 5974[T]	*Actinobacillus salpingitidis*	Chicken	-ve	-ve
NCTC 4189[T]	*A. lignieresii*	Cow	-ve	-ve
P737	*Mannheimia. glucosida*	Sheep	-ve	-ve
14R525	*Escherichia coli*	Human	-ve	-ve
SA 4461	*Heamophillus paragallinarum*	Chicken	-ve	-ve
SA 7191	*H. paragallinarum*	Chicken	-ve	-ve
4237/2sv	*Riemerella anatipestifer*	Duck	-ve	-ve
4280/2sv	*R. anatipestifer*	Duck	-ve	-ve
726–82[T]	*Coenotia anatina*	Duck	-ve	-ve
1912+pSD	*Salmonella enterica* serotype Gallinarum	Chicken	-ve	-ve
19110+pSD	*S.enterica* serotype Gallinarum	Chicken	-ve	-ve

[a] T = Type strain
[b] +++ = High signal intensity
[c] -ve = Negative, no signal observed

Table 32: Oligonucleotide probe DNA sequence of the *P. multocida* specific probe pmhyb 449 compared with selected 16S rRNA sequences of strains of bacteria within the genera *Pasteurella*, *Actinobacillus* and *Mannheimia* of *Pasteurellaceae* used for *in situ* hybridization

Probe sequence/ Bacterial species	16S rRNA gene sequence[A] (3′ -- 5′)	Accession number	Ref[B].
Pmhyb-449	CTTCCCTACAACAATTTATC		
Non-pmhyb449	GATAAATTGTTGTAGGGAAG		
Bacteria			
P. multocida subsp *multocida*	.....[D]	M35018	Unpub[C]
P. multocida subsp *gallicida*	.....	AF294412	Unpub
P. multocida subsp *septica*	.....	AF294423	Unpub
P. avium biovar 2	.....	L06085	19
P. canis biovar 2	.....	Unpublished	Unpub
P. canis biovar 1	.....G.A..A..G.....	M75049	19
P. gallinarum	.....CGGTAGTG.TN....[E]	M75059	19
P. anatis	.....CGGTAGTG.T.....	M75054	19
P. avium biovar 1	.....TTG.AGTG.T.....	M75058	19
A. lignieresii	.....TA.CAAA..T.....	M75068	19
A. salpingitidis	.....TTNA.ATG.T.....	L06077	19
M. glucosidal	.....CGAT.GT..T.....	AF053889	6

[A] Pairing to pmhyb 449 probe is denoted by dots.
[B] Reference publication: 19 - Dewhirst et al., 1993; 6 – Angen et al., 1999
[C] Unpublished sequence
[D] ..... Same base pairs as *P.multocida*
[E] CGGTA different base pairs from *P.multocida*

Table 33: The results of *in situ* hybridization on the lung tissues showing the anatomical location where *P. multocida* was detected by probe pmhyb449

Tissue source	Chicken[a]			Pig[a]
	Hours post-infection period			
	12	24	48	
Bronchi	++	-	-	+
Parabronchi	++	++	-	++ [b]
Interstitium spaces	+	-	-	+
Smooth muscles	-	-	+	+
Infundibulum spaces	+	+	+	N/A [c]
Aircapillary or alveoli spaces	+++[d]	++	+	+++
Air capillary or alveolar septa	+++	++[e]	+	+++
Pleura	+[f]	-[g]	+	-
Vascular areas	+	+	-	-

[a] chicken lungs from fowl cholera and bacteria injected into pig lung on post mortem
[b] Bronchiole lumen
[c] Not applicable
[d] +++ Many bacteria detected
[e] ++ Moderate number of bacteria detected
[f] + Few bacteria detected
[g] - No bacteria detected

Figure 5: Photomicrographs demonstrating specific detection of *P. multocida* in pure culture and formalin-fixed lung tissues by use of the FISH technique **(see legend on page 114b)**

Figure 5: Photomicrographs demonstrating specific detection of *P.multocida* in pure culture and formalin-fixed lung tissues by use of the FISH technique **(see page 115)**

Legend:

A - A smear of *P. multocida* subspecies *multocida* strain 40605-1 from a culture showing red bacterial fluorescence after hybridization with CY3 labeled probe pmhyb449.

B - Chicken lung from fowl cholera showing *in situ* location of *P. multocida* subspecies *multocida* bacterial cells near two blood vessels (artery and arteriole) after hybridization with CY3 labeled probe pmhyb449.

C - Normal chicken lung showing location on the lungs, where the bacteria were detected in fowl cholera. 1- parabronchi, 2– Infundibulum, 3– Air capillary, 4– Pleura, and 5- Arteriole (Heamatoxylin / Eosin stain).

D - Chicken lung from fowl cholera showing *in situ* location of *P. multocida* subspecies *multocida* bacterial cells within the lumen and walls of aircapillaries and perivascular spaces after hybridization with CY3 labeled probe pmhyb449.

E - Chicken lung from fowl cholera showing *in situ* location of individual organisms or microcolonies of *P. multocida* subspecies *multocida* bacterial cells after hybridisation with CY3 labeled probe pmhyb449.

F - Control lung tissue section from fowl cholera hybridised with probe nonpmhyb449.

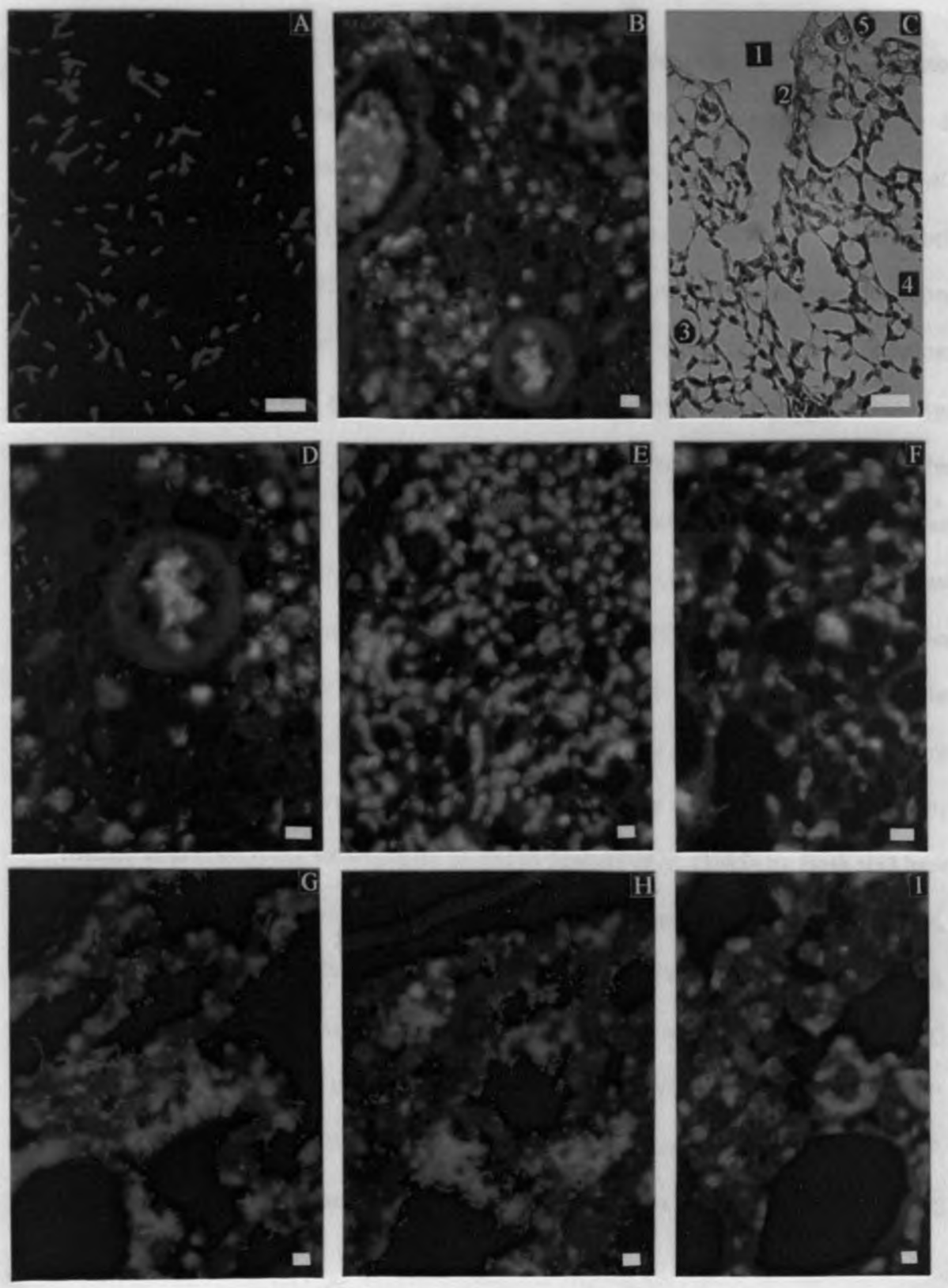
G - Lung of a pig showing *P. multocida* subspecies *septica* bacterial cells in the lumen and wall of the alveoli after hybridisation with CY3 labeled probe pmhyb449.

H - Lung of a pig showing *P. multocida* subspecies *septica* bacterial cells in the lumen, wall of the alveoli and close to the pleura after hybridisation with CY3 labeled probe pmhyb449.

I - Control lung tissue section from a pig injected with *P.multocida* subspecies *septica* after hybridised with probe nonpmhyb449.

Bar = 10 µm for Fig7 A, B, D, E, F, G, H, and I and 100 µm for C.

Figure 5: Photomicrographs demonstrating specific detection of P. multocida in pure culture and formalin-fixed lung tissues by use of the FISH technique (see legend on page 114)



9.4 Discussion

Fluorescent *in situ* hybridization (FISH) of whole cells with rRNA-targeted oligonucleotide probes has been extensively used as a tool for specific detection of bacteria (Delong *et al.*, 1989; Amann *et al.*, 1991 and 1995; Nuovo, 1997; Fussing *et al.*, 1998; Christensen *et al.*, 1999; Boye *et al.*, 2000; Jansen *et al.*, 2000; Moter and Göbel, 2000; Bojesen *et al.*, 2003). To my knowledge no FISH procedure has been developed and used for detection or diagnosis of *P. multocida.* To aid in diagnosis of infections caused by *P. multocida,* a species-specific probe pmhyb449 targeting 16S rRNA was designed and used for fluorescent *in situ* hybridization. The specificity of this oligonucleotide probe was examined by whole-cell hybridization against selected species representing both 16S rRNA clusters of Dewhirst *et al.* (1993) outlined within genus *Pasteurella sensu stricto* as defined by Mutters *et al.* (1989) and other bacterial species commonly associated with respiratory tract infections and septicaemia in poultry. The probe was able to differentiate bacteria by at least four base pair mismatches. Exceptions were *P. avium* biovar 2, and *P. canis* biovar 2. These taxa were originally described by Madsen *et al* (1985) and subsequently named by Mutters *et al.* (1985a,b). Both taxa have been reported from pneumonia in calves in several countries (Bisgaard *et al.*, 1991a, b). 16S rRNA sequence studies of members of the family *Pasteurellacea* (Pohl, 1981), however, indicated that taxon 13 of Bisgaard should be re-investigated since CCUG 16497 (*P. avium* biovar 2) clustered with the type strain of *P. multocida* and not with *P. avium* (Dewhirst *et al.*, 1993). Similarly it was found that *P. canis* biovar 2 is related to *P. multocida* and not to *P. canis* biovar 1. However, similarities of organisms based on 16S

rRNA sequence comparison are insufficient, per se, for species separation (Fox *et al.*, 1992); just as outlining of species based upon a few selected DNA:DNA hybridizations might result in uncertain species definitions (Angen *et al.*, 1999). Recently it was found that the 16S rRNA similarity between *P. multocida* and biovars 2 of *P. avium* and *P. canis* are higher than 98.6% compared to 94 and 96% between *P. multocida* and biovars 1 of *P. avium* and *P. canis* (Petersen *et al.*, 2001a; Christensen and Bisgaard, 2003). On this background it appears that biovars 2 of *P. avium* and *P. canis* are misclassified and that future re-classification will place them with *P. multocida*. With this view, probe hyb449 is concluded to be specific for *P. multocida* except for these two biovars till their re-classification.

The *in situ* hybridization assay was used for detection of *P. multocida* in formalin–fixed, paraffin–embedded tissues of a pig lung injected with a pure culture of *P. multocida* subspecies *septica* and in lungs from chickens that developed clinical fowl cholera infection with *P. multocida* subspecies *multocida*. The pmhyb449 probe developed was able to detect single cells of *P. multocida in situ* in the respective lung tissues, whereas no signal was observed from control lung tissue sections from non–infected birds that contained no organisms or infected lung tissues that were hybridized with the complementary non–pmhyb449 probe.

16S rRNA based methods are advantageous in detection and identification of micro-organisms due to the fact that each bacterial cell contains multiple copies of the 16S rRNA in its ribosomes, that ease its detection, with evolutionary highly conserved 16S rRNA regions common to bacteria, and other regions which might be species specific (Delong *et al.*, 1989; Krimmer *et al.*, 1999). 16S rRNA based methods allow

identification of microorganisms independently of bacterial growth rates and metabolic activities (Tolker-Nielsen *et al.*, 1997; Krimmer *et al.*, 1999) although cellular ribosomes are more abundant in rapidly growing bacteria (Delong *et al.*, 1989). The amount of bound probe is directly correlated to the cellular rRNA content, which is dependent on physiological activity at the time of fixation (Amann *et al.*, 1991; Giovannoni *et al.*, 1988). In cases of fowl cholera, the *P. multocida* cells could have been highly active within the lung tissue as they gave strong signals with probe pmhyb449. In fast–growing bacteria, the rRNA content correlates directly with the growth rate (Delong *et al.*, 1989) although it may not be valid in the case of slow growing or starved organisms (Kemp *et al.*, 1993; Tolker-Nielsen *et al.*, 1997). Starved and dormant bacteria can, however, be detectable with FISH as active bacteria if they still possess relatively high 16S rRNA levels as reported for *S. typhimurium* (Tolker-Nielsen *et al.*, 1997).

Probe pmhyb449 clearly demonstrated short rods of the bacteria in various parts of the chicken lungs under mild or severe lung inflammation. In the chickens *P. multocida* was shown to occur singly, in aggregates probably representing microcolonies or in masses occupying large areas of the lungs. Their individual morphology was clearly demonstrated by the pmhyb449 probe as reported in similar FISH procedures of other bacteria (Delong *et al.*, 1989; Giovannoni *et al.*, 1988; Amann *et al.*, 1991).

There are limitations as the test depends on the number of copies of rRNA in the cell (Amann *et al.*, 1995), and hence on the physiological activity of the microorganisms prior to fixation of samples; it is restricted to eutrophic environments such as the lungs of the chickens. Another limitation is the autofluorescence background of the eucaryotic tissue. However, in contrast to immunological methods that rely on the expression of specific

antigenic markers that may not be constant, phenotypic variation does not pose a problem when rRNA is used as a target (Boye *et al.*, 1998b and 2000). Furthermore autolysis of the tissue, as a result of the material being shipped unfixed to the laboratory, does not hinder the detection of bacteria by FISH (Nuovo, 1997) nor does the inflammatory reactions as observed with *P. multocida* infections during this study. The stability of the ribosome target allows for the detection of single cells even in clinical material, as well as identification in smears of pure culture (Nuovo, 1997) that correlates with our observations here.

Muhairwa *et al.* (2000) in their study of *P. multocida* carriers in commercial poultry in Denmark found mouse inoculation to be more effective in recovery of this organism as compared with the commonly used selective media. Yet mouse passage may only select strains pathogenic for the mice. The developed test can offer a complementary role in active clinical case diagnosis and can be applied in pathogenesis and pathogenicity studies of *P. multocida*.

The probe pmhyb449 is recommended as our results suggest that the probe might be applied with advantage to studies of *P. multocida* pathogenesis and its infections in poultry, pig and maybe other animals. Furthermore, results can be obtained quickly since pure cultures of the bacteria are not needed. It can also be used to confirm and differentiate *P. multocida* (except the two biovars) from other *Pasteurellaceae* in culture. The test is simple and can be applied in most research and diagnostic laboratories.

CHAPTER 10

10.0 EXPERIMENT 8: PATHOGENESIS OF *PASTEURELLA MULTOCIDA* IN NON-IMMUNOSUPPRESSED AND IMMUNOSUPPRESSED INDIGENOUS CHICKENS

10.1 Introduction

P.multocida organisms can enter the chicken's body through various routes to eventually cause fowl cholera, a disease that occurs either as paracute and acute septicaemic condition or as localized chronic infections (Christensen and Bisgaard, 2000; Glisson *et al.*, 2003). Upon infection, the organism spreads to various organs, especially the respiratory system, to cause variable pathology (Glisson, 1998). The spread of these organisms from the point of infection into the various organs of indigenous chickens is, however, not well studied. There is, therefore, a need to simulate the infection by infecting the birds through the most common point of entry, the respiratory tract (Matsumoto *et al.*, 1991; Petersen *et al.*, 2001b), and observe the resultant pathology in various organs at various intervals. Further, the FISH test developed in chapter 9 was used to identify *P.multocida* in many chicken tissues.

As for experiment 4, chapter 6, of this thesis, two groups of chickens, one normal and one immunosuppressed, were used to find out if immunosuppression, which is one manifestation of stress, has any effect on the pathogenesis of the organisms and the pathology after infection. This was occasioned by the fact that scavenging village chickens are normally subjected to different types of stressful conditions (Aini, 1999a) that may modulate the pathology altering the tissue reaction to *P.multocida* infection.

The aim of this experiment was to study the pathogenesis of *P.multocida* infection in non-immunosuppressed and immunosuppressed indigenous chickens. The parameters used included: - gross and microscopic pathology of respective organs, *P.multocida* isolation and its detection in respective tissues using fluorescent *in situ* hybridization (FISH) test.

10.2 Materials and methods

10.2.1 Experimental chickens

Chickens at their most susceptible age of 12 weeks, as determined earlier (Chapter 4, Experiment 2 of this thesis) were used in this experiment. Some birds were bought at day-old from farmers, while others were hatched from indigenous bird-eggs purchased locally. They were reared, kept and screened for *P.multocida* organisms as given on chapter 4 section 4.2.1. Infection trials were undertaken when birds reached 12 weeks of age.

10.2.2 Bacteria used to infect chickens

P.multocida type strain (NCTC 10322^T) maintained on Dorset egg agar, in our laboratory that had low virulence than other *P.multocida* was used in this study. It was spread onto BA with 5 % citrated calf blood, incubated aerobically at 37 °C, for 24 hours to check for purity prior to preparation of the inoculum. Individual colony of this was inoculated into brain heart infusion broth, incubated aerobically at 37 °C, for 24 hours. A ten fold serial dilution of one milliliter of the bacteria in BHI was made and 25µl of each dilution spotted onto BA plates for colonies that were translated into colony forming units.

10.2.3 Immunosuppression of chickens

Birds to be immunosuppressed were injected intramuscularly using dexamethasone (Agar Holland, 3760 AL Soest, Holland), 4mg/Kg body weight per day as done elsewhere for 6 days prior to experimental infection (Birrenkott and Wiggins, 1984; Corrier and DeLoach, 1990; Corrier *et al.*, 1991; Nakamura *et al.*, 1994).

10.2.4 Experimental procedure

Experimental birds were randomly allocated into infected and control groups as given by Steel and Torrie (1980). Twelve-week old, wing-tagged indigenous chickens were used in two separate experiments: one for the immunosuppressed (IS) chickens and one for the non-immunosuppressed (NIS) ones, each with respective controls. The birds were separated and put in the experimental rooms 48 hours prior to inoculation.

For the experiment on NIS chickens, 34 chickens were used: 24 were infected with *P.multocida* organisms and 10 were used as controls. For the experiment on IS chickens, 44 chickens were used: 24 IS ones were infected with *P.multocida* organisms, while the controls consisted of 10 IS and 10 NIS birds. Each infected bird (both NIS and IS) was inoculated with 0.5 ml of brain heart infusion (BHI) broth culture containing 1.2-1.9 x 10^8 CFU of *P.multocida* organisms, while the control (both NIS and IS) were inoculated with 0.5ml of sterile BHI broth medium. The infected and control birds were housed in different rooms located in different houses away from each other. Biosecurity was maintained during the entire period of the study.

Two birds, randomly selected from each experimental group were sacrificed at specified times, namely: - hour 0,1,3,6,12 and 24 and days 2,3,5,7,10 and 14, post-infection. The zero (0) hour was achieved by inoculating the birds and immediately

sacrificing them within 1-5 minutes. To avoid possible cross-infection, the daily sacrifices started with birds in the control houses before proceeding to the infected houses. Table 34 shows the group, number of birds, and the specified sacrificial times.

Table 34: The number of birds used in pathogenesis study and the time they were sacrificed

Birds	Hours post infection						Days post infection						Total
	0	1	3	6	12	24	2	3	5	7	10	14	
NIS Infected	2	2	2	2	2	2	2	2	2	2	2	2	**24**
NIS Control	2	-	-	2	-	2	-	-	-	2	-	2	**10**
IS Infected	2	2	2	2	2	2	2	2	2	2	2	2	**24**
IS Control	2	-	-	2	-	2	-	-	-	2	-	2	**10**
NIS Control	2	-	-	2	-	2	-	-	-	2	-	2	**10**
Total	**10**	**4**	**4**	**10**	**4**	**10**	**4**	**4**	**4**	**10**	**4**	**10**	**78**

Legend: - No bird was sacrificed at that time; IS – immunosuppressed: NIS – non-immmunosuppressed.

Post- mortem examination was done on the sacrificed birds and gross lesions on various organs and tissues noted and recorded. At the same time, swabs were taken aseptically from the oropharynx, cloaca, lungs, caecal tonsils, pruning gland, liver and spleen, for *P.multocida* re-isolation and characterization.

Tissue samples, including: - lung, trachea, lower conjunctiva, airsacs, spleen, liver, heart, thymus, Harderian gland, pancreas, brain, sciatic nerve, kidney, testis or ovary, adrenal gland, bursa of Fabricius, proventriculus, duodenum, midgut (at the Merkel's diverticulum), caecal tonsils, large intestine, pruning gland, comb and pectoral muscle, were removed, immediately immersed in formalin, where they were kept for histological processing. Some paraffin-embedded tissue sections were mounted on adhesive super-frost plus slides for the FISH processing and examination. For each organ examined, the

resultant histological examination, bacterial re-isolation of *P.multocida* and FISH results were recorded.

10.2.5 Post-mortem examination

Birds were killed through cervical dislocation. Post mortem examination was done as described by Bermudez and Stewart-Brown (2003). The dead birds were opened aseptically, tissues and organs observed individually, and gross lesions noted and recorded. For microscopic lesions, six tissue sections of a particular organ per group were examined. The severity of the lesions was semi- quantitatively scored on the basis of: no minimal or slight lesions (- or 0), minimum, slight or few lesions (1 or mild), many lesions in a localized area or in many tissue sections (2 or moderate) and severe or diffuse lesions and in all tissue sections (3 or severe) as described by others (Gross and Siegel, 1965; Gross and Colmano, 1967; Shivaprasad and Droual, 2002).

10.2.6 Histological processing of tissues

The tissue samples were kept fully immersed in labeled bottles containing 10% formalin solution for 24 hours. They were then transferred into 70% alcohol, where they remained until trimming was done (Brown, 1998). The fixed tissues were manually trimmed to a thickness of 2 to 3 millimeters. The trimmed tissues were then placed in an automatic tissue processor for the following treatments: - (1) dehydration using: - (i) 80% ethyl alcohol for the first 4 hours, (ii) 96% alcohol for the next 4 hour, and (iii) 100% alcohol for 4.5 hour; (2) clearing with xylene for a total of 5 hours; and (3) impregnating with molten paraffin wax at 60 °C for a total of 6 hours. The tissues were then removed from the processor and were embedded into wax blocks using a molten wax dispenser. The individual tissue blocks were separated and fixed onto microtome

chunks using a searing spatula, after which they were sectioned to 3-5 µm thickness, floated on a water bath at 50 °C to flatten out, placed on a microscope slide and dried in an oven at 60 °C for about one hour. The sections were then quickly dewaxed in xylene and washed in alcohol before re-hydrating in water. They were then stained using haematoxylin and eosin, mounted in destrene 80, dibutylphthalate and xylene (DPX) and the slides left to dry before examination under the microscope.

10.2.7 Fluorescent *in situ* hybridization technique

Tissue sections were processed as given in 10.2.5 above up to the stage of tissue sectioning. The paraffin-embedded sections were then mounted on super-frost plus slides (Menzel-Gläser, Germany) and hybridized as described on chapter 9, section 9.2.7.

10.2.8 Statistical analysis

Data obtained during the study up to 14 days post infection was analyzed using the analysis of variance procedures of the statistical analysis systems (SAS, 1996) and chi-squared test (X^2) (Steel and Torrie, 1980).

10.3 RESULTS

10.3.1 Pathogenesis of *P.multocida* organisms as observed through gross lesions

10.3.1.1 Gross lesions of NIS chickens

Organs from NIS chickens showing gross lesions and their respective severities at specific periods are presented in Table 35. Lesions were present on 23 of the 24 organs examined; pruning gland was negative for the whole of the study period. Gross lesions were absent from all organs at 0 (1-5 minutes) hour and on day 14 post inoculation (p.i). Lesions were commonly observed on the lung (75%), airsacs (66.7%), trachea (58.3%),

spleen (58.3%), caecal tonsils (50%), heart (41.7%), kidney (41.7%), liver (37.5%), and conjunctiva, thymus, Harderian gland, bursa of Fabricius, duodenum, midgut and large intestines (33.3%, each); other organs had lesions less than one third of the observation time.

At one hour p.i, there were 4 (33.3%) organs with inflammatory lesions: the lungs, trachea, airsacs and heart, while at third hour 20 (83.3%) organs were positive except bursa of Fabricius, proventriculus, pruning gland and the comb. By the sixth hour p.i 22 (91.7%) organs had developed a lesion, except proventriculus and the pruning gland. On the twelveth hour, the proventriculus had a lesion to make a total of 23 (95.8%) organs with lesions. Most organs showed lesions between 3 to 24 hours post infection. By the second day p.i, there were few organs with lesions: the lungs, trachea, airsacs, spleen, liver, kidney, bursa of Fabricius, and caecal tonsils only. These lesions became limited to the lung, trachea or caecal tonsils, airsacs and spleen on the third and fifth day and to the lungs alone on days 7 and 10 p.i. In the second week post infection only the lung had a lesion. Thus, the number of organs with lesions increased from a total of 4 at the first hour p.i, to a maximum of 22 at the sixth hour and decreased gradually to 1 at day 10 p.i.

Lesions on these organs were mild 69 (67.7%) others moderate 24 (23.5%) and the rest were severe 9 (8.8%). Severe and moderate lesions were observed mostly on the lungs, airsacs, trachea, conjunctiva, spleen, liver, heart, kidneys, duodenum, midgut, caecal tonsils, and pectoral muscle. Moderate to severe lesions were observed on the lungs at one hour - 7 days; airsacs 3 hours - 2 days; trachea 3-12 hours; liver, spleen, and kidney 12-24 hours; heart 6 –12 hours; pectoral muscle 3 – 6 hours; and duodenum, midgut, and caecal tonsils at the 12 hours.

The various gross pathological lesions observed and their respective severities at specific period are presented in Table 36. A total of 39 inflammatory lesions were recorded from 23 of the 24 organs examined per bird over the 14 days' study period. These were: congestion, haemorrhage, oedema, purulent exudate, fibrin or fibrino-purulent exudate, necrosis, emphysema, fibrosis, froth, paleness of an organ, thickened airsacs, hydropericardium, mucus exudate, urate deposition, collapsed or hepatized lungs, splenomegally, hepatomegally, pneumonia, air sacculitis, tracheitis, sinusitis, pleuritis, peritonitis, conjunctivitis, pericarditis, myocarditis, hepatitis, perihepatitis, splenitis, splenic capsulitis, splenic atrophy, nephritis, enteritis, meningitis, sciatic nerve myelitis, bursitis and pectoral muscle myositis.

Congestion was found on the carcass and most organs, while haemorrhages were on the sinuses, trachea, heart, lungs, pleura, airsacs, peritoneum and intestines. Oedema was conspicuous on the lungs, pleura and peritoneal cavities, trachea, conjunctiva, heart, brain, bursa of Fabricius, and intestinal serosa. Fibrin, purulent exudation (micro-abscess), fibrino-purulent exudation, necrosis and fibrosis were common in many organs. Mucus exudation was common in sinuses, trachea, bronchi, and intestines. Paleness was obvious on the heart and kidneys. Sinusitis was more conspicuous in the choanae and infraorbital sinuses. Other lesions were specific for each organ. No lesions were observed at 0 hour and the 14th day post infection. During the study period the commonly observed lesions were pneumonia (83.3%); fibrin exudation on the lungs, pleura, peritoneum and other organs (75%); congestion, haemorrhage, airsacculitis and tracheitis (66.7%); fibrosis, mucus exudation, pleuritis, peritonitis, hepatitis, and splenitis (58.3%); and

oedema, necrosis, emphysema, splenomegally, hepatomegally and nephritis (50%), in that order of occurrence.

At one hour p.i, 12 (30.8%) lesions were observed, namely; congestion, haemorrhage, oedema, pneumonia and air sacculitis, together with mild deposition of fibrin, emphysema in the lungs, lung hepatization (with marbling appearance), tracheitis, sinusitis, pleuritis and peritonitis. On the third hour p.i, 27 (70.0%) lesions were encountered after further observation of purulent exudate, fibrino-purulent exudate, froth from trachea, sinuses, and bronchi, mucus exudates from sinuses, bronchi, trachea and intestines, splenomegally, hepatomegally, conjunctivitis, pericarditis, hepatitis, splenitis, nephritis, enteritis, meningitis, sciatic nerve myelitis and sternal muscle myositis. After 6 hours p.i, necrosis, hydropericardium, collapsed lungs and myocarditis were observed, making a total of 33 (84.6%) observed inflammatory lesions. On the twelve hour, p.i, 38 (97.4%) lesions observed were fibrosis, paleness, thick airsacs, urate deposition and splenic capsulitis. Splenic atrophy was observed on the third day post infection.

Most organs showed lesions between 3 hours and three days but by the fifth day, there were few lesions observed. On the second week of observation only occasional haemorrhages, fibrosis and pneumonia were recorded. The number of lesions increased from 12 at 1 hour, to a maximum of 38 at 12 hours and decreased gradually to 3 on day 10 p.i. Lesions were mild {129 (62.3%)}, moderately severe {48 (23.2%)} and severe {30 (14.5%)} ones. The severe lesions were pneumonia (3-24 hours); congestion (3-12 hours); airsacculitis, purulent exudates, and fibrino-purulent exudation (6 – 24 hours); oedema (3 – 6 hours); heamorrhage, froth, hepatomegally, tracheitis and pleuritis (6

hours); collapsed lungs, lung hepatization and peritonitis (12 hours); and mucus exudates and necrosis (24 hours)(Table 36).

All the control chickens had no lesions observed on their organs.

10.3.1.2 Gross lesions of IS chickens

Immunosuppression affected the lymphoid organs of chickens as shown by examples of the bursal of Fabricius and caecal tonsils in Figure 6. Organs from IS chickens showing gross lesions and their respective severities at specified periods are presented in Table 35. Lesions were present on 22 of the 24 organs sampled; the adrenal and pruning glands were negative during the study period. Gross lesions were absent from all organs on 0 (1-5 minutes) hour, day 10 and 14 p.i but present other sampling times. Over the study period lesions were commonly observed on the lung (75%), airsacs, liver (58.3% each), spleen (50%), trachea, kidney (41.7%), and conjunctiva, heart (33.3%). At one hour, p.i, there were 6 (25%) organs with lesions, namely; lungs, trachea, airsacs, spleen, liver, and heart only. The organs increased to 14 (58.3%) by the third hour, as there were lesions on the conjunctiva, thymus, Harderian gland, pancreas, brain, kidney, large intestine, and the pectoral muscle. At the sixth hour, organs with lesions increased to 16 (66.7%) due to lesions on the sciatic nerve, sex organ, and bursa of Fabricius. On the twelveth hour, 22 organs had shown a lesion as proventriculus, duodenum, midgut, caecal tonsils and the comb were positive. Most organs expressed lesions between 3 to 12 hours post infection. By the second day p.i, there were few organs with lesions that were observed on the lungs, airsacs, spleen, liver and the kidney. These lesions became limited to the lung, airsacs and liver on the third day and to the lungs alone on days 5 and 7 p.i. The second week post infection had no lesion observed on the organs. The organs with

lesions increased from a total of 6 at one hour, p.i, to a maximum of 20 at the 12 hours and declined to 1, at day 7. Lesions were mild 60 (78.9%) others moderate 10 (13.2%) and the rest were severe 6 (7.9%) ones. Moderate to severe lesions were observed mostly on the lung (1hour – day 2), airsacs (3 –24 hours), trachea (3 – 12 hours), spleen, liver, and the kidney (12 hours).

A total of 39 inflammatory lesions were recorded from 22 of the 24 organs examined per bird over the 14 days study period as in NIS chickens, as presented on Table 36. Their distribution was like in NIS chickens. No lesions were observed on 0 hour, 10th and the 14th day p.i but were present other sampling times. During the study period the commonly observed lesions were pneumonia (75%); air sacculitis (66.7%); tissue necrosis, splenitis and nephritis (58.3%); fibrin deposition, fibrino-purulent exudation, fibrosis, and hepatitis (50%).

At one hour, p.i, 13 (33.3%) lesions were observed, namely; congestion, haemorrhage, oedema, emphysema, pneumonia, air sacculitis, tracheitis, pleuritis, peritonitis pericarditis, hepatitis, splenitis and nephritis. On the third hour p.i, 25 (64.1%) lesions were encountered after recording of fibrin deposition, froth, hydropericardium, mucus exudate, collapsed and hepatization of the lungs, sinusitis, conjunctivitis, myocarditis, meningitis, bursitis and pectoral muscle myositis. After six hours, p.i, purulent exudate, necrosis, fibrino-purulent exudate, perihepatitis and splenic capsulitis were observed, making a total of 28 (71.8%) observed inflammatory lesions. On the twelveth hour p.i 36 (92.3%) lesions had been observed due to the recording of fibrosis, paleness, thick airsacs, urates deposition, splenomegally, hepatomegally, and enteritis. The last lesions to be recorded were sciatic nerve myelitis at 24 hours and splenic atrophy

on the third day p.i. Most organs expressed lesions between 3 to 24 hours p.i. By the fifth day, there were few lesions observed. On the second week of observation there were no lesions recorded. The number of lesions increased from 13 at 1 hour, to a maximum of 36 at 12 hours and gradually declined to 3 on day 7 p.i. Most lesions were mild {112 (66.3%)}, moderately severe {38 (22.5%)}, and severe {19 (11.3%)} ones. The severe lesions were airsacculitis (6 – 24 hours), pneumonia, haemorrhage, purulent exudate, fibrino-purulent exudates (6 – 12 hours), necrosis, mucus exudates, collapsed and hepatization of the lungs (6 hours), and emphysema (3 hours). There was a slight difference between organs with lesions between NIS and IS chickens, although the difference was not statistically significant ($p>0.05$; Table 35 and 36).

All the control chickens had no lesions on any of their organs.

Table 35: Organs of non-immunosuppressed (NIS) and immunosuppressed (IS) chickens with gross lesions and their severity at specified times

Organs	Chicken treatment	Hours post infection						Days post infection						% lesions per organ[d]
		0	1	3	6	12	24	2	3	5	7	10	14	
Lung	NIS	-	2	3	3	3	3[c]	2	2	2	2[b]	1[a]	-	75
	IS	-	3	3	3	3	2	2	1	1	1	-	-	75
Trachea	NIS	-	1	2	3	2	1	1	1	-	-	-	-	58.3
	IS	-	1	2	2	2	1	-	-	-	-	-	-	41.7
Conjunctiva	NIS	-	-	1	2	2	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Airsacs	NIS	-	1	2	3	3	3	2	1	1	-	-	-	66.7
	IS	-	1	3	3	2	2	1	1	-	-	-	-	58.3
Spleen	NIS	-	-	1	1	2	2	1	1	1	-	-	-	58.3
	IS	-	1	1	1	2	1	1	-	-	-	-	-	50
Liver	NIS	-	-	1	1	2	3	1	1	1	-	-	-	37.5
	IS	-	1	1	1	2	1	1	1	-	-	-	-	58.3
Heart	NIS	-	1	1	2	2	1	-	-	-	-	-	-	41.7
	IS	-	1	1	1	1	-	-	-	-	-	-	-	33.3
Thymus	NIS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	-	-	-	-	-	-	-	25.5
Harderian gland	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
Pancreas	NIS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
	IS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
Brain	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	1	1	1	-	-	-	-	-	-	-	25
Sciatic nerve	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Kidney	NIS	-	-	1	1	2	2	1	-	-	-	-	-	41.7
	IS	-	-	1	1	2	1	1	-	-	-	-	-	41.7
Testis/ovary	NIS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Adrenal	NIS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Bursa Fabricius	NIS	-	-	-	1	1	1	1	-	-	-	-	-	33.3
	IS	-	-	1	1	1	-	-	-	-	-	-	-	16.7

Table 35 (Continued on the next page)

Legend: [a] - Mild lesions; [b] - Moderate lesions; [c] - Severe lesions; - No lesion seen; [d] – percentage each organ was positive during 12 samplings at the specified times

Table 35 (Continued)

Proventriculus	NIS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
	IS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
Duodenum	NIS	-	-	1	1	2	1	-	-	-	-	-	-	33.3
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Midgut	NIS	-	-	1	1	2	1	-	-	-	-	-	-	33.3
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Caecal tonsils	NIS	-	-	1	1	2	1	1	-	1	-	-	-	50
	IS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
Large intestine	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	-	1	-	-	-	-	-	-	-	16.7
Pruning gland	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Comb	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Pectoral muscle	NIS	-	-	2	2	1	-	-	-	-	-	-	-	25
	IS	-	-	1	1	1	-	-	-	-	-	-	-	25
Total mild [a]	NIS	0	3	16	15	9	11	6	3	4	0	1	0	68
	IS	0	5	12	13	13	9	4	3	1	1	0	0	61
Total moderate[b]	NIS	0	1	3	3	9	2	2	1	1	1	0	0	23
	IS	0	0	1	1	5	2	1	0	0	0	0	0	10
Total severe [c]	NIS	0	0	1	3	2	3	0	0	0	0	0	0	9
	IS	0	1	2	2	1	0	0	0	0	0	0	0	6

Legend: [a] - Mild lesions; [b] - Moderate lesions; [c] - Severe lesions; - No lesion seen; [d] – percentage positive samplings out the 12 in the specified times

X^2 calculated (^{a,b,c}) = 3.17 ($p>0.05$)

Table 36: Gross lesions and their severity as observed in NIS and IS chickens at specified times

Lesions	Chicken treatment	Hours post infection						Days post infection						% lesions per organ[j]
		0	1	3	6	12	24	2	3	5	7	10	14	
Congestion	NIS	-	2	3	3	3[f]	2	2[e]	1[d]	-	1	-	-	66.7
	IS	-	1	2	3	2	1	-	-	-	-	-	-	41.7
Haemorrhage	NIS	-	2	2	3	2	2	1	-	1	-	1	-	66.7
	IS	-	1	2	3	3	1	-	-	-	-	-	-	41.7
Oedema	NIS	-	2	3	3	2	2	1	-	-	-	-	-	50
	IS	-	1	2	2	2	1	-	-	-	-	-	-	41.7
Purulent exudate	NIS	-	-	2	3	3	3	2	-	-	-	-	-	41.7
	IS	-	-	-	3	3	2	2	1	-	-	-	-	41.7
Fibrin exudates	NIS	-	1	2	3	3	3	2	1	1	1	-	-	75
	IS	-	-	2	3	3	2	2	1	-	-	-	-	50
Fibrinopurulent	NIS	-	-	2	3	3	3	2	-	-	-	-	-	41.7
Exudates	IS	-	-	-	3	3	2	2	2	1	-	-	-	50
Necrosis	NIS	-	-	-	1	2	3	2	1	1	-	-	-	50
	IS	-	-	-	3	2	2	2	2	2	1	-	-	58.3
Emphysema	NIS	-	1	1	1	1	-	-	-	-	-	-	-	50
	IS	-	1	3	2	1	-	-	-	-	-	-	-	33.3
Fibrosis	NIS	-	-	-	-	1	1	1	2	2	1	1	-	58.3
	IS	-	-	-	-	1	1	1	1	1	1	-	-	50
Froth	NIS	-	-	1	3	2	1	-	-	-	-	-	-	33.3
	IS	-	-	1	-	2	1	-	-	-	-	-	-	25
Paleness	NIS	-	-	-	-	1	2	1	1	-	-	-	-	33.3
	IS	-	-	-	-	2	1	-	1	-	-	-	-	25
Thick airsac	NIS	-	-	-	-	1	1	1	1	1	-	-	-	41.7
	IS	-	-	-	-	1	1	2	1	-	-	-	-	33.3
Hydropericardium	NIS	-	-	-	1	2	1	1	-	-	-	-	-	33.3
	IS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
Mucus exudate	NIS	-	-	1	1	2	3	1	1	1	-	-	-	58.3
	IS	-	-	1	3	2	1	-	-	-	-	-	-	33.3
Urate deposits	NIS	-	-	-	-	1	2	1	1	-	-	-	-	33.3
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Collapsed lung	NIS	-	-	-	1	3	2	1	1	-	-	-	-	41.7
	IS	-	-	1	3	2	1	-	-	-	-	-	-	33.3
Lung hepatization	NIS	-	1	2	1	3	1	-	-	-	-	-	-	41.7
	IS	-	-	1	3	2	-	-	-	-	-	-	-	25
Splenomegally	NIS	-	-	1	2	2	2	1	1	-	-	-	-	50
	IS	-	-	-	-	1	2	1	-	-	-	-	-	25
Hepatomegally	NIS	-	-	1	3	2	2	1	1	-	-	-	-	50
	IS	-	-	-	-	1	1	1	1	-	-	-	-	33.3
Pneumonia	NIS	-	2	3	3	3	3	2	1	1	1	1	-	83.3
	IS	-	1	2	3	3	2	2	1	1	1	-	-	75

Table 36 (Continued on the next page)

Legend: [d] - Mild lesions; [e] - Moderate lesions; [f] - Severe lesions; - No lesion seen; [j] – percentage each organ was positive during 12 samplings at the specified times

Table 36 (Continued)

Airsacculitis	NIS	-	2	2	3	3	3[f]	2	2[e]	1[d]	-	-	-	66.7
	IS	-	1	2	3	3	3	2	1	1	-	-	-	66.7
Tracheitis	NIS	-	1	1	3	2	2	1	-	1	1	-	-	66.7
	IS	-	1	1	2	2	1	-	-	-	-	-	-	41.7
Sinusitis	NIS	-	1	1	2	1	1	-	-	-	-	-	-	41.7
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Pleuritis	NIS	-	1	1	3	2	2	1	1	-	-	-	-	58.3
	IS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
Peritonitis	NIS	-	1	1	3	2	1	1	1	-	-	-	-	58.3
	IS	-	1	1	2	1	1	-	-	-	-	-	-	41.7
Conjunctivitis	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Pericarditis	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
Myocarditis	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	1	2	1	-	-	-	-	-	-	-	25
Hepatitis	NIS	-	-	1	2	2	2	1	1	1	-	-	-	58.3
	IS	-	1	1	1	1	1	1	-	-	-	-	-	50
Perihepatitis	NIS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Splenitis	NIS	-	-	1	2	2	1	1	1	1	-	-	-	58.3
	IS	-	1	1	2	1	1	1	1	-	-	-	-	58.3
Splenic capsulitis	NIS	-	-	-	-	1	-	1	-	-	-	-	-	16.7
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Splenic atrophy	NIS	-	-	-	-	-	-	-	1	1	-	-	-	16.7
	IS	-	-	-	-	-	-	-	1	1	-	-	-	16.7
Nephritis	NIS	-	-	1	1	1	1	1	-	-	-	-	-	50
	IS	-	1	1	1	2	1	1	1	-	-	-	-	58.3
Enteritis	NIS	-	-	1	1	1	1	1	-	-	-	-	-	41.7
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Meningitis	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Nervermyelitis	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
Bursitis	NIS	-	-	-	1	1	1	1	-	-	-	-	-	33.3
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Myositis	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Total mild [d]	NIS	0	6	18	15	16	18	20	17	11	5	3	0	123
	IS	0	13	18	11	20	25	6	10	5	3	0	0	111
Total moderate [e]	NIS	0	5	6	4	13	11	8	2	1	0	0	0	50
	IS	0	0	6	6	10	6	7	2	1	0	0	0	38
Total severe [f]	NIS	0	0	3	13	8	7	0	0	0	0	0	0	31
	IS	0	0	1	11	6	1	0	0	0	0	0	0	19

Legend: [d] - Mild lesions; [e] - Moderate lesions; [f] - Severe lesions; - No lesion seen; [j] – percentage each organ was positive during 12 samplings at the specified times

X^2 calculated (^{d,e,f}) = 1.66 ($p>0.05$)

FIG.6. CAECAL TONSILS AND BURSA OF FABRICIUS FROM NORMAL (CT1, BS1) AND IMMUNOSUPPRESSED (BS2, CT2) CHICKEN

CT1 BS1 BS2 CT2

10.3.2 Pathogenesis of *P.multocida* organisms as evaluated from histopathological lesions of chicken organs

10.3.2.1 Histopathological lesions in NIS chickens

Organs from NIS chickens with histopathological lesions, and their respective severities at specified periods are presented on Table 37. All organs had histopathological lesions distributed over the study period, except at 0 (1-5 minutes) hr and day 14 p.i. Over the study period lesions were commonly observed on the lungs (83.3%), trachea, heart (75%), airsacs, spleen, liver (66.7%), proventriculus (58.3%), conjunctiva, large intestine (50%), and thymus, brain, kidney, bursa of Fabricius, midgut, and caecal tonsils (41.7%).

Within one hour p.i, lesions were observed in 13 (54.2%) organs, namely: lung, trachea, conjunctiva, airsacs, spleen, liver, heart, brain, kidney, adrenal gland, proventriculus, caecal tonsils and the large intestine. By the third hour p.i, organs with lesions increased to 18 (75%), due to pathology in the thymus, Harderian gland, sciatic nerve, bursa of Fabricius, and duodenum. Organs with lesions further increased to 22 (91.7%) by the sixth hour due to pathology in the pancreas, midgut, pruning gland and the comb. At the twelve hours p.i, all 24 organs had pathological lesions including the sex organ and pectoral muscles.

Organs with histopathological lesions increased from a total of 13 at one hour, p.i, to a maximum of 22 at the twelveth hour and decreased gradually to 1 at day 10 of the study. Most organs expressed lesions between 1 and 24 hours p.i. Thereafter organs with lesions decreased to eight namely: lungs, trachea, airsacs, spleen, liver, proventriculus and midgut or comb on day 3 and 5 p.i, and to 3 organs on day 7 p.i, namely:- lungs,

trachea, and heart and to lung alone on day 10 p.i. Most histopathological lesions were mild 107 (82.3%), moderately severe 15 (11.5%) and severe 8 (6.2%) ones. Severe lesions were observed mostly on the lung (6 hours – day 2), airsacs (6 – 12hours), trachea (3 hours), and spleen (6 hours) in that order.

A total of 20 different types of histopathological lesions were recorded from the 24 organs over the study period as presented on Table 38. These were congestion, oedema (in tissues and around blood vessels), haemorrhages, emphysema, atelectasis, fibrin deposition, infiltration of tissues by heterophils, mononuclear cells (Fig. 7), and giant cells, granuloma formation, mucus exudate, urate deposition, degeneration of the epithelium and blood vessels (especially arteries and arterioles), thrombosis formation in blood vessels, muscular degeneration (especially on the heart with predominant zenker's degeneration), depleted lymphoid tissues, necrosis, fibrosis and serositis (mainly on the pleura, pericardium, and intestinal serosa). As in gross lesions no lesions were observed at zero hour and day 14 p.i. Commonly observed lesions were infiltration with heterophils, and mononuclear cells (83.3%, each), congestion, lymphoid cells depletion, necrosis (66.7%, each); degeneration of blood vessels (58.3%), and oedema, giant cells, and fibrosis (50%).

One hour p.i, 10 (50%) observed lesions, were congestion, oedema, haemorrhages, emphysema, atelectasis, infiltration by heterophil, mononuclear cells and giant cells, degeneration of blood vessels, and tissue necrosis. By the third hour p.i lesions increased to 15 (75%) due to fibrin deposition, mucus exudation, degeneration of the epithelium, muscular degeneration and lymphoid cells depletion observed in various organs. After six hours p.i, lesions increased further to 17 (85%) as fibrinous thrombosis in blood vessels,

and serositis were recorded. On the twelveth hour p.i, granulomas were observed in the lungs and airsacs and a total of 16 (80%) lesions were recorded and others (emphysema and atelectasis) were not seen. At 24 hrs p.i, the lesions were 17 (85%) as urate deposits and fibrosis were observed.

The number of lesions increased from 10 at one hour, p.i, to a maximum of 17 at six hours and decreased gradually to 3 on day 10 p.i. Different lesions had been recorded by twenty four hours p.i. Lesions reached a peak from three to twenty four hours p.i. By the third day, there were few lesions observed.

On the second week of the study, only occasional infiltration with heterophils and mononuclear cells, and fibrosis were recorded. Majority of the lesions were mild {63 (58.3%)}, moderate {28 (25.9%)} and severe {17 (15.8%)} ones. Most of the severe lesions were observed between 3 hours and 2 days post infection. The severest lesions were congestion (3-24hours), oedema (6-12hours), heterophil infiltration (6-24hours), granuloma formation (12-24 hours), and necrosis (6-24hours).

No lesions were observed on any of the organs of the control chickens.

10.3.2.2 Histopathological lesions in IS chickens

Organs from IS chickens with histopathological lesions, type and their respective severities at specified times are presented in Table 37. A total of 20 out of 24 organs had histopathological lesions distributed over the study period, but the pancreas, sciatic nerve, pruning gland, and pectoral muscle were negative. There were no lesions observed at 0 (1-5 minutes) hour and day 14 p.i. Lesions were commonly observed in lungs (83.3%),

airsacs (75%), trachea, liver, bursa of Fabricius (58.3%), spleen (50%), heart, kidney, proventriculus and duodenum (33.3%).

Within one hour p.i, 8 (33.3%) organs with lesions, were lung, trachea, airsacs, spleen, liver, kidney, sex organ and bursa of Fabricius. By the third hour p.i, organs with lesions had increased to 14 (58.3%), due to pathology in the conjunctiva, heart, thymus, brain, adrenal gland, midgut and the comb. On the sixth hour p.i, 11 (45.8%) organs that had lesions were: midgut, proventriculus, duodenum, caecal tonsils, and large intestine. At the twelveth hour p.i, 12 (50%) organs had expressed pathology at one time or another. At twenty four hours 16 organs had lesions. The last organ to show a lesion was Harderian gland on second day p.i.

Organs with histopathological lesions increased from a total of 8 by the 1st hour p.i, to a maximum of 16 at the 24th hour and decreased gradually to 3 at day 10 of the study. Most organs showed lesions between 3 and 24 hours post infection. Thereafter organs with lesions decreased gradually to eight, namely: lungs, airsacs, spleen liver, thymus, Harderian gland, bursa of Fabricius and the comb on day 2 and to 3 organs on day 10 p.i, namely: - lungs, liver and bursa of Fabricius . Most histopathological lesions were mild 59 (67.1%), moderate severe 18 (20.5%) and severe 11 (12.5%) ones. Severe lesions were observed mostly on the lungs (3-24hours), airsacs (12th hour), trachea, spleen, liver (6-12hours), and heart (12th hour).

A total of 20 different types of inflammatory lesions were recorded from the 24 organs on histopathological examination over the study period as presented in Table 38. As in gross signs, no lesions were observed on any organ at zero (1-5minutes) hour and day 14 p.i. Commonly encountered lesions were lymphoid cells depletion (100%),

infiltration with heterophils (58.3%), fibrin deposition (58.3%), mononuclear cells, necrosis (50%), congestion, and oedema (41.7%).

At one hour p.i, 5 (25%) lesions observed were congestion, oedema, emphysema, infiltration by heterophil, and lymphoid cell depletion. By the third hour p.i, lesions increased to 10 (50%) as haemorrhage, atelectasis, fibrin deposition, degeneration of the epithelium and muscular tissues were recorded. After 6 hours p.i lesions increased to 16 (80%) as fibrinous thrombosis in blood vessels, mononuclear cells, giant cells, granuloma, mucus exudate, urate deposits, necrosis and fibrosis were recorded. On the twelveth hour p.i muscular degeneration and serositis were observed to make a total of 17 (85%) lesions recorded. All the lesions had been observed at 12 hours p.i. At 24 hours p.i, the lesions were 14 (70%).

The number of lesions increased from 10 at 1hour p.i, to a maximum of 17 at 12 hours and decreased gradually to 1 on day 10 p.i. The main peak of the lesions were on the third hour to the second day p.i. By the third day p.i, there were few lesions observed.

On the second week of observations only lymphoid cell depletion was recorded. Majority of the lesions were mild {63 (72.7%)} others were moderately severe {18 (20.5%)}, and severe {6 (6.8%)} ones. Most of the severe lesions were observed between 3 to 24hours p.i. The severest lesions were congestion (3-12hours), oedema (3-6hours), heterophil infiltration (3hours - 2 days), granuloma formation (6 hours), and necrosis (12 hours - 2 days). NIS chickens had significant different total number organs with lesions; and severe, and mild lesions than IS birds ($p<0.05$).

No lesions were observed on any of the organs of the control chickens.

Table 37: Histopathological lesions and their severity in organs of NIS and IS chickens at specified times

Organs	Chicken treatment	Hours post infection						Days post infection						%lesions perorgan[j]
		0	1	3	6	12	24	2	3	5	7	10	14	
Lung	NIS	-	2	2	3	3	3	3[i]	2[h]	1[g]	1	1	-	83.3
	IS	-	1	3	2	2	1	1	1	1	1	-	-	83.3
Trachea	NIS	-	1	3	2	2	1	1	1	1	1	-	-	75
	IS	-	1	1	2	2	1	-	1	1	-	-	-	58.3
Conjunctiva	NIS	-	1	1	2	2	1	1	-	-	-	-	-	50
	IS	-	-	1	-	3	2	-	-	1	-	-	-	16.7
Airsacs	NIS	-	1	2	3	2	1	1	1	1	-	-	-	66.7
	IS	-	1	1	2	3	2	1	1	1	1	-	-	75
Spleen	NIS	-	1	2	3	2	1	1	1	1	-	-	-	66.7
	IS	-	1	2	3	3	1	1	-	-	-	-	-	50
Liver	NIS	-	1	2	2	1	1	1	1	1	1	-	-	66.7
	IS	-	2	2	3	3	2	1	-	-	-	1	-	58.3
Heart	NIS	-	1	2	2	1	1	1	1	1	1	-	-	75
	IS	-	-	1	-	3	2	-	-	-	-	-	-	33.3
Thymus	NIS	-	-	1	1	1	1	1	-	-	-	-	-	41.7
	IS	-	-	1	-	-	1	1	-	-	-	-	-	25
Harderian gland	NIS	-	-	1	1	-	1	1	-	-	-	-	-	33.3
	IS	-	-	-	-	-	-	1	-	-	-	-	-	16.7
Pancreas	NIS	-	-	-	1	1	-	1	-	-	-	-	-	25
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Brain	NIS	-	1	1	2	1	1	-	-	-	-	-	-	41.7
	IS	-	-	1	-	1	1	-	-	-	-	-	-	25
Sciatic nerve	NIS	-	-	1	-	1	1	1	-	-	-	-	-	33.3
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Kidney	NIS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
	IS	-	1	1	-	1	1	-	-	-	-	-	-	33.3
Testis/ovary	NIS	-	-	-	-	1	1	1	-	-	-	-	-	25
	IS	-	1	-	1	-	-	-	-	-	-	-	-	16.7

Table 37 (Continued next page)

Legend: [g] - Mild lesions; [h] - Moderate lesions; [i] - Severe lesions; - No lesion seen; [j] – percentage each organ was positive during 12 samplings at the specified times

Table 37 (Continued)

Adrenal	NIS	-	1	1	-	-	1	1	-	-	-	-	-	33.3
	IS	-	-	1	-	-	1	N	-	-	-	-	-	16.7
Bursa of	NIS	-	-	1	1	1	1	1	-	-	-	-	-	41.7
Fabricius	IS	-	1	2	1	1	2	1	-	-	-	-	-	58.3
Proventriculus	NIS	-	1	1	1	1	1	-	1	1	-	-	-	58.3
	IS	-	-	-	2	-	2	-	-	1	1	-	-	33.3
Doudenum	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	-	1	-	2	-	1	1	-	-	-	33.3
Midgut	NIS	-	-	-	1	1	1	1	-	1	-	-	-	41.7
	IS	-	-	1	-	1	2	-	-	-	-	-	-	25
Caeacal	NIS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
tonsils	IS	-	-	-	1	1	1	-	-	-	-	-	-	25
Large	NIS	-	1	1	1	1	1	1	-	-	-	-	-	50
intestines	IS	-	-	-	2	-	-	-	1	-	-	-	-	16.7
Pruning gland	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	-	-	-	N	-	-	N	-	-	0
Comb	NIS	-	-	-	1	1	-	-	1	-	-	-	-	25
	IS	-	-	1	N	-	-	1	-	N	-	N	-	16.7
Pectoral	NIS	-	-	-	-	1	-	1	-	-	-	-	-	16.7
muscle	IS	-	-	-	N	-	-	-	N	-	-	N	N	0
Total mild [g]	NIS	0	12	12	12	17	19	16	7	8	4	1	0	108
	IS	0	7	10	4	5	8	8	5	5	3	1	0	56
Total	NIS	0	1	5	5	4	0	0	1	0	0	0	0	16
moderate [h]	IS	0	1	3	5	2	8	0	0	0	0	0	0	19
	NIS	0	0	1	3	1	1	1	0	0	0	0	0	7
Total severe [I]	IS	0	0	1	2	5	0	0	0	0	0	0	0	8

Legend: [g] - Mild lesions; [h] - Moderate lesions; [i] - Severe lesions; - No lesion seen; N – Not done; [j] – percentage each organ was positive during 12 samplings at the specified times

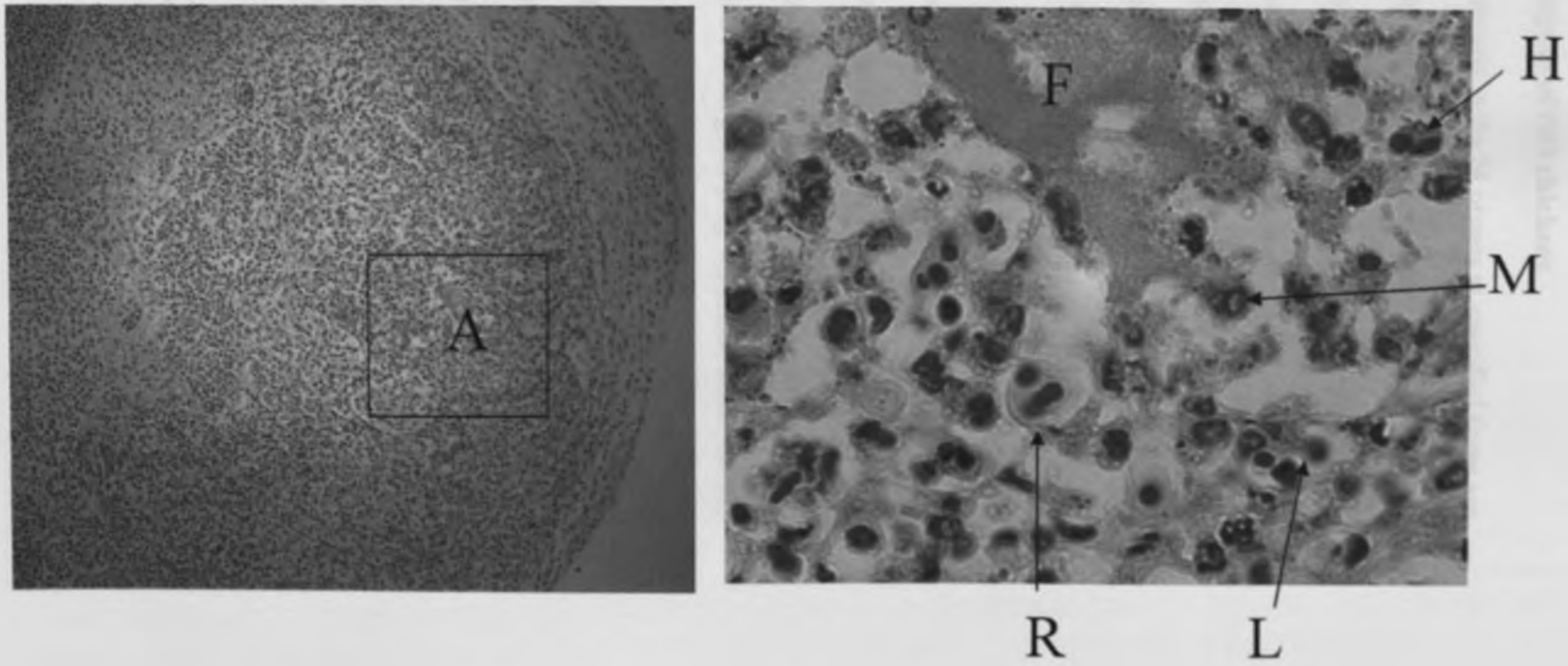
X^2 calculated (^{g,h,i}) = 6.37 ($p<0.05$)

Table 38: Histopathological lesions and their severity as observed in NIS and IS chickens at specified times

Lesions	Chicken treatment	Hours post infection						Days post infection						% lesions per organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Congestion	NIS	-	1	3	3	3	3	1	1	1	-	-	-	66.7
	IS	-	1[a]	3[c]	3	2[b]	1	-	-	-	-	-	-	41.7
Oedema	NIS	-	1	2	3	3	2	1	-	-	-	-	-	50
	IS	-	1	2	2	1	1	-	-	-	-	-	-	41.7
Haemorrhages	NIS	-	1	2	2	1	1	-	-	-	-	-	-	41.7
	IS	-	-	1	1	2	-	-	-	-	-	-	-	25
Emphysema	NIS	-	1	2	1	-	-	-	-	-	-	-	-	25
	IS	-	1	1	-	-	-	-	-	-	-	-	-	16.7
Atelectasis	NIS	-	1	1	1	-	-	-	-	-	-	-	-	25
	IS	-	-	1	1	1	-	-	-	-	-	-	-	25
Fibrin exudates	NIS	-	-1	2	2	3	1	-	-	-	-	-	-	41.7
	IS	-	-	1	2	2	2	1	1	1	-	-	-	58.3
Hetrophils	NIS	-	1	2	3	3	3	2	1	1	1	1	-	83.3
	IS	-	1	3	3	3	2	2	1	1	-	-	-	66.7
Mononuclear cells	NIS	-	1	1	1	2	2	2	3	1	1	1	-	83.3
	IS	-	-	-	1	1	1	2	1	1	-	-	-	50
Giant cells	NIS	-	1	1	1	2	3	1	-	-	-	-	-	50
	IS	-	-	-	1	1	1	-	-	-	-	-	-	25
Granuloma	NIS	-	-	-	-	1	3	3	2	-	-	-	-	33.3
	IS	-	-	-	2	1	1	1	1	-	-	-	-	41.7
Mucus exudates	NIS	-	-	1	2	1	-	-	-	-	-	-	-	25
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Urate deposits	NIS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
	IS	-	-	-	1	-	-	1	-	-	-	-	-	16.7
Ephithelium degeneration	NIS	-	-	1	2	2	1	-	-	-	-	-	-	33.3
	IS	-	-	1	-	-	1	1	-	-	-	-	-	33.3
Blood vessel degeneration	NIS	-	1	1	2	2	2	1	1	-	-	-	-	58.3
	IS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
Thrombosis	NIS	-	-	-	1	1	1	-	-	-	-	-	-	25
	IS	-	-	-	1	1	1	-	-	-	-	-	-	25
Muscular degeneration	NIS	-	-	1	1	2	1	1	-	-	-	-	-	1.7
	IS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
Lyphoid deplction	NIS	-	-	1	1	2	2	1	1	1	1	-	-	66.7
	IS	-	2	1	1	1	1	1	1	1	1	1	-	83.3
Necrosis	NIS	-	1	2	3	3	3	2	1	1	-	-	-	50
	IS	-	-	-	1	2	3	2	1	1	-	-	-	50
Fibrosis	NIS	-	-	-	-	-	1	2	2	2	1	1	-	25
	IS	-	-	-	1	2	2	1	1	2	1	-	-	58.3
Serositis	NIS	-	-	-	1	1	1	-	-	-	-	-	-	25
	IS	-	-	-	-	1	1	1	-	-	-	-	-	25
Total mild [a]	NIS	0	11	8	8	5	8	6	5	5	4	3	0	63
	IS	0	4	7	10	10	9	7	7	5	2	1	0	62
Total moderate [b]	NIS	0	0	6	5	6	4	4	2	1	0	0	0	28
	IS	0	1	1	4	5	3	3	0	1	0	0	0	18
Total severe [c]	NIS	0	0	1	4	4	5	1	1	0	0	0	0	16
	IS	0	0	2	2	1	1	0	0	0	0	0	0	6

Legend: [a] - Mild lesions; [b] - Moderate lesions; [c] - Severe lesions; - No lesion seen; X^2 calculated (^{a,b,c}) = 4.49 ($p>0.05$)

FIG.7. CHICKEN LUNG FROM NON-IMMUNOSUPPRESSED BIRDS SHOWING PYOGRANULOMATOUS FIBRINOUS INFLAMMATION (X40) ON THE LEFT ; AND ON THE RIGHT HIGH MAGNIFICATION OF "A" (X400) . F = FIBRIN EXUDATE; H = HETEROPHIL; M = MACROPHAGE; L = LYMPHOCYTE; R = ERYTHROCYTE. (HAEMATOXYLIN AND EOSIN STAIN).



10.3. 3 Pathogenesis of *P.multocida* organisms as evaluated through signals of fluorescent *in situ* hybridization

10.3.3.1 *P.multocida* FISH signals in organs of NIS chickens

Occurence of *P.multocida* FISH signals in the 24 organs at specified times are presented in Table 39. All organs had P.*multocida* FISH signal during the 14 days' study period, although some organs were missed at some stages during the processing of the FISH test. Lungs and tracheal lumen of various birds had P. *multocida* FISH signal observed from zero (1-5minutes after inoculation) hour up to 14th day of the study, a sign of its high sensitivity. Other organs commonly found to have P. *multocida* signals were spleen and liver (83.3%, each), airsacs (75%), bursa of Fabricius (58.3%), and thymus gland (50%).

By one hour p.i, 10 (41.7%) organs with P.*multocida* signal, were - lung (Fig. 8), trachea, conjunctiva, airsacs, liver, thymus, spleen, bursa of Fabricius (Fig. 9), midgut and large intestine. On the third hour, organs with *P. multocida* signals increased to 16 (66.7%) due to signal observation in the Harderian gland (Fig. 9), brain, sciatic nerve, kidney, sex organ, and the proventriculus. At six hours, the heart, pancreas, duodenum, and caecal tonsils showed *P. multocida* signals, making the total observed organs to be 14 (58.3%). The drop in number of organs with signals was due to some earlier positive organs being negative at this hour. After 12 hours, organs with *P. multocida* increased to 16 (66.7%) as adrenal gland was positive. At 24 hours the pectoral muscles were positive and some organs dropped the signal such that 11 (45.8%) organs were positive. At this time, only pruning gland had no signal but it showed a signal on the 14th day p.i (Fig.10). Organs with *P. multocida* were 2 at zero (1-5minutes after inoculation) hour and they

increased to a maximum of 16 at the third hour. They stayed high from 1hour - 24 hours but decreased to about 25% of the organs being positive for the rest of the period. All the organs of the control birds had no *P. multocida* FISH signals.

10.3.3.2: *P.multocida* FISH signals in organs of IS chickens

Occurence of FISH P.*multocida* signals in the 24 organs at specified times are presented in Table 39. A total of 21 out of 24 organs had the signal observed during the 14 days' study period, although some organs were missed at some stages during the processing of the FISH test. The sciatic nerve, pruning gland and the comb were negative. Lung of various birds had *P. multocida* signal from 1 hour until the 14th day (91.7%) of the study. Other organs where the bacterial signal was commonly detected were spleen and liver (83.3%), airsacs (75%), trachea (66.7%), thymus gland, bursa of Fabricius and duodenum (41.7).

At one hour p.i, 14 (58.3%) organs with *P. multocida* signals, were: - lungs, conjunctiva, airsacs, spleen, liver (Fig. 11), thymus, Harderian gland, pancreas, brain (Fig. 12), adrenal gland, bursa of Fabricius, duodenum, midgut and large intestine. On the 3rd hour, organs with P. *multocida* signals decreased to 11 (45.8%) although more signals were observed on the trachea, heart, and caecal tonsils.

At six hours, organs with FISH signal were 11 (45.8%) and decreased to 8 (33.3%) at 12 hours, although the pectoral muscle was positive. Organs with P. *multocida* were highest at 14 at one hour p.i, and stayed high from one hour to 24 hours to decrease to 4 at the 10th day. All the organs from the control chickens had no *P. multocida* FISH signal.

The NIS had slightly more signals than IS chickens though not different statistically ($p>0.05$).

Table 39: Fluorescent *in situ* hybridization (FISH) signals observed in organs of NIS and IS chickens at specified times

Organs	Chicken treatment	Hours post infection						Days post infection						Signals per organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Lung	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
	IS	-	+	+	+	+	+	+	+	+	+	+	+	11
Trachea	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
	IS	-	-	+	+	+	+	+	-	+	+	+	-	8
Conjunctiva	NIS	-	+	+	+	+	-	-	-	-	-	-	N	4
	IS	-	+	+	+	-	-	-	-	-	-	-	-	3
Airsacs	NIS	-	+	+	+	+	+	-	+	-	+	+	+	9
	IS	-	+	-	+	+	+	+	+	+	+	-	+	9
Spleen	NIS	-	+	+	+	+	+	+	+	+	+	-	+	10
	IS	-	+	+	+	+	+	+	+	+	-	+	+	10
Liver	NIS	-	+	+	+	+	+	+	-	+	+	+	+	10
	IS	-	+	+	+	+	N	+	+	+	-	+	+	10
Heart	NIS	-	-	-	+	+	-	-	-	+	+	-	-	4
	IS	-	-	+	+	-	-	+	-	-	-	-	-	3
Thymus	NIS	-	+	+	+	+	+	-	+	-	-	-	-	6
	IS	-	+	+	+	N	N	+	+	-	-	-	-	5
Harderian gland	NIS	-	-	+	+	+	-	-	-	-	-	-	-	3
	IS	-	+	-	-	-	-	-	-	-	-	-	-	1
Pancreas	NIS	-	-	-	+	+	+	-	-	-	-	-	-	3
	IS	-	+	-	+	N	-	-	-	-	-	-	+	3
Brain	NIS	-	-	+	+	+	-	N	-	N	-	-	-	3
	IS	-	+	-	-	-	+	+	-	-	+	-	-	4
Sciatic nerve	NIS	-	-	+	-	-	-	-	-	N	-	-	+	2
	IS	-	-	N	-	-	-	-	N	-	-	-	-	0
Kidney	NIS	-	-	+	-	+	-	-	-	-	-	-	+	3
	IS	-	-	-	-	-	-	+	-	-	N	-	-	1
Testis/ovary	NIS	-	-	+	-	-	-	-	-	-	-	-	+	2
	IS	-	-	+	-	+	-	-	-	+	+	-	N	4
Adrenal	NIS	-	-	-	-	+	-	-	N	-	-	-	-	1
	IS	-	+	-	N	-	-	-	-	-	-	-	-	1
Bursa of Fabricius	NIS	-	+	+	+	+	+	-	-	+	-	-	+	7
	IS	-	+	+	+	-	+	N	-	-	-	-	+	5
Proventriculus	NIS	-	-	+	-	-	-	N	-	-	-	-	+	2
	IS	-	N	-	-	-	-	-	+	-	-	-	+	1
Duodenum	NIS	-	-	-	+	+	+	-	-	-	+	-	-	4
	IS	-	+	+	+	-	+	-	-	-	-	-	+	5
Midgut	NIS	-	+	-	N	N	+	-	+	-	-	-	-	3
	IS	-	+	N	-	+	-	-	-	+	-	-	N	3
Caecal tonsils	NIS	-	N	-	+	+	-	+	-	-	+	-	-	4
	IS	-	-	+	-	-	-	+	-	N	-	-	+	3
Large intestines	NIS	-	+	+	-	-	N	-	-	-	-	-	-	2
	IS	-	+	N	-	-	-	+	-	N	N	-	-	2
Pruning gland	NIS	-	-	-	-	-	-	-	-	-	-	-	+	1
	IS	-	-	N	-	-	-	-	N	-	-	-	-	0
Pectoral muscle	NIS	-	N	N	N	-	+	-	N	N	-	N	-	1
	IS	-	N	N	+	+	-	-	-	-	N	-	-	2
Comb	NIS	-	N	+	N	-	-	-	N	-	-	-	-	1
	IS	-	N	-	-	-	-	-	-	-	-	-	-	0

Legend: + Positive signal; - Negative signal; N – Not done

FIG.8. CHICKEN LUNGS FROM NON-IMMUNOSUPPRESSED BIRDS SHOWING: (A) BACTERIA IN AIR CAPILLARIES AND (B) IN PNEUMONIC LESIONS POSITIVE FOR *P.MULTOCIDA* ON FISH TEST; AND (C) CONTROL LUNG.

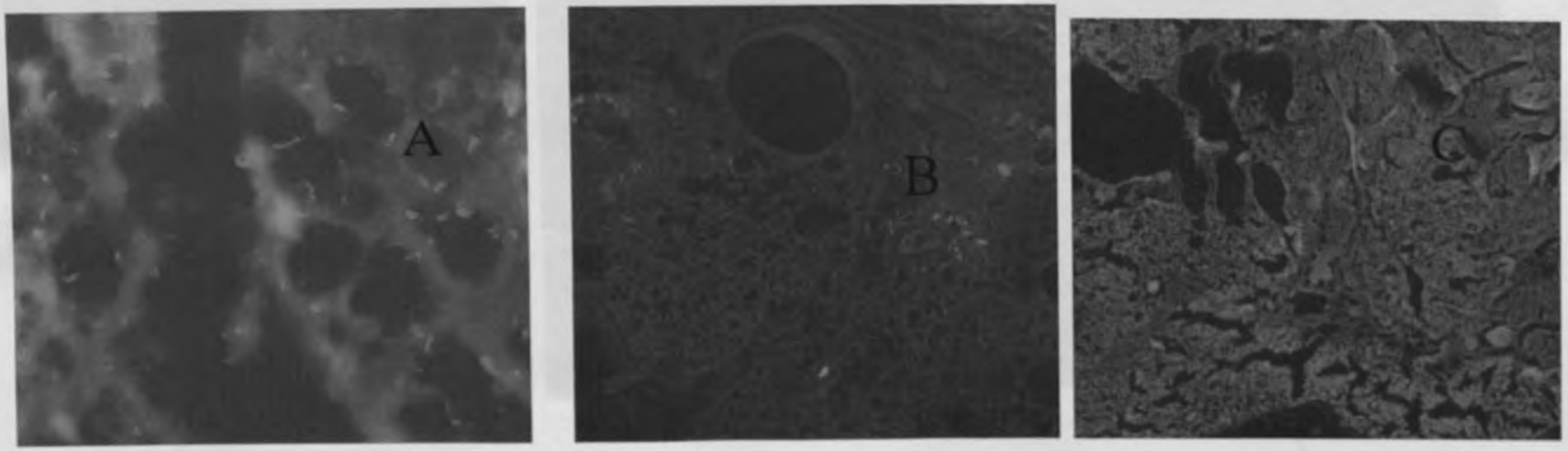


FIG.9. BURSA OF FABRICIUS (A), SPLEEN (B), AND HARDERIAN GLAND (C) FROM NON-IMMUNOSUPPRESSED CHICKENS POSITIVE FOR *P. MULTOCIDA* ON FISH TEST (ARROWS).

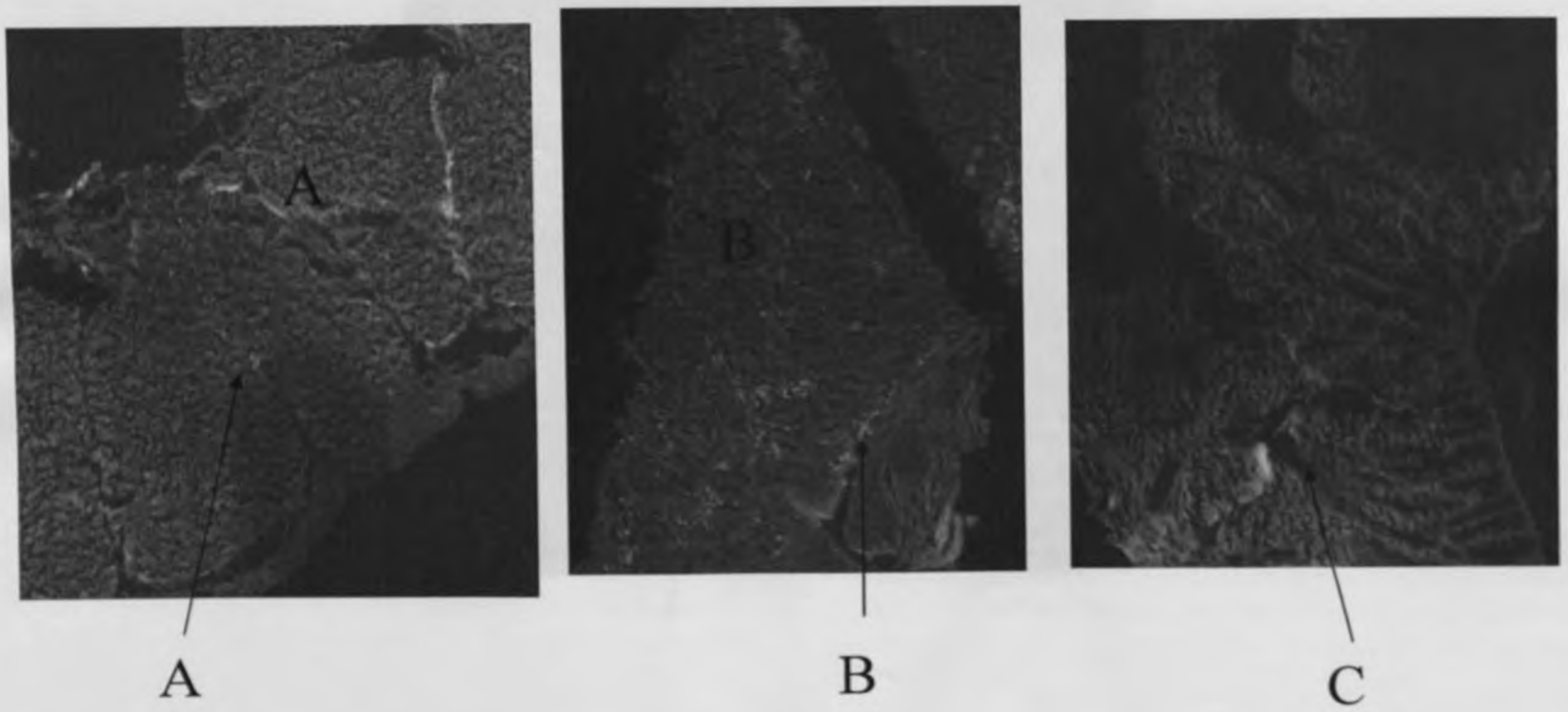


FIG.10. PRUNING GLAND FROM NON-IMMUNOSUPPRESSED CHICKEN POSITIVE FOR *P. MULTOCIDA* ON FISH TEST (ARROW).

FIG.11. LIVER FROM IMMUNOSUPPRESSED CHICKEN : (A) POSITIVE FOR *P. MULTOCIDA* (ARROW) , AND (B) A NEGATIVE CONTROL.

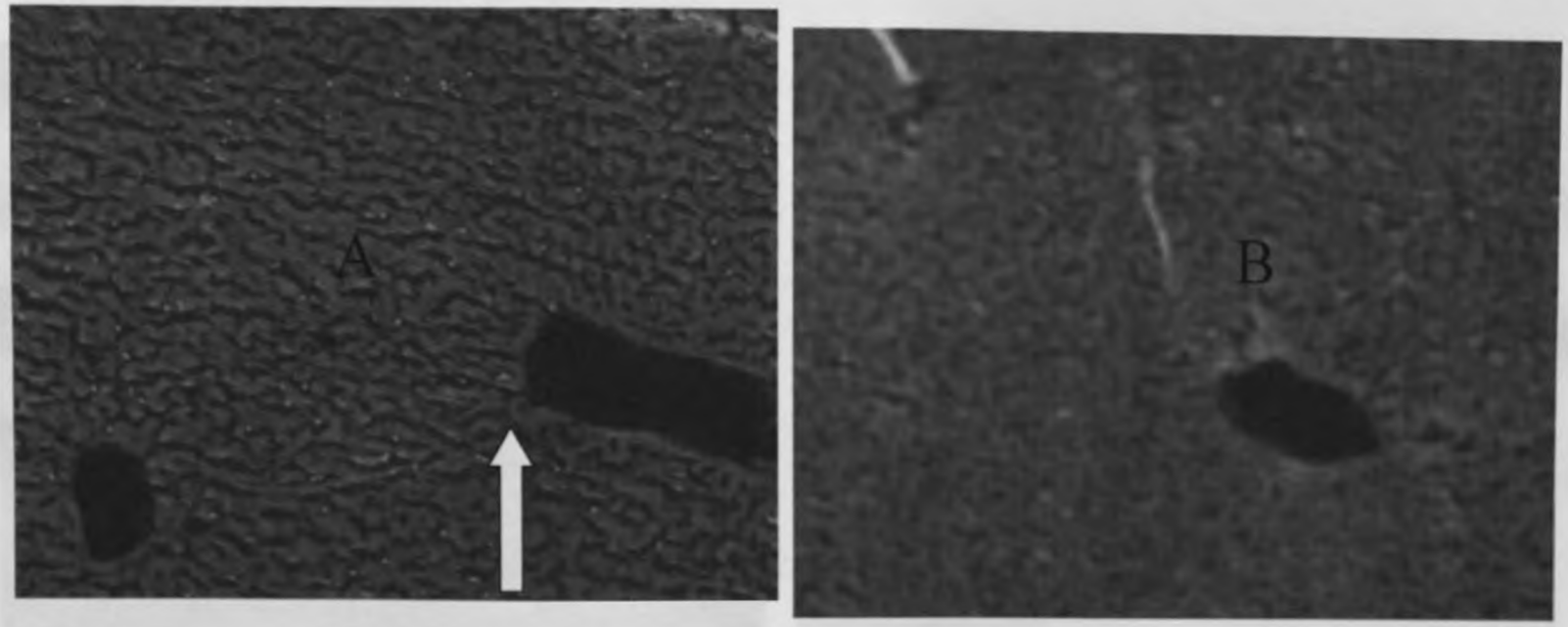
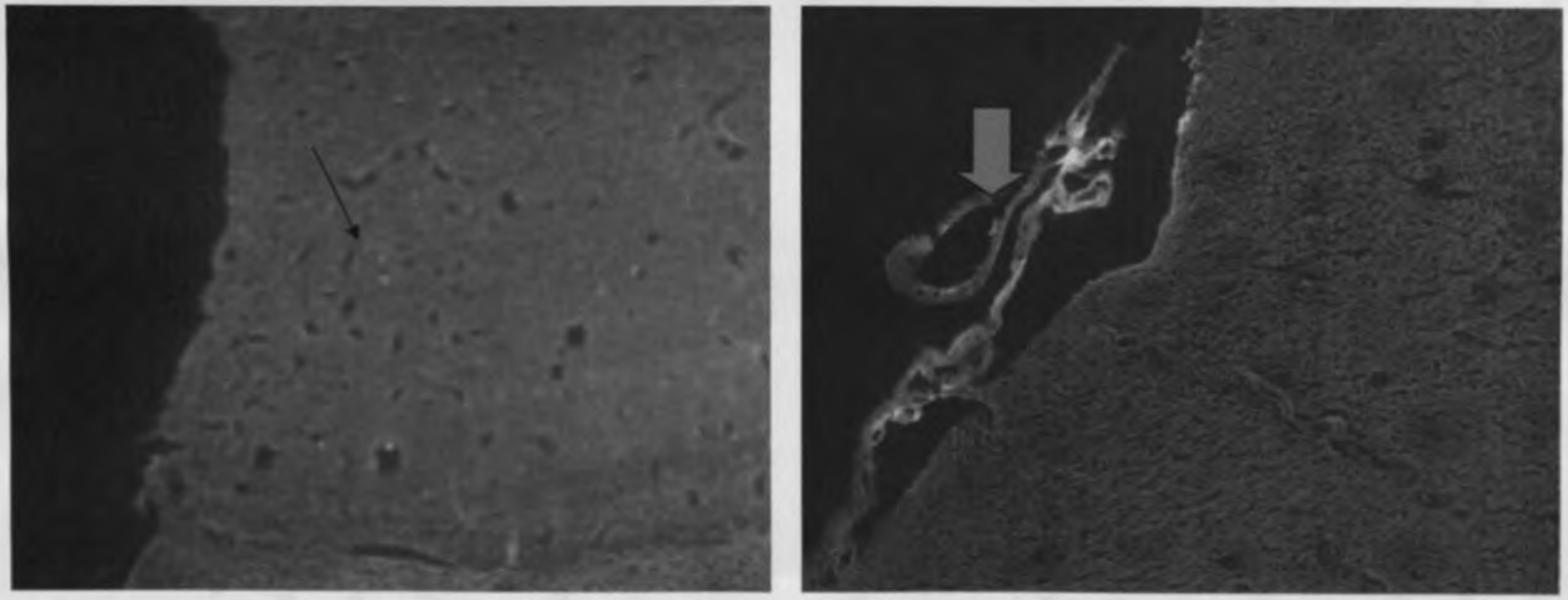


FIG.12. BRAIN (THIN ARROW) AND MENINGES (THICK ARROW) FROM IMMUNOSUPPRESSED CHICKEN SHOWING *P. MULTOCIDA* POSITIVE ON FISH TEST.



10.3.3.3 Comparison between the *P. multocida* FISH signals in the organs of NIS and IS chickens at specified times

On comparison between the expression of *P.multocida* FISH test signals (NIS and IS chickens) most organs had almost the same number of signals over the study period ($p>0.05$). However, signals were recorded more in NIS chickens trachea, sciatic nerve, kidney, pruning gland, bursa of Fabricius, and the comb. Similarly more signals were observed in IS chickens sex organs, and pectoral muscle. The NIS organs had 7 % more signals than IS chickens organs. At various sacrificial periods, the NIS chickens had most signals expressed between 1 hour to 24 hour p.i, while IS chickens had *P. multocida* signals that peaked at 1 hour p.i, and stayed high up to 6 hours and decreased thereafter. Whereas it was possible to detect organisms at 0 hour from the NIS chickens, none was observed in IS chickens.

10.3.4 Pathogenesis of *P. multocida* organisms as evaluated through bacterial re-isolation from selected organs

10.3.4.1 *P.multocida* organisms re-isolated from NIS chickens

Pasteurella multocida organisms re-isolated from seven organs from NIS chickens at the specified periods are presented in Table 40. The organs swabbed were lungs, oropharynx, cloaca, caecal tonsils, spleen, liver and pruning gland. *P. multocida* organisms were recovered from the oropharynx (75%), lungs (58.3%), spleen (50%), liver (25%) and caecal tonsils (25%). Isolates from the oropharynx and lungs were recovered up to the 7th day p.i, while those from other organs were recovered up to the second day p.i. Most of the *Pasteurella* isolates were recovered within 24 hours p.i. No

isolates were recovered from pruning gland and cloaca. At zero hour (1-5minutes after inoculation) 2 isolates were recovered from the oropharynx. At one hour, p.i, the isolates increased to 9, as they were recovered from the lungs, oropharynx, caecal tonsils, spleen, and liver. At 3-6 hours p.i, 3 and 2 isolates respectively, were recovered from the lungs and spleen, while at 12th hour p.i, 3 isolates were recovered from lung, spleen and liver. At 24 hours p.i, 2 isolates were cultured from the oropharynx only and two days p.i, 2 isolates from the lungs and caecal tonsils. On the 3rd and 7th day, isolates were cultured from the lungs and oropharynx. There were no isolates recoverable from any organ on the second week of the study from infected chickens.

All organs of the control chickens were negative for *P. multocida* organisms.

10.3.4.2 *P. multocida* organism re-isolation from IS chickens

P.multocida organisms isolated from seven organs of IS chickens at various periods are presented on Table 40. Same organs as for NIS chickens were swabbed. *P. multocida* organisms were recovered from the oropharynx (83.3%), lungs (58.3%), caecal tonsils (25%), liver and spleen (8.3%, each). Isolates from the oropharynx were recovered up to the 5th day while those from other organs were recovered up to the 3rd day post infection. Most *P.multocida* organisms were recovered within 24 hours p.i. No isolates were recovered from pruning gland and cloaca. At zero, 1, and 3 hours p.i, 4 isolates each were recovered from various organs and 6 isolates at 24 hours from the lungs, oropharynx, caecal tonsils, and liver. A total of 4 isolates each were recovered from lungs and oropharynx at zero hour p.i, 1hour, p.i, from the lungs, oropharynx and spleen and at 3 hours p.i, from the lungs, oropharynx and caecal tonsils. At 6 and 12 hours p.i, 1 isolate each, was recovered from the oropharynx. By 24 hours p.i, 6 isolates were recovered

from the lungs, oropharynx, caecal tonsils and the liver. Thereafter one isolate was recovered from caecal tonsils on day 3 and another from oropharynx on day 5. There were no isolates recoverable from any of the organs on the second week of the study period.

NIS chicken had slightly more isolates than IS birds but the difference was not statistically significant ($p>0.05$).

No *P. multocida* organisms were isolated from the control chickens.

10.3.5 Comparison between the four tests for *P.multocida* organisms in NIS and IS chickens

10.3.5.1 Among the NIS chickens

Comparison of the results of the four main tests undertaken on a few NIS chicken organs, where all tests were done at specified periods are presented in Table 41. FISH test results compared very well with gross and histopathology results. Detection of *P. multocida* by culture and FISH test were comparable up to day 3 p.i, there after, the FISH test was found to detect the bacteria better in the lungs, trachea (oropharynx), spleen, liver and caecal tonsils. *P.multocida* FISH signals were found in the pruning gland and large intestines, but no bacteria were isolated from the cloaca and pruning gland.

10.3.5.2 Among the IS chickens

Comparison of the results of the four tests undertaken on a few IS chicken organs, where all tests were done at specified period are presented in Table 41. FISH test results compared well with those of gross and histopathology. Detection of P. *multocida* FISH

signals and culture method were comparable up to 24 hours p.i, thereafter, the FISH test was found to detect the bacteria better in the lungs, trachea (oropharynx), spleen, liver, caecal tonsils and large intestines (cloaca). Whereas, *P.multocida* FISH signals were found in the large intestine, no bacteria were isolated from the cloaca swabs.

Immmediately after inoculation, *P. multocida*, could only be detected by FISH test or bacterial culture. After one hour, p.i, all tests were able to detect the organism. Detection of *P. multocida* was possible in NIS chicken organs up to the third day and up to the second day for IS chicken organs, using all the tests. On the 14th day p.i, only FISH test was able to detect the *P.multocida*.

There was no significant difference between NIS and IS chickens ($p>0.05$).

Table 40: *P.multocida* organisms re-isolated by culture from non-immunosuppressed (NIS) and immunosuppressed (IS) chickens at specified times

Organs	Chicken treatment	Hours post infection						Days post infection						Isolates per organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Lungs	NIS	-	+	+	+	+	-	+	+	-	+	-	-	7
	IS	++	+	++	-	-	++	-	-	-	-	-	-	7
Oropharynx	NIS	++	++	-	-	-	++	-	+	-	++	-	-	9
	IS	++	++	+	+	+	++	-	-	+	-	-	-	10
Caecal tonsils	NIS	-	++	-	-	-	-	+	-	-	-	-	-	3
	IS	-	-	+	-	-	+	-	+	-	-	-	-	3
Spleen	NIS	-	++	++	+	+	-	-	-	-	-	-	-	6
	IS	-	+	-	-	-	-	-	-	-	-	-	-	1
Liver	NIS	-	++	-	-	+	-	-	-	-	-	-	-	3
	IS	-	-	-	-	-	+	-	-	-	-	-	-	1
Pruning gland	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Cloaca	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Total Isolates	**NIS**	**2**	**9**	**3**	**2**	**3**	**2**	**2**	**2**	**0**	**3**	**0**	**0**	**28[a]**
	IS	**4**	**4**	**4**	**1**	**1**	**6**	**0**	**1**	**1**	**0**	**0**	**0**	**22[a]**

Legend: - or 0 – No *P. multocida* isolate
+ *P.multocida* isolated from one chicken
++ *P.multocida* isolated from two chickens
X^2.calculated ([a]) = 0.72 ($p>0.05$)

Table 41: Comparison between the gross and histopathological lesions, FISH signals and *P.multocida* re-isolation from NIS and IS chickens

Organs	Chicken treatment		Hours post infection						Days post infection						+ results per organ
			0	1	3	6	12	24	2	3	5	7	10	14	
Lung	Gross	NIS	-	+	+	+	+	+	+	+	+	+	+	-	10
		IS	-	+	+	+	+	+	+	+	+	+	-	-	9
	Histo	NIS	-	+	+	+	+	+	+	+	+	+	+	-	10
		IS	-	+	+	+	+	+	+	+	+	+	+	-	10
	FISH	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
		IS	-	+	+	+	+	+	+	+	+	+	+	+	11
	B/isola	NIS	-	+	+	+	+	-	+	+	-	+		-	7
		IS	+	+	+	-	-	+	-	-	-	-	-	-	4
Trachea	Gross	NIS	-	+	+	+	+	+	+	+	-	-	-	-	7
		IS	-	+	+	+	+	+	-	-	-	-	-	-	5
	Histo	NIS	-	+	+	+	+	+	+	+	+	+	-	-	9
		IS	-	+	+	+	+	+	-	+	+	-	-	-	7
	FISH	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
		IS	-	-	+	+	+	+	+	-	+	+	+	-	8
	B/isola	NIS	+	+	-	-	-	+	-	+	-	+	-	-	5
		IS	+	+	+	+	+	+	-	-	+	-	-	-	7
Caecal tonsils	Gross	NIS	-	-	+	+	+	+	+	-	+	-	-	-	6
		IS	-	-	-	-	+	-	-	-	-	-	-	-	1
	Histo	NIS	-	+	+	+	+	+	-	-	-	-	-	-	5
		IS	-	-	-	+	+	+	-	-	-	-	-	-	3
	FISH	NIS	-	N	-	+	+	-	+	-	-	+	-	-	4
		IS	-	-	+	-	-	-	+	-	N	-	-	+	3
	B/isola	NIS	-	+	-	-	-	-	+	-	-	-	-	-	2
		IS	-	-	+	-	-	+	-	+	-	-	-	-	3
Spleen	Gross	NIS	-	-	+	+	+	+	+	+	+	-	-	-	7
		IS	-	+	+	+	+	+	+	-	-	-	-	-	6
	Histo	NIS	-	+	+	+	+	+	+	+	+	+	-	-	9
		IS	-	+	+	+	+	+	+	-	-	-	-	-	6
	FISH	NIS	-	+	+	+	+	+	+	+	+	-	+	+	10
		IS	-	+	+	+	+	+	+	+	+	-	+	+	10
	B/isola	NIS	-	+	+	+	+	-	-	-	-	-	-	-	4
		IS	-	+	-	-	-	-	-	-	-	-	-	-	1

Table 41 (Continued next page)

Legend: Gross – gross lesions; Histo – histological lesions; FISH - fluorescent *in situ* hybridization signals; B/isola – *P. multocida* isolation by culture; + - positive result; - negative result; N – not done

Table 41 (Continued)

Liver	Gross	NIS	-	-	+	+	+	+	+	+	+	-	-	-	7
		IS	-	+	+	+	+	+	+	+	-	-	-	-	7
	Histo	NIS	-	+	+	+	+	+	+	+	+	-	-	-	8
		IS	-	+	+	+	+	-	+	-	-	-	+	-	6
	FISH	NIS	-	+	+	+	+	+	+	-	+	+	+	+	10
		IS	-	+	+	+	+	N	+	+	+	+	+	+	10
	B/isola	NIS	-	+	-	-	+	-	-	-	-	-	-	-	2
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Pruning gland	Gross	NIS	-	-	+	+	+	+	-	-	-	-	-	-	4
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0
	Histo	NIS	N	-	-	+	+	-	-	-	-	-	N	N	2
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0
	FISH	NIS	-	-	N	N	-	-	-	N	-	-	-	+	1
		IS	-	-	N	-	-	-	-	N	-	-	-	-	0
	B/isola	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
		IS	-	-	-	-	-	+	-	-	-	-	-	-	1
Large intestine	Gross	NIS	-	-	+	+	+	+	-	-	-	-	-	-	4
		IS	-	-	+	-	+	-	-	-	-	-	-	-	2
	Histo	NIS	-	+	+	+	+	+	+	-	-	-	-	-	6
		IS	-	-	-	+	-	-	+	-	-	-	-	-	2
	FISH	NIS	-	+	+	-	-	N	-	-	-	-	-	-	2
		IS	-	+	N	-	-	-	+	-	N	N	-	-	2
	B/isola	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Totals	Gross	NIS	0	2	3	7	7	7	5	4	4	1	1	0	36
		IS	0	4	5	4	6	4	3	2	1	1	0	0	30
	Histo	NIS	2	6	6	7	7	6	5	4	4	3	1	0	51
		IS	0	4	5	6	5	4	4	2	2	1	2	0	35
	FISH	NIS	2	5	5	5	5	4	5	3	4	4	4	5	51
		IS	0	4	5	4	4	3	6	3	4	3	4	4	44
	B/isola	NIS	1	5	2	2	3	1	2	2	0	2	0	0	20
		IS	2	3	3	1	1	4	0	1	0	0	0	0	16

Legend: Gross – gross lesions; Histo – histological lesions; FISH - fluorescent *in situ* hybridization signals; B/isola – *P. multocida* isolation by culture; + - positive result; - negative result; N – not done

X^2 calculated = 0.6421 ($p>0.05$)

10.4 DISCUSSION

The *P.multocida* strain NCTC 10322^T (Mutter *et al.*, 1985 b) used in this study, caused clinical fowl cholera in indigenous chicken without mortality. The bacteria were preserved at – 80 ^{0}C (Denmark) and in Dorset egg agar (Kabete, Kenya), prior to inoculation. It caused gross lesions in 23 NIS and 22 IS chicken organs out of 24 that were examined. The pruning and adrenal glands (IS) and pruning gland (NIS) appeared less susceptible grossly. *P. multocida* induced gross lesions in multiple organ systems with severest ones being seen in the respiratory system as reported by Glisson (1998). Respiratory tract, lymphoid, hepatic, cardiovascular, nervous, urinary, reproductive, endocrine and gastro-intestinal tract, cutaneous, and muscular tissues were examined in this study and found to have lesions. The infection of the gastro-intestinal tract raises a possibility that *P. multocida,* can enter or be shed through it, as suggested by Lee *et al* (2000), and Muhairwa *et al* (2001) and as observed in experiment 1, chapter 3 of these studies. Organs with lesions during most of the study period were lungs, airsacs, trachea, spleen, liver, and caecal tonsils. Those organs of NIS birds were more frequently affected than the IS chickens. Gross lesions were common on the 1st week mainly and only a few were observed on the lungs of NIS chickens on the 2nd week. Peak lesions were recorded at the 6th hour for NIS and 12th hour for IS chickens. The observed severe lesions were slightly more in NIS as compared to IS chickens.

Whereas the *P.multocida* established itself in chicken organs causing obvious lesions, the latter were few 3 days post infection. On the 7th day both NIS and IS had lesions only in the lungs. Generally the NIS birds had more severe lesions than the IS chickens, a

possibility therefore, that immunosuppression had an affect on formation of the *P.multocida* lesions.

Infection of the reproductive organs (testis and ovary) as observed in this study, were as reported in oviducts of ducks by Bisgaard (1995). The respiratory system had a more prolonged moderate to severe *P.multocida* infection in NIS than in IS chickens. These lesions were due to circulatory disturbances, degeneration and necrosis of the tissues.

Histopathological lesions observed in all NIS were more outstanding than the 20 out of the 24 organs with lesions for IS birds. The severest lesions were in the respiratory, cardiovascular, hepatic, lymphoid, gastrointestinal tract, nervous and urinary systems in a descending order. The pancreas, sciatic nerve, pruning gland and superficial pectoral muscle from IS birds were negative. As in gross lesions, all tissue systems examined had lesions although NIS chicken organs had lesions for a long period as compared with IS ones. Histopathological lesions were commonly noted in the first week p.i, with a few observed on the lungs, liver and bursa of Fabricius on the second week. Lesions peaked at the 12^{th} hour in NIS and 24^{th} hour for IS birds. Observed total organs with lesions were significantly more in NIS than IS birds ($p<0.05$). Histopathological lesions comprised of cellular and humoral exudates, emphysema. atelectasis, urate deposition, thrombosis of blood vessels, degeneration, necrosis, granulomatous formation, fibrosis and serositis in combinations. These lesions were intermediate to those of acute and chronic infections reported elsewhere (Glisson *et al.*, 2003). The more frequently encountered lesions in both NIS and IS were those due to cellular infiltration (heterophils, mononuclear cells, multinucleated giant cells) and fibrin exudation. The inflammatory changes were more severe in NIS than in IS birds. In the IS birds, lymphoid depletion and atrophy of the

lymphoid organs was more marked and widely spread than NIS birds due to the immunosuppressive effect of dexamethasone. Birds are reported to rely on non-specific cellular defense that is activated by replicating bacteria that involves poly-morphonuclear neutrophils, heterophils, and macrophages (Toth, 2000; Qureshi *et al.*, 2000). Stress in poultry has been shown to increased the infected birds' nonspecific resistance to *Escherichia coli* and *Staphylococcus aureus* resorting in less disease manifestations unlike in viral (New castle virus), *Mycoplasma gallisepticum* (Gross and Siegel, 1965; Gross and Colmano, 1967,1969, 1970; Freeman, 1976) and *Salmonella enteritides* infections (Nakamura *et al.*, 1974). Stress lowers the total leukocyte counts (leukopenia), impair immunological functions, reduce appetite and reduces the weight of the bursa of Fabricius (Siegel and Gross, 1965; Freeman, 1971). The dexamethasone immunosuppressed birds infected with *P. multocida* NCTC 10322^T strain in this study appear to have the nonspecific resistance as reported elsewhere for other bacteria when chickens are stressed (Gross and Siegel, 1965; Gross and Colmano, 1967,1969, 1970; Nakamura *et al.*, 1974; Freeman, 1976). Therefore, some of these cellular defense may be affected by the dexamethasone used to immunosupress the IS birds.

Studies with turkeys have shown that bacteria multiply extensively in trachea and lungs within 2hour p.i, to reach high numbers by 6hours in the respiratory tissues (Matsumoto *et al.*, 1991). In some cases the *P.multocida* organisms instantaneously reached the internal organs via blood by some unknown mechanism (Matsumoto *et al.*, 1991). This agrees with these studies as lesions and bacteria were observed as early as 1 hour p.i in many chicken organs. Other studies have reported systemic invasion of *P.multocida* by 12 hours but not 6 hours p.i (Rhoades and Rimler, 1990). In this study,

lesions and bacterial signals were highest at 6 to 24 hours p.i. Fast multiplying *P. multocida* organisms may die during incubation in BHI broth culture to release some endotoxins that may have a role in instantaneous spread of the organisms into the blood stream and inflammation.

Using the *P.multocida* probe pmhyb 449 FISH test developed during these studies (Mbuthia *et al.*, 2001), *P.multocida* NCTC 10322^{T} was detectable in all the 24 NIS and in 22 IS organs, thereby fulfilling the aim of diagnostic microbiology. This provides a tool for accurate identification of bacteria in diseased birds and in the natural environment (Moter and Göbel, 2000). Bacteria were observed at all sacrificial intervals in lungs and trachea (100%), and various times in spleen and livers (83.3%), airsacs (75%), bursa of Fabricius (58.3%), and Thymus (50%) in NIS chickens and the trend was matched in IS organs although lower in frequency. Immediately, after inoculation, the bacterial FISH signals were eminent in the tracheal and bronchial lumen close to the epithelium. The number of organs with the bacteria increased from 2 (lung and trachea) after inoculation to 10 in one hour to a maximum of 16 organs at 3 hours in NIS chickens unlike IS chickens which rose to 14 organs at one hour. Majority of organs had signals between 1 to 24 hrs and 1hr to 2 days in IS chickens. This may be a possible indication of acute or subacute septicaemic phase of the disease before a chronic phase. The respiratory, hepatic, lymphoid, cardiovascular, gastrointestinal tract, urinary, nervous, reproductive, endocrine, muscular and cutaneous system, in a descending order appear to be preferred predilection sites for *P. multocida* organisms in both NIS and IS chickens.

As the disease progresses, it appears to provoke an intensive inflammatory reaction that resolves within 2 days. After 14 days period, bacterial lesions wane off, but using

probe pmhyb 449, *P.multocida* were still seen localized in tissue sections of most organs examined (except sciatic nerve, pruning gland and the comb of the immunosuppressed birds). Possibly an indication of the sensitivity of this probe.

This technique that had previously localized *P.multocida* in lung tissues (Mbuthia *et al.*, 2001) has been confirmed to detect the bacteria in all tissues of chicken for a period of 2 weeks post-inoculation. The FISH technique has been used previously to demonstrate other bacteria (Kimmer *et al.*, 1999; Jansen *et al.*, 2000; Boye *et al.*, 2000; Bojesen *et al.*, 20003) but not in as many tissues of an animal, as in this study. The FISH results correlated well with gross and histological lesions as reported by others (Moter and Göbel, 2000). This test allows the evaluation of occurrence, number, morphology and spatial distribution of *P.multocida* in various chicken tissues.

Bacterial recovery was done from 5 out of seven organs of infected NIS and IS chickens but not pruning gland and cloaca. Oropharynx yielded the majority isolates, then lungs, spleen, caecal tonsils and liver in that order. There were slightly more *P.multocida* re-isolated from the NIS than the IS chickens. This was possibly due to the stress effect of dexamethasone on immunosuppressed birds as reported earlier.

In NIS, more isolates were recovered in the first hour p.i, while in IS, they were high immediately p.i, to 3 hrs and a single peak at 24hrs. Recovery of *P.multocida* after 24 hrs was scanty in the IS but NIS chickens had many isolates up to day 7 post inoculation. This general trend is as reported elsewhere; recovery after 48hrs being scanty (Christensen and Bisgaard, 2000; Petersen *et al.*, 2001b). Bacterial isolation overlapped with time of severest inflammation, gross and histological lesions and the demonstration of FISH signals. Generally, the 4 different types of tests, showed that using *P.multocida*

NCTC 10322^T in *in vivo* studies, lesions were expressed as sub-acute septicaemia within 24hrs p.i, but they tended to wards the chronic nature, 48 hrs onwards. There was good correlation between all the tests up to day 2, post inoculation. Thereafter, the FISH test appeared to correlate well with gross and histopathology in NIS and IS chickens, up to day 10 and but had more signals thereafter.

CHAPTER 11

11.0 EXPERIMENT 9: PATHOGENESIS OF *PASTEURELLA MULTOCIDA* IN NON-IMMUNOSUPPRESSED AND IMMUNOSUPPRESSED INDIGENOUS DUCKS

11.1 Introduction

P.multocida organisms infect waterfowl populations to cause fowl cholera and subsequent massive mortality in many countries (Hunter and Wobeser, 1980; Rhoades and Rimler, 1989; Glisson *et al.*, 2003). These organisms can enter the duck's body through various routes to spread to various organs, especially the respiratory system, to cause variable pathology (Glisson, 1998; Pehlivanoglu *et al.*, 1999). The spread of these organisms from the point of infection into the various organs of indigenous ducks is, however, not well documented. There is, therefore, need to simulate the infection by infecting the birds through the most common point of entry, the respiratory tract (Matsumoto *et al.*, 1991; Petersen *et al.*, 2001b), and observe the resultant pathology in various organs at various intervals. Further, the FISH test developed in chapter 9 would be used to identify *P.multocida* in duck tissues.

As for experiment 4 (chapter 6) of this thesis, in this experiment, two groups of ducks, one normal and one immunosuppressed, were used to see if immunosuppression, which is one manifestation of stress, has any effect on the pathogenesis of the organisms and the resultant gross and microscopic pathology. This was occasioned by the fact that scavenging village ducks are normally subjected to different types of stressful conditions (Aini, 1999b) that may modulate the pathology and alter the tissue reaction of *P.multocida* infection.

The aim of this experiment was to study the pathogenesis of *P.multocida* infection in immunosuppressed and non-immunosuppressed indigenous ducks. The parameters used included: gross and microscopic pathology in respective organs, *P.multocida* isolation and its detection in the respective tissues using fluorescent *in situ* hybridization (FISH) test.

11.2 Materials and methods

11.2.1 Experimental ducks

Ducks at their most susceptible age of 8 weeks, (Chapter 5, Experiment 3, of this thesis) were used in this experiment, as described in chapter 10, section 10.2.1.

11.2.2 Bacteria used to infect ducks

P.multocida type strain (NCTC 10322^T) maintained on Dorset egg agar, in our laboratory that had low virulence than other *P.multocida* was used in this study. It was spread onto BA with 5 % citrated calf blood, incubated aerobically at 37 °C, for 24 hours to check for purity prior to preparation of the inoculum. Individual colony of this was inoculated into brain heart infusion broth, incubated aerobically at 37 °C, for 24 hours. A ten fold serial dilution of one milliliter of the bacteria in BHI was made and 25µl of each dilution spotted onto BA plates for colonies that were translated into colony forming units.

11.2.3 Immunosuppression of ducks

Birds to be immunosuppressed were injected intramuscularly using dexamethasone (Agar Holland, 3760 AL Soest, Holland), 4mg/Kg body weight per day as done elsewhere for 6

days prior to experimental infection (Birrenkott and Wiggins, 1984; Corrier and DeLoach, 1990; Corrier *et al.*, 1991; Nakamura *et al.*, 1994).

11.2.4 Experimental procedure

Experimental birds were randomly allocated into infected and control groups as given by Steel and Torrie (1980). Eight-week old, wing-tagged indigenous ducks were used in two separate experiments: one for the immunosuppressed (IS) ducks and one for the non-immunosuppressed (NIS) ones, each with respective controls. The birds were separated and put in the experimental rooms 48 hours prior to inoculation.

For the experiment on NIS ducks, 34 ducks were used: 24 were infected with *P.multocida* organisms and 10 were used as controls. For the experiment on IS ducks, 44 ducks were used: 24 IS ones were infected with *P.multocida* organisms, while the controls consisted of 10 IS and 10 NIS birds. Each infected bird (both NIS and IS) was inoculated with 0.5 ml of brain heart infusion (BHI) broth culture containing 1.2-1.9 x 10^8 CFU of *P.multocida* organisms, while the control (both NIS and IS) were inoculated with 0.5ml of sterile BHI broth medium. The infected and control birds were housed in different rooms located in different houses away from each other. Biosecurity was maintained during the entire period of the study.

Two birds, randomly selected from each experimental group were sacrificed at specified times, namely: - hour 0,1,3,6,12 and 24 and days 2,3,5,7,10 and 14, post-infection. The zero hour was achieved by inoculating the birds and immediately sacrificing them within 1-5 minutes. To avoid possible cross-infection, the daily sacrifices started with birds in the control houses before proceeding to the infected houses. Table 42 shows the group, number of birds, and the specified sacrificial times.

Table 42: The number of birds used in pathogenesis study and the time they were sacrificed

Birds	Hours post infection						Days post infection						Total
	0	1	3	6	12	24	2	3	5	7	10	14	
NIS Infected	2	2	2	2	2	2	2	2	2	2	2	2	**24**
NIS Control	2	-	-	2	-	2	-	-	-	2	-	2	**10**
IS Infected	2	2	2	2	2	2	2	2	2	2	2	2	**24**
IS Control	2	-	-	2	-	2	-	-	-	2	-	2	**10**
NIS Control	2	-	-	2	-	2	-	-	-	2	-	2	**10**
Total	**10**	**4**	**4**	**10**	**4**	**10**	**4**	**4**	**4**	**10**	**4**	**10**	**78**

Legend: - No bird was sacrificed at that time; IS – immunosuppressed; NIS – non-immmunosuppressed.

Post- mortem examination was done on the sacrificed birds and gross lesions on various organs and tissues noted and recorded. At the same time, swabs were taken aseptically from the oropharynx, cloaca, lungs, caecal tonsils, pruning gland, liver and spleen, for *P.multocida* re-isolation and characterization.

Tissue samples, including: - lung, trachea, lower conjunctiva, airsacs, spleen, liver, heart, thymus, Harderian gland, pancreas, brain, sciatic nerve, kidney, testis or ovary, adrenal gland, bursa of Fabricius, proventriculus, duodenum, midgut (at the Merkel's diverticulum), caecal tonsils, large intestine, pruning gland, comb and pectoral muscle, were removed, immediately immersed in formalin, where they were kept for histological processing. Some paraffin-embedded tissue sections were mounted on adhesive super-frost plus slides for the FISH processing and examination. For each organ examined, the resultant histological examination, bacterial re-isolation of *P.multocida* and FISH results were recorded.

11.2.5 Post-mortem examination

Birds were killed through cervical dislocation. Post mortem examination was done as described by Bermudez and Stewart-Brown (2003). The dead birds were opened aseptically, tissues and organs observed individually, and gross lesions noted and recorded. For microscopic lesions, six tissue sections of a particular organ per group were examined. The severity of the lesions was semi- quantitatively scored on the basis of: no minimal or slight lesions (- or 0), minimum, slight or few lesions (1 or mild), many lesions in a localized area or in many tissue sections (2 or moderate) and severe or diffuse lesions and in all tissue sections (3 or severe) as described by others (Gross and Siegel, 1965; Gross and Colmano, 1967; Shivaprasad and Droual, 2002).

11.2.6 Histological processing of tissues

The tissue samples were kept fully immersed in labeled bottles containing 10% formalin solution for 24 hours. They were then transferred into 70% alcohol, where they remained until trimming was done (Brown, 1998). The fixed tissues were manually trimmed to a thickness of 2 to 3 millimeters. The trimmed tissues were then placed in an automatic tissue processor for the following treatments: - (1) dehydration using: - (i) 80% ethyl alcohol for the first 4 hours, (ii) 96% alcohol for the next 4 hour, and (iii) 100% alcohol for 4.5 hour; (2) clearing with xylene for a total of 5 hours; and (3) impregnating with molten paraffin wax at 60 °C for a total of 6 hours. The tissues were then removed from the processor and were embedded into wax blocks using a molten wax dispenser. The individual tissue blocks were separated and fixed onto microtome chunks using a searing spatula, after which they were sectioned to 3-5 µm thickness, floated on a water bath at 50 °C to flatten out, placed on a microscope slide and dried in

an oven at 60 °C for about one hour. The sections were then quickly dewaxed in xylene and washed in alcohol before re-hydrating in water. They were then stained using haematoxylin and eosin, mounted in destrene 80, dibutylphthalate and xylene (DPX) and the slides left to dry before examination under the microscope.

11.2.7 Fluorescent *in situ* hybridization technique

Tissue sections were processed as given in 10.2.5 above up to the stage of tissue sectioning. The paraffin-embedded sections were then mounted on super-frost plus slides (Menzel-Gläser, Germany) and hybridized as described on chapter 9, section 9.2.7.

11.2.8 Statistical analysis

Data obtained during the study up to 14 days post infection was analyzed using the analysis of variance procedures of the statistical analysis systems (SAS, 1996) and chi-squared test (X^2) (Steel and Torrie, 1980).

11.3 Results

11.3.1 Pathogenesis of *P.multocida* organisms as observed through gross lesions

11.3.1.1 Gross lesions of NIS ducks

Organs from NIS ducks with gross lesions, type and their respective severities at specified periods are presented in Table 43. Lesions occurred in 22 of the 24 organs examined, except pruning gland and the skin. There were no gross lesions in all organs at 0 hour post inoculation (p.i). Lesions were commonly observed throughout the in the lung and airsacs (91.7%), liver and heart (58.3%), conjunctiva, spleen and pectoral muscle (50%), trachea, kidney, bursa of Fabricius, duodenum and large intestine (41.7%). At one hour p.i, 11 (45.8%) organs with lesions were; the lungs, trachea, conjunctiva,

airsacs, spleen, liver, heart, kidney, bursa of Fabricius, duodenum and pectoral muscles, while at 3rd hour 18 (75%) of these organs were positive except pancreas, sciatic nerve, adrenal gland and proventriculus. By the 6th hour p.i 22 of 24 (91.7%) organs had clear lesions. Most organs expressed lesions between 1 to 24 hours p.i but decreased by the second day p.i; with lesions only in the lungs, conjunctiva, airsacs, spleen, liver, heart, large intestine, and the pectoral muscles. These lesions were confined to the lung, airsacs, liver or the heart on the 3rd and 5th day and to the lungs and airsacs on days 7 to 14 (second week) p.i. The organs with lesions increased from a total of 11 at the 1st hour p.i, to a maximum of 22 at the 6th hour and decreased gradually to 2 at day 14 p.i. Lesions varied from mild 73 (67.6%) to moderate 24 (19.4%) and a few being severe 14 (13%). Severe and moderate lesions were observed mostly on the lung, airsacs, trachea, conjunctiva, spleen, liver, heart, thymus, kidneys, duodenum, caecal tonsils, large intestine, and pectoral muscles. Moderate to severe lesions were observed on the lungs (1hour - 5th day), airsacs (3 hours – 5th day), trachea (1 – 6 hours), conjunctiva (3-6 hours), liver (6-24 hours), spleen and heart (6-12 hours), duodenum (3-6 hours), and thymus, large intestine, caecal tonsils, kidney, and pectoral muscle (6 hours).

Table 44 shows 39 inflammatory lesions that were recorded from 22 of the 24 organs examined per bird over the 14 days study period. They were similar in nature and distribution but more severe than those in chicken. No lesions were observed on 0 hour post inoculation. After that the commonly observed lesions were pneumonia and air sacculitis (91.7%) (Fig.13), fibrin deposition (83.3%), necrosis (Fig.14) and thick airsacs (66.7%), congestion, fibrosis, pleuritis, myocarditis, hepatitis (58.3%), pus exudation, fibrinopurulent exudation, hydropericardium, sinusitis, peritonitis, splenitis and myositis

(50%). At one hour, p.i, 17 (43.6%) lesions were; congestion, haemorrhage, oedema, emphysema pneumonia, air sacculitis, tracheitis, sinusitis, pleuritis, peritonitis conjunctivitis, myocarditis, hepatitis, splenitis, nephritis, enteritis, and myositis. On the 3rdhour p.i 27 (70.0%) lesions were encountered, on further observation of purulent exudate, fibrin exudate, thick airsacs, hydropericardium, mucus exudate from trachea and intestines, collapsed lungs and, meningitis, sciatic nerve myelitis and lung hepatization. After 6 hours p.i, fibrinopurulent exudate, necrosis, froth, paleness, urate deposits, splenomegally, hepatomegally, and pericarditis were observed, making a total of 35 (89.7%)of the inflammatory lesions. On the 12th hour p.i 36 (92.3%) lesions had been observed, and included splenic capsulitis. The last lesions recorded were fibrosis, perihepatitis at 24th hour and splenic atrophy that was observed on the 3rd day post infection. Most organs showed lesions between 1 hour and the 2nd day. By the 5th day, there were few lesions observed. On the second week of observation fibrin deposition, fibrinopurulent exudate, necrosis, fibrosis, thick airsacs, pneumonia, airsacculitis, splenic atrophy were recorded. The number of lesions increased from 17 at 1 hour peaking to 36 at 12 hours and decreased gradually to 4 on day 14 p.i. Most lesions were mild {127 (59.4%)}, moderately severe {57 (26.6%)} and severe {30 (14.1%)} ones. The severe lesions were pneumonia (6 hours to day 2), congestion and sinusitis, (6 to 12 hours), airsacculitis, and fibrin deposition (6 to 24 hours), oedema (3rd hour), haemorrhage, emphysema, (3 to 6 hours), tracheitis (6th hour), and pleuritis (12th hour), lung hepatization and peritonitis (12th hour), and mucus exudate and necrosis (24th hour). All the control ducks were negative.

11.3.1.2 Gross lesions of IS ducks

Organs from IS ducks with gross lesion, type and their respective severities at specified periods are presented on Table 43. Lesions occurred in 20 of the 24 organs sampled except the adrenal gland, proventriculus, skin and pruning gland. There were no gross lesions in all organs on 0 hour, p.i. Lesions were commonly observed on the lung (83.3%) airsacs, (91.7%) liver (66.7%), spleen (41.7%), trachea, (41.7%) kidney (33.3%), and conjunctiva, (33.3%) heart (41.7%), brain, caecal tonsils and pectoral muscle (33.3%).

At one hour p.i, there were 4 (16.7%) organs with lesions, namely, lungs, trachea, airsacs, and liver. The organs increased to 11 (45.8%) by the 3rd hour as there were lesions on the conjunctiva, spleen, heart, thymus, brain, kidney, and the pectoral muscle. At the 6th hour organs with lesions increased to 16 (66.7%) due to lesions on the Harderian gland, sciatic nerve, bursa of Fabricius and large intestine. On the 12th hour, 18 (75%) organs had shown a lesion, as pancreas, duodenum, and midgut were positive while the sex organs were the last to be positive on the second day, p.i. Most organs expressed lesions between 3 to 24 hours p.i. By the 2nd day p.i lesions were only present in the lungs, airsacs, spleen, liver, heart, and sex organ. These lesions became limited to the lung, airsacs, liver and kidney on the 3rd day and to the lungs, airsacs and liver or kidney on days 5 and 7; and to lung and airsacs on days 10 to 14 p.i. The organs with lesions increased from a total of 4 at the 1st hour p.i, to a maximum of 18 at the 12th hour and declined to 2 at day 14. Lesions were mild 62 (75.6%), moderate 12 (14.6%) and severe 6 (9.8%). Moderate to severe lesions were observed mostly on the lung (1hr – day 5), airsacs (3 hours –5th day), trachea (3 – 12 hours), and the heart (3-24 hours).

Inflammatory lesions as in NIS ducks were recorded from 20 of the 24 organs per bird over the 14 days study period as shown in Table 44. Their distribution was like in the NIS ducks. No lesions were observed on 0 hour, p.i. The commonly observed lesions were pneumonia, fibrin deposition (Fig.15), airsacculitis (91.7%), thick air sacs, hepatitis (75%), congestion, necrosis, fibrosis, hydropericardium, fibrinopurulent exudate, (66.7%), purulent exudate, pleuritis, peritonitis, myocarditis (58.3%), haemorrhage, oedema, urate deposition, tracheitis, sinusitis, splenitis, and myositis (50%).

At one hour, p.i 12 (43.6%) lesions observed, were; congestion, haemorrhages, oedema, fibrin, emphysema, hydropericardium, lung hepatization, pneumonia, air sacculitis, tracheitis, sinusitis, peritonitis, conjunctivitis, myocarditis, hepatitis, splenitis and nephritis. On the 3rd hour p.i, 26 (66.7%) lesions were encountered on further recording of fibrinopurulent exudate, fibrin exudate, thick airsac, mucus exudate, collapsed lungs, pleuritis, meningitis, and pectoral muscle myositis. After 6 hours p.i, necrosis, froth, paleness, urate deposits, splenomegally, hepatomegally, pericarditis, nerve myelitis, and bursitis, were observed, a total of 35 (89.7%) inflammatory lesions. On the 12th hour p.i 37 (94.9%) lesions had been observed after the recording of fibrosis, and splenic capsulitis. The last lesions to be recorded were perihepatitis at 24 hours and splenic atrophy on the 2nd day p.i.

Most organs expressed lesions between 1 hour and the 5th day p.i decreasing to a few by the 7th day. The number of lesions increased from 17 at 1 hour, to a maximum of 37 at 12 hours and gradually declined to 4 on day 14 p.i. Most lesions were mild {125 (53.3%)}, moderate {68 (30.1%)}, and severe {33 (14.6%)}. The severe lesions were thick airsacs (3-24hrs), pneumonia, haemorrhage (3-12 hr), congestion, purulent exudate

(6-24 hours), oedema, fibrin deposition, air sacculitis, tracheatis (6-12 hr), pleuritis (12hr-day 2), emphysema (6^{th} hour), and fibrinopurulent exudate (12^{th} hour). Lesions in NIS and IS did not differ significantly ($p>0.05$).

Table 43: Organs of NIS and IS ducks with gross lesions and their severity at specified times

Organs	Ducks treatment	Hours post infection						Days post infection						% lesions per organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Lung	NIS	-	3	3	3	3	3[f]	2	2	2[e]	1[d]	1	1	91.7
	IS	-	3	3	3	2	2	2	2	2	1	1	1	83.3
Trachea	NIS	-	3	2	2	1	1	-	-	-	-	-	-	41.7
	IS	-	1	2	3	2	1	-	-	-	-	-	-	41.7
Conjunctiva	NIS	-	1	3	3	1	1	1	-	-	-	-	-	50
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Airsacs	NIS	-	1	3	3	3	3	2	2	2	1	1	1	91.7
	IS	-	1	3	3	3	3	2	1	2	1	1	1	91.7
Spleen	NIS	-	1	1	2	2	1	1	-	-	-	-	-	50
	IS	-	-	1	1	1	1	1	-	-	-	-	-	41.7
Liver	NIS	-	1	1	3	2	3	1	-	1	-	-	-	58.3
	IS	-	1	1	1	1	1	1	1	1	-	-	-	66.7
Heart	NIS	-	1	1	2	2	1	1	1	-	-	-	-	58.3
	IS	-	-	2	1	2	2	1	-	-	-	-	-	41.7
Thymus	NIS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	-	-	-	-	-	-	-	25
Harderian gland	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Pancreas	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
Brain	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Sciatic nerve	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Kidney	NIS	-	1	1	2	1	1	-	-	-	-	-	-	41.7
	IS	-	-	1	1	-	1	-	1	-	-	-	-	33.3
Testis/ovary	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
Adrenal gland	NIS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0

Table 43 (Continued next page)

Legend: [d] - Mild lesions; [e] - Moderate lesions; [f] - Severe lesions; - No lesion seen

Table 43 (Continued)

Bursa of Fabricius	NIS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
	IS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
Proventriculus	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Duodenum	NIS	-	1	2	2	1	1	-	-	-	-	-	-	41.7
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Midgut	NIS	-	-	1	1	1	-	-	-	-	-	-	-	25
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Caecal tonsils	NIS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Large intestines	NIS	-	-	1	2	1	1	1	-	-	-	-	-	41.7
	IS	-	-	-	1	1	1	-	-	-	-	-	-	25
Pruning gland	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Comb	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Pectoral muscle	NIS	-	1	1	2	1	1	1	-	-	-	-	-	50
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Total mild [d]	NIS	0	9	13	8	17	12	6	1	1	2	2	2	73
	IS	0	3	8	13	14	12	3	3	1	2	2	2	63
Total moderate [e]	NIS	0	0	2	9	3	0	2	2	2	0	0	0	20
	IS	0	0	2	2	3	2	2	1	2	0	0	0	14
Total severe [f]	NIS	0	2	3	4	2	3	0	0	0	0	0	0	14
	IS	0	1	2	3	1	1	0	0	0	0	0	0	8

Legend: [d] - Mild lesions; [e] - Moderate lesions; [f] - Severe lesions; - No lesion seen

X^2 calculated = 0.922 ($p>0.05$)

Table 44: Gross lesions and their severity as observed in non-immunosuppressed (NIS) and immunosuppressed (IS) ducks at specified times

Lesions	Ducks treatment	Hours post infection						Days post infection						% lesions per organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Congestion	NIS	-	1[g]	2[h]	3[i]	3	2	1	1	-	-	-	-	58.3
	IS	-	1	2	3	3	3	2	1	1	-	-	-	66.7
Haemorrhages	NIS	-	2	3	3	2	1	-	-	-	-	-	-	41.7
	IS	-	1	3	3	3	2	1	-	-	-	-	-	50
Oedema	NIS	-	2	3	2	2	1	-	-	-	-	-	-	41.7
	IS	-	2	2	3	3	2	1	-	-	-	-	-	50
Suppurate exudates	NIS	-	-	2	3	3	2	1	1	-	-	-	-	50
	IS	-	-	2	3	3	3	2	1	1	-	-	-	58.3
Fibrin exudates	NIS	-	-	2	3	3	3	2	2	1	1	1	1	83.3
	IS	-	1	3	3	3	2	2	1	1	1	1	1	91.7
Fibrino-suppurative	NIS	-	-	-	2	3	3	2	2	1	1	1	-	50
Exudates	IS	-	-	1	2	3	2	-	1	1	1	-	-	58.3
Necrosis	NIS	-	-	-	2	3	3	2	1	1	1	1	-	66.7
	IS	-	-	-	1	3	2	1	1	1	1	1	-	66.7
Emphysema	NIS	-	2	3	3	1	-	-	-	-	-	-	-	33.3
	IS	-	1	2	3	2	1	-	-	-	-	-	-	41.7
Fibrosis	NIS	-	-	-	-	-	1	2	1	1	1	1	1	58.3
	IS	-	-	-	-	1	2	2	1	1	1	1	1	66.7
Froth	NIS	-	-	-	2	1	1	-	-	-	-	-	-	25
	IS	-	-	-	1	2	1	-	-	-	-	-	-	25
Paleness	NIS	-	-	-	1	1	2	-	-	-	1	-	-	33.3
	IS	-	-	-	1	1	2	1	1	-	-	-	-	41.7
Thick airsacs	NIS	-	-	2	3	3	2	1	-	1	1	1	-	66.7
	IS	-	-	3	3	3	3	2	2	1	1	1	-	75
Hydropericardium	NIS	-	-	1	2	2	2	1	1	-	-	-	-	50
	IS	-	1	1	2	2	2	1	1	1	-	-	-	66.7
Mucus exudates	NIS	-	-	3	2	2	1	1	-	-	-	-	-	41.7
	IS	-	-	1	2	2	1	-	-	-	-	-	-	33.3
Urate deposits	NIS	-	-	-	2	1	1	-	-	-	-	-	-	25
	IS	-	-	-	1	2	2	1	1	-	-	-	-	50
Collapsed lungs	NIS	-	-	2	2	1	1	1	-	-	-	-	-	41.7
	IS	-	-	3	2	2	2	1	-	-	-	-	-	41.7
Lung hepatization	NIS	-	-	1	2	1	-	-	-	-	-	-	-	25
	IS	-	2	3	2	1	-	-	-	-	-	-	-	33.3
Splenomegally	NIS	-	-	-	1	2	1	1	-	-	-	-	-	33.3
	IS	-	-	-	2	2	2	1	-	-	-	-	-	33.3

Table 44 (Continued next page)

Legend: [g] - Mild lesions; [h] - Moderate lesions; [i] - Severe lesions; - No lesion seen

Table 44 (Continued)

Hepatomegally	NIS	-	-	-	1	2	1	1	-	-	-	-	-	33.3
	IS	-	-	-	2	2	1	1	-	-	-	-	-	33.3
Pneumonia	NIS	-	1	2	3	3	3	3	2	1	1	1	1	91.7
	IS	-	2	3	3	3	2	2	2	1	1	1	1	91.7
Airsacculitis	NIS	-	1	2	3	3	3	2	2	1	1	1	1	91.7
	IS	-	1	2	3	3	2	2	1	1	1	1	1	91.7
Tracheitis	NIS	-	1	2	3	1	1	-	-	-	-	-	-	41.7
	IS	-	1	2	3	3	2	1	-	-	-	-	-	50
Sinusitis	NIS	-	1	2	1	3	3	1	1	-	-	-	-	50
	IS	-	1	1	2	2	1	1	-	-	-	-	-	50
Pleuritis	NIS	-	1	1	2	3	2	1	1	-	-	-	-	58.3
	IS	-	-	1	2	3	3	3	2	1	-	-	-	58.3
Peritonitis	NIS	-	1	1	2	2	2	1	-	-	-	-	-	50
	IS	-	1	2	2	2	2	1	1	-	-	-	-	58.3
Conjunctivitis	NIS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
	IS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
Pericarditis	NIS	-	-	-	1	2	2	1	-	-	-	-	-	33.3
	IS	-	-	-	1	2	1	1	-	-	-	-	-	33.3
Myocarditis	NIS	-	1	1	2	2	2	1	-	-	-	-	-	58.3
	IS	-	1	1	1	2	1	1	1	-	-	-	-	58.3
Hepatitis	NIS	-	1	1	2	2	2	1	-	1	-	-	-	58.3
	IS	-	1	2	2	2	2	1	-	-	-	-	-	58.3
Perihepatitis	NIS	-	-	-	-	-	1	1	-	-	-	-	-	16.7
	IS	-	-	-	-	1	2	-	-	-	-	-	-	16.7
Splenitis	NIS	-	1	1	2	1	1	1	-	-	-	-	-	50
	IS	-	1	2	2	2	1	1	-	-	-	-	-	50
Splenic capsulitis	NIS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
	IS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
Splenic atrophy	NIS	-	-	-	-	-	-	-	1	1	1	1	-	33.3
	IS	-	-	-	-	-	-	1	1	1	-	-	-	256
Nephritis	NIS	-	1	2	2	1	1	-	-	-	-	-	-	41.7
	IS	-	1	1	2	2	1	1	1	-	-	-	-	41.7
Enteritis	NIS	-	1	1	1	1	1	-	-	-	-	-	-	41.7
	IS	-	-	1	1	1	1	-	-	-	-	-	-	41.7
Meningitis	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
Nerve myelitis	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	-	1	1	-	-	-	-	-	-	-	33.3
Bursitis	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	-	1	1	-	-	-	-	-	-	-	33.3
Myositis	NIS	-	1	1	1	1	1	1	-	-	-	-	-	50
	IS	-	-	1	1	1	1	-	-	-	-	-	-	50
Total mild [g]	NIS	0	14	13	14	19	18	14	10	10	8	8	4	132
	IS	0	14	11	10	12	17	19	14	9	6	6	4	122
Total moderate [h]	NIS	0	3	10	12	9	10	4	0	0	0	0	0	48
	IS	0	3	9	14	20	11	2	1	0	0	0	0	41
Total severe [i]	NIS	0	0	4	9	7	2	0	0	0	0	0	0	22
	IS	0	0	6	11	5	1	1	0	0	0	0	0	24

Legend: [g] - Mild lesions; [h] - Moderate lesions; [i] - Severe lesions; - No lesion seen

X^2 calculated = 0.4534 ($p>0.05$)

FIG.13. LUNGS FROM NON- IMMUNOSUPPRESSED DUCKS SHOWING HAEMORRHAGES (A, B); AIR-SACCULITIS (C) AND CONTROL LUNG (D).

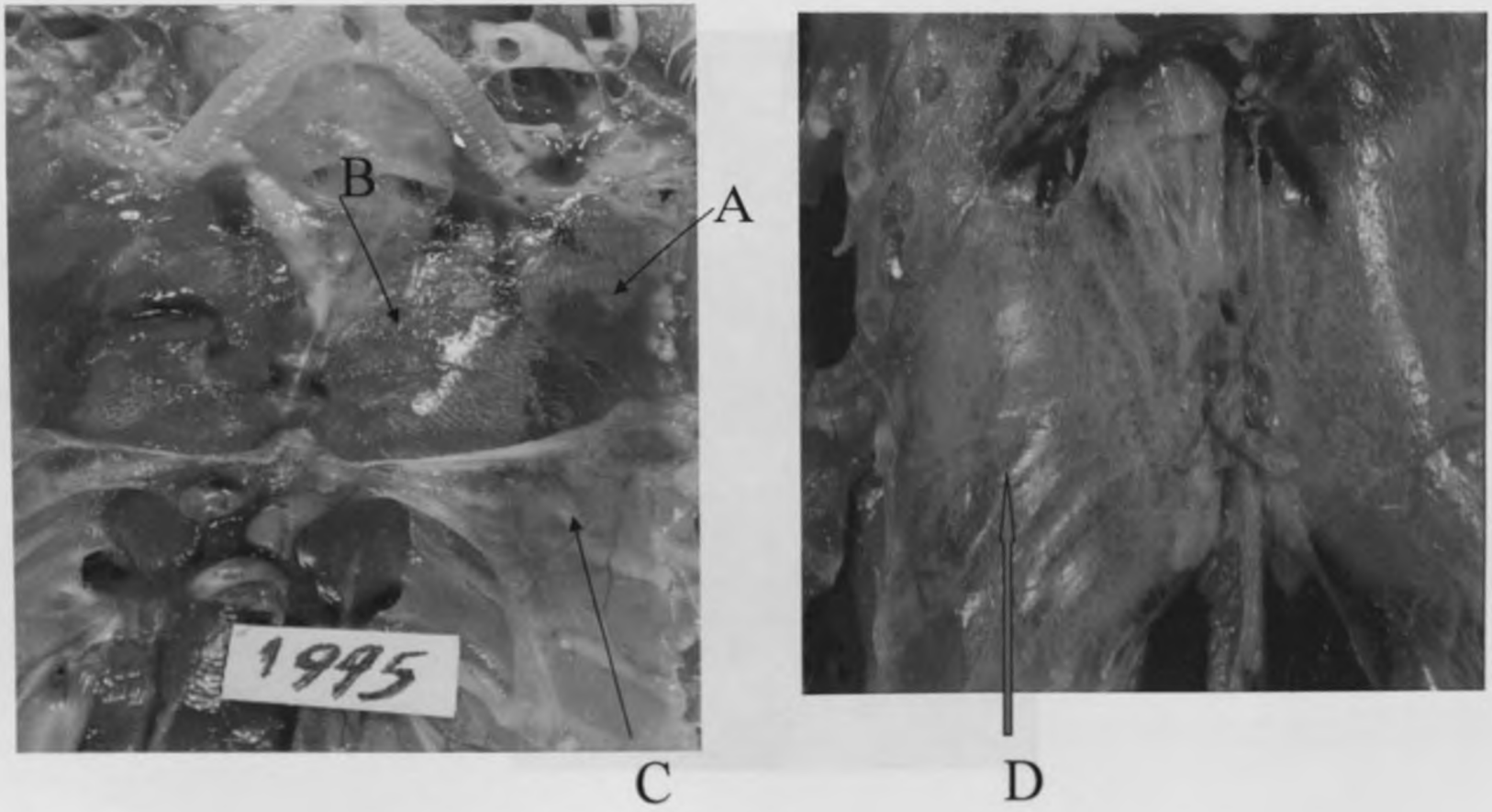


FIG. 14. PNEUMONIC LUNGS FROM NON-IMMUNOSUPPRESSED DUCKS SHOWING HEAVY FIBRIN DEPOSITS (ARROW).

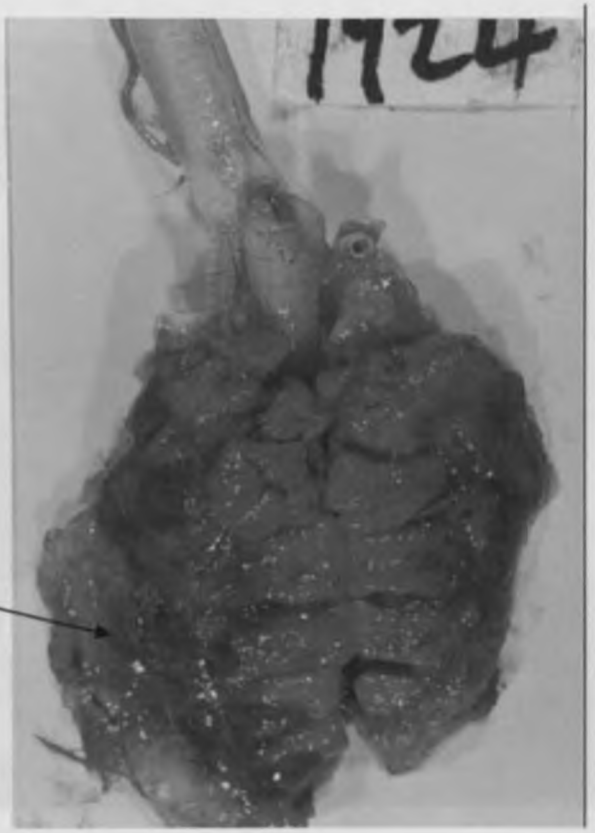
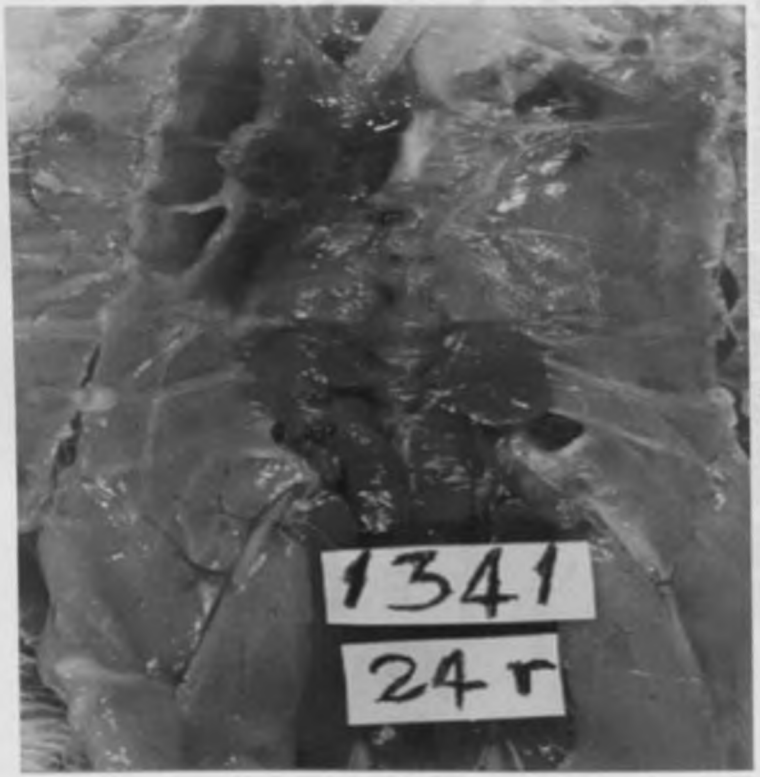


FIG.15. LUNGS FROM IMMUNOSUPPRESSED DUCKS SHOWING CONSOLIDATION AND FIBRIN STRANDS (LEFT) COMPARED TO LUNGS FROM NORMAL CONTROL NON – IMMUNOSUPPRESSED DUCKS (RIGHT).



11.3.2 Pathogenesis of *P.multocida* organisms as evaluated from histopathological lesions of duck organs

11.3.2.1 Histopathological lesions in NIS ducks

Organs from NIS ducks with histopathological lesions, and their respective severities at specified periods are presented in Table 45. A total of 21 out of 24 organs had histopathological lesions. However, there were no lesions on the sciatic nerve, skin and pectoral muscles and in all organs at 0hr p.i. Starting from 1 hour onwards lesions were commonly observed on the lungs (91.7%), liver (75%), airsacs, conjunctiva, spleen, thymus (66.7%), trachea (58.3%), kidneys, bursa of Fabricius (50%), heart, and midgut (41.7%), in that order.

Within 1hour p.i, lesions were observed in 5 (20.8%) organs, namely: lung, trachea, conjunctiva, airsacs, liver, and thymus. By the 3rd hour p.i, organs with lesions increased to 13 (54.2%), due to lesions in the spleen, kidney, adrenal gland, bursa of Fabricius, and midgut, caecal tonsils, and large intestine. Organs with lesions were 11 (45.8%) by the 6th hour due to pathology in the heart and pruning gland. At the 12th hour p.i, 15 (62.5%) organs had showed lesions with a positive Harderian gland and duodenum. The last organs to show lesions were testis/ovary, and proventriculus (24 hour), and brain tissue (2nd day). Organs with histological lesions increased from a total of 5 on the 1st hour p.i, to a maximum of 15 at the 12th hour and decreased gradually to 1 at day 14 of the study. Most organs showed lesions between 3rd hour and day 10 p.i. Thereafter only the lung had lesions. Most histopathological lesions were mild 69 (69.7%), moderate 19.(19.2%), and severe 11 (11.1%) ones. Severe lesions were observed mostly on the lung (6th hour),

airsacs (6 – 24hours), trachea (6th hour), spleen (6th -24hour), thymus (24th hour), and brain (2nd day).

A total of 20 different types of histopathological lesions were recorded from the 21 out of 24 organs as shown in Table 46. These were congestion, oedema (in tissues and around blood vessels), haemorrhages, emphysema, atelectasis, fibrin deposition, infiltration of tissues by heterophils, mononuclear and giant cells, granuloma formation, mucus exudate, urate deposition, degeneration of the epithelium and blood vessels (especially arteries and arterioles), thrombosis formation in blood vessels, muscular degeneration (especially on the heart with predominant zenker's degeneration), depleted lymphoid tissues, necrosis, fibrosis and serositis (mainly on the pleura, pericardium, and intestinal serosa). As in gross lesions no lesions were observed at zero hour p.i. Commonly observed lesions were infiltration with heterophils, serositis, and mononuclear cells, fibrin exudates, and congestion.

One hour p.i, 2 (10%) observed lesions were congestion, and infiltration by heterophils. By the 3rd hour p.i, 12 (60%) lesions were seen due to further recording of oedema, haemorrhages, emphysema, atelectasis, fibrin and mucus exudate, epithelial, blood vessel and muscular degeneration, and serositis. After 6 hours p.i, lesions increased further to 18 (90%) as mononuclear cells, granulomas, urate deposits, thrombosis, lymphoid cell depletion, and necrosis were recorded. On the 12th hour p.i, the lesions decreased gradually to17 (85%) at 24 hours p.i, and 2 (10%) at day 14 p.i.

The number of lesions increased from 2 at one hour p.i, to a maximum of 20 at 12th hour and decreased gradually to 2 on day 14 p.i. Different lesions had been recorded by

the 24th hour and the peak lesions time were from the 3rd hour to the 24th hour. By the 7th day, there were few lesions observed.

On the second week of the study, only occasional infiltration with heterophils and mononuclear cells, serositis and fibrosis were recorded. Majority of the lesions were mild 71 (68.9%), while others were moderate 19 (18.5%), and severe 13 (12.6%) ones. Most of the severe lesions were observed within 24 hours post infection. The severest lesions were congestion (3-6 hours), oedema (6th hour), emphysema (3rd hour), fibrin exudate (6-12 hours), heterophil infiltration (3-24hour), serositis (3rd hour) and necrosis (6thhour).

No lesions were observed on any of the organs of the control ducks.

11.3.2.2 Histopathological lesions in IS ducks

Organs from IS ducks with histopathological lesions, and their respective severities at specified times are presented in Table 45. A total of 21 out of 24 organs had histopathological lesions, except the pruning gland, skin and pectoral muscle. There were no lesions observed at 0hr and day 14 p.i. Lesions commonly observed were in the lungs (83.3%), liver (75%), airsacs (66.7%), trachea, heart, Harderian gland (41.7%), conjunctiva, bursa of Fabricius, midgut, and kidney (33.3%).

Within one hour p.i, 4 (16.7%) organs with lesions were lung, airsacs, liver, and pancreas. By the 3rd hour p.i, organs with lesions had increased to 6 (25%), due to lesions in the trachea, thymus, and Harderian gland. On the 6th hour p.i, 10 (41.7%) organs with lesions were conjunctiva, kidney, adrenal gland and bursa of Fabricius. At the 12th hour p.i, 15 (62.5%) organs had lesions due to lesions in the spleen, brain, midgut, caecal tonsils, and large intestine. On the 24th hour, 19 (79.2%) organs had lesions due to lesions

in sciatic nerve, sex organ, and proventriculus. The last organ to express a lesion was duodenum on the 3rd day p.i.

Organs with histological lesions increased from a total of 4 by the 1st hour p.i, peaking to19 at the 24th hour, and decreased gradually to 2 at day 10 of the study. Most organs showed lesions between 3rd hour and 3rd day p.i; and decreased gradually to 5 namely: lungs, liver, brain, kidney, and bursa of Fabricius on day 5 and to 4 organs on day 7 p.i, namely: - lungs, airsacs, liver and sex organs and finally to 2 organs with lesions at day 10 p.i, namely the lung and heart. Most histopathological lesions were mild 59 (73.8%), moderate 15 (18.7%) and severe 6 (7.5%) ones. Severe lesions were observed mostly on the lungs (6-12 hours), trachea, spleen, conjunctiva (12th hour), and liver (24th hour).

A total of 20 different types of inflammatory lesions were recorded from the 21 organs on histopathological examination as shown in Table 46. The commonly encountered lesions were lymphoid cell depletion, infiltration with heterophils, granuloma formation, serositis, fibrin exudate, mononuclear cells, necrosis, congestion, and fibrosis.

Lymphoid cell depletion was present at zero hour, while at one hour p.i, 2 (10%) lesions were congestion, and lymphoid cell depletion. By the 3rd hour p.i, lesions increased to 9 (45%) as oedema, haemorrhages, emphysema, atelectasis, fibrin exudate, heterophil infiltration, and necrosis were recorded. After 6 hours p.i, lesions increased to 14 (70%) as granuloma, mucus exudate, urate deposits, epithelial and muscular degeneration, and serositis were recorded. On the 12th hour p.i, a total of 18 (90%) lesions were observed due to further recording of mononuclear cells, giant cells, blood vessel

degeneration, and thrombosis. All the lesions had been observed at 12 hours p.i. At 24 hrs p.i, the lesions were 18 (70%).

The number of lesions increased from 1 at zero hour p.i, peaking to 18 at 12th hour and decreased gradually to 1 on day 14 p.i. The lesions reached a peak on the 3rd hour to 3rd day p.i. By the 5th day p.i, there were few lesions observed.

On the second week only mononuclear cells, granuloma, lymphoid cell depletion, and fibrosis were recorded. Majority of the lesions were mild 72 (76%), and the remaining were moderate 11 (11.5%), and severe (12.5%). Most of the severe lesions were observed between 6 to 24hours p.i. The severest lesions were congestion (12-24hours), oedema (12th hour), fibrin exudate (3rd hour), heterophil infiltration (12-24hours), giant cells (24 hour), granuloma formation (6-24hours), and necrosis (24thhour). Lesions in NIS and IS ducks were not different statistically ($p>0.05$), although those of NIS were more than the IS.

No lesions were observed on any of the organs of the control ducks.

Table 45: Histopathological lesions and their severity in organs of non-immunosuppressed (NIS) and immunosuppressed (IS) ducks at specified times

Organs	Ducks treatment	Hours post infection						Days post infection						% lesions per organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Lung	NIS	-	1[a]	3[c]	3	3	2[b]	1	1	1	1	1	1	91.7
	IS	-	1	2	3	3	2	1	1	1	1	1	-	83.3
Trachea	NIS	-	1	2	3	2	1	1	1	-	-	-	-	58.3
	IS	-	-	1	2	3	1	1	-	-	-	-	-	41.7
Conjunctiva	NIS	-	-	2	2	1	-	1	1	1	1	1	-	66.7
	IS	-	-	-	1	3	1	-	1	-	-	-	-	33.3
Airsacs	NIS	-	1	1	3	2	3	2	2	-	1	-	-	66.7
	IS	-	2	1	2	2	1	1	1	-	1	-	-	66.7
Spleen	NIS	-	-	1	3	2	3	-	1	1	1	1	-	66.7
	IS	-	-	-	-	1	3	2	-	-	-	-	-	25
Liver	NIS	-	1	1	2	1	2	1	1	-	1	1	-	75
	IS	-	1	1	1	2	3	2	1	1	1	-	-	75
Heart	NIS	-	-	-	1	2	1	1	-	-	1	-	-	41.7
	IS	-	-	-	1	1	1	1	-	-	-	1	-	41.7
Thymus	NIS	-	2	1	1	2	3	2	1	1	-	-	-	66.7
	IS	-	-	1	-	-	2	-	1	-	-	-	-	25
Harderian gland	NIS	-	-	-	-	1	1	-	-	1	-	-	-	25
	IS	-	-	1	2	1	1	-	1	-	-	-	-	41.7
Pancreas	NIS	-	-	-	-	-	-	-	-	1	-	-	-	8.3
	IS	-	1	-	-	1	1	-	-	-	-	-	-	25
Brain	NIS	-	-	-	-	-	-	3	2	1	-	-	-	25
	IS	-	-	-	-	1	1	-	-	1	-	-	-	25
Sciatic nerve	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	1	-	-	-	-	-	8.3
Kidney	NIS	-	-	1	-	1	1	1	-	1	-	1	-	50
	IS	-	-	-	2	1	1	-	-	1	-	-	-	33.3
Testis/ovary	NIS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
	IS	-	-	-	-	-	2	-	-	1	-	-	-	16.7
Adrenal gland	NIS	-	-	1	-	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	1	-	-	-	-	-	-	-	-	8.3
Bursa of Fabricius	NIS	-	-	2	2	2	-	1	1	-	-	1	-	50
	IS	-	-	-	1	2	1	-	-	1	-	-	-	33.3

Table 45 (Continued next page)

Legend: [a] - Mild lesions; [b] - Moderate lesions; [c] - Severe lesions; - No lesion seen

Table 45 (Continued)

Proventriculus	NIS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
	IS	-	-	-	-	-	1	-	-	-	-	-	-	8.3
Duodenum	NIS	-	-	-	-	1	-	-	-	-	-	-	-	8.3
	IS	-	-	-	-	-	-	1	-	-	-	-	-	8.3
Midgut	NIS	-	-	1	1	-	1	-	1	-	-	1	-	41.7
	IS	-	-	-	-	1	1	1	1	-	-	-	-	33.3
Caecal tonsils	NIS	-	-	1	-	1	1	-	-	-	-	-	-	25
	IS	-	-	-	-	2	1	-	-	-	-	-	-	16.7
Large intestine	NIS	-	-	1	-	-	-	-	-	-	-	-	-	8.3
	IS	-	-	-	-	1	1	-	-	-	-	-	-	16.7
Pruning gland	NIS	-	-	-	1	1	-	-	-	-	-	-	-	16.7
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Skin	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Pectoral muscle	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Total mild [a]	NIS	0	4	9	4	8	8	7	8	8	6	7	1	62[d]
	IS	0	3	5	5	8	12	7	6	6	3	1	0	56[d]
Total moderate[b]	NIS	0	1	3	3	6	2	2	2	0	0	0	0	19[d]
	IS	0	1	1	4	4	3	2	0	0	0	0	0	15[d]
Total severe[c]	NIS	0	0	1	4	1	3	0	0	0	0	0	0	9[d]
	IS	0	0	0	1	3	2	0	0	0	0	0	0	6[d]

Legend: [a] - Mild lesions; [b] - Moderate lesions; [c] - Severe lesions; - No lesion seen

X^2 calculated [d] = 0.366 ($p>0.05$)

Table 46: Histopathological lesions and their severity as observed in non-immunosuppressed (NIS) and immmunosuppressed (IS) ducks at specified times

Lesions	Ducks treatment	Hours post infection						Days post infection						% lesion / organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Congestion	NIS	-	1[d]	3[f]	3	2[e]	2	1	-	-	-	-	-	50
	IS	-	-	1	2	3	3	1	1	1	-	-	-	41.7
Oedema	NIS	-	-	1	3	2	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	3	1	-	-	-	-	-	-	33.3
Haemorrhages	NIS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	2	1	-	-	-	-	-	-	33.3
Emphysema	NIS	-	-	3	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	2	-	-	-	-	-	-	-	25
Atelectasis	NIS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
	IS	-	-	1	1	1	-	-	-	-	-	-	-	25
Fibrin exudates	NIS	-	-	2	3	3	2	1	1	1	-	-	-	58.3
	IS	-	-	1	2	3	2	1	-	-	-	-	-	50
Heterophils	NIS	-	1	3	3	3	3	2	2	1	1	1	-	83.3
	IS	-	-	1	2	3	3	1	1	1	1	-	-	66.7
Mononuclear cells	NIS	-	-	-	1	2	2	1	1	1	1	1	-	66.7
	IS	-	-	-	-	1	1	1	1	-	-	1	-	41.7
Giant cells	NIS	-	-	-	-	1	1	-	1	-	-	-	-	25
	IS	-	-	-	-	1	3	1	-	-	-	-	-	25
Granuloma	NIS	-	-	-	1	2	2	-	-	1	-	-	-	33.3
	IS	-	-	-	3	3	3	1	1	1	1	1	-	66.7
Mucus exudates	NIS	-	-	1	2	2	-	-	-	-	-	-	-	25
	IS	-	-	-	1	1	1	-	-	-	-	-	-	25
Urate deposits	NIS	-	-	1	1	-	-	-	-	-	-	-	-	16.7
	IS	-	-	-	1	-	1	-	-	-	-	-	-	16.7
Epithelium degn	NIS	-	-	1	1	1	1	-	-	-	-	-	-	33.3
	IS	-	-	-	1	1	1	1	-	-	-	-	-	33.3
Blood vessel	NIS	-	-	1	1	2	1	-	-	1	-	-	-	41.7
Degeneration	IS	-	-	-	-	1	1	1	-	-	-	-	-	25
Thrombosis	NIS	-	-	-	1	1	1	1	-	1	-	-	-	41.7
	IS	-	-	-	-	1	2	1	-	-	-	-	-	25

Table 46 (Continued next page)

Legend: [d] - Mild lesions; [e] - Moderate lesions; [f] - Severe lesions; - No lesion seen
Epithelium degn – Epithelium degeneration

Table 46 (Continued)

Muscular degeneration	NIS	-	-	1	2	1	1	-	-	-	-	-	-	33.3
	IS	-	-	-	1	1	1	-	-	-	-	-	-	25
Lymphiod depletion	NIS	-	-	-	1	2	-	1	-	1	-	-	-	33.3
	IS	1	1	2	1	1	1	1	-	-	-	-	-	58.3
Necrosis	NIS	-	-	-	3	2	1	1	-	1	-	-	-	41.7
	IS	-	1	-	-	2	3	1	1	-	-	-	-	41.7
Fibrosis	NIS	-	-	-	-	1	1	1	1	1	1	1	1	66.7
	IS	-	-	-	-	-	1	1	1	1	-	1	-	41.7
Serositis	NIS	-	-	3	2	2	1	1	1	1	1	1	1	75
	IS	-	-	-	2	3	2	1	1	1	-	-	-	50
Total mild [d]	NIS	0	2	8	8	8	12	8	5	10	4	4	2	71
	IS	1	2	7	9	9	10	13	7	5	2	3	0	68
Total moderate [e]	NIS	0	0	1	5	9	4	1	1	0	0	0	0	21
	IS	0	0	1	4	3	3	0	0	0	0	0	0	11
Total severe [f]	NIS	0	0	4	5	2	1	0	0	0	0	0	0	12
	IS	0	0	0	1	6	5	0	0	0	0	0	0	12

Legend: [d] - Mild lesions; [e] - Moderate lesions; [f] - Severe lesions; - No lesion seen

X^2 calculated [d, e, f] = 2.3334 ($p>0.05$)

11.3. 3 Pathogenesis of *P.multocida* organisms as evaluated through signals of fluorescent *in situ* hybridization

11.3.3.1 *P.multocida* FISH signals in organs of NIS ducks

Occurence of *P.multocida* FISH signals in the 24 organs at specified times are presented in Table 47. All organs had P.*multocida* FISH signal during the 14 days study period, although some organs were missed at some stages during the processing of the FISH test.

Lungs and tracheal lumen of various birds had P. *multocida* FISH signals from zero hour (Fig.16) up to 14th day of the study. Other organs commonly found to have P. *multocida* signals were spleen (91.7%), liver (83.3%), heart (66.7%), airsacs, bursa of Fabricius, and thymus gland (58.3%).

By one hour p.i, 13 (54.2%) organs with P.*multocida* signals were: - lung, trachea, conjunctiva, airsacs, spleen, liver, heart, thymus, sciatic nerve, bursa of Fabricius, proventriculus, duodenum and caecal tonsils (Fig. 16), and pruning gland. On the 3rd hour, organs with *P. multocida* signals increased to 15 (62.5%) due to signal observation in conjunctiva, kidney, sex organ, adrenal gland, and pectoral muscle. At 6th hour, the Harderian gland, brain, midgut, and head skin, showed *P. multocida* signals, making the total observed organs to be 17 (70.8%). After 12 hours p.i, organs with *P. multocida* increased to 19 (79.2%) as large intestine and pruning gland were positive. At 24 hours p.i, some organs had no signal such that 11 (45.8%) organs were positive. At this time, only pancreas had no signal, which it had on the second day. Organs with *P. multocida* were 2 at zero (1-5minutes after inoculation) hr and they increased to a maximum of 19 at

the 12th hour. They remained high from the 1st hour to 3rd day but stabilized at about 6 positive organs for the rest of the period.

11.3.3.2 *P.multocida* FISH signals in organs of IS ducks

Occurence of FISH P.*multocida* signals in the 24 organs at specified times are presented in Table 47. All the 24 organs had the signal observed during the 14 days study period, although some organs were missed at some stages during the processing of the FISH test. Lung and trachea of various birds had *P. multocida* signal from the 1st hour until the 14th day (91.7%, each) of the study. Other organs where the bacterial signals were commonly detected were liver (83.3%), bursa of Fabricius (75%), spleen (50%) (Fig.17), airsacs (66.7%), heart, (50%), brain, kidney, and adrenal glands (41.7%, each). At one hour p.i, 13 (54.2%) organs with *P. multocida* signals, were: - lungs, trachea, airsacs, spleen, liver, heart, pancreas, sex organ, adrenal gland, bursa of Fabricius, duodenum, caecal tonsils, and large intestine. On the 3rd hour, organs with P. *multocida* signals decreased to 12 (50%) although more signals were observed on the thymus and the brain. At 6th hour, the organ with signals, were 10 (41.7%) although Harderian gland and the kidney became positive. After 12 hours, organs with *P. multocida* increased to 15 (62.5%) as sciatic nerve and head skin were then positive. At 24th hour, 17 (70.8%) organs had signals after the last two organs, proventriculus and pruning gland became positive. Organs with *P. multocida* were high at 13 at 1 hour p.i, and stayed high to a peak of 17, at 24 hrs to decrease to 2 at the 14th day.

The NIS ducks had more signals compared with the IS but the difference was not significant ($p>0.05$).

Table 47: Fluorescent *in situ* hybridization signals observed in various organs of non-immunosuppressed (NIS) and immunosuppressed (IS) ducks at specified times

Organs	Duck	Hours post infection						Days post infection						Lesions
	Treatment	0	1	3	6	12	24	2	3	5	7	10	14	per organ
Lung	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
	IS	-	+	+	+	+	+	+	+	+	+	+	+	11
Trachea	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
	IS	-	+	+	+	+	+	+	+	+	+	+	+	11
Conjunctiva	NIS	-	-	+	+	+	-	N	N	+	-	+	N	5
	IS	-	-	+	+	+	+	-	N	-	-	-	-	4
Airsacs	NIS	-	+	+	+	+	+	+	-	-	+	-	-	7
	IS	-	+	+	+	+	+	+	-	N	+	+	N	8
Spleen	NIS	-	+	+	+	+	+	+	+	+	+	+	+	11
	IS	-	+	-	+	+	+	-	-	+	-	+	-	6
Liver	NIS	-	+	+	+	+	+	+	+	+	+	-	+	10
	IS	-	+	+	+	+	+	+	+	+	+	+	-	10
Heart	NIS	-	+	+	+	+	+	+	+	+	-	N	-	8
	IS	-	+	-	+	-	+	+	+	+	N	-	-	6
Thymus	NIS	-	+	+	+	+	-	-	+	+	-	-	+	7
	IS	-	-	+	-	-	+	+	-	-	-	-	-	3
Harderian gland	NIS	-	-	-	+	-	N	-	+	+	N	-	-	3
	IS	-	-	-	+	-	-	-	-	-	-	-	-	1
Pancreas	NIS	-	-	-	-	-	N	+	+	-	-	-	N	2
	IS	-	+	+	-	+	+	-	-	-	-	-	-	4
Brain	NIS	-	-	-	+	+	+	-	+	+	-	-	N	5
	IS	-	-	+	-	+	+	+	+	-	-	-	-	5
Sciatic nerve	NIS	-	+	-	-	-	N	-	-	-	N	-	-	1
	IS	-	-	N	-	+	-	-	N	N	-	-	-	1
Kidney	NIS	-	-	+	+	-	N	-	+	-	-	+	-	4
	IS	-	-	-	+	+	+	+	+	-	-	-	-	5
Testis/ovary	NIS	-	-	+	N	+	N	-	N	-	-	+	N	3
	IS	N	+	-	-	-	-	-	-	-	N	-	-	1
Adrenal gland	NIS	-	-	+	+	+	-	-	N	-	-	-	-	3
	IS	-	+	+	-	+	+	-	-	-	+	-	-	5

Table 47 (Continued next page)

Legend: + positive FISH signals; - negative FISH signals; N – organ not examined

Table 47 (Continued)

Bursa of Fabricius	NIS	-	+	+	+	+	+	+	+	-	-	-	-	7
	IS	-	+	+	+	+	+	+	+	+	+	-	N	9
Proventriulus	NIS	-	+	-	-	+	-	-	+	-	-	+	+	5
	IS	-	-	-	-	-	+	-	+	-	-	-	-	2
Duodenum	NIS	-	+	+	-	-	-	+	+	-	-	N	-	4
	IS	-	+	N	-	+	+	+	N	-	-	-	-	4
Midgut	NIS	-	-	-	+	+	+	+	-	-	-	+	N	5
	IS	-	N	+	-	N	+	-	-	-	-	-	-	2
Caecal tonsils	NIS	-	+	+	+	+	-	+	-	-	-	-	-	5
	IS	-	+	+	-	N	-	-	-	+	-	-	-	3
Large intestine	NIS	-	-	-	-	+	+	-	-	-	-	-	-	2
	IS	-	+	-	-	+	-	-	N	-	-	-	-	2
Pruning gland	NIS	-	+	-	-	+	-	N	N	-	N	-	N	2
	IS	-	-	-	-	-	+	-	-	-	+	-	-	2
Pectoral muscle	NIS	-	N	+	+	+	-	N	N	-	N	-	N	3
	IS	N	-	N	N	N	N	+	-	-	N	-	N	1
Head skin	NIS	-	-	-	+	+	-	+	N	-	-	N	N	3
	IS	N	-	N	-	+	-	-	N	N	-	+	N	2
Total	**NIS**[g]	**2**	**13**	**15**	**17**	**19**	**10**	**12**	**13**	**9**	**5**	**8**	**6**	**129**
	IS[g]	**0**	**13**	**12**	**10**	**15**	**16**	**11**	**8**	**7**	**7**	**6**	**2**	**107**

Legend: + positive FISH signals; - negative FISH signals; N – organ not examined

X^2 calculated [g] = 2.051 ($p>0.05$)

FIG.16. TRACHEA (A) AND CAECAL TONSILS (B) FROM NON-IMMUNOSUPPRESSED DUCKS POSITIVE FOR *P. MULTOCIDA* ON FISH TEST (ARROWS).

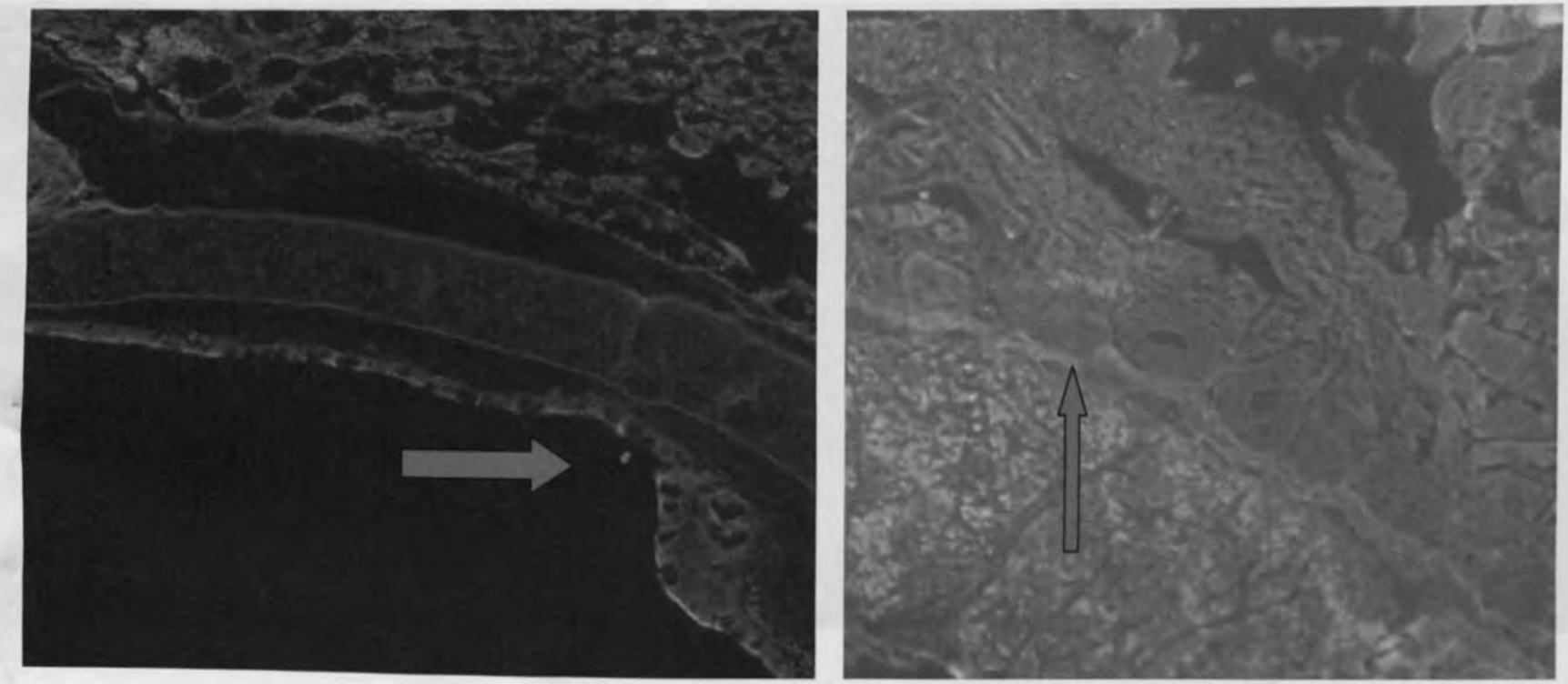
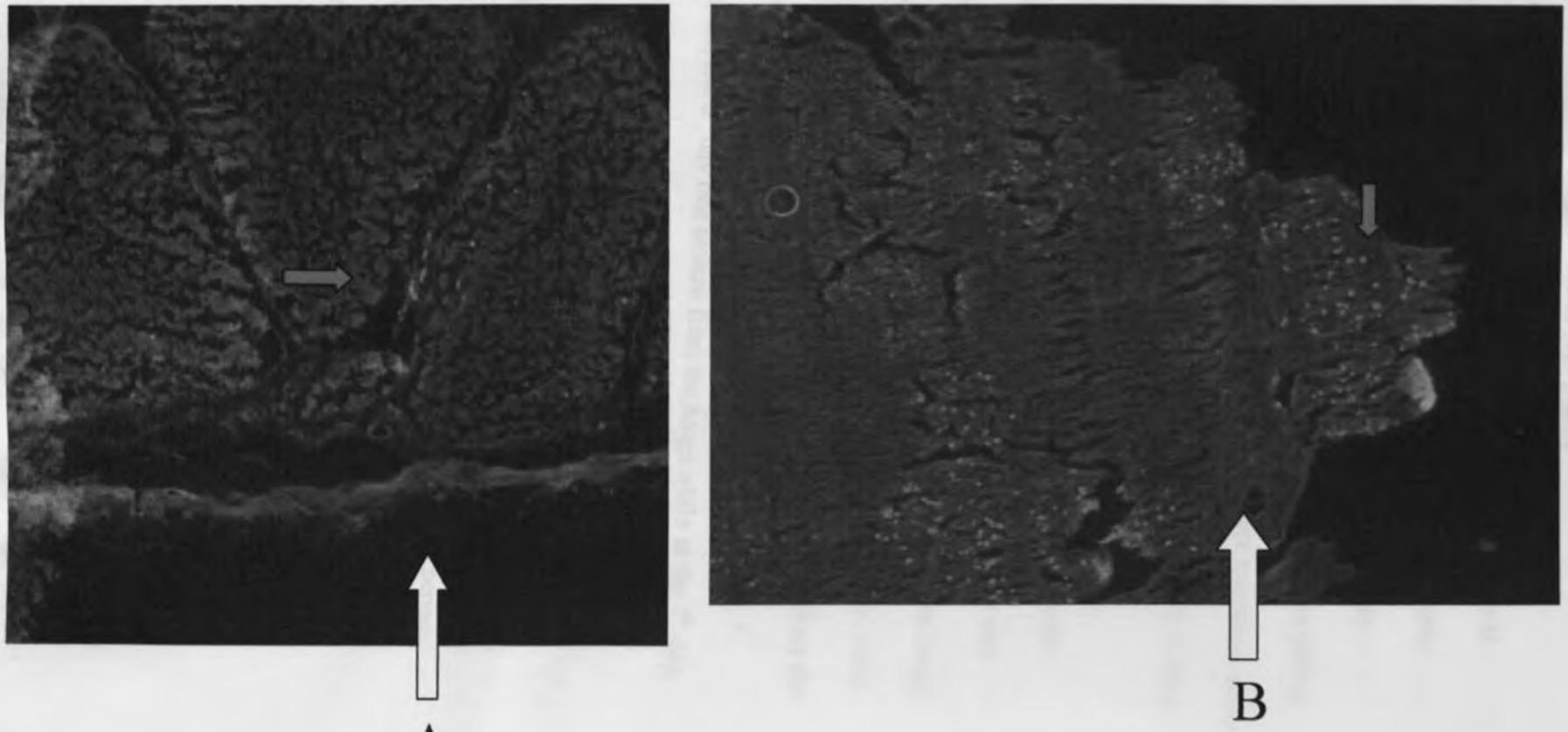


FIG.17. BURSA OF FABRICIUS (A) AND SPLEEN (B) FROM IMMUNOSUPPRESSED DUCKS POSITIVE FOR *P. MULTOCIDA* ON FISH TEST (ARROWS)



A

B

11.3.4 Pathogenesis of P. *multocida* organisms as evaluated through bacterial re-isolation from selected organs

11.3.4.1 *P.multocida* organisms re-isolated from NIS ducks

P. multocida organisms re-isolated from seven organs from NIS ducks at specified periods are presented in Table 48. The organs swabbed were lungs, oro-pharynx, cloaca, caecal tonsils, spleen, liver and the pruning gland. *P. multocida* organisms were recovered from the oro-pharynx (58.3%), lungs (37.5%), spleen (25%), liver (42%), and caecal tonsils (42%). Isolates from the oro-pharynx were recovered up to the 14th day p.i, while those from lungs up to the 5th day, and the spleen, 7th day p.i. Most of *Pasteurella* isolates were recovered within 24 hours p.i.

No isolates were recovered from pruning gland and cloaca. At zero hour (ducks killed 1-5 minutes after inoculation), 2 isolates were recovered from lung and the oro-pharynx. At 1hour p.i, the isolates increased to 7, as they were recovered from the lungs, oro-pharynx, caecal tonsils, and spleen. At 3-6 hr p.i, 3 and 2 isolates, respectively, were recovered from the lungs and spleen, while at 12th hr p.i, 1 isolate was recovered from the spleen. At 24 hrs p.i, 4 isolates from lungs, liver and oro-pharynx and 2-3 days p.i, 1 isolate each from the lungs. On the 5th day one isolate from the lungs while at the 7th day, isolates were cultured from the spleen and oro-pharynx of infected duck.

All organs of the control ducks were negative for *P. multocida.*

11.3.4.2 *P. multocida* organism re-isolated from IS ducks

P.multocica organisms isolated from seven organs of IS ducks at specified periods are presented in Table 48. Same organs as those for NIS ducks were swabbed. *P. multocida* organisms were recovered from the oropharynx (45.8%), lungs (33.3%), and

spleen (16.7%). Isolates from the oropharynx were recovered up to the 4th day while those from other organs were recovered up to the 3rd day post infection. Most *P.multocida* organisms were recovered within 24 hours p.i.

No *P.multocida* isolates were recovered from caecal tonsils, liver, pruning gland and cloaca. At zero hr, 4 isolates were recovered from the lungs and oropharynx. By 1 hour, p.i 5 isolates were recovered from lung, oro-pharynx and spleen, while at the 3rd hour they were isolated from lungs and spleen. At the 6th, 12th, 24th hour and 2nd day p.i, one isolate recovered from oro-pharynx and lungs, respectively. At the 3rd day p.i, there were isolates from lung and oro-pharynx.

All organs of the control ducks were negative for *P. multocida.*

NIS ducks had more isolates than IS ducks but the difference was not significant ($p>0.05$).

Table 48: Re-isolation of *P.multocida* organisms by culture method from organs of non-immunosuppressed (NIS) and immunosuppressed (IS) ducks at specific times

Organs	Ducks treatment	Hours post infection						Days post infection						isolates / organ
		0	1	3	6	12	24	2	3	5	7	10	14	
Lungs	NIS	++	++	++	+	-	+	-	-	+	-	-	-	9
	IS	++	++	+	+	-	+	-	+	-	-	-	-	8
Oropharynx	NIS	++	++	-	-	-	++	+	++	-	++	+	++	14
	IS	++	+	-	-	+	-	+	++	-	+	+	++	11
Caecal tonsils	NIS	-	+	-	-	-	-	-	-	-	-	-	-	1
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Spleen	NIS	-	++	+	+	+	-	-	-	-	+	-	-	6
	IS	-	++	++	-	-	-	-	-	-	-	-	-	4
Liver	NIS	-	-	-	-	-	+	-	-	-	-	-	-	1
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Pruning gland	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Cloaca	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
	IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Total isolates	**NIS[h]**	**4**	**7**	**3**	**2**	**1**	**4**	**1**	**2**	**1**	**3**	**1**	**2**	**31**
	IS[h]	**4**	**5**	**3**	**1**	**1**	**1**	**1**	**3**	**0**	**1**	**1**	**2**	**23**

Legend: + positive isolation of *P.multocida;* - negative isolation of *P.multocida*

X^2 calculated[h] = 1.185 ($p>0.05$)

11.3.5 Comparison between the four tests used in the NIS and IS ducks in the *P. multocida* pathogenesis study

11.3.5.1 Among the NIS ducks

Comparison of the results of the four tests undertaken on a few NIS duck organs, where all tests were carried out at specified periods are presented in Table 49. FISH test results compared very well with gross and histopathology and bacterial isolation up to day 3 p.i. There after the FISH test had same results as gross and histological lesions for the lungs and spleen but it was able to detect the bacteria better in the liver, caecal tonsils, and other organs than the bacterial culture and other tests. Most organs had higher frequency of *P. multocida* detection with FISH test than expression of gross, histopathological lesions and isolation by culture. At zero hour only FISH and bacterial culture could detect *P. multocida.*

11.3.5.2 Among the IS ducks

Comparison of the results of the four tests undertaken on a few IS duck organs, at specified periods are presented in table 49. FISH test results compared well with those of gross and histopathology. Detection of P. *multocida* FISH signals and culture method were comparable up to 24 hrs p.i, thereafter, the FISH test was found to detect the bacteria better in the lungs, trachea (oro-pharynx), spleen, liver, caecal tonsils and large intestines (cloaca). Whereas, *P.multocida* FISH signals were found in the large intestines, and pruning gland, no bacteria were isolated by culture from the cloaca swabs and the pruning gland.

NIS ducks had more positive results in all tests than the IS birds but the difference was not significant ($p>0.05$).

Table 49: Comparison between the gross and histopathological lesions, bacterial re-isolation and FISH signals from organs of non-immunosuppressed (NIS) and immunosuppressed (IS) ducks

Organs	Duck treatment		Hours post infection						Days post infection						Positive results
			0	1	3	6	12	24	2	3	5	7	10	14	
Lung	Gross	NIS	-	+	+	+	+	+	+	+	+	+	+	+	11
		IS	-	+	+	+	+	+	+	+	+	+	+	-	10
	Histo	NIS	-	+	+	+	+	+	+	+	+	+	+	+	11
		IS	-	+	+	+	+	+	+	+	+	+	+	-	10
	FISH	NIS	+	+	+	+	+	+	+		+	+	+	+	12
		IS	-	+	+	+	+	+	+		+	+	+	+	11
	B/isola	NIS	+	+	+	+	-	+	-	-	+	-	-	-	6
		IS	+	+	+	+	-	+	-	+	-	-	-	-	6
Trachea	Gross	NIS	-	+	+	+	+	+	-	-	-	-	-	-	5
		IS	-	-	+	+	+	+	-	-	-	-	-	-	4
	Histo	NIS	-	+	+	+	+	+	+	+	-	-	-	-	7
		IS	-	-	+	+	+	+	+	-	-	-	-	-	5
	FISH	NIS	+	+	+	+	+	+	+	+	+	+	+	+	12
		IS	-	+	+	+	+	+	+	+	+	+	+	+	11
	B/isola	NIS	+	-	-	-	-	+	+	+	-	+	+	+	7
		IS	+	+	-	-	-	-	+	+	-	+	+	+	7
Spleen	Gross	NIS	-	+	+	+	+	+	+	-	-	-	-	-	6
		IS	-	-	+	+	+	+	+	-	-	-	-	-	5
	Histo	NIS	-	-	+	+	+	+	-	+	+	+	+	-	8
		IS	-	-	-	-	+	+	+	-	-	-	-	-	3
	FISH	NIS	-	+	+	+	+	+	+	+	+	+	+	+	11
		IS	-	+	-	+	+	+	+	-	-	+	-	-	6
	B/isola	NIS	-	+	+	+	+	+	-	-	-	+	-	-	6
		IS	-	+	+	-	-	-	-	-	-	-	-	-	2
Liver	Gross	NIS	-	+	+	+	+	+	+	-	+	-	-	-	7
		IS	-	-	+	+	+	+	+	+	+	+	-	-	8
	Histo	NIS	-	+	+	+	+	+	+	+	+	-	+	-	9
		IS	-	+	+	+	+	+	+	+	+	+	-	-	9
	FISH	NIS	-	+	+	+	+	+	+	+	+	+	-	+	10
		IS	-	+	+	+	+	+	+	+	+	+	+	-	10
	B/isola	NIS	-	-	-	-	-	+	-	-	-	-	-	-	1
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0

Table 49 (Continued next page)

Legend: Gross – gross lesions; Histo – histological lesions; FISH - fluorescent *in situ* hybridization signals; B/isola – *P. multocida* isolation by culture; + - positive result; - negative result; N – not done

Table 49 (Continued)

Caecal tonsils	Gross	NIS	-	-	+	+	+	+	-	-	-	-	-	-	4
		IS	-	-	+	+	+	+	-	-	-	-	-	-	4
	Histo	NIS	-	-	+	-	+	-	-	-	-	-	-	-	2
		IS	-	-	-	-	+	+	-	-	-	-	-	-	2
	FISH	NIS	-	+	+	+	+	-	+	+	-	-	-	-	5
		IS	-	+	+	-	N	-	-	-	+	-	-	-	3
	B/isola	NIS	-	+	-	-	-	-	-	-	-	-	-	-	1
		IS	-	-	-	-	+	-	-	-	-	-	-	-	0
Pruning gland	Gross	NIS	-	-	+	+	+	+	+	-	-	-	-	-	5
		IS	-	-	-	-	-	-	-	-	-	-	-	-	3
	Histo	NIS	-	-	+	-	-	-	-	N	-	-	-	-	1
		IS	-	-	-	-	-	-	-	-	-	-	-	-	2
	FISH	NIS	-	-	-	-	+	+	-	-	-	-	-	-	2
		IS	-	-	-	-	-	+	-	-	-	+	-	-	2
	B/isola	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Large intestine	Gross	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
		IS	-	-	-	+	+	+	-	-	-	-	-	-	0
	Histo	NIS	-	-	-	+	-	-	-	N	-	-	-	-	1
		IS	-	-	-	-	+	+	-	-	-	-	-	-	0
	FISH	NIS	-	+	-	-	+	-	N	N	-	N	-	N	2
		IS	-	+	-	-	+	-	-	N	-	-	-	-	2
	B/isola	NIS	-	-	-	-	-	-	-	-	-	-	-	-	0
		IS	-	-	-	-	-	-	-	-	-	-	-	-	0
Total	Gross [a]	NIS	0	4	6	6	6	6	4	1	2	1	1	1	38
		IS	0	1	6	5	6	6	3	2	2	2	1	0	24
	Histo [a]	NIS	0	3	6	5	5	4	3	4	3	2	3	1	39
		IS	0	2	3	3	6	6	4	2	2	2	1	0	31
	FISH [a]	NIS	2	6	5	5	7	5	5	4	5	4	3	3	54
		IS	0	6	4	4	5	5	4	2	4	4	3	2	44
	B/isol [a]	NIS	2	4	2	2	1	4	1	1	1	2	1	1	22
		IS	2	3	2	1	1	1	1	2	0	1	1	1	16

Legend: Gross – gross lesions; Histo – histological lesions; FISH - fluorescent *in situ* hybridization signals; B/isol – *P. multocida* isolation by culture; + - positive result; - negative result; N – not done

X^2 calculated [a] = 0.6687 ($p>0.05$)

11.4 Discussion

The *P.multocida* strain NCTC 10322^T (Mutters *et al.*, 1985b) used in this study, caused clinical signs, pathology but no mortality in indigenous ducks. The gross and histopathological lesions observed in this study were similar to those previously reported by Hunter and Wobeser (1980) and Glisson *et al.* (2003) for intermediate to chronic fowl cholera. The lesions were further supported by the findings of FISH test using a *P.multocida* specific probe on the same organs and the bacteria were re-isolated by culture from selected organs. Furthermore no other disease conditions were detected by histopathologic or bacteriological examination in infected and the control ducks. Less virulent isolate of *P.multocida,* as used in this study produce chronic infection in waterfowl (Hunter and Wobeser, 1980; Pehlivanoglu *et al.*, 1999).

The lungs, airsacs, liver, heart, spleen, conjunctiva, pectoral muscle, trachea, kidney, bursal of Fabricius, duodenum and large intestine were frequently involved in that order for NIS and IS ducks. However, the NIS ducks were more affected than IS ducks in terms of number of infected organs, severity of the lesions and re-currency of lesion or infection in organs at various intervals. The high frequency of lesions in the lungs and liver than other organs, has also been reported by Rhoades and Rimler (1989) for fowl cholera.

The strain of *P.multocida* and route of inoculation may affect the pathogenicity (Pehlivanoglu *et al.*, 1999) and pathogenesis of *P.multocida* (Hunter and Wobeser, 1980). In their detailed work on pathology in Mallard ducks, Hunter and Wobeser (1980) gave an account on the lesions for acute and chronic fowl cholera as seen on lungs, heart, spleen, liver, intestines, airsacs, eye, joint, serous membranes, brain, kidneys and peri-

bronchiolar lymphoid aggregates in the lungs from 12 hrs p.i up to 5 days p.i. In the current studies we have further observed lesions in the thymus, Harderian gland, pancreas, sciatic nerve, testis or ovary, adrenal gland, bursa of Fabricius, pruning gland, and the pectoral muscle immediately after inoculation and up to 14 days post inoculation. These organs had mild to moderate lesions varying from congestion, haemorrhages, oedema, necrosis, fibrinopurulent exudate, myositis, bursitis, nerve myelitis to fibrosis in the 2nd week of infection. They were infected as early as 1hour post inoculation and lesions were recorded up to the 7th day in some ducks. Events immediately after inoculation up to 14 days post inoculation are similar to those described by Aye *et al.* (2000) in turkeys, who reached 14 days p.i and gave a detail of the gastrointestinal, respiratory, kidney, brain, spleen, bursa of Fabricius lesions. Unlike the turkey experiments events after immediate inoculation and one hour and the 10th day p.i are also reported. Whereas we have not compared the different *P.multocida* strains as done by others (Hunter and Wobeser, 1980; Pehlivanoglu *et al.*, 1999; Aye *et al.*, 2000), the study compares the events in normal and immunosuppressed village ducks. The lesions have also been correlated to the bacterial culture re-isolation from infected birds at various intervals when the infection is active and further compared with the *P.multocida* organisms detected using the species specific probe in the fluorescent *in situ* hybridization test (Mbuthia *et al.*, 2001). The severity of infection in various organs and lesions are also described.

Organs with gross and histopathological lesions at various sacrificial intervals were lungs, airsacs, liver (NIS and IS), heart, spleen, conjunctiva, and pectoral muscle for NIS ducks. The lesions were observed as early as the 1st hour p.i for NIS and 3rd hour p.i for

IS, but peaking at 24 hrs p.i, for the two treatments. The main lesions were pneumonia, airsacculitis, fibrin exudation, pleuritis, congestion, myocarditis, hepatitis, suppurative exudation, fibrinopurulent, hydropericardium, sinusitis, peritonitis, splenitis and myositis for both NIS and IS ducks. *P.multocida* organisms were frequently observed on the lungs, trachea, spleen, liver, heart, airsacs, thymus and bursa of Fabricius, in NIS and IS ducks at various sacrificial intervals, using the FISH test. This correlated closely with the lesions observed previously on pathology and the *P.multocida* re-isolation from the oro-pharynx for the whole period, but up to the 3rd (NIS) and 5th (IS) day for lung isolates and earlier than the 12th hour for isolates from the spleen. Cultural method of isolation of the *P.multocida* was found inferior to the FISH method in the detection of this bacterium especially after 24 hrs p.i. Compared to the pathological lesions, the FISH test was comparable up to the 10th day for NIS birds and up to the 7th day p.i, for IS birds; thereafter the FISH test detected the organisms better. While as the pathological lesions are not always specific for *P.multocida*, the signals observed with FISH test refers to this bacteria. FISH test has been used to detect various aetiological agents (McNicol and Farquharson, 1997; Moter and Göbel, 2000) and as a tool in pathological diagnosis (Brown, 1998; Bojesen *et al.*, 2003), and was developed in these studies for *P.multocida.* Earlier studies showed its use in detecting *P.multocida* in lung tissues (Mbuthia *et al.*, 2001) but this study shows that it can pick this bacterium from all the 24 organs of ducks. It is therefore a useful diagnostic tool for *P.multocida*.

The FISH test proved to be an adequate and accurate diagnostic microbiology tool for detection of the bacteria in duck tissues (Moter and Göbel, 2000). The bacterium was detected immediately after inoculation (1-5 minutes) in the tracheal and lung bronchial

epithelium, and as late as 14 days p.i. Studies with turkey and raptors have shown that bacteria multiply extensively in trachea and lungs within 2 hours p.i, and almost instantaneously reach the internal organs via blood by some unknown mechanism (Matsumoto *et al.*, 1991; Morishita *et al.*, 1997). This may not be unique to these birds alone, as pathological lesions, and *P.multocida* were identified in many tissues as early as one hour p.i, in this study. There is a need therefore to investigate the bacterial events within 1hour post inoculation, using the FISH test. Most bacteria were isolated by culture immediately after inoculation and thereafter.

P.multocida was found in various organs of normal and immunosuppressed ducks. It is possible, therefore, to excrete it from various secretions, and excretions of these ducks, hence the isolation of *P.multocida* from the gastrointestinal tract and cloaca as reported elsewhere (Lee *et al.*, 2000; Muhairwa *et al.*, 2001b; and in experiment 1, Chapter 3 of this thesis).

The *P.multocida* used was of low virulence as demonstrated by mild nature of the lesions, almost 65% of all the cases, 20% moderate and only 15% severe in both NIS and IS ducks. Most gross and histopathological lesions occurred between 1hr and 3 days p.i, for NIS and 3rd hour to 2nd day for IS ducks. This strain of *P.multocida* appears to provoke cellular and fibrinous exudate that is non-specific cellular defense in the ducks (Toth, 2000; Qureshi *et al.*, 2000). The inflammation appears to be modulated by immunosuppression, like in studies of *Escherichia coli* (Gross, 1976) lowering the inflammatory reaction as well as delaying it and making the bird to recover faster than in NIS ducks.

In conclusion, the pathogenesis of *P.multocida* (NCTC 10322^T) has been documented in village ducks, where it provokes mild to moderate lesions in tissues for 2 days or more p.i, and where the organisms appear to be overwhelmed by the host defense mechanisms, thereafter. FISH test proved to be a useful tool in detecting this strain in tissues with or without inflammatory lesions and in normal and immunosuppressed ducks. The NIS ducks had more severe reaction to this *P.multocida* than IS ducks, which may indicate that immunosuppression modulates the *P.multocida* infections in ducks.

CHAPTER 12

12.0 GENERAL DISCUSSION AND CONCLUSION

12.1 Discussion

Healthy-looking farmed, slaughter and market indigenous chickens and ducks were screened for the presence of *P.multocida*, from swabs collected from oropharynx and cloaca, using the mice passage and culture methods as described by others (Mutters *et al.*, 1989; Muhairwa *et al.*, 2001a,b). Of the 216 birds sampled (162 chickens and 54 ducks), 24 (11.11%) yielded *Pasteurella* organisms; 10 (4.63%) from chickens and 14 (6.48%) from ducks. Most of the isolates were *P.m.gallicida,* followed by *P.m.multocida,* and *P.m.septica* and mainly from oropharyngeal swabs. All the three subspecies of *P.multocida* were recovered from indigenous chickens and ducks that were reared in peri-urban villages, marketed alive, or being slaughtered in various trading centres in Nairobi. Previous investigations of occurrence of *P.multocida* were done on farm chickens and ducks but not on traded (slaughter and market) birds (Curtis and Ollerhead, 1981; Muhairwa *et al.*, 2000 and 2001b). Such a study has not been carried out previously in Kenya.

Slaughter and market birds in this study originated from rural and urban districts. *P.multocida* organisms were recovered from birds that originated from Kiambu, Kisii, Machakos, Makueni, and Nairobi districts. The study agrees with the findings of Muhairwa *et al.* (2001b) that ducks yielded more isolates of the organisms than chickens. It is possible that ducks are better reservoirs of *P. multocida* organisms than chickens. Previous reports of *P. multocida* and fowl cholera in Kenya were in wild birds (Hudson, 1959; Miringa, 1975) and our study is the first on indigenous chickens and ducks. This

study confirms that *P. multocida* organisms are present among the indigenous flocks that are of cultural, social, and economic importance to the rural and peri-urban communities in Kenya. An apparently healthy *P. multocida* carrier bird in form of a gift can easily introduce and maintain *P. multocida* on the respective farms and pose a risk to both indigenous and commercial flocks. A nation wide epidemiological survey can show the extent of *P.multocida* of occurrence and distribution, and the possible risk of fowl cholera to the poultry industry.

Having recorded the occurrence of carriers of *P. multocida* among the indigenous flocks in Kenya, it was prudent to investigate the most susceptible age group among birds, to *P. multocida*. *P.multocida* has a wide range of avian hosts, with variable degrees of susceptibility among different types of birds and age groups (Faddoul *et al.*, 1967; Rhoades and Rimler, 1989). To determine the most susceptible age for chickens and ducks, the birds were grouped into four sets 4,8,12 and 16 week-old birds and experimentally inoculated with a known *P.multocida* strain NCTC 10322^{T} intratracheally as previously done (Matsumoto *et al.*, 1991; Petersen *et al.*, 2001b). For both experimental and control birds clinical signs, temperatures, and weights were monitored for 2 weeks. This *P.multocida* strain was found to be of low virulence with birds expressing various clinical signs, fever, and weight changes but no mortality. In the chicken experiment, the 12 week-old indigenous chickens showed more severe clinical signs that reached a peak within a few days post inoculation than those of the other age groups that is 4, 8, and 16 week-old birds. The clinical signs were similar to those reported by others (Christensen and Bisgaard, 2000; Glisson *et al.*, 2003). Previously, it has been reported that mature chickens were more susceptible than young ones

(Heddleston 1962; Heddleston and Watko, 1965) to *P.multocida* organism. This could be true when comparison is made between young birds (9 -30 weeks) and mature ones (52 weeks). However, among the grower chickens of between 4 –16 week-old, the 12 week-old chickens appear more susceptible than the other age groups. This compares well with previous works that have shown that the onset of fowl cholera in turkeys was 12 weeks, in Georgia, U.S.A. (Morris *et al.*, 1989). In the current study, none of the control chickens had clinical signs or fever through out the 14 days of observations.

All the infected indigenous chickens had lower weight gain than the control birds and hence *P. multocida* was influencing the weight gains. The findings agree with those of Morris *et al.* (1989) that one of the effects of fowl cholera is decrease in feed efficiency among turkeys. From the foregoing results, it can rightfully, be assumed, that the 12 week-old indigenous chickens are more susceptible to *P.multocida* than the other grower chicken age groups.

In the duck experiment, a similar picture was observed, 8 week-old ducks had more severe signs than those of 4, 12, and 16 weeks old. This is in agreement with the work of Hunter and Wobeser (1980) that young Mallard ducks less than 11 weeks old were more susceptible than older ducks of 16 to 18 weeks old. None of the control ducks had clinical manifestations of fowl cholera.

Infected ducks had lower weight gains than the control birds, although older birds lost marginally more than the younger birds; a possibility therefore that infection with *P.multocida* decreased feed efficiency (Morris *et al.*, 1989). Infected wild birds have been reported to have below normal weight (Faddoul *et al.*, 1967), a finding confirmed in these

studies. The afore-named parameters supported the conclusion that 8 week-old indigenous ducks were more susceptible than other grower age groups.

Chicken and duck susceptibility studies confirm previous reports that different types of birds and different age groups within a type vary in their susceptibility to the same strain of *P.multocida* organisms (Rhoades and Rimler, 1989). As a result the 12 week-old chickens and the 8 week-old indigenous ducks appeared more susceptible to *P. multocida* than other age groups for these indigenous birds.

At farm level, marketing points and during transportation of birds to and from the markets, chickens and ducks are subjected to various stresses such as rough handling, stressful transportation methods, congestion in cages and coops, poor feeding, housing, parasitic infestations, subclinical infections, and threat to predators, all of which may induce causing flare-up of infection. Stress can precipitate outbreaks of fowl cholera at farm level and in market areas (Blackall *et al.*, 1995). With the determination of the susceptible age to *P.multocida* organisms among the indigenous birds, it was necessary to document the clinical picture of fowl cholera in these birds, and observe whether immunosupression, one of the effects of stress has any effect on their manifestation. Same strain of *P.multocida* was used and inoculation and observations were as previously done for age susceptibility. The birds were grouped and separated into non-immunosuppressed and immunosuppressed with their respective controls. Immunosuppression was carried out as done by other authors (Corrier and DeLoach, 1990; Corrier *et al.*, 1991; Nakamura *et al.*, 1994). Chickens and ducks of the susceptible age groups were used. Grossly and histologically the lymphoid organs of IS birds were atrophied and depleted of the lymphoid cells an indication that dexamethasone had an

effect. The birds expressed signs intermediate between those of subacute and chronic fowl cholera as described by others (Rimler *et al.*, 1998; Christensen and Bisgaard, 2000; Glisson *et al.*, 2003) such as depression, ruffled feathers, fever, nasal discharges, sneezing, dyspnoea, diarrhoea, spontaneous tracheal rales, mucous discharges from the mouth, nose and eyes, cyanosis, and anorexia. Nervous tics, ataxia, and head scratching were additional clinical signs that were expressed by both IS and NIS birds. Torticollis from meningeal infection in chronic fowl cholera (Glisson *et al.*, 2003) was not observed. The nervous tics could be a reaction to mild or severe laryngitis, or an effort to clear the nostrils and the upper respiratory tract, or may be a sign of meningitis or meningo-encephalitis. Ataxia was observed as wobbling movements (staggering, unsteady gaits shivering, flipping ever) that could be a central nervous disturbance, while head scratching was common in chickens but not ducks. This could be a further sign of irritation of the head region (otitis and or sinusitis).

Stress in poultry has been shown to increased nonspecific resistance to some bacteria (*Escherichia coli*) resorting in less disease manifestations unlike in viral and *Mycoplasma* species infections (Gross and Siegel, 1965; Gross and Colmano, 1967,1969, 1970; Freeman, 1976). Stress lowers the total leukocyte counts (leukopenia), impair immunological functions, reduce bird's appetite and weight of some lymphoid organs (Siegel and Gross, 1965; Freeman, 1971). In this study *Pasteurella multocida* infections appear to have a similar effect in chickens and ducks as *Escherichia coli*. NIS birds had more severe clinical signs than the IS birds. Up to 5 different clinical signs were observed on a single bird during the observation period. On post mortem IS birds appeared qualitatively to have more fat deposition than NIS birds. The main post mortem findings

were caseated fibrino-suppurative exudates, fibrosis on lungs and airsacs. Chickens expressed more clinical signs than ducks. However, dyspnoea was more marked in ducks than in chickens.

In these studies, the carrier status, most susceptible grower bird age group and clinical picture in normal and immunosuppressed birds infected with *P.multocida* have been established. However, small-scale farmers often keep mixed flocks with indigenous chickens and ducks (Minga *et al.*, 1989; Nyaga *et al.*, 2002; Mbuthia *et al.*, 2003) under the same management. This type of management can easily facilitate cross transmission of common pathogens. To evaluate this, 2 experiments were conducted where indigenous chickens were infected and mixed with naïve ducks and another where ducks were infected and mixed with naïve chickens. The bacterial inoculations were as previously done (in this thesis) and infected and sentinel birds were monitored for the presence of *P.multocida* by swabbing of the oropharynx and the cloaca on a daily basis for 14 days. At the same time some sentinel birds were sacrificed at various intervals after mixing with the infected ones.

In the case of chicken to duck transmission, 40% of sentinel susceptible indigenous ducks had picked *P.multocida* after 24 hrs, and 80% had the organism by the 2nd day, while all had the infection on the 6th day post inoculation. For the duck to chicken transmission, 80% of the sentinel susceptible indigenous chickens had picked *P.multocida* after 24 hrs, while all the chickens had the organisms by the 2nd day post inoculation. The direct contact through birds pecking on the bills of each other (chicken to chicken; chicken to ducks; duck to duck) and indirect contact through exposure to water and feed contaminated by infected birds, successfully transmitted infection

between the different species of birds (Rhoades and Rimler, 1989; Gooderham, 1999). Besides, the infected excretions from the head regions, infected faecal materials may also play a role in cross transmission of the organisms; as *P.multocida* have been isolated from cloacal swabs (Muhairwa *et al.*, 2000 and 2001b; Chapter 3, Experiment 1 of this thesis). Elsewhere, it has been reported that poultry strain acquired by or inoculated into wildlife have also been shown to be transmitted back to poultry (Snipes *et al.*, 1988). It can, therefore, be concluded that *P.multocida* NCTC 10322^T can be transmitted between the indigenous chickens and ducks. All birds infected by cross transmission expressed clinical signs (Chapter 6 of this thesis) and lesions as reported elsewhere (Hunter and Wobeser, 1980; Glisson *et al.*, 2003). The contact cross transmission, may play a role in the maintenance of *P.multocida* at village level, especially due to the lack of all-in all-out management systems for the scavenging village birds.

Diagnosis of fowl cholera in sick birds is dependent on cultural methods and subsequent characterization of *P.multocida* (Christensen and Bisgaard, 2000). This is limited by presence of V- factor requirement, non-typable strains, and viable but non-culturable cells (Krause *et al.*, 1987; Moter and Göbel, 2000). There is a need to develop a culture-independent test for precise localization of *P.multocida* in culture and within histological sections (Brown, 1998). Fluorescent *in situ* hybridization is such a test, and it had not been developed for *P.multocida.* A specific oligonucleotide probe pmhyb 449 (Alm et al., 1996) for specific detection of *P.multocida* was selected based on 16S rRNA sequence comparison. The probe was later optimized for hybridization conditions with fixed bacterial strains. The optimized probe was further tested, initially with pig lung injected with *P.multocida* to establish whether it can detect the organism in tissues and

later in lung tissues from chicken with fowl cholera (Christensen *et al.*, 1998). In both cases, the probe emitted specific and narrow fluorescence that distinguish *P. multocida* bacterial cells by a red fluorescence, while the lung tissue cells fluoresced greenish to brownish and red blood cells were yellow on examination after *in situ* hybridization. The probe was later tested against twenty two fixed bacterial strains and it gave good signals for *P. multocida* comparable to probe EUB338, while its complementary probe did not give any signals with the same bacterial strain. All other bacteria tested, except *P. avium* biovar 2 and *P. canis* biovar 2 were negative with probe Pmhyb449. These two taxa that were described by Madsen *et al* (1985) and subsequently named by Mutters *et al* (1985 a,b) clustered with the type strain of *P. multocida* and not with *Pasteurella avium* (Dewhirst *et al.*, 1993) or *P. canis*. Their similarity to *P. multocida* are higher than 94-98.6%, and possibly in future they may be placed together with *P. multocida* (Petersen *et al.*, 2001a).

In this way, the FISH test allowed simultaneous visualization, identification and localization of *P. multocida* cells in the lung tissues (Moter and Göbel, 2000). The test was superior to culture-based methods as it was less time consuming and can be used for fastidious, yet to be cultured bacteria and could pick out the specific target microorganism in a mixture of bacterial communities or microbial diversity in infections. Whileas other microbial staining methods, molecular techniques like PCR (Polymerized chain reaction) are good, but they do not provide information about morphology, number, spatial distribution or the cellular environment of the organisms (Moter and Göbel, 2000). The 16S rRNA based methods are advantageous in detection and identification of microorganisms due to the fact that each bacterial cell contains multiple copies of the 16S

rRNA in its ribosomes, that eases its detection, with evolutionary highly conserved 16S rRNA regions common to bacteria and other regions which might be species specific (Delong *et al.*, 1989; Krimmer *et al.*, 1999). The amount of bound probe is directly correlated to the cellular rRNA content, which is dependent on physiological activity at the time of fixation (Giovannoni *et al.*, 1988; Amann *et al.*, 1991), and directly correlates with the bacterial growth rate (Delong *et al.*, 1989). Using rRNA as a target in FISH test, phenotypic variations do not pose a problem, unlike immunological methods (Boye *et al.*, 2000). Probe Pmhyb449 clearly demonstrated the *P. multocida* bacterial rods in various chicken lungs under mild, moderate or severe lung inflammation. The bacteria occurred singly, in pairs, in aggregates like micro-colonies and in masses occupying large areas of the lungs. Their individual morphology was clearly demonstrated by this probe as reported in FISH procedures of other bacteria (Delong *et al.*, 1989; Amann *et al.*, 1991; Bojesen *et al.*, 2003). The test limitations are its dependence on the number of copies of rRNA in the target bacterial cell (Amman *et al.*, 1995) and hence on the physiological activity of the microorganisms prior to fixation of samples. Autolysis of the tissues does not hinder the detection of bacteria by FISH (Nuovo, 1997) nor the inflammatory reaction due to *P. multocida*. In this study, probe pmbyb 449 was developed and is recommended for studies of *P. multocida* pathogenesis and its infection in animals. It can also be used to confirm and differentiate *P. multocida* from other *Pasteurellaceae* in culture. It is a simple test applicable in most research and diagnostic laboratories.

To add more insight on *P. multocida* infections it is worthwhile to understand some aspects of the spread of this bacterium in various tissues of indigenous chickens and ducks that are immunosuppressed and non-immunosuppressed. The type and severity of

the lesions and location of the bacteria in organs at various intervals in NIS and IS indigenous birds was undertaken to help understand the pathology and pathogenesis of *P. multocida* using a less virulent strain of the bacteria. To achieve this, the indigenous chickens and ducks of the susceptible age groups were inoculated with *P. multocida* and sacrificed at specified intervals. Immonusuppression was done as previously described (Birrenkott and Wiggins, 1984; Corrier and DeLoach, 1990; Corrier *et al.*, 1991; Nakamura *et al.*, 1994). Thorough post mortem examination was undertaken, 24 tissues taken for histopathological examination and FISH test and *P. multocida* bacterial re-isolation done from swabs taken from selected 7 organs. The gross and histopathological picture for the indigenous chickens and ducks were as described by others (Hunter and Wobeser, 1980; Rhoades and Rimler, 1989; Pehlivanoglu *et al.*, 1999; Glisson *et al.*, 2003). The NIS were more affected than IS birds in terms of number of infected organs, severity of the lesions and re-currency of lesions or infection in organs at various intervals. Although in some cases these differences were not statistically significant.

More organs were included in this study than examined by Hunter and Wobeser (1980) for ducks, while they were similar to those given by Rhoades (1964), although the latter used a highly virulent *P. multocida* that gave microscopic lesions of acute fowl cholera and cannot be compared with the findings of this study.

In the chicken experiments, grossly, the lungs, airsacs, trachea, spleen, liver and caecal tonsils had lesions most of the time in that order. Those of NIS birds were marginally more affected (95.8%), than IS chickens (91.7%). Lesions were observed in the gastrointestinal tract, a possibility that *P. multocida* can be shed through it as suggested elsewhere (Lee *et al.*, 2000; Muhairwa *et al.*, 2001b and Chapter 3 of this

thesis). Lesions were present in most organs by the 6th hour in NIS and 12th hour for IS chickens.

The picture was similar for histopathological lesions, all organs in NIS having a lesion while 83.3% of IS organs had lesions. Severest lesions were in respiratory system, cardiovascular, hepatic system, lymphoid system, gastro-intestinal tract, nervous, and urinary systems as reported elsewhere (Rhoades and Rimler, 1989). Peak lesions were observed at 6th hour in NIS and 12th hour for IS and most of them were recorded in the first week of observation. Previous studies with turkeys have shown that the *P. multocida* is able to instantaneously reach the internal organs via blood by some unknown mechanism (Matsumoto *et al.*, 1991). This was confirmed in this study, lesions being observed as early as 1hour post inoculation and the *P. multocida* organisms were demonstrated immediately (1-5 minutes) after inoculation on the epithelium of the trachea and lung bronchi. A number of organs had the bacteria by the 1st hour post inoculation. A similar picture to the distribution of lesions was observed when FISH test was performed. It was easier to demonstrate *P. multocida* organism in the tissue of NIS than IS chickens. The occurrence of the bacteria was more in the lungs, trachea, spleen, airsacs, bursa of Fabricius and thymus in NIS and IS using FISH test other organs. FISH test was successfully used to demonstrate *P. multocida* in all tissues of the chicken examined. On recovery of *P. multocida* from tissues, most bacterial isolations were done at the time of peak lesions and in the first week. NIS birds had slightly more *P. multocida* isolates from them than IS birds. The picture in ducks is similar to that of chickens except that the lesions were intensive and spread for a longer period in ducks than in chickens. *P. multocida* organisms appear to provoke cellular and fibrinosuppurative exudates after

non-specific cellular defense by the host duck (Toth, 2000). In both the ducks and chickens the inflammation appear to be modulated by immunosuppression, lowering the inflammatory reaction as well as delaying it, making the bird to recover faster than in NIS ducks. Severity of the lesions in various organs and at specified time intervals have been documented in both NIS and IS birds. While severe lesions were earlier seen in NIS they disappeared early in IS birds.

FISH test correlated well with gross and histopathological lesions and with bacterial recovery by culture (up to 24 hours p.i). After the 2nd day post inoculation FISH test was observed to be more sensitive and hence superior to the other tests in NIS and IS birds. Although FISH test is specific for *P.multocida*, lesions alone are not specific as other aetiologies can provoke similar pathology (Glisson, 2003). This study has demonstrated for the first time the distribution of *P. multocida* in various organs of indigenous chickens and ducks at various intervals.

12.2 Conclusions and Scope for further work

12.2.1 Conclusions

In this study the following were achieved:

1. For the first time, the carrier-status of *P. multocida* (involving all the three *P. multocida* subspecies) in the local scavenging indigenous chickens and ducks.
2. The susceptible age for *P. multocida* and its clinical manifestations among the grower indigenous chickens and ducks.
3. *Pasteurella multocida* organisms can easily be transmitted from infected to non-infected birds; and ducks were more susceptible than chickens and seem to act as the natural reservoirs.

4. A 16S rRNA oligonucleotide probe for *P. multocida* organisms was developed and used successfully, for the first time.
5. The pathogenesis of *P. multocida* infection in immunosuppressed and normal indigenous chickens and ducks was established; that lesions were more in the non-immunosuppressed birds.
6. A species-specific FISH probe was developed as diagnostic tool for *P. multocida* detection in cultures and tissues and was able to detect the bacterium in 24 organs of indigenous chickens or ducks.
7. That FISH test was a better tool in detecting *P. multocida* organisms than the other diagnostic tests evaluated.
8. Immunosuppression modulates clinical signs and lesion expression in *P. multocida* infections.

12.2.2 Scope for further work

1. Country wide *P.multocida* studies are needed to establish the distribution of this organism in indigenous birds and commercial poultry.
2. Study the socio-economic impact of fowl cholera and *P.multocida* infections in indigenous poultry farming establishments.
3. Further studies on cross transmission involving other avian species under the smallholder poultry farms.
4. Test the virulence of the Kenyan *P.multocida* isolates.
5. Establish the role of various phagocytes in the inflammation of *P.multocida* and expression of fowl cholera clinical signs and pathological lesions.

CHAPTER 13

13.0 REFERENCES

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CHAPTER 14

14.0 APPENDICES

Appendix 1: *Pasteurella multocida* recovery from swabs of healthy-appearing farm birds: their flock size, type of bird, division and districts of origin in Kenya

District	Division	Type of bird	Farm code	Flock size	Total birds sampled	*P. multocida* isolates	
						C	P
Nairobi	Embakasi	Chicken	1	31	6	0/6	0/6
		"	2	20	6	0/6	0/6
		"	3	36	6	0/6	0/6
		"	4	52	6	0/6	0/6
Machakos	Athi River	"	5	25	12	0/12	0/12
		"	6	56	11	0/11	0/11
		"	7	74	6	0/6	0/6
Kiambu	Githunguri	"	8	35	35	0/35	1/35
Nairobi	Embakasi	Ducks	9	30	12	0/12	0/12
		"	10	18	12	0/12	0/12
		"	11	85	6	0/6	2/6
	Dagoreti	"	12	10	3	1/3	0/3
Kiambu	Kikuyu	"	13	11	11	1/11	5/11
	Githunguri	"	14	14	3	0/3	1/3
Total				481	135	2/135	9/135

Legends:
C – Cloacal swab; P - oropharyngeal swab; *P.multocida – Pasteurella multocida*

Appendix 2: *Pasteurella multocida* recovery from swabs taken from rural indigenous chickens at the time of slaughter in two slaughterhouses in Nairobi

Slaughterhouse	Rural district of origin	Total chicken sampled	*P. multocida* isolates	
			C	P
Kariokor	Kitui	12	0/12	0/12
	Bomet	10	0/10	0/10
	Kericho	6	0/6	0/6
	Uasin Gishu	4	0/4	0/4
Burma Maziwa	Makueni	13	0/13	1/13
Total		45	0/45	1/45

Legends:
C – Cloacal swab; P - Oro-pharyngeal swab; *P.multocida – Pasteurella multocida*

Appendix 3: *Pasteurella multocida* isolated from swabs taken from rural indigenous birds from various trading centres in Nairobi

Market trading Center	Rural district of origin	Total birds sampled	*P. multocida* isolates	
			C	P
A. Chickens				
Jogoo Road	Bomet	4	0/4	0/4
	Kisii	3	1/3	1/3
	Makueni	2	0/2	0/2
Kariokor	Kitui	1	0/1	0/1
	Makueni	2	0/2	0/2
	Mwingi	2	0/2	0/2
	Uasin Gishu	2	0/2	0/2
Nairobi South	Machakos	8	2/8	2/8
	Makueni	3	1/3	1/3
Westlands	Mwingi	2	0/2	0/2
B.Ducks				
Kasarani	Nairobi	7	1/7	3/7
Total		**36**	**5/36**	**7/36**

Legends:

C – Cloacal swab; P - oropharyngeal swab; *P.multocida – Pasteurella multocida*

Appendix 4: Type, total number and severity of the clinical signs in duplicate experiments conducted on 4 week-old chickens

Clinical Signs	Number and severity of clinical signs in expt.1			Number and severity of clinical signs in expt. 2			Average number and severity of clinical signs		
	1	2	3	1	2	3	1	2	3
Depression	13	20	18	43	5	2	28	17	10
N/tics	-	-	-	-	-	-	-	-	-
R/feathers	12	21	15	5	6	7	9	16	11
Sneezing	12	10	1	2	-	-	7	5	1
Ataxia	1	-	-	-	-	-	1	-	-
N/discharge	2	1	1	-	-	-	1	1	1
Dyspnoea	4	-	-	2	-	-	3	1	-
M/discharge	-	-	-	-	-	-	-	-	-
Diarrhoea	1	-	2	12	1	1	7	1	2
Cyanosis	-	-	-	-	-	-	-	-	-
Rales	17	9	1	9	-	-	13	8	1
Fever	13	20	1	12	5	2	13	14	2
H/scratching	-	-	-	-	-	-	-	-	-
Total signs	**75**	**71**	**39**	**85**	**17**	**12**	**82**	**63**	**28**

Legend: – No observed clinical sign; severity of clinical signs: 1- mild, 2 – moderate, 3 – severe sign; N/tics - nervous tics; R/feathers - ruffled feathers; N/discharge - nasal discharges; M/discharge - mouth discharges; H/scratching - head scratching.

Appendix 5: Type, total number and severity of the clinical signs in duplicate experiments conducted on the 8 week-old chickens

Clinical Signs	Number and severity of clinical signs in expt.1			Number and severity of clinical signs in expt.2			Average number and severity of clinical the signs		
	1	2	3	1	2	3	1	2	3
Depression	31	9	18	16	26	16	28	18	17
Nervous tics	2	-	-	3	-	-	3	-	-
R/feathers	25	11	17	17	26	15	26	19	16
Sneezing	14	11	17	3	1	-	12	6	9
Ataxia	-	1	-	6	1	-	3	2	1
N/ discharge	4	-	5	-	-	-	3	-	3
Dyspnoea	9	2	3	15	-	-	14	1	2
M/ discharge	-	-	-	3	-	-	2	1	-
Diarrhoea	3	-	-	4	-	-	4	1	-
Cyanosis	-	-	-	-	-	-	-	-	-
Rales	18	9	4	11	-	-	20	3	1
Fever	10	15	13	20	32	9	25	18	10
H/scratching	1	3	-	4	-	-	3	1	-
Total signs	**117**	**61**	77	**102**	**86**	**40**	**143**	**70**	**59**

Legend: 1 – mild; 2 – moderate; 3 – severe sign; - No observed clinical sign; N/tics - nervous tics; R/feathers - ruffled feathers; N/discharge - nasal discharges; M/discharge - mouth discharges; H/scratching - head scratching; expt. 1 or 2 – experiment 1 or 2.

Appendix 6: Type, total number and severity of clinical signs after duplicate experimentation on the 12 week-old chickens

Clinical Signs	Number and severity of clinical signs in expt.1			Number and severity of clinical signs in expt. 2			Average and severity of clinical signs		
	1	2	3	1	2	3	1	2	3
Depression	59	14	6	38	10	18	52	15	12
N/tics	12	1	2	6	-	-	9	2	2
R/feathers	55	14	10	30	11	13	47	15	12
Sneezing	3	-	-	33	15	12	20	9	7
Ataxia	9	1	2	2	3	4	8	3	3
N/discharges	-	-	-	26	17	18	16	9	9
Dyspnoea	13	-	-	12	1	-	13	2	1
M/discharge	5	-	-	2	1	1	4	1	1
Diarrhoea	9	2	3	3	2	3	8	3	3
Cyanosis	-	-	-	4	-	-	3	-	-
Rales	7	-	-	5	-	-	6	2	-
Fever	21	30	12	23	17	9	23	24	13
H/scratching	9	5	3	6	4	3	8	5	2
Total signs	**192**	**67**	**38**	**190**	**81**	**81**	**217**	**90**	**65**

Legend: 1 – mild; 2 – moderate; 3 – severe sign; – No observed clinical sign; N/tics - nervous tics; R/feathers - ruffled feathers; N/discharge - nasal discharges; M/discharge - mouth discharges; H/scratching - head scratching; expt. 1 or 2 – experiment 1 or 2.

Appendix 7: Type, total number and severity of clinical signs after duplicate experimentation on the 16 week-old chickens

Clinical Signs	Number and severity of clinical signs in expt.1			Number and severity of clinical signs in expt. 2			Average and severity number of clinical signs		
	1	2	3	1	2	3	1	2	3
Depression	24	10	3	20	8	12	23	10	9
N/tics	11	1	-	18	5	-	15	3	3
R/feathers	24	10	3	1	4	3	13	10	4
Sneezing	4	-	-	20	6	3	15	3	2
Ataxia	3	-	-	2	-	-	4	-	-
N/discharge	-	-	-	1	-	-	1	-	-
Dyspnoea	4	-	1	14	2	-	10	2	1
M/discharge	3	-	-	-	-	-	2	-	-
Diarrhoea	9	-	-	2	-	2	6	2	1
Cyanosis	2	-	-	-	-	-	1	-	-
Rales	1	-	-	1	-	-	2	-	-
Fever	20	11	-	20	16	5	20	15	3
H/scratching	4	2	-	4	2	1	4	2	1
Total signs	**109**	**34**	**6**	**103**	**43**	**26**	**116**	**47**	**24**

Legend: 1 – mild; 2 – moderate; 3 – severe sign; – No observed clinical sign; N/tics- nervous tics; R/feathers- ruffled feathers; N/discharge- nasal discharges; M/discharge- mouth discharges; H/scratching- head scratching; expt. 1 or 2 – experiment 1 or 2.

Appendix 8: Weight change in grams for control and infected 4 week-old chickens

a.Control birds

Chicken Number	Starting weight	Final weight	Gain in weight
1st batch			
1169	150	250	100
1170	175	200	25
1171	150	250	100
1172	75	100	25
1173	137	200	63
2nd batch			
1247	125	275	150
1248	75	125	50
1249	187	325	138
1250	150	250	100
Total			**750**
Average			**83.3**

a.Infected birds

Wing band	Starting weight	Final weight	Gain in weight
1st batch			
1174	162	225	63
1175	150	225	75
1176	100	87	-13
1177	125	200	75
1178	112	162	50
1179	115	175	60
1180	100	175	75
1181	75	162	87
1182	100	175	75
1183	100	175	75
2nd batch			
1237	75	300	225
1238	137	100	-37
1239	75	125	50
1240	75	125	50
1241	125	250	125
1242	100	162	62
1243	100	150	50
1244	100	187	87
1245	100	175	75
1246	100	175	75
Total			**1384**
Average			**69.2**

Appendix 9: Weight change in grams for control and infected 8 week-old chickens

a.Control birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1162	425	525	100
1164	400	600	200
1166	325	475	150
1167	375	675	300
2nd batch			
1192	225	325	100
1194	375	575	200
1195	325	575	250
1196	275	450	175
1223	300	450	150
Total			**1625**
Average			**180.5**

a.Infected birds

Wingband	Starting weight	Final weight	Gain in weight
1st batch			
1156	325	475	150
1157	275	425	150
1158	375	475	100
1159	475	650	175
1160	475	725	250
1161	425	600	175
1163	325	450	125
1165	325	500	175
1168	550	725	175
2nd batch			
1193/1206	300	400	100
1197	225	362	137
1198	350	575	225
1199	375	500	125
1200	325	525	200
1201	250	425	175
1202	250	400	150
1203	287	450	163
1204	325	475	150
1205	325	575	250
Total			**3050**
Average			**160.5**

Appendix 10: Weight change in grams for control and infected 12 week-old chickens

a.Control birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1107	725	900	175
1120	1250	1625	375
1136	662	850	188
1101	775	875	100
1133	675	850	175
2nd batch			
1232	500	662	162
1227	250	487	237
1220	700	1000	300
1235	450	600	150
Total			**1862**
Average			**206.9**

a.Infected birds

Wingband	Starting weight	Final weight	Weight gain
1st batch			
1102	1200	1225	25
1108	1075	1300	225
1116	1300	1400	100
1129	775	1025	250
1130	750	1025	275
1141	825	1000	175
1142	750	1000	250
1143	800	1075	275
1105	550	575	25
1121	975	1275	300
2nd batch			
1194	700	850	150
1222	512	712	200
1210	600	800	200
1233	450	612	162
1234	550	750	200
1228	375	512	137
1230	600	750	150
1219	450	600	150
1212	500	700	200
1213	675	850	175
Total			**3624**
Average			**181.2**

Appendix 11: Weight change in grams for control and infected 16 week-old chickens

a.Control birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1086	1125	1425	300
1095	1025	1325	300
1149	925	1100	175
1153	725	850	125
1154	600	850	250
2nd batch			
1131	1500	1775	275
1139	875	1125	250
1126	1400	1675	275
1106	1100	1287	187
1122	825	1150	325
Total			**2462**
Average			**246.2**

a.Infected birds

Wingband	Starting weight	Final weight	Weight gain
1st batch			
1087	900	900	0
1090	1075	1200	125
1145	1075	1150	75
1100	1075	1125	50
1151	1100	1000	-100
1099	975	1200	225
1091	1150	1325	175
1144	875	900	25
1148	800	875	75
1155	900	1000	100
2nd batch			
1134	875	1025	150
1103	1475	1550	75
1115	850	1000	150
1113	1275	1312	37
1137	650	712	62
1114	1075	1150	75
1123	1100	1225	125
1140	1200	1300	100
1135	1200	1212	12
1111	900	950	50
Total			**1586**
Average			**79.3**

Appendix 12: Type, total number and severity of the clinical signs in each experiment conducted on the 4 week-old ducks

Clinical Signs	Experiment 1			Experiment 2			Average number signs		
	Severity level			Severity level			Severity level		
	1	2	3	1	2	3	1	2	3
Depression	11	2	2	11	4	-	11	3	1
Nervous tics	-	-	-	-	-	-	-	-	-
Ruffled feathers	11	2	-	11	4	2	11	3	1
Sneezing	1	1	-	3	1	-	2	1	-
Ataxia	-	-	-	-	-	-	-	-	-
Nasal discharges	4	2	2	9	2	12	7	2	7
Dyspnoea	3	3	1	26	4	4	15	4	3
Mouth discharges	3	-	1	1	-	1	2	-	1
Diarrhoea	2	-	-	-	-	2	1	-	1
Cyanosis	-	-	-	-	-	-	-	-	-
Rales	9	2	1	10	3	2	10	3	2
Fever	22	8	4	3	4	9	13	6	7
Head scratching	-	-	-	-	-	-	-	-	-
Coughing	-	-	-	-	-	-	-	-	-
Eye discharges	-	-	-	-	-	-	-	-	-
Total signs	**66**	**21**	**11**	**74**	**22**	**32**	**72**	**22**	**23**

Legend: – No sign recorded; Severity -1: mild sign; 2: moderate sign; 3: severe sign

Appendix 13: Type, total number and severity of the clinical signs in each experiment conducted on the 8 week-old ducks

Clinical signs	Experiment 1			Experiment 2			Average signs		
	Severity level			Severity level			Severity level		
	1	2	3	1	2	3	1	2	3
Depression	6	4	3	6	3	2	6	4	3
Nervous tics	-	-	-	-	-	-	-	-	-
Ruffled feathers	6	4	3	6	3	2	6	4	3
Sneezing	20	2	3	18	4	3	19	3	3
Ataxia	5	3	-	4	1	1	5	2	1
Nasal discharges	11	8	26	34	10	25	23	9	26
Dyspnoea	12	9	5	15	5	5	14	7	5
Mouth discharges	2	4	1	6	3	2	4	4	2
Diarrhoea	1	-	-	-	-	-	1	-	-
Cyanosis	-	-	-	-	-	-	-	-	-
Rales	-	2	-	2	-	-	1	1	-
Fever	15	7	6	6	8	1	11	8	4
Head scratching	-	-	-	-	-	-	-	-	-
Coughing	4	1	3	-	5	-	2	3	2
Eye Discharges	-	-	-	1	1	-	1	1	-
Total signs	**82**	**44**	**50**	**98**	**43**	**41**	**93**	**46**	**49**

Legend: – No sign recorded; Severity -1: mild sign; 2: moderate sign; 3: severe sign

Appendix 14: Type, total number and severity of the clinical signs in each experiment conducted on 12 week-old ducks

Clinical signs	Experiment 1:signs			Experiment 2: signs			Average signs		
	Severity level			Severity level			Severity level		
	1	2	3	1	2	3	1	2	3
Depression	3	2	-	4	2	-	4	2	-
Nervous tics	-	-	-	-	-	-	-	-	-
Ruffled feathers	3	2	-	4	2	-	4	2	-
Sneezing	11	2	1	13	1	2	12	2	2
Ataxia	-	-	-	-	-	-	-	-	-
Nasal discharges	13	4	4	20	1	19	17	3	12
Dyspnoea	1	-	-	-	-	-	1	-	-
Mouth discharges	2	-	-	2	-	-	2	-	-
Diarrhoea	-	-	-	-	-	-	-	-	-
Cyanosis	-	-	-	-	-	-	-	-	-
Rales	-	-	-	-	-	-	-	-	-
Fever	9	3	5	7	3	3	8	3	4
Head scratching	1	-	-	-	-	-	1	-	-
Coughing	1	-	-	-	-	-	1	-	-
Eye Discharges	-	-	-	-	-	-	-	-	-
Total signs	**44**	**13**	**10**	**50**	**9**	**24**	**50**	**12**	**18**

Legend: – No sign recorded; Severity -1: mild sign; 2: moderate sign; 3: severe sign

Appendix 15: Type, total number and severity of the clinical signs in each experiment conducted on the 16 week-old ducks

Clinical signs	Experiment1: signs			Experiment 2: signs			Average signs		
	Severity level			Severity level			Severity level		
	1	2	3	1	2	3	1	2	3
Depression	3	1	-	2	1	-	3	1	-
Nervous tics	-	-	-	-	-	-	-	-	-
Ruffled feathers	3	1	-	2	-	-	3	1	-
Sneezing	10	6	1	8	2	1	9	4	1
Ataxia	-	-	-	-	-	-	-	-	-
Nasal discharges	21	10	21	13	6	13	17	8	17
Dyspnoea	-	1	-	3	-	-	2	1	-
Mouthdischarges	-	-	-	1	-	-	1	-	-
Diarrhoea	-	-	-	-	-	-	-	-	-
Cyanosis	-	-	-	-	-	-	-	-	-
Rales	-	-	-	-	-	-	-	-	-
Fever	10	4	3	2	4	5	6	4	4
Head scratching	-	-	-	-	-	-	-	-	-
Coughing	-	-	-	-	1	-	-	1	-
Eye discharges	-	-	-	-	-	-	-	-	-
Total signs	**47**	**23**	**25**	**31**	**14**	**19**	**41**	**20**	**22**

Legend: – No sign recorded; Severity -1: mild sign; 2: moderate sign; 3: severe sign

Appendix 16: Weight change in grams for control and infected 4 week-old ducks

a.Control birds

Wing band	Starting weight	Final weight	Gain in weight
1st batch			
1184	375	700	275
1185	500	800	300
1186	537	887	350
2nd batch			
1597	212	400	188
1578	150	350	200
1587	325	575	250
1591	250	487	237
1590	325	512	187
Total			**1987**
Average			**248.4**

a. Infected birds

Wing band	Starting weight	Final weight	Gain in weight
1st batch			
1187	437	575	138
1188	400	675	275
1189	475	775	300
1190	500	875	375
1191	450	700	250
2nd batch			
1589	187	375	188
1584	325	625	300
1596	275	562	287
1576	275	487	212
1577	287	537	250
1582	350	600	250
1595	112	200	88
1594	300	575	275
1583	437	662	225
1592	312	512	200
Total weight			**3613**
Average weight			**240.9**

Appendix 17: Weight change in grams for control and infected 8 week-old ducks

a.Control birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1934	1025	1625	600
1928	837	1237	400
1941	800	1387	587
1948	700	1112	412
1945	750	1050	300
2nd batch			
1921	1300	1700	400
2000	875	1250	375
1564	812	1300	488
1915	875	1225	350
1913	750	1150	400
Total			**4312**
Average			**431.2**

a.Infected birds

Wing band	Starting weight	Final weight	Weight gains
1st batch			
1938	825	1075	250
1952	575	1075	500
1932	1125	1600	475
1937	662	1125	463
1939	800	900	100
1940	712	1125	413
1931	662	1000	338
1933	887	1175	288
1929	1012	1462	450
1944	975	1412	437
2nd batch			
1568	787	1125	338
1907	1425	1987	562
1566	750	1200	450
1916	912	1487	575
1919	875	1325	500
1994	787	1200	413
1909	437	800	363
1914	750	1312	562
1925	725	1200	475
1997	775	1200	425
Total			**8377**
Average			**418.9**

Appendix 18: Weight change in grams for control and infected 12 week-old ducks

a.Control birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1508	1075	1375	300
1511	1150	1475	325
1523	1025	1225	200
1513	1075	1600	525
1520	1450	1850	400
2nd batch			
1954	887	1200	313
1951	1050	1450	400
1993	1025	1325	300
1946	1487	1837	450
1947	1075	1525	450
Total			**3663**
Average			**366.3**

a. Infected birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1516	1200	1700	500
1505	1200	1425	225
1514	1625	2037	412
1515	1275	1862	587
1503	1275	1800	525
1512	1200	1687	487
1501	1450	2025	575
1517	1300	1850	550
1504	1000	1275	275
1521	1150	1662	512
2nd batch			
1943	1925	2200	275
1934	1950	2150	200
1950	900	950	50
1930	1150	1450	300
1949	1087	1387	300
1936	825	1062	237
1935	1700	2037	337
1942	1250	1425	175
1928	1425	1337	-88
1927	1800	2125	325
Total			**6757**
Average			**337.95**

Appendix 19: Weight change in grams for control and infected 16 week-old ducks

a.Control birds

Wing band	Starting weight	Final weight	Weight gains
1st batch			
1506	1637	1650	13
1525	1487	1387	-100
1524	2700	2800	100
1504	1550	1550	0
2nd batch			
1311	2500	3087	587
1307	3175	3112	-63
1300	1700	1700	0
1303	1562	1700	138
1306	2550	2950	400
Total			**1075**
Average			**119.44**

a. Infected birds

Wing band	Starting weight	Final weight	Weight gain
1st batch			
1511	1500	1612	112
1510	2262	2200	-62
1519	1500	1600	100
1509	1350	1612	262
1518	2187	2200	13
1508	1387	1500	113
1520	2150	2300	150
1522	1337	1487	150
1507	2337	2700	363
2nd batch			
1304	1375	1362	-13
1299	1350	1250	-100
1310	1537	1600	63
1309	2425	2362	-63
1308	2450	2437	-13
1301	2300	2400	100
1298	2375	2550	175
1305	2400	2612	212
1312	2337	2562	225
1302	1350	1375	25
Total			**1817**
Average			**90.6**

Appendix 20: Combinations of the daily number of clinical signs observed in the immunosuppressed indigenous chickens

Clinical Signs	Days post infection														Total Signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
D+S+Ata	-	-	-	-	-	1	1	-	-	-	-	-	-	-	2
D+RF	1	2	2	-	2	-	-	2	-	-	1	2	-	-	12
D+S+RF	1	-	-	-	-	-	-	-	-	1	-	-	-	-	2
Dys+NT	-	-	1	1	2	-	-	-	2	-	-	-	-	-	6
D+ND+Ata+MD	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
S+Cys	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
S+Ata+NT	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1
D+Dys	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
ND+Ata	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1
D+S+NT	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
S+Ata	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1
D+S+Dys	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
S+NT+RF	-	-	1	1	1	-	-	-	1	-	-	-	-	1	5
D+ND	-	-	1	-	-	-	-	-	-	-	-	-	-	1	2
D+Ata	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1
D+NT+RF	-	-	-	2	1	2	-	-	-	-	-	-	-	-	5
Ata+NT	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1
S+ND	-	-	-	1	-	-	-	-	-	-	1	-	1	-	3
D+Dys+Ata+NT	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
Total signs	**4**	**6**	**6**	**8**	**9**	**6**	**6**	**5**	**5**	**4**	**5**	**5**	**5**	**3**	**77**

Legend: D-Depression; Ata-Ataxia; S-Sneezing; R-Rales; ND-Nasal discharges; MD-Mouth discharges; RF-Ruffled feathers; Dia-Diarrhoea; Dys-Dyspnoea; Cys-Cyanosis; NT-Nervous tics

Appendix 21: Combinations of the daily number of clinical signs observed in the non-immunosuppressed indigenous chickens

Clinical Signs	Days post infection														Total Signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
D+S	2	-	-	2	1	-	-	1	1	-	-	2	-	-	9
D+ND	2	1	1	-	-	-	2	-	-	1	-	-	-	1	8
D+S+ND+RF	4	4	3	1	2	1	-	1	1	1	-	1	3	-	22
D+S+Dys	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
D+S+ND+Dys+RF	-	1	-	-	-	1	-	-	-	-	-	1	-	-	3
D+S+ND+Dys+R	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
D+Dys+R	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
D+RF	-	-	1	1	-	-	1	1	1	-	2	-	-	1	8
D+S+ND+R	-	-	1	-	-	-	-	-	-	-	-	-	-	-	1
S+ND+Dys+RF	-	-	1	-	-	-	1	-	-	-	-	-	-	-	2
D+ND+Ata+RF	-	-	1	1	1	-	-	1	-	-	-	-	-	-	4
S+ND	-	-	-	1	1	-	-	-	1	1	1	-	-	-	5
D+ND+Dia	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1
D+S+ND+Ata+RF	-	-	-	-	1	-	-	-	-	1	-	-	-	-	2
D+S+ND+NT+RF	-	-	-	-	-	1	1	-	-	-	-	-	-	-	2
S+ND+NT	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
D+ND+Ata+Cys	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
NT+Cy	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
S+Ata+Cy	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1
S+R	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1
D+S+R	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1
ND+Dys+NT	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1
D+S+ND+Ata+Cy	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1
S+MD+Ata+Dia	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
ND+MD+RF	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
Total signs	**9**	**8**	**8**	**7**	**7**	**7**	**8**	**6**	**9**	**7**	**6**	**6**	**4**	**5**	**97**

Legend: D-Depression; Ata-Ataxia; S-Sneezing; R-Rales; ND-Nasai discharges; MD-Mouth discharges; RF-Ruffled feathers; Dia-Diarrhoea; Dys-Dyspnoea; Cys-Cyanosis; NT-Nervous tics

Appendix 22: Combinations of the daily number of clinical signs observed in the immunosuppressed indigenous ducks

Clinical Signs	Days post infection														Total Signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
ND+R	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1
S+Dys+ND	1	1	1	-	-	-	-	1	-	-	-	-	-	-	4
S+Dys+R+Ata	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
S+Dys+ND+Ata+MD	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
ND+Dys	-	2	1	2	-	-	-	1	-	1	2	-	-	1	10
ND+S+Dys	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
S+Dys	1	-	-	1	-	1	-	-	-	1	-	-	-	-	4
Total signs	**6**	**8**	**6**	**6**	**7**	**5**	**4**	**5**	**4**	**5**	**5**	**3**	**2**	**2**	**68**

Legend: D-Depression; Ata-Ataxia; S-Sneezing; R-Rales; ND-Nasal discharges; MD-Mouth discharges; RF-Ruffled feathers; Dia-Diarrhoea; Dys-Dyspnoea; Cys-Cyanosis; NT-Nervous tics

Appendix 23: Combinations of the daily number of clinical signs observed in the non-immunosuppressed indigenous ducks

Clinical Signs	Days post infection														Total Signs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
ND+MD	1	-	1	-	-	1	-	-	-	-	-	-	-	-	3
ND+Dys+R	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
ND+S	1	2	-	-	-	-	1	1	1	1	-	-	1	-	8
ND+S+Dys	-	-	1	1	-	1	-	-	-	-	-	-	-	-	3
ND+S+ Ata	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1
ND+Dys+Ata	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1
ND+Dys	-	-	-	-	-	3	1	-	-	-	-	-	-	-	4
ND+MD+ED	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
Total signs	**7**	**6**	**10**	**7**	**7**	**9**	**8**	**7**	**6**	**6**	**5**	**3**	**2**	**0**	**83**

Legend: D-Depression; Ata-Ataxia; S-Sneezing; R-Rales; ND-Nasal discharges; MD-Mouth discharges; RF-Ruffled feathers; Dia-Diarrhoea; Dys-Dyspnoea; Cys-Cyanosis; NT-Nervous tics

Appendix 24: Comparison between the combinations and numbers of clinical signs observed in IS and NIS chickens and ducks

Clinical signs	Chickens		Ducks	
	IS	NIS	IS	NIS
D+S+RF	2	9	-	-
Dys+NT	5	-	-	-
ND+D+MD+Ata	1	-	-	-
S+Dys	1	-	4	-
S+NT+Ata	1	-	-	-
D+Dys	1	-	-	-
D+S+Ata	2	-	-	-
D+S+NT	1	-	-	-
S+Ata+Cy	-	1	-	-
S+Ata	1	-	-	-
D+S+Dys	1	1	-	-
S+NT+RF	5	-	-	-
D+ND	2	8	-	-
D+Ata	1	-	-	-
D+NT+RF	5	-	-	-
NT+Ata	1	-		
S+ND	3	5	8	-
S+R	-	1	-	-
D+Dys+Ata+NT	1	-	-	-
D+S+ND+RF	-	22	-	-
D+S+ND+Dys+RF	-	3	-	-
D+S+ND+Dys+R	-	1	-	-
D+S+R	-	1	-	-
D+Dys+R	-	1	-	-
D+S+ND+Ata+Cy	-	1	-	-
D+S+ND+R	-	1	-	-
ND+Dys+NT	-	1	-	-
S+ND+Dys+RF	-	2	-	-
D+ND+Ata+RF	-	4	-	-
N+ND+Dia	-	1	-	-
D+S+ND+Ata+RF	-	2	-	-
D+S+ND+NT+RF	-	2	-	-
S+ND+NT	-	1	-	-
D+ND+Ata+Cy	-	1	-	-
NT+Cy+RF	-	1	-	-
S+MD+Ata+Dia		1	-	-
ND+Ata	1	-	-	-
ND+MD+RF	-	1	-	-
R+ND	-	-	2	-
S+Dys+R+Ata	-	-	1	1
ND+S+Dys	-	-	5	3
ND+Dys	-	-	9	4
ND+MD	-	-	3	-
ND+Dys+R	-	-	1	-
ND+Dys+Ata	-	-	1	-
ND+S+Ata	-	-	-	1
ND+MD+ED	-	-	1	-
ND+MD+S+Dys+Ata	-	-	1	-
Total signs	77	97	68	83

Legend: D-Depression; Ata-Ataxia; S-Sneezing; R-Rales; ND-Nasal discharges; MD-Mouth discharges; RF-Ruffled feathers; Dia-Diarrhoea; Dys-Dyspnoea; Cys-Cyanosis; NT-Nervous tics

Appendix 25: Gross pathological lesions and bacterial isolation from the ducks and chickens at the end of cross transmission (chicken to duck) study

Duck number	Day of sacrifice	Organ with gross lesions				*P.multocida* Re-isolated
		Airsacs	Liver	Lungs	Pleura	
1417	1	-	+	-	-	+
1421	1	-	-	-	-	+
1418	3	-	-	+	-	-
1422	3	+	-	+	+	+
1431	5	-	-	-	-	+
1434	5	-	-	-	-	-
1430	7	-	-	+	-	-
1436	7	-	-	-	-	-
1419	10	-	-	+	-	+
1424	10	+	-	+	-	-
1414	14	-	+	-	-	+
1425	14	+	+	+	-	+
1427	14	-	-	-	-	+
1428	14	-	-	-	-	-
1433	14	-	-	+	-	-
Chicken number						
1571	14	+	-	+	-	+
1572	14	-	-	-	-	-
1573	14	-	-	+	-	-
1574	14	+	-	+	-	+
1598	14	-	-	-	-	-
1599	14	+	-	-	-	-

Legends: + Lesions on organs or bacterial recovered from the oro-pharynx and cloaca swabs; - No significant findings.

Appendix 26: Gross pathological lesions and bacterial isolation from the chickens and ducks at the end of cross transmission study (duck to chickens)

Chicken number	Day of sacrifice	Organs with gross lesions				*P.multocida* Re-isolated
		Airsacs	Liver	Lungs	Pleura	
1285	1	+	-	+	-	+
1290	1	+	-	+	-	+
1292	3	-	-	-	-	-
1293	3	-	-	-	-	-
1295	5	+	-	-	-	+
1297	5	-	-	-	-	-
1286	7	-	-	-	-	-
1294	7	-	-	-	-	+
1296	10	-	-	-	-	-
1284	14	+	-	-	-	+
1287	14	-	-	-	-	-
1288	14	-	-	-	-	-
1289	14	-	-	-	-	-
1291	14	-	-	-	-	-
Ducks number						
1901	14	-	-	-	-	+
1902	14	-	-	-	-	-
1903	14	+	-	+	-	+
1904	14	+	-	+	-	+
1905	14	+	-	+	-	+
1906	14	-	-	-	-	-

Legend: + Lesions on organs bacterial recovered from the oro-pharynx and cloaca swabs; - No significant findings.

Appendix 27: Specific detection of *Pasteurella multocida* in chickens with fowl cholera and in pig lung tissues using fluorescent rRNA *in situ* hybridization

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Specific Detection of *Pasteurella multocida* in Chickens with Fowl Cholera and in Pig Lung Tissues Using Fluorescent rRNA In Situ Hybridization

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A *Pasteurella multocida* species-specific oligonucleotide probe, pmhyb449, targeting 16S rRNA was designed and evaluated by whole-cell hybridization against 22 selected reference strains in animal tissues. It differentiated *P. multocida* from other bacterial species of the families *Pasteurellaceae* and *Enterobacteriaceae* and also from divergent species of the order *Cytophagales* (except biovar 2 strains of *Pasteurella avium* and *Pasteurella canis*, which have high 16S rRNA similarity to *P. multocida*). The potential of the probe for specific identification and differentiation of *P. multocida* was further detected in formalin-fixed paraffin-embedded lung tissues from experimental fowl cholera in chickens and infections in pigs. In chicken lung tissues *P. multocida* cells were detected singly, in pairs, as microcolonies, and as massive colonies within air capillaries (septa and lumen), parabronchial septa, and blood vessels (wall and lumen). In pig lung, postmortem-injected *P. multocida* was detected in the alveoli (lumen and wall), and in both animals the bacterial cells were seen in the bronchi. The results showed that with the oligonucleotide probe pmhyb449, fluorescent in situ hybridization is a suitable and fast method for specific detection of *P. multocida* in histological formalin-fixed tissues. The test was replicable and reproducible and is recommended as a supplementary test for diagnosis and as a tool in pathogenesis studies of fowl cholera and respiratory tract infections in pigs due to *P. multocida*.

Pasteurella multocida is an important pathogen that infects many production animals and is an opportunistic human pathogen (7, 21). In poultry, infection with *P. multocida* may result in fowl cholera, a disease of economic importance in commercial production that may occur in different forms, such as peracute, acute, and chronic infections (16, 43). In pigs, *P. multocida* is commonly associated with atrophic rhinitis, pneumonia, and septicemic pasteurellosis. Pneumonic pasteurellosis due to *P. multocida* is common and is of major economic importance for industrial pig production (41). The histology, clinical signs, and macroscopic lesions associated with *P. multocida* infections in poultry and pigs are not pathognomonic and can be mixed up with other respiratory system infections characterized by upper respiratory tract inflammations, pneumonia, airsacculitis, polyserositis, and septicemia (14, 33, 41, 43), and hence the diagnosis depends on specific detection of the causative organism.

Detection and characterization of *P. multocida* by phenotypic characteristics including serotype have been dependent on the ability to cultivate and purify the bacteria in the laboratory (16). Cultivation and identification by standard bacteriological methods can be ambiguous because of V-factor requirements or nontypeable strains, including cross-reaction in serotyping and viable but nonculturable cells (30).

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In situ hybridization (ISH) allows precise localization of a specific segment of nucleic acid within a histologic section (13) or detection of specific rRNA in morphologically intact bacteria cells (5). ISH combines basic molecular biological techniques and the ability to appreciate subtle histomorphologic changes (13). The key feature distinguishing ISH from other molecular methodologies (filter hybridization and PCR) is that the sample DNA or RNA is detected directly in the intact cell rather than being extracted from the cell before testing (40). The cell morphology and its abundance and spatial distribution can be analyzed in situ (5, 42).

With fluorescent-labeled probes, in situ hybridization (FISH) has excellent spatial resolution (42). FISH has been used to visualize the spatial distribution of *Escherichia coli* in intestinal mucosa (42); to evaluate colonization of mouse intestines by *Salmonella enterica* serovar Typhimurium (32); and to specifically detect *Salmonella* serovars in pig intestines and mouse lungs (40), *Actinobacillus pleuropneumoniae* strains in diseased porcine tissues (23, 26), *Brachyspira (Serpulina) pilosicoli* infections in the intestines of growing pigs (11, 27), *Lawsonia intracellularis* in porcine proliferative enteropathy (10), *Streptococcus suis* infection in pigs (12), and bacteria in blood cultures (25, 29).

The aim of the present study was to develop a culture-independent FISH test for *P. multocida* based upon hybridization of tagged oligonucleotide probes to bacterial rRNA. The diagnostic potential of this test was evaluated using lungs from chickens infected with *P. multocida* that developed clinical fowl cholera and with pig lung tissues injected with *P. multocida*.

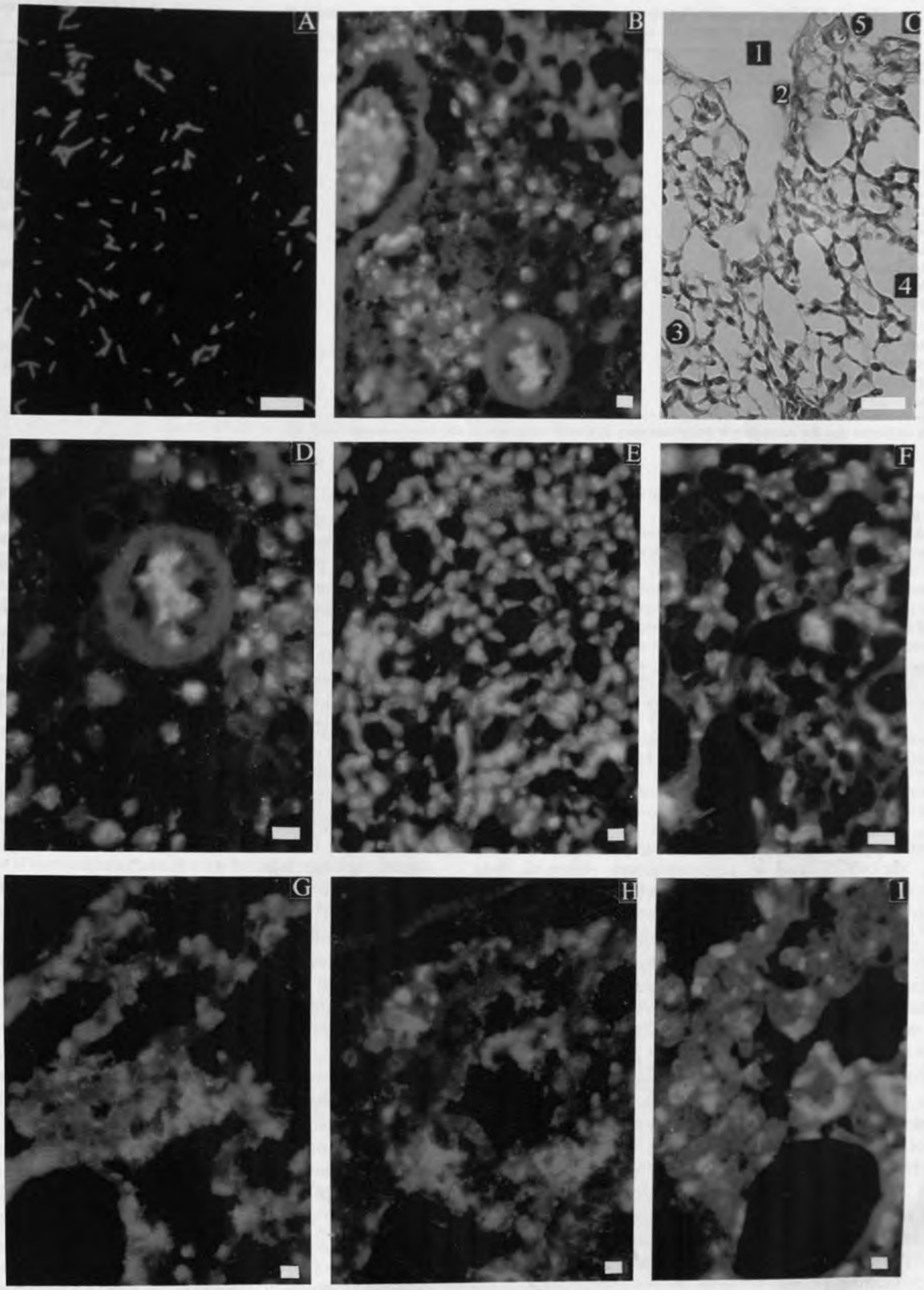

FIG. 1. Photomicrographs demonstrating specific detection of *P. multocida* in pure culture and formalin-fixed lung tissues by use of the FISH technique. Shown are a pure culture of *P. multocida* subsp. *multocida* strain 40605-1 (A) and lung tissue sections of chicken (B, C, D, E, and F) or pig (G, H, and I) following light microscopy (C) or epifluorescence microscopy (A, B, and D through I) and ISH with the *P. multocida*-specific Cy3-labeled probe pmhyb449 (A, B, D, E, G, and H) and a nonsense Cy3-labeled probe, non-pmhyb449 (F and I), used as a control. A normal chicken lung (C) shows the location of the bacteria detected in cases of fowl cholera with an indication of the parabronchi (1), infundibulum (2),

TABLE 3. Results of ISH lung tissues showing the anatomical location where *P. multocida* was detected by probe pmhyb449

Tissue source	Bacteria detection in[a]			
	Chicken after an infection period (h) of:			Pig[b]
	12	24	48	
Bronchi	++	−	−	+[c]
Parabronchi	++	++	−	++
Interstitium spaces	+	−	−	+
Smooth muscles	−	−	+	+
Infundibulum spaces	+	+	+	NA[d]
Air capillaries or alveoli spaces	+++	++	+	+++
Air capillaries or alveolar septa	+++	++	+	+++
Pleura	+	−	+	−
Vascular areas	+	+	−	−

[a] −, no bacteria detected; +, few bacteria detected; ++, moderate number of bacteria detected; +++, many bacteria detected.
[b] Bacteria were injected into pig lung postmortem.
[c] From bronchiole lumen.
[d] NA, not applicable.

respiratory atria. Some bacteria were seen in pairs or in fours in a ring formation in phagocytic cells (macrophages or heterophils) within the parabronchial spaces and blood vessels and in aggregation of lymphoid tissues within the lung tissues. At 48 h most bacteria were detected in the air capillaries, air capillary walls, and infundibulum spaces, and a few were detected on the spiral smooth muscles of the parabronchi (Table 3). Some bacteria were found in a ring formation in phagocytes (in bronchi and blood vessels) and in lymphoid aggregation areas of the lung tissue, indicating either phagocytosis or the presence of circulating phagocytes with bacteria. The role of these cells in pathogenesis of *P. multocida* needs further investigation.

In the pig lung, ISH with probe pmhyb449 clearly detected *P. multocida* among tissues and cells of the lung. The bacteria were found mainly in the alveoli, terminal bronchioles, and interstitial areas, and a few bacteria were observed in the bronchi, bronchioles, and terminal bronchioles (Table 3 and Fig. 1G and H).

Infected as well as noninfected tissues were negative when hybridized with the non-pmhyb449 probe (Fig. 1F and I). The inflammatory reaction of the lung tissues in response to the bacteria did not hinder the binding of the probe in fowl cholera. The results of this study indicate that probe pmhyb449 is suitable for detection and determination of the in vivo localization of *P. multocida* in tissues.

DISCUSSION

FISH of whole cells with rRNA-targeted oligonucleotide probes has been extensively used as a tool for specific detection of bacteria (4, 5, 12, 15, 18, 23, 25, 39). To our knowledge no FISH procedure has been developed and used for detection or diagnosis of *P. multocida*. To aid in diagnosis of infections caused by *P. multocida*, a species-specific probe, pmhyb449, targeting 16S rRNA was designed and used for FISH. The specificity of this oligonucleotide probe was examined by whole-cell hybridization against selected species representing both 16S rRNA clusters described by Dewhirst et al. (19) outlined within genus *Pasteurella* sensu stricto as defined by Mutters et al. (38) and other bacterial species commonly associated with respiratory tract infections and septicemia in poultry. The probe was able to differentiate bacteria by at least four base pair mismatches. Exceptions were *P. avium* biovar 2 and *P. canis* biovar 2. These taxa were originally described by Madsen et al. (34) and subsequently were named by Mutters et al. (36, 37). Both taxa have been reported from pneumonia in calves in several countries (8, 9). 16S rRNA sequence studies of members of the family *Pasteurellaceae* Pohl 1981, however, indicated that taxon 13 of Bisgaard should be reinvestigated, since CCUG 16497 (*P. avium* biovar 2) clustered with the type strain of *P. multocida* and not with *P. avium* (19). Recently it was found that the 16S rRNA similarities between *P. multocida* and biovar 2 of *P. avium* and *P. canis* are greater than 98.6%, while similarities between *P. multocida* and biovar 1 of *P. avium* and *P. canis* are 94 and 96%, respectively (data not shown). However, similarities of organisms based on 16S rRNA sequence comparison are insufficient per se for species separation (20). Just as the outlining of species based upon a few selected DNA-DNA hybridizations might result in uncertain species definitions (6), on this background it appears that biovar 2 of *P. avium* and *P. canis* are misclassified and that future reclassification will place them with *P. multocida*. With this view, probe pmhyb449 is concluded to be specific for *P. multocida*.

The ISH assay was used for detection of *P. multocida* in formalin-fixed, paraffin-embedded tissues of a pig lung injected with a pure culture of *P. multocida* subsp. *septica* and in lungs from chickens that developed clinical fowl cholera infection with *P. multocida* subsp. *multocida*. The pmhyb449 probe was able to detect single cells of *P. multocida* in situ in the respective lung tissues, whereas no signal was observed for control lung tissue sections from noninfected birds that contained no organisms or infected lung tissues that were hybridized with the complementary non-pmhyb449 probe.

Methods based on 16S rRNA are advantageous in the detection and identification of microorganisms due to the fact that each bacterial cell contains multiple copies of the 16S rRNA that eases its detection, with evolutionarily highly conserved 16S rRNA regions common to bacteria and other regions which might be species specific (18, 31). 16S rRNA-based methods allow identification of microorganisms independently

air capillary (3), pleura (4), and arterioles (5) (hematoxylin and eosin stain). Also shown are chicken lung infected with fowl cholera (experimentally infected with *P. multocida* subsp. *multocida* strain 40605-1), with the location of *P. multocida* cells near two blood vessels (artery and arteriole) (B), with *P. multocida* cells within the lumen and the walls of the air capillaries and perivascular space (D), and with *P. multocida* cells as individual organisms or as microcolonies (E); a control tissue section hybridized with probe non-pmhyb449 is also shown (F). Also shown is lung of a pig infected postmortem with *P. multocida* subsp. *septica* strain HIM 746-6^T showing *P. multocida* bacteria in the lumen and wall of the alveoli (G), the area close to the pleura (H), and a control tissue section hybridized with non-pmhyb449 (I). Bars = 10 μm (A, B, D, E, F, G, H, and I) and 100 μm (C).

of bacterial growth rates and metabolic activities (31, 44), although cellular ribosomes are more abundant in rapidly growing bacteria (18). The amount of bound probe is directly correlated to the cellular rRNA content, which is dependent on physiological activity at the time of fixation (4, 24). In cases of fowl cholera the *P. multocida* cells could have been highly active within the lung tissue, as they gave strong signals with probe pmhyb449. In fast-growing bacteria, the rRNA content correlates directly with the growth rate (18), although it may not be valid in the case of slow-growing or starved organisms (28, 44). Starved and dormant bacteria can, however, be detected with FISH as active bacteria if they still possess relatively high 16S rRNA levels, as reported for *S. enterica* serovar Typhimurium (44).

Probe pmhyb449 clearly detected short rods of bacteria in various parts of the chicken lungs under mild or severe lung inflammation. In chickens, *P. multocida* was shown to occur singly, in aggregates probably representing microcolonies, or in masses occupying large areas of the lungs. Their individual morphologies were clearly detected by the pmhyb449 probe, as reported in similar FISH procedures with other bacteria (4, 18, 24).

There are limitations to the ISH assay, as the test depends on the number of copies of rRNA in the cell (5) and hence on the physiological activity of the microorganisms prior to fixation of samples and as it is restricted to eutrophic environments such as the lungs of the chickens. Another limitation is the autofluorescence background of the eucaryotic tissue. However, in contrast to immunological methods that rely on the expression of specific antigenic markers which may not be constant, phenotypic variation does not pose a problem when rRNA is used as a target (11, 12). Furthermore, the inflammatory reactions observed with *P. multocida* infections during this study did not hinder the detection of bacteria by FISH. The stability of the ribosome target allowed for the detection of single cells even in clinical material as well as identification in smears of pure culture (39), which correlates with our observations here.

In their study of *P. multocida* carriers in commercial poultry in Denmark, Muhairwa et al. (35) found mouse inoculation to be more effective in the recovery of this organism than the commonly used selective media. Yet mouse passage may only select strains pathogenic for mice. The developed test can offer a complementary role in active clinical case diagnosis and can be applied in pathogenesis and pathogenicity studies of *P. multocida*.

The probe pmhyb449 is recommended, as our results suggest that the probe might be applied with advantage to studies of *P. multocida* pathogenesis and its infections in poultry, pigs, and maybe other animals. Furthermore, results can be obtained quickly, since pure cultures of the bacteria are not needed. It can also be used to confirm and differentiate *P. multocida* from other *Pasteurellaceae* in culture. The test is simple and can be applied in most research and diagnostic laboratories.

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