OCCURRENCE AND CHARACTERISTICS OF *AVIBACTERIUM PARAGALLINARUM* IN UGANDA

By

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ABSTRACT

Investigations were conducted to establish the occurrence and characteristics of *Avibacterium paragallinarum* in Uganda. Seven hundred and ten bacteriological and serum samples (642 from healthy birds and 68 from chickens with coryza-like signs) were collected from chickens and turkeys for isolation of bacteria and demonstration of antibodies. Isolates were characterised by phenotypic (serotyping, biotyping and antimicrobial susceptibility) and genotypic methods (enterobacterial repetitive intergenic sequence based polymerase chain reaction, ERIC-PCR and distribution of resistance genes). The potential role of gallinaceous birds in transmission of *A.paragallinarum* was evaluated experimentally and the efficacy of a commercial vaccine against the disease was assessed. Only five isolates were recovered and all were serotype C and NAD-dependent. Overall seropositivity was 40.5%, with 18%, 0.5% and 22 % against serotypes A, B and C, respectively. No antibodies were demonstrated in turkey sera. Multidrug resistance was demonstrated in three isolates and resistance genes for sulphamethoxazole (*sul2*), ampicillin (*blaTEM*), tetracycline (*tetC* and *tetA*) and streptomycin (*strA)* were demonstrated. Only antimicrobial resistance markers differentiated isolates according to their epidemiological background. Commercial and local chickens were equally susceptible to challenge while turkeys and guinea fowls showed transient mild signs and did not transmit infection neither did they pick infection from infected chickens. The isolates were resistant in normal chicken serum at both 3 and 6 hours of incubation but resistant at 3 hours and sensitive at 6 six hours in turkey and guinefowl sera. The susceptibility of the isolates in serum correlated with their pathogenicity in the different poultry. No

carrier status was demonstrated in this study using PCR and culture. Vaccinated birds were partially protected. This is the first report of isolation and characterisation of *A. paragallinarum* from Uganda. Resistance genes in *A. paragallinarum* are also reported for the first time. Studies based on a wider collection of isolates would be important to elucidate the mechanisms behind their persistence in carrier chickens and the potential exchange of resistance genes among respiratory tract pathogens.

DECLARATION

I, Denis Karuhize Byarugaba do hereby declare to the Senate of Sokoine University of Agriculture, that this thesis is my own original work and to the best of my knowledge, has never been submitted for any degree in any other university.

Signature.....

Date: 24 July 2006

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Dedication

To my children

Diana Byarugaba and Derrick Byarugaba

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LIST OF ABBREVIATIONS AND SYMBOLS

AAC	Aminoglycoside acetyltransferases
AFLP	Amplification fragment length polymorphism
ANT	Aminoglycoside nucleotidyltransferases
Apg	*Avibacterium paragallinarum*
APH	Aminoglycoside phosphotransferases
AP-PCR	Arbitrarily primed polymerase chain reaction
bp	Base pairs
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferases
cfu	Colony forming units
dATP	di-adenine triphosphate
dCTP	di-cytosine triphosphate
dGTP	di-guanine triphosphate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dNTPs	di-nucleotide triphosphates
dTTP	di-thymine triphosphate
ELISA	Enzyme linked immunosorbent assay
ENRECA	Enhancement of Research Capability
ERIC	Enterobacterial repetitive intergenic consensus sequences
FAO	Food and Agriculture Organisation
GA	Glutaraldehyde

GMT	Geometric mean titers
HA	Haemagglutinating Antigen
HI	Haemagglutination inhibition
HP-2 PCR	*Haemophilus paragallinarum*-2 Polymerase chain reaction
HU	Haemagglutination unit
IC	Infectious coryza
Kb	Kilo base
kg	Kilogram
LC	Local council
LPS	Lipopolysaccharide
MDR	Multi drug resistance
mg	Milligram
mm	Millimeter
MW	Molecular weight
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
OMP	Outer membrane protein
ONPG	Ortho-nitro-phenyl β-D galactosidase
OTR	*Ornithobacterium rhinotracheale*
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with thimerosal
PCR	Polymerase chain reaction

PFGE	Pulsed-field gel electrophoresis
RAPD	Randomly amplified polymorphic DNA
REA	Restriction enzyme analysis
REP	Repetitive extragenic palindromic sequences
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SE	Standard error
TAE	Tris-acetate EDTA buffer
TM/SN	Test medium supplemented with serum and NADH
UV	Ultraviolet
vol/vol	volume in volume
µg	Microgram
µl	Microliter

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Avibacterium paragallinarum (Apg) (Basonym; *[Haemophilus] paragallinarum*) is an important avian pathogen world wide causing a highly contagious acute respiratory disease in chickens called infectious coryza (IC) (Blackall *et al.*, 2005). The chicken is the natural host while turkeys, ducks and other birds are refractory to infection (Yamada, 1981; Blackall *et al.*, 1997). All ages are susceptible and may become carriers (Namgoong *et al.*, 1982). Infectious coryza occurs mainly on farms keeping multi-aged birds and the severity of the disease is influenced by several factors including; the virulence of the bacteria, environmental factors, as well as other concurrent infections (Delaplane *et al.*, 1934; Page, 1962a; Raggi *et al.*, 1967; Sandoval *et al.*, 1994). The interactions of these different entities within and among flocks make the epidemiology of the disease complex.

The disease is of economic significance (Blackall, 1999) as it is associated with decrease in egg production of up to 40% in layer flocks (Mouahid *et al.*, 1989; Sandoval *et al.*, 1994) and condemnation of carcases due to upper respiratory disorders in broilers (Droual *et al.*, 1990). Infectious coryza is normally acute and spreads rapidly with high morbidity of up to 60-80% in chicken flocks (Uchida *et al.*, 1990; Sandoval *et al.*, 1994). The disease is characterised by facial swelling, nasal discharges, anorexia, lacrimation and sometimes diarrhoea. The mortality may range from 1 to 15% and tends to increase when complicated by other infections (Linzitto *et*

al., 1988; Mouahid *et al.*, 1989; Srithar *et al.*, 1997). Under field conditions, outbreaks are usually accompanied by complicating infections that are persistent in nature and frequently re-occur (Verma, *et al.*, 1985; Sandoval *et al.*, 1994).

Extensive investigations of the disease in many countries have been frustrated by the difficulties associated with the conventional diagnostic methods that are based on clinical signs, the isolation of the organism by culture and confirmation of the isolates by extensive biochemical characterisation (Blackall *et al.*, 1997). Clinically, chickens with signs suggestive of infectious coryza, may also be due to infection with *Ornithobacterium rhinotracheale*, (Amonsin *et al.*, 1997). When complicated by infectious agents, clinical signs of the disease become confusing and this frustrates accurate diagnosis (Blackall, 1999).

Isolation and biochemical characterisation require specialised complex media (Blackall and Yamamoto, 1998). *A. paragallinarum* are relatively slow growing and difficult to recover in pure cultures because they can be overgrown by related organisms such as *Avibacterium volantium*, *Avibacterium avium*, *Avibacterium* species A that are part of the normal flora in chicken and less nutritionally demanding (Blackall, 1999). High failure rates in detection by culture of *A. paragallinarum* from field samples may also be due to poor sample collection, delayed transport or poor storage (Blackall and Yamamoto, 1998).

The recent advances in molecular biology have offered great opportunities for handling fastidious organisms. Nucleic acid amplification techniques have

particularly found wide applications for the detection of a wide variety of infectious agents especially those that cannot grow *in-vitro* or where current culture techniques are less sensitive or where prolonged incubation times and complex media are required (Ieven and Goossens, 1997). A new PCR technique has been recently developed for the diagnosis of *A. paragallinarum* (Chen *et al.*, 1996). This PCR is more useful in the diagnosis of field clinical infections than culture (Chen *et al.*, 1998b) and is hoped to facilitate extensive studies on this organism for a better understanding of the disease epidemiology. Advances in molecular technology have also enabled appropriate classification of the organism. Until recently, the taxonomic position of *A. paragallinarum* was uncertain and had a temporary name *[Haemophilus] paragallinarum* (*species incertae sedis*; a preliminary name) (Mutters *et al.*, 1985). *A. paragallinarum* now belongs to a genus ***Avibacterium*** proposed by Blackall *et al.*, (2005) together with former so-called avian haemophili based on sequence analysis of 16S rRNA.

Infectious coryza is usually controlled either by vaccination or by therapeutic means using antibiotic treatment during outbreaks of infection. Like for many infectious diseases, vaccination would be the most ideal control strategy. However, vaccine failures have been reported due to occurrence of serotypes different from the strains used in most commercial vaccines and the difference in the virulence of different strains (Blackall *et al.*, 1994a; Sandoval *et al.*, 1994; Bragg 2005).

Antibiotics have also been extensively used to control the disease when it occurs. Treatment failures, however, have been reported due to antibiotic resistance (Lu *et al.*, 1983; Reece and Coloe, 1985; Verma *et al.*, 1985; Blackall, 1988; Blackall *et al.*,

1989; Takahashi *et al.*, 1990; Prasad *et al.*, 1999). Although this resistance is likely to have sustained the presence of the disease in many countries, it has received relatively little attention and the resistance mechanisms have not been described.

The main source of infection is said to be clinically infected or carrier birds although the carrier status has not been clearly demonstrated (Blackall *et al.*, 1990d). Periodic outbreaks have been associated with stressful factors such as cold weather or physiological changes in the body. For example birds at the point of lay have been reported to be more susceptible to Apg due to associated physiological changes in the body and the energy demands on the body (Blackall, 1999).

In Uganda, infectious coryza has been reported in chickens basing on clinical symptoms and pathological lesions (Ojok, 1993). However, no information on surveys or isolation of the organisms is available and the epidemiological features of the disease in poultry in Uganda are still obscure. Whereas the causative agent *A. paragallinarum* has been isolated and characterised in many developed countries, the difficulties associated with its isolation and handling have constrained studies of this organism in many developing countries due to the lack of good microbiological facilities for culture mainly because of high costs.

The diagnosis of the disease in the developing world has therefore, depended on the clinical picture of the disease and pathological lesions. Because of reports of other bacteria such as *Ornithobacterium rhinotracheale* causing similar clinical signs (Bragg *et al.*, 1997), clinical diagnosis of infectious coryza is no longer reliable. Thus

proper control measures based on the clinical picture are not always rational and sometimes do result in failures in the management of the disease. With advancement in diagnostic methods such as the polymerase chain reaction, it has been possible to develop specific diagnostic tools for many infectious diseases including infectious coryza. It was therefore important that investigations be carried out to ascertain the occurrence of *A. paragallinarum* in Uganda and characterise the isolates using modern techniques so that rational control strategies can be implemented.

1.2 OBJECTIVES OF THE STUDY

1.2.1 Overall Objective

The overall objective of this study was to determine the occurrence of *A. paragallinarum* in Uganda and characterise the isolates as a basis for making rational decisions for control of the disease.

1.2.2 Specific objectives

The specific objectives were to: -

1. Determine the occurrence *A. paragallinarum* in poultry in Uganda.
2. Isolate and characterise *A. paragallinarum* isolates obtained in the study
3. Establish the role of gallinaceous birds (turkeys and guinea fowls) in the transmission of *A. paragallinarum* infection.
4. Evaluate the efficacy of the infectious coryza vaccines available in the country against the disease.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Introduction

Domestic fowl are the most important type of poultry kept in many African countries, a practice which stems from ancient traditions. In these countries, poultry production is dominated by free-range local poultry raised on a free-range system, which comprise about 80% in most African countries (Kitalyi, 1996). Village producers keep small flocks of between five and twenty birds per household, where women and children play a key role in their management (Scola, 1992; Gueye, 1998). Rudimentary coops or shelters may be provided to give some protection against bad weather and night predators. Thus, village fowl are maintained on small land with little labour and capital inputs (described as low-input-low-output system) and can therefore be kept even by the poorest social strata of the rural population. Because of low productivity, indigenous fowl production in Africa has been neglected and is frequently considered by farmers as an insignificant occupation compared with other agricultural activities. Nevertheless, these poultry provide the population with a vital source of protein and income. In addition, they play an important role within the context of many social and/or religious ceremonies (Gueye, 1998).

Commercial poultry, on the other hand, has undergone tremendous improvement in terms of management and disease control (Zander *et al.*, 1997). The rapid growth of the commercial poultry industry is attributed to the efficiency of poultry in converting

vegetable protein into animal protein and the relative ease with which new technologies can be transferred between countries for improved and efficient production. This has resulted in intensified production systems to meet consumer demands and has necessitated efficient management and control of diseases (Zander *et al.*, 1997)

The total fowl population in Africa was estimated at 1,868 million in 1995, producing 1,695,620 metric tones of eggs and 2,096,000 metric tones of meat (FAO, 1996). In Uganda, the poultry population was estimated at about 26 million, of which rural free-range local poultry were estimated to represent 80- 90 % of the total, and yet very little attention is paid to them (FAO, 1996). The majority who keep this type of poultry are the rural poor (Byarugaba *et al.*, 2002).

Many developing countries have a great challenge to uplift their rural poor out of abject poverty. Improvement of productivity of rural poultry could possibly help lift these poor people to another better economic level. This has been proved in other areas such as Bangladesh, where improvement in management by a semi-scavenging poultry system, has had a positive impact on women and children's lives (Alam, 1997).

Despite the potential of free-range local poultry to alleviate poverty and improve the quality of life of the hardcore poor and their children, little attention has been paid to it as judged by records of limited funding and expertise, and less regard of smallholder poultry as an area of importance in terms of political aspects or scientific

prestige (Sorensen, 1999). Research in the area of free-range local poultry has gained momentum recently, although it is yet to be well streamlined into many national and international agricultural research systems. The development of the commercial sector in developing countries is also still limited by occurrence of diseases, expensive feeds and poor management.

One of the major constraints to village and commercial poultry production in Africa is undoubtedly diseases (Minga and Nkini, 1986; Ojok, 1993). Diseases caused by infectious and parasitic agents are frequently complex and depend upon the characteristics of the host, the disease agents and their interaction with the environment.

The most prevalent diseases among poultry include Newcastle disease, respiratory disease complexes, fowl pox, diarrhoea, salmonellosis, collibacilosis, fowl cholera, and parasitic conditions (Minga and Nkini, 1986; Ojok, 1993; Byarugaba *et al.*, 2002). Rearing losses are generally severe especially due to high mortalities. It is estimated that mortality of indigenous poultry under free-range conditions may reach as high as 70% in chicks up to 8 weeks of age (Sonaiya, 1990) and infectious diseases are reported to be the main cause of death (Thitisak *et al.*, 1988). Cumulative levels of mortality influence the structure of the flocks whereby 30-50% of flocks are normally chicks (Wilson *et al.*, 1987). Understanding the limitation to village free-range poultry systems in order to improve production is critical to improvement of the livelihoods of the rural folk. In addition, knowledge of the epidemiology and

management of important diseases in poultry is essential in order to improve the production and health of poultry in developing countries.

2.2 Infectious coryza

Infectious coryza is a serious disease in poultry production systems as it results in production losses and mortality (Thitisak,*et al.*, 1988; Srinivasa *et al.*, 1989). The disease has received little attention because of its notoriety in the isolation of the causative agent. The correct identification of bacteria isolated from chicken showing signs of coryza is an important requirement for development, application and monitoring of sustainable prevention and control measures for the disease.

2.2.1 Occurrence of infectious coryza

A. paragallinarum occurs worldwide. Isolation and characterisation of the organism has been described in a number of countries on all the continents (Blackall, 1999). The isolation of Apg in Africa has only been reported in Zimbabwe (Bragg, 2002a), South Africa (Bragg *et al.*, 1997) and Morocco (Mouahid *et al.*, 1989). In many other countries there are reports of the disease, based on clinical symptoms but the causative agent has not been isolated.

In India, infectious coryza has been reported as the second most important bacterial disease after salmonellosis (Srinivasa *et al.*, 1991) and is reported to be the most common cause of deaths in chickens less than 8 weeks old or those over 6 months old

in Thailand (Thitisak *et al.*, 1988). The impact of the disease in many developing countries is not well documented because the organism has not been isolated and characterised. Often vaccinations are conducted without isolating the organism.

Although the disease has been reported in Uganda basing on clinical signs and pathological lesions (Ojok, 1993), no isolation of the causative agent has ever been done. Over a period of 15 years, only eight cases of IC have been recorded at the pathology department, Makerere University (Makerere University, Department of Veterinary Pathology Laboratory Records; unpublished data). Even then, these cases were never confirmed by isolation of *A. paragallinarum* despite the fact that the pathological lesions are usually not pathognomonic.

2.2.2 Aetiology

Infectious coryza is caused by *Avibacterium paragallinarum* (Basonym: *Haemophilus paragallinarum)* (Blackall *et al.*, 2005). The first isolation of the causative agent of infectious coryza was first reported in 1932 and was then named *Bacillus haemoglobinophilus coryzae gallinarum* by De Blieck, (1932). Most isolates show symbiotic growth and a requirement for 5–10% CO_2 for growth in early *in vitro* passages. The organisms are catalase negative, with variable phosphatase reaction. No acid is produced from glycerol, D-arabitol, L-arabinose, D-arabinose, meso-inositol, D-galactose, lactose, trehalose or raffinose. The production of acid from D-ribose, D-xylose, L-fucose, maltose, D-mannitol, D-sorbitol and dextrin is variable. Some require the nicotinamide adenine dinucleotide (NAD), while others do not (Mouahid *et al.*, 1992a).

A. paragallinarum was originally defined as "Gram-negative coccobacilli or rods that require accessory growth factor V (Co-enzyme 1: nicotinamide adenine dinucleotide, NAD or its phosphate)" before the occurrence of the NAD-independent isolates (Blackall *et al.*, 1997). This has been overtaken by more frequent isolation of strains that do not require the V-factor; also called the NAD-independent; which have been reported in South Africa (Mouahid *et al.*, 1992a) and South America (Garcia *et al.*, 2004) with increasing frequency.

2.2.2.1 Taxonomic position of *A. paragallinarum*

A. paragallinarum belongs to the family *Pasteurellaceae* as defined by Olsen *et al* (2004). The family *Pasteurellaceae* Pohl 1981 is currently composed of over 70 species in 11 genera namely; *Pasteurella* Trevisan 1887, *Actinobacillus* Brumpt 1910, *Haemophilus* Winslow *et al.* 1917, *Lonepinella* Osawa *et al* 1996, *Mannheimia* Angen *et al* 1999, *Phocoenaocater* Foster *et al* 2000, *Gallibacterium* Christensen et *al* 2003, *Histophilus* Angen *et al* 2003, *Volucribacter* Christensen *et al* 2004, *Nicoletella* Kuhnert *et al* 2005, and *Avibacterium* Blackall *et al* 2005 (ICSP, 2005). Over 70 species or taxa from human, avian, other mammals and reptilian sources have been described belonging to these genera. The family *Pasteurellaceae* as a whole and of its component genera has undergone dynamic taxonomic changes with over five genera being named in the last two years including that of genus *Avibacterium*.

Until recently, the taxonomic position of *Avibacterium paragallinarum* was uncertain. *A. paragallinarum* is one of the five species under the new genus *Avibacterium* proposed by Blackall *et al.*, (2005) based on sequence analysis of 16sRNA. On the basis of DNA:DNA hybridisation studies it did not belong to the genera *Haemophilus, Actinobacillus or Pasteurella* as defined by Mutters *et al* (1985). Therefore for a long time it has had the temporary name *[Haemophilus] paragallinarum* (*species incertae sedis*) (Mutters *et al.*, 1985). The organism was previously named *Haemophilus gallinarum* (Eliot and Lewis, 1934; Delaplane *et al.*, 1934) because it was found to require both X factor (haemin) and V factor (nicotinamide adenine dinucleotide, NAD) for growth at that time. However, Page (1962a) and Biberstein and White (1969), found their isolates were dependent only on NAD for growth and not on both X and V factors. Hence Biberstein and White (1969) proposed the name *Haemophilus paragallinarum* for those haemin independent and NAD-dependent haemophili causing infectious coryza in chickens.

Haemin-independent and NAD-dependent haemophili, which did not produce infectious coryza and were different from *H. paragallinarum* in their physiologic, biochemical and serological properties were reported in 1962. Page, (1962a) referred to them as *Haemophilus avium*. These were later transferred to genus *Pasteurella* basing on DNA:DNA hybridisation studies (Mutters *et al.*, 1985). Horner *et al* (1992) reported for the first time, the isolation of NAD independent isolates from chickens showing signs of infectious coryza in South Africa. These were later confirmed as *A. paragallinarum* basing on extensive phenotypic characterisation and DNA base

composition (Mouahid *et al.*, 1992a) and later by the species-specific PCR (Miflin *et al.*, 1999)

2.2.2.1.1 Phenotypic based phylogenetic relationships

Historically, microbial taxonomy has been conducted using a variety of physical and biochemical tests that allow the grouping of microbial isolates into genera and species. Members of the family *Pasteurellaceae* were originally classified on the basis of a limited number of phenotypic characteristics (Bisgaard, 1995). In particular, organisms were assigned to the family on the basis of their requirements for the growth factors hemin and/or nicotinamide adenine dinucleotide and on the basis of their ability to cause disease in vertebrates.

Major studies based on phenotypic traits included those on *Haemophilus*, *Actinobacillus* and *Pasteurella* (Sneath and Stevens, 1985). The studies by Sneath and Stevens (1985) indeed suggested overlapping interrelationships between *Pasteurella* and *Actinobacillus*. The discovery of *A. paragallinarum* organisms that do not require NAD (Mouahid *et al.*, 1992a, Garcia *et al.*, 2004) have discounted the earlier basis for classifying these organisms as requiring the V-factor for growth and revealed the complexity of using phenotypically expressed characteristics for classifying organisms. This has, therefore, justified the use of genetic information for identifying isolates.

2.2.2.1.2 Molecular taxonomy of *A. paragallinarum*

More recent genotypic studies have shown that many members of the family *Pasteurellaceae* had been misclassified (Mutters *et al.*, 1985). The use of molecular markers has improved analysis of phylogenetic relationships among the members of the family *Pasteurellaceae* and indeed many other organisms. Several markers have been used to examine the relationships among these bacteria. Studies with genetic transformation, restriction enzyme analysis, restriction fragment length polymorphism, DNA-DNA hybridisation, DNA-rRNA hybridisation, and 16S rRNA sequencing have provided various levels of classification and understanding of the relationships of these organisms with varying levels of accuracy. Full sequencing of proteins and nucleic acids provides a more powerful approach for measuring evolutionary relationships. Comparative sequence analysis of ribosomal RNAs or their corresponding genes, is currently the most widely used approach for the reconstruction of microbial phylogeny (Korczak *et al.*, 2004).

Ribosomal genes such as the *rpoB* gene and other housekeeping genes have been used in the phylogenetic analysis of the family *Pasteurellaceae* (Christensen *et al.*, 2004b). The *rpoB* gene is strongly conserved within the various species of the family *Pasteurellaceae* and has been found valuable, in combination with 16S rRNA sequencing, for phylogenetic studies of the *Pasteurellaceae*. It was suggested that it could be used as a basis for reorganizing the current taxonomy of this family (Korczak *et al.*, 2004). Other studies on speciation derived from 16S rRNA and housekeeping gene sequence comparisons have resulted in different evolutionary scenarios for members of the *Pasteurellaceae*. The phylogeny based on the housekeeping genes support observed host-parasite associations between

Mannheimia, *Actinobacillus sensu stricto* and *[Pasteurella] trehalosi* and animals with paired hooves (Christensen *et al.*, 2004b).

Major advances in understanding the phylogeny of the members of the family *Pasteurellaceae* started with studies involving DNA-DNA hybridisation (Mutters *et al.*, 1985) and rRNA:DNA hybridisation (De Ley *et al.*, 1990). These studies defined species belonging to *sensu stricto* definitions of the genera *Pasteurella*, *Haemophilus* and *Actinobacillus* as well as species belonging to *species incertae sedis* as was the case with *[Haemophilus] paragallinarum* (Mutters *et al.*, 1985). These studies showed that the phylogenetic structure of the *Pasteurellaceae* is complex and needed further reclassification. Up to now, many taxa still do not fall into well-defined clusters, and the branching of genus-level clusters remains unclear.

Studies involving full 16S rRNA sequencing of strains representing many described species or taxa have proved extremely useful for determining phylogenetic relationships among eukaryotic and prokaryotic organisms (Woese, 1987). Unlike hybridization studies, complete distance matrices, where the similarity of every sequence to every other sequence is determined, can be used. Treeing algorithms that correctly account for differing branch lengths are then applied to the distance matrix data to produce phylogenetic trees (Woese *et al.*, 1985). The use of 16S rRNA gene sequencing has revealed that many of the avian members of the family *Pasteurellaceae* form phylogenetically related clusters (Dewhirst *et al.*, 1993; Olsen *et al.*, 2004). Dewhirst *et al.* (1993) recognized avian clusters 3A, 3D and 7. The

avian 3A and 3D clusters have been re-termed avian 16S rRNA cluster 18, while cluster 7 is now termed 16S rRNA cluster 21 (Olsen *et al.*, 2004).

Several of these avian members of *Pasteurellaceae*, have been found to belong to phylogenetically related clusters that have led to re-classification of 16S rRNA cluster 18 into several new genera. Christensen *et al* (2003b) showed that avian 16S rRNA cluster 18 consisted of at least 11 species or species-like taxa. Christensen *et al* (2003a) also proposed a new genus *Gallibacterium* for the avian 16S rRNA cluster 18, which incorporates organisms once known as [*Pasteurella haemolytica*], '*Actinobacillus salpingitidis*' and *Pasteurella anatis*. Similarly, Bisgaard taxon 33 has been reclassified as *Volucribacter psittacicida* and *V. amazonae* (Christensen *et al.*, 2004a).

Using similar 16S rRNA sequences, Blackall *et al* (2005) have recently proposed a new genus *Avibacterium* for former avian haemophili. This genus now includes *Avibacterium paragallinarum* (Basonym: *[Haemophilus] paragallinarum*), *Avibacterium avium* (Basonym: *Haemophilus avium*), *Avibacterium gallinarum* (Basonym: *Pasteurella gallinarum*), *Avibacterium volantium* (Basonym: *Pasteurella volantium*), and *Avibacterium* species A. (Basonym: *Pasteurella* species A).

While 16S rRNA sequence comparison analysis results in complex phylogenetic trees, it allows determination of closely related strains to be grouped together into

species. Previous 16S rRNA phylogenetic studies of the family *Pasteurellaceae* by Dewhirst *et al* (1992) revealed rapidly and slowly evolving strains presenting clear phylogenetic relationships, which are now being proposed as new genera. Besides, there are still a number of them not yet allocated to a genus or species.

Previous classification of the genus *Avibacterium* by Biberstein and White (1969) led into the designation of infectious coryza-causing bacterium as *Haemophilus paragallinarum* because they required V-factor for growth. With more and more NAD-independent isolates being reported, it has become difficult to identify with precision organisms reported in previous studies. This is further complicated by recent description of other new organisms causing similar clinical signs being reported such as *Ornithobacterium rhinotracheale* (Amonsin *et al.*, 1997) and further reclassification of former avian haemophili into a new genus (Blackall *et al.*, 2005). This calls for thorough investigations of the susceptibility and the role of *A. paragallinarum* in chickens and other related poultry in which reports of infectious coryza was made. Earlier observations indicated these related poultry to be refractory to the disease (Blackall *et al.*, 1997).

2.3 Pathogenesis

The pathogenesis of *A. paragallinarum* infections is still not well understood. Attachment of bacteria to mucosal surfaces is the initial event in the pathogenesis of most infectious diseases that gain their entry through the mucosal system (Beachy, 1981). The extent to which these mucosal pathogens adhere, influences the amount of colonisation and thus the virulence of the organisms. Surface components of a bacterial cell are important in its ecology since they mediate the contact of the bacterium with its environment. Microorganisms use such surface components to assess the environment and respond in a way that supports its own existence and survival. The surface properties of a bacterium are determined by the exact molecular composition of its membrane and cell wall, including lipopolysaccharide (LPS), and the other surface structures such as fimbriae and capsules (Saylers and Whitt, 2002).

2.3.1 Adherence of *A. paragallinarum*

A. paragallinarum adheres and multiplies preferentially on the surface of the chicken's nasal mucosa (Nakamura *et al.*, 1993). Adherence of the organism to mucous membranes has been demonstrated both *in vitro,* using chicken embryo fibroblasts, and *in vivo,* in the nasal mucosa and trachea by means of electron microscopy (Ueda *et al.*, 1982; Sawata *et al.*, 1985a). This adherence is thought to be mediated by the capsule (Sawata and Kume, 1983). Haemagglutinating antigens have also been suspected to play a role (Yamaguchi *et al.*, 1993). For both Page's serotype A and C, the assessment of mutants lacking the haemagglutination activity has shown

that the haemagglutinin antigens play a role in colonisation (Sawata and Kume, 1983; Yamaguchi *et al.*, 1993). Nakamura *et al* (1993) also identified *A. paragallinarum* organisms on the epithelium of the nasal passages in formalin-fixed and paraffin-embedded sections by immunoperoxidase methods. This correlated with bacteriological isolation. *A. paragallinarum* strains which are able to adhere to and colonise the mucosa thereby causing disease (Sawata *et al.*, 1985a).

2.3.2 Colonisation by *A. paragallinarum*

Colonisation of the upper respiratory tract and the sinuses by *A. paragallinarum* results into an acute catarrhal inflammation of the mucous membranes (Reid and Blackall, 1984). In uncomplicated cases, this progresses into mucous sloughing off with loss of cilia; edema and congestion of the sinus mucosa with infiltration of heterophils and mucous and desquamated epithelial cells in the lumen (Sawata *et al.*, 1985a). There is mild hyperplasia of the tracheal mucosa and lesions are restricted to the upper respiratory tract (Reid and Blackall, 1984). When complicated by other organisms like *Mycoplasma gallisepticum*, *Escherichia coli*, *Salmonella pullorum*, *Salmonella gallinarum*, *Pasteurella multocida* and respiratory viral infections, extensive lesions are observed (Raggi *et al.*, 1967). Sandoval *et al.* (1994) isolated *A. paragallinarum* from the heart, liver, ovary, intra-abdominal abscesses, eye contents, and also from purulent contents of tarsal tenosynovitis. The significance and contribution of *A. paragallinarum* in such pathology is not known. Kume *et al.* (1984) suggested that the major clinical effects, usually seen in infectious coryza, may be due to the production of a toxin released from the capsule.

Most birds recover from the disease (Blackall *et al.*, 1997) but many of them remain carriers. The organisms seem to persist in the respiratory tract without causing disease until there are stress factors that make the birds start shedding the bacteria again, thereby precipitating re-occurrence of the disease. The ability to attach to the mucus without mucociliary clearance has been reported in other respiratory pathogens such as *H. influenzae* causing chronic symptoms (Kubiet *et al.*, 2000). There may be similar mechanisms in infectious coryza. Extensive investigations are, therefore, important to elucidate what sparks off infection and mediate the persistence of the organisms in the carrier birds.

2.3.3 Virulence Factors

Virulence has been redefined as the relative capacity of a microbe to cause damage in a host (Casadevall and Pirofski, 1999). This ability is genetically determined and recent advances in molecular biology have enabled deeper understanding of the mechanisms of the factors that enable organisms to cause such damage. The first virulence factors to be characterised at the molecular level were bacterial toxins (Saylers and Whitt, 2002). Certain proteins or enzymes displayed on the surface of bacteria significantly contribute to pathogenesis and play a role in the disease process. Often, these proteins are involved in direct interactions with host tissues or in concealing the bacterial surface from the host defence mechanisms. Invasive pathogenic bacteria have, in common, the capacity to overcome the defence mechanisms of their animal host and to proliferate in tissues (Boyle and Finlay, 2003). They each have their own mechanisms and target organs and cause a variety of

clinical signs and diseases, which suggests the existence of great diversity among the bacterial virulence strategies (Saylers and Whitt, 2002).

There is paucity of information concerning the virulence factors of *A. paragallinarum*. Some workers have investigated the association of the characteristics of *A. paragallinarum* with the virulence of this organism in respiratory tract infections (Hinz, 1976; Sawata *et al.*, 1979; Sawata and Kume, 1983; Sawata *et al.*, 1985a; Sawata *et al.*, 1985b). It is well known that *A. paragallinarum* isolates differ in their virulence (Delaplane *et al.*, 1934; Page, 1962a; Kume *et al.*, 1984) and a number of factors have been suspected to be responsible and are discussed further.

2.3.3.1 Capsule and somatic antigens

The capsules of bacteria are generally associated with virulence and it has been demonstrated that encapsulated bacteria are more virulent than non-encapsulated ones (Finlay and Falkow, 1997). The capsule of *A. paragallinarum* has been associated with colonisation (Sawata *et al.*, 1985a) but its role in the development of the lesions associated with coryza is still controversial (Sawata and Kume, 1983; Sawata *et al.*, 1985b). There is evidence that encapsulated organisms are responsible for the disease and produce gross coryza lesions in chickens whose degree of severity is well correlated with the amount of capsule (Sawata and Kume, 1983), while non-encapsulated organisms are said to be avirulent. Sawata and Kume (1983) have

demonstrated that the capsule is also related to resistance of *A. paragallinarum* against chicken blood bactericidal activity.

The presence of the capsule has been demonstrated on culture by iridescence (Sawata *et al.*, 1980) and this has been used to estimate the virulence of the organism *in vitro* (Sawata and Kume, 1983). The significance of the capsule against phagocytosis and after entry of the organism has been thoroughly investigated in other organisms (Snipes and Hirsh, 1986) and demonstrated to have varying roles. In *A. paragallinarum*, there have been limited studies to demonstrate this. In one study, Ueda *et al.*, (1982) demonstrated Apg in the cytoplasm of chicken embryo fibroblasts using encapsulated strains which were always enclosed in membrane bound vesicles, indicating that they were resistant to killing mechanisms by the cells.

The major component of the capsule of bacteria is hyaluronic acid, a high molecular weight polymer consisting of alternating residues of N-acetylglucosamine and glucoronic acid (Finlay and Falkow, 1997). Hyaluronic acid has been reported in some *A. paragallinarum* strains (Iritani *et al.*, 1980) but its function is not known. Encapsulated *A. paragallinarum* organisms treated with hyaluronidase result in complete disappearance of the capsule implying that a hyaluronic acid-like substance might be an important component of the capsule (Kume *et al.*, 1984). While the capsule of *A. paragallinarum* is a very important antigen for the attachment and colonisation of the mucosa (Sawata and Kume 1983), it is still controversial whether it is directly involved in the pathological changes. On the other hand, some somatic antigens seem to be responsible for the inefficient adherence of the non-encapsulated

strains (Sawata and Kume, 1983). These somatic antigens are, however, known to be responsible for protective immunity (Kume *et al.*, 1980b; Sawata *et al.*, 1982; Kume and Sawata, 1984).

2.3.3.2 Outer Membrane Proteins (OMPs)

Outer-membrane proteins of pathogenic bacteria are of particular interest in terms of their potential as vaccine candidates and for their roles as virulence determinants (Confer *et al.*, 1995; Prado *et al.*, 2005). The haemagglutinin antigens of *A. paragallinarum* have been suggested to be potential candidates for vaccines against infectious coryza (Iritani *et al.*, 1980). In addition, the *A. paragallinarum* haemagglutinin is a key component of the serotyping schemes which are based on inhibition of haemagglutination activity (Page, 1962a; Kume *et al.*, 1983). The haemagglutinin antigen of *A. paragallinarum* plays a key role not only in serotyping and immunity but also in pathogenicity.

There have been very few studies concerning OMPs of *A. paragallinarum*. Much of the interest has been due to its suspected role in induction of protective immunity (Blackall, 1999). However, outer membrane proteins have also been shown to contribute to the virulence of certain bacteria (Ikeda and Hirsh, 1988). Blackall *et al.*, (1990e) analysed the OMPs of four selected strains of *A. paragallinarum* and some of them were recognised in immunoblots by immune serum. One of them which was strongly recognised in all the isolates by the immune sera had a molecular weight

similar to that of OMPs associated with iron regulation in pathogens like *Pasteurella multocida* (Ikeda and Hirsh, 1988) and *E.coli* (Chart *et al.*, 1988). Confirmation of the role of this OMP in iron regulation using depletion and iron saturation conditions has yet to be done. Other antigens of *A. paragallinarum* which have been demonstrated to be protective (Kume *et al.*, 1980a, Sawata *et al.*, 1982; Kume and Sawata, 1984) are said to be components of the outer membrane of the bacterial cell (Sawata *et al.*, 1984a).

2.3.3.3 Adhesins

A necessary step in the successful colonization and, ultimately, production of disease by microbial pathogens is the ability to adhere to host surfaces (Kerr, 1999). This fundamental property has led to a lot of research over the past two decades in trying to understand how bacterial pathogens adhere to host cells (Beachy, 1981). Data on adhesin biogenesis, the regulation of adhesin factors and, to a lesser extent, the identity of host receptors that are the targets of microbial adhesin factors have of recent become available (Saylers and Whitt, 2002). The majority of adhesins are surface-exposed proteins with a special structure that enables them to interact with host cells due to specific carbohydrate or polypeptide receptors. The biochemical and biophysical processes of adhesin-receptor interaction are similar to those observed in interaction between antibody-antigen, carbohydrate-lectin, hormone-receptor or granulocyte-adhesion molecules (Saylers and Whitt, 2002).

Not all adhesins are essential virulence factors. The specific role of a particular adhesin in disease has been surprisingly difficult to define, since a single pathogen expresses many adherence factors. A wide array of bacterial adhesins has been found in a variety of pathogenic microbes. Some pathogens have been reported to possess no less than a dozen different adhesins expressed at one time or another or in different strains of the same species (Beachy, 1981).

Bacterial adhesins can be divided into two major groups, namely; pili (fimbriae) and nonpilus adhesins (afimbrial adhesins). One of the major features among diverse pili is the conservation of the molecular machinery needed for pilus biogenesis and assembly onto the bacterial surface. The host receptor that a pathogenicity-associated adhesin recognizes probably determines the tissue specificity for that adhesin and bacterial colonization or persistence. It has been demonstrated that bacteria lacking some pilus gene cluster have decreased adherence to mucins, supporting a role of pili in the binding of organisms like *H. influenzae* to human respiratory mucins (Kubiet *et al.*, 2000). While the ability of *A. paragallinarum* to adhere to mucous membranes has been shown to occur, no definitive adhesins have been demonstrated and characterized (Sawata and Kume, 1983).

2.3.3.4 Toxins

Pathogenic bacteria produce many substances that are directly or indirectly toxic to host cells. Secreted microbial proteins, usually enzymes, which kill host cells at exquisitely low concentrations are called exotoxins (Gyles and Theon, 1993). Other

non-proteinaceous toxic bacterial substances, like lipopolysaccharide (LPS) or endotoxin, do not use a direct enzymatic mechanism to injure host cells and are biologically active at much higher concentrations. Endotoxin is a critical component of the gram-negative cell wall. All gram-negative pathogens make the endotoxin, although its toxicity varies among species (Saylers and Whitt, 2002).

The role of toxins in the pathogenesis of *A. paragallinarum* infections has not been well studied. Sawata and Kume (1983) suggested that appearance of signs in chickens is related to a certain substance in the capsule or a substance that is specifically produced by encapsulated *A. paragallinarum*. Later Kume *et al.*, (1984) suggested that the toxic substance responsible for the signs might be released from the encapsulated organisms during proliferation. The definitive toxic substance and its role, has yet to be defined. Crude extracts of *A. paragallinarum* polysaccharide have been demonstrated to be toxic to chickens and may be responsible for the toxic signs that may follow administration of a bacterin (Iritani *et al.*, 1981). However, the role of this component in the natural occurrence of the disease is still unknown.

2.3.3.5 Iron acquisition mechanisms

The low concentration of free iron in physiological fluids in animals (about 10^{-18} $mol.L^{-1}$) is not sufficient to allow bacterial growth which requires a concentration of about 10^{-6} $mol.L^{-1}$ (Andrews, 1998). Microorganisms have to adapt to the iron limitation present in mammalian hosts by evolving diverse mechanisms for the

assimilation of iron sufficient for growth. In addition, many bacterial pathogens have used the low concentration of iron present in the host as an important signal to enhance the expression of a wide variety of bacterial toxins and other virulence determinants (Litwin and Calderwood, 1993). The molecular basis of coordinate regulation by iron has been most thoroughly studied in *E.coli*. In this organism, coordinate regulation of gene expression by iron depends on the regulatory gene, *fur* gene (Escolar *et al.*, 1998). Regulation of gene expression by iron in a number of pathogenic organisms is coordinated by proteins homologous to the fur protein of *E. coli*. Additional regulatory proteins may be superimposed on the *fur* repressor to provide the fine-tuning necessary for the precise regulation of individual virulence genes in response to iron and other environmental signals (Litwin and Calderwood, 1993). Studies of the mechanisms of regulation of iron acquisition systems and virulence determinants by iron have led to a better understanding of the adaptive response of bacteria to the low-iron environment of the host and its importance in virulence.

Numerous bacteria with invasive abilities have developed high affinity iron-acquisition systems, which can compete with the host siderophores such as transferrin, and thus favour bacterial growth in low iron concentration (Dho-Moulin and Fairbrother, 1999). Ogunnariwo and Schryvers (1992) demonstrated that *A. paragallinarum* is capable of acquiring iron from chicken and turkey transferrin, suggesting that iron sequestration by the chicken host may not be an adequate host defence mechanism against this organism. However, detailed studies to demonstrate

the responsible chelating siderophores has not been done and no information is available.

2.3.3.6 Resistance to bactericidal effects of host serum

Resistance to the lytic action of host complement is a well-known virulence associated parameter in many bacteria (Taylor, 1983). This allows such bacteria to resist bactericidal action of the serum. Bactericidal activity of mammalian serum is known to involve activation of complement system. The complement system of the chicken has been reported to be responsible for serum haemolytic activity in a manner analogous to that of the mammals (Rose and Orlans, 1962). The resistance to this action is therefore very essential for bacteria to survive in the host (Gyles and Thoen, 1993). This ability has been associated with the bacterial capsules, which are able to interact with the C-3 to C-3b activators in the classical and alternative complement pathways (Gyles and Thoen, 1993). Although avian complement activation pathways have not been well established, bactericidal action of chicken sera is said to involve complement, as has been observed about the loss of bactericidal activity against bacteria including *A. paragallinarum* which is completely lost by heating at 56^{0}C for 30 min and could be restored by the addition of fresh sera (Sawata *et al.*, 1984b; Sawata *et al.*, 1984c).

Sawata and Kume (1983) and Sawata *et al* (1984b) reported that fresh normal chicken serum with no demonstrable specific antibodies could inactivate non-encapsulated organisms of *A. paragallinarum*, which the encapsulated ones resisted. These data

indicate that serum resistance may play a role in the pathogenesis of infectious coryza in the chicken, although septicaemia has not been reported.

2.3.3.7 Molecular determinants of virulence

In the course of infection, pathogenic microorganisms encounter different types of environments to which they have to adapt in order to survive and multiply (Saylers and Whitt, 2002). Thus, pathogenic bacteria need to synthesize virulence factors that will allow them to colonise a hostile environment and to survive the host immune and non-immune defences. To survive and multiply within a host organism, bacterial pathogens require the coordinated expression of a range of genes. Pathogens continually alter their gene expression profiles in response to the innate immune system and environment of their host as they move from one host niche to another (Boyce *et al.*, 2002). Microorganisms sense the environment and, in response to the signals that they receive, act accordingly by turning off or on the expression of their virulence genes (Gyles and Thoen, 1993). For the majority of virulence factors, the specific host signals that the regulatory proteins detect are not fully understood, although the environmental signals that modulate the expression of virulence genes in many cases have been identified *in vitro*. Parameters such as temperature, osmolarity, pH, source of nitrogen and concentrations of iron, sugars and amino acids, are known to affect the regulation of virulence genes *in vitro* (Dho-Moulin and Fairbrother, 1999).

Very little is known about the molecular basis of the pathogenesis of *A. paragallinarum* infections, its virulence factors and their expression. This has been due to lack of well-developed systems to genetically manipulate this organism. More and more manipulations of the organisms are being reported. Gonzales *et al* (1996) developed a transposon mutagenesis system for *A. paragallinarum* using the Gram-positive transposon Tn916. This was thought to open numerous possibilities for unravelling the genetics of this bacterium. Recently, De Smidt *et al* (2004) characterised the genetic organisation of the capsule transport gene region from *A. paragallinarum*. The region involved in export of the capsule polysaccharides to the cell surface was cloned and the genetic organisation determined. This will enable generation of genetically defined non-capsulated mutants that will facilitate further characterisation of the role of the capsule in the virulence of *A. paragallinarum* isolates. Knowledge of capsule export genes in *P. multocida,* enabled the construction of mutants impaired in capsule export by disruption with a tetracycline resistance cassette, through allelic replacement resulting into a genetically defined acapsular phenotype. Using this well characterised decapsulated mutant, it was possible to unequivocally demonstrate that the presence of the capsule was crucial for virulence (Boyce *et al.*, 2000a; Boyce *et al.*, 2000b).

Various molecular techniques using DNA microarrays, signature-tagged mutagenesis (STM), *in vivo* expression technology (IVET) and proteomics are being used to identify novel virulence factors of various bacteria to determine their roles in pathogenesis (Badger *et al.*, 2000; Angelichio and Camilli, 2002; Harper *et al.*, 2003). The development of DNA microarray methods for studying gene expression on a

whole-genome scale has provided the opportunity to analyse gene expression changes directly in response to growth within a host (Boyce *et al.*, 2002). Genes that are differentially regulated *in vitro* under conditions that mimic those within host organisms have been demonstrated in a number of studies (Mahan *et al.*, 1993). Comparison of bacterial gene expression during growth in the natural host (*in vivo*) can be compared with growth *in vitro* using DNA microarray techniques enhancing deeper understanding of how organisms cause disease.

The DNA microarray is a surface that contains an ordered arrangement of identified open reading frames of a sequenced genome and it is the engine of functional genomics (Kellam, 2000). Its expression profile, provides a genome wide snapshot of the transcriptome (Eisen *et al.*, 1998). Using these technologies, the expression profile discloses, at the transcriptional level, how the microbe adapts to new conditions of growth (Relman, 2002). Adaptation to host microenvironments underlies the capacity of infectious agents to persist in and damage host tissues. The persistence of *A. paragallinarum* in chickens or the environment has been puzzling with speculations of carrier status in recovered birds, but only few studies have reported this (Yamamoto and Somersett, 1964). Yet understanding these persistence mechanisms is central in designing better methods of control.

Unleashing these current state-of-the-art technologies to monitor the whole genome transcriptional response of *A. paragallinarum* within infected tissues has not been attempted. The complex, tissue-specific response, which are the sum of individual responses of the bacteria to specific physicochemical features characterise the host

milieu and could reveal significant data that could help in the understanding of how this organism generates a compromise for its survival in the nasal passages or elsewhere in the chickens for a long time. The genetic basis for pathogenicity can be studied by using microarray-based comparative genomics to characterise and quantify the extent of genetic variability within natural populations at the gene level of resolution (Finlay and Falkow 1997).

Boyce *et al.*, (2002) have used whole genome-scale micro-array analysis to assess the differential gene expression in the host that enables *P. multocida* to survive in the host. The combined use of bacterial and host microarrays to study the same infected tissue may reveal the dialogue between *A. paragallinarum* and the chicken in a gene-by-gene and, site- and time-specific manner that can facilitate the understanding of persistent *A. paragallinarum*. Such technologies have already found their application in related organisms such as *P. multocida* (Paustian *et al.*, 2001) and they could be applied to *A. paragallinarum* to shed some light on some of these questions. Boyce *et al.*, (2004) applied these technologies further to follow the genes expressed in different tissues, which may enable *P. multocida* to survive host defence mechanisms.

2.3.3.8 Role of plasmids in virulence of *A. paragallinarum*

Early in the search for virulence genes, researchers discovered that many of the genes resided on plasmids or phages. However, it was also clear that these genes did not

produce all the physiological changes induced in host cells by various pathogens (Finlay and Falkow, 1997). There have been a few reports of plasmids in *A. paragallinarum* (Blackall, 1999). Bragg *et al* (1993) used crude plasmid extracts from nicotinamide adenine dinucleotide (NAD)-independent strains of *A. paragallinarum* isolated in South Africa to convert NAD-dependent strains of *A. paragallinarum* into NAD-independent isolates, indicating that NAD-independence is carried on a plasmid. Comparison of the pathogenicity of the NAD-dependent with the NAD-independent revealed that the NAD independent isolates were less virulent (Bragg, 2002a; Bragg, 2002b).

Further work has been done with some plasmids isolated from *A. paragallinarum* revealing more relationships of plasmids with virulence characteristics. Characterisation by fully sequencing one of the plasmids indicated that the sequence included a haemocin-producing locus, which expressed a protein capable of killing a range of other Gram-negative bacteria (Terry *et al.*, 2003). The plasmid contained seven open reading frames (ORFs), five of which were highly similar to the haemocin production locus of *H. influenzae* (LiPuma *et al.*, 1990). The mechanism of action of the haemocin protein is unclear. However, haemocin production in *H. influenzae* is strongly associated with type b encapsulated members of this species and it plays a role in host nasopharyngeal colonization by this pathogen (LiPuma *et al.*, 1990)

Haemocin produced by *A. paragallinarum* may play a role in enhancing colonization of the sinus of the chicken by inhibiting the growth of some Gram-negative bacteria that are associated with respiratory disease in chickens. All the isolates of *A. avium*,

A. volantium and *Avibacterium* species A, which are all non-pathogenic and are commonly found in the upper respiratory tract of chickens suffering from respiratory disease due to other agents, were sensitive to the *A. paragallinarum* haemocin (Blackall *et al.*, 1997).

2.3.4 Chicken defence mechanisms

Host defence mechanisms play an important role in the outcome of an infection process. Chicken defense mechanisms are like in other animals, composed of non-specific and specific defenses. The non-specific defense mechanisms protect the host by excluding pathogens, or by creating conditions within the host, which provide an inhospitable environment for a wide range of pathogens. Barriers to entry and survival of pathogens in the respiratory system include the mucus coat (Fedde, 1998). Once a pathogen has overcome the entry barriers and gained entry to the host, the initial response is an inflammatory response.

Macrophages are part of the first line of defense against invading organisms, and several elements of bacteria enable them to circumvent the microbicidal action of these phagocytes. Upon interaction with macrophages, bacteria impair phagocytosis, inhibit the respiratory burst, trigger apoptosis, and suppress the normal release of tumor necrosis factor alpha (TNF-α) (Saylers and Whitt, 2002). Each of these four aspects has an important effect in enabling the bacteria to survive and invade host tissues, hence causing damage.

Poultry are able to express antimicrobial peptides, classified as beta-defensins, which have been identified from epithelial cells of chicken and turkeys (Zhao *et al.*, 2001). The peptides are an essential component of innate immunity in a range of species. Beta-defensins are a family of antimicrobial peptides characterized by six cystein residues forming beta-defensin motifs that are also found in bovines, ovines, swines, and humans (Sugiarto and Yu, 2004). These peptides are active against a wide range of microorganisms including Gram-positive and negative bacteria, fungi, and yeast. Antimicrobial peptides are generally thought to act by disrupting the membrane integrity of microbes (Kagan *et al.*, 1990). There have been newly characterized epithelial beta defensins in chicken (*Gallus gallus*) and turkeys (*Meleagris gallopavo)* called gallinacin-3 and gallopavin-1 (GPV-1), respectively. Zhao *et al* (2001) demonstrated significant increase in the expression of gallinacin-3 after experimental infection of chickens with *A. paragallinarum,* implying that it may play a role in the defense against infectious coryza.

2.4 Clinical signs

Infectious coryza is characterised by catarrhal inflammation of the nasal sinuses, manifested as nasal discharges, lacrimation and facial swelling. This normally results into decreased feed and water intake that translates into poor growth in the young stocks and reduced egg production in layers that may be as high as 40% (Blackall *et al.*, 1997). Anorexia and diarrhoea may set in at later stages especially when complicated by other organisms (Sawata and Kume, 1983, Verma *et al.*, 1985).

Infectious coryza has also been reported in broilers, with the typical signs leading to condemnation of carcases at slaughter (Uchida *et al.*, 1990). Droual *et al* (1990) reported an unusual swollen-head-like syndrome where *A. paragallinarum* organisms were recovered with no association of other infectious agents. Infectious coryza takes a different form when complicated by other bacterial or viral infections. This has been reported where *A. paragallinarum* was isolated along with other organisms such as *Mycoplasma gallisepticum, M. synoviae, E.coli, Pasteurella multocida and Salmonella spp*, and infectious bronchitis virus (Sandoval *et al.*, 1994). In these cases, septicaemia, arthritis and other unusual signs were reported, followed by recovery of the organism in distal organs such as the liver, kidney and synovial fluid. These divergent clinical manifestations of the disease make diagnosis difficult, especially where facilities for isolation of the organism may not be easily available.

Differential clinical diagnosis of the disease has become more complicated by the recent reports of isolation of other bacteria causing similar clinical signs. *O.rhinotracheale* was isolated from the sinuses of chickens showing clinical signs similar to those associated with infectious coryza (Bragg *et al.*, 1997). The similarity in the clinical signs is likely to create confusion as to the causative agent, which re-emphasizes the requirement for isolataion and precisely identifying the causatve agent.

2.5 Diagnosis

The diagnosis of infectious coryza has been based on clinical signs in many developing countries because of the difficulties associated with the isolation and identification of the causative agent. While the clinical signs described give a tentative diagnosis it must be differentiated from other infections with similr clinical manifestation caused by different organisms. Pathological lesions are not pathognomonic either; therefore, definitive diagnosis is still dependent on the isolation and precise characterisation of the causative agent (Blackall *et al.*, 1997)

The correct and confident identification of bacteria isolated from chickens showing signs of coryza is important for development, application and monitoring of sustainable prevention and control measures for the disease. Conventional diagnosis of infectious coryza has been based on typical clinical signs, the isolation of the organism by culture and confirmation of the isolate by extensive biochemical characterisation (Blackall *et al.*, 1997). However, chickens with clinical signs suggestive of infectious coryza, may also yield *O. rhinotracheale*, NAD independent; *A. volantium*, (NAD-dependent and NAD-independent); *A. avium*, (NAD-dependent and NAD-independent); *Avibacterium* species A, (NAD-dependent and NAD-independent) (Mutters *et al.*, 1985; Amonsin *et al.*, 1997). The presence of many other bacteria in complicated cases, further frustrate accurate clinical diagnosis of the disease further. Differential diagnosis from such diseases as chronic fowl cholera,

chronic respiratory disease, fowl pox, swollen head syndrome, and A-avitaminosis, which may produce related clinical signs, should always be considered.

2.5.1 Isolation of the bacteria

The biochemical characterisation requires the availability of specialised media that can support the NAD dependent strains and such media are often expensive (Blackall *et al.*, 1997). High failure rates in detection by culture of *A. paragallinarum* from field samples have been frequently associated with problems of poor sampling, delayed transport and poor storage (Blackall, 1999).

Several attempts have been made to improve the survival of the organisms during sample transportation and storage. Chen *et al* (1998b) compared the survival of *A. paragallinarum* in different storage media and conditions from sinus samples collected from artificially infected chicken. The organisms survived longest in glycerol-enriched phosphate buffered saline (3 days) when stored at 4°C or –20°C, whereas other media were not as effective. Recently Bragg *et al* (2004) have also established that the bacterium remains viable for up to 18 days in Amies Transport Medium containing all the supplements, when stored at 4°C or 37°C.

2.5.2 Serology

There have been several attempts to detect antibodies against *A. paragallinarum* in chickens using haemagglutination inhibition and ELISA techniques (Blackall, 1999, Zhang *et al.*, 1999). However, these have not found wide applications because of the

occurrence of different serotypes in different countries and unavailability of a single test for their detection. Little work, therefore, has been done to harmonise the various assays for detection of antibodies to *A.paragallinarum*. While ELISA showed good potential, it still requires development and commercialisation of a panel of monoclonal antibodies to all the serotypes. Verschoor *et al* (1989) were the first to establish a panel of monoclonal antibodies against *A. paragallinarum* and since then other monoclonal antibodies have been established and evaluated (Yamaguchi *et al.*, 1990; Blackall *et al.*, 1990b, 1991a). The ability of monoclonal antibodies to differentiate between different serotypes and serovars has been variable and their commercial development for routine diagnosis has not been achieved.

Three types of haemagglutination inhibition tests termed; simple, extracted, and treated haemagglutination inhibition (HI) tests; have also been described which vary in the type of red blood cell treatment and the preparation of the haemagglutinins (Blackall, 1999). The most extensively used HI test for the detection of antibodies against all the serotypes of *A. paragallinarum* is the treated HI test. This is based on hyaluronidase-treatment of bacterial cells of *A. paragallinarum* for preparation of haemagglutinins (Yamaguchi *et al.*, 1989) and formalin fixed chicken red blood cells (RBCs). It has been used to detect antibodies to Page serovars A, B, and C in vaccinated chickens with high titers (Yamaguchi *et al.*, 1991) and in seroprevalence studies of infectious coryza in Indonesia (Tagaki *et al.*, 1991).

The simple HI test is based on whole bacterial cells of Page serovar A *A. paragallinarum* and fresh chicken erythrocytes (Iritani *et al.*, 1977). This test is

simple to perform, but has been reported to detect antibodies only to serovar A and thus its application has been limited. The extracted HI test, on the other hand, is based on potassium thiocyanate-extracted and sonicated cells of *A. paragallinarum* and glutaraldehyde-fixed chicken erythrocytes and has found more application than the above (Sawata *et al.*, 1982).

2.5.3 Molecular methods

In recent years, identification of organisms, has favoured analyses that reflect one of the most fundamental properties, namely, their genetic information. Genotypic identification and characterisation possess versatility surpassing that of conventional phenotypic methods because nucleic acid analyses facilitate identification and rapid detection of an organism, determination of its taxonomic position, and investigation of intra-species genetic relationships. Molecular approaches, such as DNA hybridisation and nucleic acid amplification, have allowed bacteria detection directly from clinical samples, dramatically reducing the time required for identification (Tang *et al.*, 1997). These diagnostic techniques have found great application in situations where the organisms cannot be grown *in vitro* or for which current culture techniques are too insensitive, or for organisms requiring prolonged incubation times and expensive media, such as is the case with *A. paragallinarum* (Ieven and Goosens, 1997).

Since the initial development of PCR in 1985 by Kull Mullis (Saiki *et al.*, 1985), *in vitro* nucleic acid amplification through repetitive cycling has had extensive

applications in all aspects of fundamental and applied clinical sciences. Recently, a PCR termed HP-2 PCR, was developed for the specific diagnosis of infectious coryza (Chen *et al.*, 1996). This test was found to be rapid (results available in 6 hours) and recognised all isolates including the NAD-independent strains. It was negative for the related bacteria; *O. rhinotracheale*, A. *volantium*, A. *avium* and *Avibacterium* species A. On samples from artificially infected chickens, the PCR had the same detection rate as culture, but superior to culture when tested on natural field outbreak samples (Chen *et al.*, 1998a; Chen *et al.*, 1998b). Majority of positive samples remained positive in the PCR for 180 days when kept at 4 ^{0}C or –20^{0}C, while culture failed to detect *A. paragallinarum* after 3 days of storage at 4^{0}C or –20^{0}C. This, therefore, represented a significant step in diagnosis of infectious coryza based on this PCR.

Species-specific DNA probes have also been developed and used for the detection of *A. paragallinarum* (Chen *et al.*, 1996). These probes could detect as little as 7.8 ng of the organism. DNA-based tests, therefore, represent attractive alternative technologies that might alleviate the difficulties of isolation and conventional identification of *A. paragallinarum*, which are faced by many laboratories in the developing countries.

2.6 Characterisation of *A. paragallinarum*

Understanding the characteristics and epidemiology of a disease (its aetiology and transmission patterns) is crucial for the development and implementation of effective management strategies. To achieve this, reliable markers are required to thoroughly characterise the organisms (Olive and Bean, 1999). Correct identification of microorganisms and the ability to differentiate between closely related microbial strains is essential in epidemiological studies. A number of methods currently used for these purposes are based on phenotypic properties such as biochemical parameters, phage sensitivity, antibiotic resistance, or reactions with specific antisera. In contrast, molecular typing methods are based on the genetic composition of the organism.

2.6.1 Phenotypic characterisation

Since its first isolation in 1932 (De Blieck, 1932), detection, identification and characterisation of *A. paragallinarum* have relied on the ability to cultivate or purify the organism in the laboratory. The purified organism is subsequently classified according to phenotypic traits such as morphology, carbohydrate fermentation patterns, and serological properties. However culture conditions can influence the expression of these attributes thus diminishing the stability and reliability of the phenotypic methods for strain identification. Phenotypic characterisation of *A. paragallinarum* has been based mainly on serology using specific antigen, antibiotic

resistance, protein, fatty acid and carbohydrate constituents and biochemical tests for carbohydrate fermentation and enzyme activity (Blackall *et al.*, 1989)

2.6.1.1 Serotyping

There are three main serotyping schemes that have been used to type *A. paragallinarum* namely; the Page scheme (Page, 1962a), the Kume scheme (Kume *et al.*, 1983), and the Hinz scheme (Hinz, 1980). The Page scheme has been widely used for serotyping *A. paragallinarum* and was developed based on plate or slide agglutination test to recognise the three serovars; A, B and C (Page, 1962a). The use of the haemagglutination inhibition (HI) technique has, however, been shown to be a much better method for identifying the Page serovars (Blackall *et al.*, 1990a). The three serovars are said to represent distinct immunovars since inactivated vaccines against one serovar do not provide protection against the other (Blackall, 1999).

The Kume scheme was originally based on haemagglutination inhibition tests that recognised seven serovars organised into three serogroups I, II, and III (Kume *et al.*, 1983). These have since been reorganised into three serogroups equivalent to the Page's A, C and B, respectively, with four serovars within both Kume serogroup A and C and one serovar in B to allow easy addition of more serovars as they are reported. Thus, the nine current Kume serovars are recognised as A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3 and C-4 (Blackall *et al.*, 1990c).

The Hinz scheme recognises six serotypes, which do not correlate with immunotypes and has not received much attention (Hinz, 1980). Cross protection has been variably reported within the Kume serogroup (Terzolo *et al.*, 1993). Various methods have been used to improve these serological typing methods including use of monoclonal antibodies (Blackall *et al.*, 1991a). This approach, however, has been limited by unavailability of monoclonal antibodies and the inability of the monoclonal antibodies to recognise some strains. Attempts have also been made to type *A. paragallinarum* isolates into the Kume serogroups using a serum bactericidal test (Sawata *et al.*, 1984c). While the Kume scheme allows more discrimination among isolates, it is more complex and demanding to carry out.

2.6.1.2 Other phenotyping methods

Other phenotypic characterisation methods have been used to charactrise *A.paragallinarum* isolates. These have included:- biochemical characterisation based on fermentation of carbohydrates and antibiotic resistance (Blackall *et al.*, 1989); and, chemotyping based on protein (Baeza *et al.*, 1987; Blackall and Yamamoto, 1989; Blackall *et al.*, 1990e; Munar *et al.*, 1992; Hartmann *et al.*, 1996), carbohydrate and fatty acid profiles (Mouahid *et al.*, 1992b). These have also been used to a reasonable extent. However, with the advent of more powerful molecular-based typing methods, which are able to detect more differences among isolates, these phenotyping methods are becoming less ferquently used.

2.6.2 Molecular characterisation

The ability to differentiate phenotypically similar isolates is critically important in epidemiology (Olive and Bean, 1999). Attempts to overcome the problems inherent in phenotypic methods led to development of systems, which rely on the detection of nucleic acids. Molecular typing enables investigation of intra-species genetic relationships that allow determination of epidemiological relationships (Owen *et al.*, 1989). Recent advances in nucleic acid technology coupled with computer-assisted data acquisition and analysis have resulted in the emergence of a number of genotyping techniques, which have enhanced the understanding of molecular epidemiology of many infectious diseases and their subsequent management.

Typing methods should have the power to differentiate unrelated strains, such as those that are geographically distinct from the source organism. At the same time, these methods should demonstrate the relationship of all the organisms isolated from individuals infected from the same source (Towner and Cockayne, 1993). Phenotypic attributes such as serological and biochemical fermentation profiles provide characterisation only if such phenotypic markers are present as expression of an antigen or phage receptors. The development of DNA- based techniques has provided alternative methods of characterisation which identify precisely, individual strains of closely related bacteria (Owen *et al.*, 1989). It encompasses a large range of methods with variable specificity and discriminatory power. These techniques have been applied for characterising *A. paragallinarum* isolates, and have included restriction enzyme analysis (REA) and ribotyping, enterobacterial repetitive intergenic

consensus sequence based polymerase chain reaction (ERIC-PCR) and others. However, their ability to differentiate between related strains has been variable.

2.6.2.1 Restriction enzyme analysis (REA)

Restriction enzyme analysis (REA) has proved to be a valuable component of bacterial epidemiological studies. REA involves analysis of the whole genome by digestion, *in vitro*, of genomic DNA of the organism and subsequent separation of the restriction fragments by electrophoresis. Strains from the same origin are supposed to have the same genome and, therefore, with same number and position of recognition sites for the same restriction enzyme. The choice of enzyme is therefore very critical in order to be able to generate fragments that are discriminatory and useful in differentiating between unrelated strains at the same time not too complicated to be interpreted. REA is a highly reproducible technique that is not influenced by the inconsistent expression of phenotypic traits that limit the sensitivity and specificity of conventional typing methods (Towner and Cockayne, 1993). The use of REA solely or with ribotyping can provide sensitive, distinctive banding profiles capable of differentiating isolates of different origins.

Several enzymes have been used for fingerprinting *A. paragallinarum* isolates by REA. Blackall *et al* (1991b), using *Hind* III restriction enzyme were able to generate restriction fragments that differentiated Australian isolates from those obtained from other countries but were unable to detect major differences among different Australian isolates. When the same enzyme was used on South African NAD-

independent *A. paragallinarum*, isolates yielded the same bands, suggesting that they were clonal, a fact which was also demonstrated by ribotyping using a different enzyme for digestion (Miflin *et al.*, 1995). Those isolates, however, were shown to be not completely identical since some of them lacked haemagglutinating activity indicating that there are some phenotypic variations among isolates not detectable by REA or ribotyping (Miflin *et al.*, 1995). Different REA profiles which correlated with antimicrobial profiles were detected and could differentiate between unrelated outbreaks and identify those that were related in Australia using *Hind*III (Blackall *et al.*, 1990d).

However, Miflin *et al.*, (1997), using *Hpa*II and *Hae*III, demonstrated more discriminatory band patterns that corresponded with the known epidemiological history of the isolates which *Hind*III had failed to detect. This confirms that different enzymes generate different REA profiles that vary in their ability to discriminate between unrelated isolates.

2.6.2.2 Ribotyping

Ribotyping like REA utilises restriction enzyme digestion of genomic DNA and agarose gel electrophoresis for DNA fragment separation. The banding patterns produced by REA are often complex, making visual interpretation of results difficult (Towner and Cockayne, 1993). The additional use of Southern blotting and hybridisation with a labelled DNA probe reduces the complexity of the restriction

patterns and highlights restriction fragment polymorphisms (RFLPs) within the bacterial genome without necessarily applying computer analysis. Ribosomal RNA (rRNA) molecules are highly conserved ubiquitous molecules that constitute the major proportion of RNA in the bacterial cell (Grimont and Grimont, 1986). As rRNA operons vary in copy number and genomic location between strains and species. DNA probes specific for rRNA gene sequences can be used to identify the RFLPs within and /or around the ribosomal operon, thus providing the basis for bacterial strain differentiation. There have been only a few reports, which have successfully utilised ribotyping in characterising and typing *A. paragallinarum* isolates. Miflin *et al* (1995) demonstrated, using ribotyping, that NAD-independent *A. paragallinarum* isolates reported in South Africa were clonal in nature, unlike the classic strains present in that country. Ribotyping was used to assess the genetic diversity of a collection of Chinese isolates using more discriminatory restriction enzymes and indeed different profiles were detected (Miflin *et al.*, 1997).

These genotypic methods have demonstrated considerable genomic heterogeneity among *A. paragallinarum* isolates, providing sufficient evidence to discount the relatedness of outbreaks previously indistinguishable by phenotyping methods. REA and ribotyping in combination with other genotyping methods could provide greater discrimination among *A. paragallinarum* and useful information about sources of infection, transmission, persistence and post-vaccination monitoring.

2.6.2.3 PCR-based methods

PCR-based methods have been developed with the aim of combining the advantages of genotyping with the benefits associated with amplification-based technologies. In comparison with other genotypic methods, PCR techniques are simpler, faster, and require less DNA for analysis (Kerr, 1994). A wide range of PCR-based typing methods are now available and have been applied on various organisms in varying formats including arbitrarily primed PCR (AP-PCR), random amplification of polymorphic DNA (RAPD), amplification fragment length polymorphism (AFLP) and others (Jonas *et al.*, 2003). Amplification products show various polymorphisms in fragment length, band intensity and presence or absence of a band that can permit the identification of differences between isolates. Strategies to improve these methods in terms of ability to discriminate among isolates are continuously being sought.

One of the PCR-based methods that has been applied in molecular typing of *A. paragallinarum* is the enterobacterial repetitive intergenic consensus sequences based PCR (ERIC-PCR). ERIC belongs to several families of short repetitive DNA sequences found in many bacterial genomes (Lupski and Weinstock, 1992). These sequences, which are widely distributed throughout the chromosome, are found in intercistronic regions and transcribed but not translated (Kerr, 1994). The nucleotide sequences of these elements are characterised by highly conserved central repeats and their function remains unclear, but are thought to participate in transcription termination or chromosomal organisation. The most characterised of these are the

repetitive extragenic palindromes (REP) and the enterobacterial repetitive intergenic consensus sequences (ERIC) (Versalovic *et al.*, 1991)

Khan *et al.*, (1998) and Soriano *et al.*, (2004a) used ERIC-PCR for typing *A. paragallinarum* strains resulting in patterns that were able to differentiate unrelated strains. ERIC-PCR is simple, rapid and reproducible using a few colonies of bacterial culture and thus will find wide application in many countries for competent genotyping of these isolates. The use of repetitive DNA sequences for genotyping bacteria has been found to compare favourably with more powerful typing methods such as pulsed field gel electrophoresis (PFGE) in other organisms (Gunawardana *et al.*, 2000).

PFGE, that has remained the "gold standard" fingerprinting method for molecular epidemiology in many pathogens, examines the polymorphisms throughout the chromosome without the complexity of the REA patterns and the restricted view of genetic variation produced by ribotyping (Goering, 1993). These comparable PCR-based methods therefore will be particularly useful for laboratories without specialised equipment for PFGE since they are easy to apply, less expensive and more rapid and reproducible.

2.6.2.4 Other molecular-based typing methods.

Other molecular-based typing methods have been used to try and characterise *A. paragallinarum* as well. These have included plasmid profiling, multilocus enzyme electrophoresis and, outer membrane protein (OMP) and whole cell protein profiling. Plasmid profiling did not find much use in typing *A. paragallinarum* because most strains of this species lack plasmids. OMP and whole cell protein profiling also did not reveal much heterogeneity among epidemiologically unrelated strains (Blackall *et al.*, 1989; Blackall *et al.*, 1990d, e). Multi locus enzyme analysis has also been used but has not had extensive application in typing of these organisms (Bowles *et al.*, 1993)

2.7 Epidemiology of infectious coryza

Infectious coryza occurs worldwide. Although the disease has been reported in other poultry other than chickens, these early reports need to be carefully re-examined following the current taxonomy and understanding of the disease. Chickens are the primary hosts and other poultry are refractory to infection. All ages are said to be susceptible. Conflicting reports, though, have been published regarding the severity among the different age-groups (Blackall *et al.*, 1997).

The main source of infection is said to be clinically infected and carrier birds, although the carrier status has been difficult to demonstrate. Birds, which recover from infection, show no clinical signs within three weeks. Besides, Apg are very

fragile and do not survive within the environment beyond five hours (Page, 1962b). The persistence of the organisms and re-occurrence of infection is still not well understood.

Periodic outbreaks have been associated with stressful factors such as winter or host physiological changes in the body (Blackall, 1999). Birds at the point of lay have been reported to be more susceptible due to the physiological changes and the energy demands on the body at the time. Stress from other organisms such as in infections or vaccinations with live organisms, may also trigger outbreaks (Blackall *et al.*, 1997). In village free-range local chickens, conditions showing infectious coryza clinical signs have been observed following vaccination agianst Newcastle disease with live vaccines although it has not been confirmed (Yongolo M, personal observations, not published).

2.8 Prevention and control of infectious coryza

Disease control strategies for many infectious diseases comprise prevention, control and eradication. Prevention refers to those measures designed to exclude disease from an unaffected population such as an infectious agent from a defined geographic area by quarantine or to protect a given population in an infected area by vaccination (Martin *et al.*, 1987). In a broad sense, however, prevention of disease also includes measures designed to detect disease processes as early as possible before clinical disease occurs and application of therapeutics to prevent spread of the agents to unaffected individuals. The prevention and control of infectious coryza have been

done using a combination of both good management practices regarding restocking, disinfection programmes, as well as vaccination and treatment in case of outbreaks supported by good diagnostic capacity.

2.8.1 Vaccination

The development of vaccines for prevention of infectious diseases has revolutionized the approach to prevention of infectious diseases. The poultry industry would not have been possible without the use of vaccines to prevent many of the serious infectious diseases especially where chemotherapeutic treatment is not available, for example most viral infections (Zander *et al.*, 1997). However, even where treatment is available, prophylactic measures are often easier and cheaper.

The control of infectious coryza has not been an exception. The development of vaccines against infectious coryza was based on the observation that chickens that recovered from natural infection showed some immunity to subsequent challenge. Various antigens have been studied for their role in protective immunity including outer membrane proteins, polysaccharides, and lipopolysaccharides (Blackall, 1995). Vaccine failures have been frequently reported of recent because of the differences in the field serotypes from the vaccine strains and lack of definitive cross protection between different serotypes (Soriano *et al.*, 2004b; Bragg, 2005).

Investigations involving the challenge of vaccinated and non-vaccinated chickens with NAD-independent *A. paragallinarum*, showed no significant difference in the

disease profiles obtained in vaccinated and unvaccinated chickens (Bragg, 2004). The ability of the NAD-independent isolates to evade the immune system, which was demonstrated in this study, suggests the current commercial vaccines available on the market may not protect against all the strains available in different countries.

There is, therefore, a need to determine the cross protection of the commercial vaccines in the different countries against the local isolates before deploying available vaccines. Vaccination still remains the best option for controlling infectious coryza, especially on farms where mixed ages of poultry are kept. Jacobs *et al.*, (2003) have reported promising results with a trivalent vaccine against all the variants and serotypes. Attempts were made towards a recombinant vaccine but no further reports have been made since (Tagaki *et al.*, 1991). Little efforts have also been made to explore other types of vaccines such as live vaccines or DNA vaccines for controlling infectious coryza.

2.8.2 Chemotherapy and antimicrobial resistance

Antimicrobial resistance has become a big challenge to modern medicine worldwide. Increasing prevalence of resistance has been reported in many pathogens over the years in different regions of the world (Byarugaba, 2005). This is attributed to changing microbial characteristics, selective pressures of antimicrobial use, and societal and technological changes that enhance the development and transmission of drug-resistant organisms. Antimicrobial resistance is a natural biological phenomenon and often occurs as a consequence of infectious agents' adaptation to exposure to

antimicrobials used in humans or agriculture and the widespread use of disinfectants at the farm and the household levels (Walsh, 2000). It is now accepted that antimicrobial use is the single most important factor responsible for increased antimicrobial resistance (Aarestrup *et al.*, 2001; Byarugaba, 2004).

There are various antimicrobial agents available for the treatment of infectious coryza. These include sulphonamides in various combinations which have been found to be very effective (Buys, 1971; Buys, 1972; Nair and Bhattacharya, 1980; Lublin *et al.*, 1993; Nikolovski *et al.*, 1997). Resistance to many of these drugs nevertheless, has been reported in many isolates.

The genetic basis and the phenotypic expression of resistance are extremely complex. This is because there are different classes of antimicrobials each with a different molecular target. Also a single bacterial species may exhibit more than one resistance mechanism against a single class of antimicrobial (Walsh, 2000). Moreover some bacterial species more readily develop (or acquire) resistance than others when exposed to apparently similar selective pressures (Ellner *et al.*, 1987). Resistance in *A. paragallinarum* is not an exception and treatment failures have been reported in various places (Lu *et al.*, 1983; Reece and Coloe, 1985; Verma *et al.*, 1985; Blackall, 1988; Blackall *et al.*, 1989; Takahashi *et al.*, 1990; Prasad *et al.*, 1999). Relapses after treatment may also occur, thus creating and sustaining carrier status within flocks. Resistances against streptomycin, tetracycline and neomycin have been reported (Blackall *et al.*, 1989).

Whether particular organisms become resistant to a particular antimicrobial agent may depend on many factors including:- (a) the basic physiology of the bacteria, (b) the characteristics of genetic mutations that occur, (c) the prevalence of resistance genes that might be acquired, or (d) the quantity and quality of exposure to the antimicrobial agent (Parry, 1989). Currently, there has not been a description of the molecular basis for the resistance that has been observed with in *A. paragallinarum* resistant isolates. Rapid spread of genes resistant to antimicrobial agents can occur in a bacterial population and from one ecosystem to another. Antibiotic resistance genes first described in human specific bacteria have been found in animal specific species of microorganisms and *vice versa*, suggesting those bacterial populations can share and exchange these genes between hosts (Walsh, 2000). Understanding the molecular basis for resistance to antibiotics in *A. paragallinarum* and the factors responsible for resistance spread could help in minimizing the resistance but also assist in the rational use of antimicrobial drugs during treatment

2.8.2.1 Antimicrobial resistance mechanisms

Resistance can be caused by a variety of mechanisms as described by Fluit *et al.*, (2001). These include but are not limited to:

a) the presence of an enzyme that inactivates the antimicrobial agent
b) the presence of an alternative enzyme for the enzyme that is targeted by the antimicrobial agent;
c) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent;

d) post-transcriptional or post translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent;

e) reduced uptake of the antimicrobial agent;

f) active efflux of the antimicrobial agent

g) overproduction of the target of the antimicrobial agent

h) expression or suppression of of a gene *in vivo* in contrast to the situation in *in vitro*.

Resistance has for instance been shown with β-lactams, which act on penicillin binding proteins (PBPs), that are involved in the bacterial cell wall synthesis. Being among the most diverse and most clinically used, resistance to many β-lactam compounds is common and is often caused by the presence of β-lactamases, but mutations in PBPs resulting in reduced affinity for β-lactam antibiotics are also commonly observed (Fluit *et al.*, 2001). Resistance is less frequently caused by reduced drug uptake due to changes in the cell wall or due to active efflux. An updated classification system for β-lactamases has been recently published (Leflon-Guibout, *et al.*, 2000).

A growing number of bacterial species have acquired resistance to the bacteriostatic activity of tetracycline. The resistance is mediated by efflux of the drug or protection of the ribosome from the action of the drug (Chopra and Roberts, 2001). Efflux is mediated by energy-dependent efflux pumps while the ribosome protection mechanism involves an elongation factor G-like protein (Schmitz and Fluit, 1999).

Oxidative destruction of tetracycline has also been found in a few species. The mechanisms are encoded in tetracycline-resistant bacteria by *tet* genes, which are found in pathogens, opportunistic pathogens, and members of the normal flora (Roberts, 1996). Most of the resistance genes code for one of the two important mechanisms of tetracycline resistance, described above (Fluit *et al.*, 2001).

Resistance to aminoglycosides such as gentamicin, tobramycin, amikacin, and streptomycin are widespread, with more than 50 aminoglycoside-modifying enzymes described (Schmitz and Fluit, 1999). Most of these genes are associated with gram-negative bacteria. Depending on their type of modification, these enzymes are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside phosphotransferases (APH) (Shaw *et al.*, 1993). Aminoglycosides modified at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes lose their ribosome-binding ability and thus no longer inhibit protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described (Quintiliani and Courvalin, 1995).

Resistance to chloramphenicol is generally due to inactivation of the antibiotic by a chloramphenicol acetyltransferase (CAT) encoded by the *cat* genes. The *cat* genes of Gram-negative and gram-positive bacteria show little homology, and a variety of different enzymes have been described (Kehrenberg and Schwartz, 2001). The gene is

most commonly found on plasmids. Sometimes decreased outer membrane permeability or active efflux is responsible for the resistance in Gram-negative bacteria (Butaye *et al.*, 2003)

Resistance has also been observed in sulfonamides and this is commonly mediated by alternative, drug-resistant forms of dihydropteroate synthase (DHPS). Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes *sul1* and *sul2*, encoding forms of dihydropteroate synthase that are not inhibited by the drug (Enne *et al.*, 2001). The *sul1* gene is normally found linked to other resistance genes in class 1 integrons, while *sul2* is usually located on small nonconjugative plasmids or large transmissible multiresistance plasmids. For Trimethoprim, resistance can be caused by a number of mechanisms including overproduction of the host DHFR, mutations in the structural gene for DHFR, and the acquisition of a gene (*dfr*) encoding a resistant DHFR enzyme (Thomson, 1993). The latter mechanism is the most important in clinical isolates. At least 15 DHFR enzyme types are known based on their properties and sequence homology (Schmitz and Fluit, 1999).

Multidrug resistance presents a serious problem in the treatment of bacterial infections. It is increasingly being reported in bacteria and is often mediated by genetic mobile elements such as plasmids, transposons and intergrons (Dessen *et al.*, 2001). Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination and they have an

intergrase gene (*int*), a nearby recombination site (*attI*), and a promoter, *Pant* (Hall, 1997). Integrons seem to have a major role in the spread of multidrug resistance in Gram-negative bacteria but integrons in Gram-positive bacteria have been recently described (Dessen *et al.*, 2001).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study area

The study was conducted in four regions; western, central, eastern and northern regions. Samples from the western region were taken from Bushenyi district, those from the central region were taken from Kampala and Mpigi districts, those from eastern region were taken from Kumi district, while those from the northern region originated from the districts of Gulu, Lira, Pader and Apac. Samples from village free-range local chickens from the northern region of Uganda were obtained from chickens that were brought to Kampala for sale. The samples from the central region were taken from farms where suspected cases of infectious coryza were reported.

3.2 Sample size

In Kumi and Bushenyi districts multistage systematic sampling was applied to obtain the number of birds required in each region for the free-range poultry population. The county (local council zones, LCs) was the primary unit of sampling, village as the secondary unit, the homestead as the tertiary unit and the individual chickens as the last unit. For each of the two districts (Kumi and Bushenyi) information was sought from the district veterinary officers on the numbers and distribution of the poultry and a list of counties was made and one county selected randomly for the sampling of village free-range chickens and turkeys. From the selected counties, a list of the LC zones was drawn and 10% of the LC selected randomly. For each LC selected a list of

homesteads was constructed with the help of the LC chairmen. Since it was not possible to establish the number of chickens in each homestead at this stage, it was decided that 20% of all the listed homesteads would be sampled in order to achieve the required sample size. The starting point was chosen randomly and every third homestead on the list was sampled until the required sample size was achieved. A list was also drawn for the commercial chicken farms in the districts of Kumi and Bushenyi and 10% of the farms selected for sampling.

The approximate sample size for determination of prevalence required per population (commercial or free-range) for Kumi and Bushenyi, was based on the formula $n = 4pq/L^2$ according to Martin *et al* (1987), where n = sample size, p = estimated prevalence, q = 1-p, and L = desired error (the required precision). Since there was no data available on the prevalence of infectious coryza in Uganda, an estimated prevalence (p) of 50% was used to calculate the sample size with a desired error (L) of 10% at 95% confidence interval to maximize the above expression. The numbers of chickens to be selected from each homestead was determined according to guidelines for detecting presence of disease (Martin *et al.*, 1987).

A similar number of samples were obtained from free-range local chickens brought to Kampala for slaughter from the northern region by sampling 10 chickens randomly on the same day once a week. Purposive sampling was done from five farms (Farm 1,2, 3, 4 and 5) where cases of infectious coryza suspected cases were reported during the study for isolation of the bacteria. Four farms; Farm 1, 2, 3 and 4 were reported in Kampala while one farm, Farm 5 was reported in Mpigi in the central region. In total,

710 samples were collected from chickens and turkeys, which were distributed as shown in Table 1. The map showing where these areas are found in Uganda is shown in the appendix.

Table 1: Sources and numbers of samples analysed in the entire study

	Species from which samples were collected			
	Chickens			
Region	Commercial	Free-range	Turkeys	Total
Western	100	98	0	198
Northern	0	118	0	118
Eastern	100	112	114	326
Central	68	0	0	68
Total	268	328	114	710

Total samples analysed: 710 each of serum and bacteriological samples

3.3 Sample collection

Samples for bacteriological examination were collected from all the selected live birds. Samples were taken from the nasal cavity, or sinuses and trachea according to standard procedures as described by Blackall and Yamamoto (1998). Briefly, the nasal area was cleaned and disinfected with 70% ethanol and a cotton swab carefully introduced into the nasal cavity and gently turned around. For the tracheal samples the swabbing was made in the trachea. Both swabs from each bird were placed together in glycerol-enriched phosphate buffered saline (30% glycerol in PBS, pH 7.2, supplemented with 1% heat inactivated chicken serum and 0.003% reduced nicotinamide adenine dinucleotide, NADH) as described by Chen *et al* (1998b) and kept under cold chain until the laboratory at Makerere University. All the bacteriological samples were cultured within 24 hours from the time of collection. Blood was collected from the wing vein from each of the selected bird and sera harvested for detection of antibodies against *A. paragallinarum*. Where it was possible to purchase sick birds, birds were sacrificed and sinus swabs were taken through a sterile incision through the infra-orbital sinus in addition to the nasal and tracheal swabs. Before any of the samples were taken each bird to be sampled was clinically examined for signs infectious coryza.

3.4 Detection of *A. paragallinarum* infection

A. paragallinarum infection was detected by two methods namely: detection of the agent by culture and PCR and, detection of antibodies to the agent by serology using the haemagglutination inhibition test.

3.4.1 Isolation of the bacterium

For isolation of the bacteria, bacteriological samples were streaked onto chocolate agar plates that were incubated in a candle jar at 37^{0}C for 18–48 hrs. Smears from pinpoint colonies, which were greyish in colour, were made and stained by the Gram's stain and observed under the microscope. Those which appeared gram-negative and pleomorphic coccobacilli in shape were suspected to be *A. paragallinarum* and were subcultured on Chen's medium as modified in this study, containing 1% polypeptone (BBL), 1% yeast extract (Oxoid), 1% sodium chloride, 0.5% trypsin casein hydrolysate (Sigma) and 0.02% glucose supplemented with 1% heat inactivated chicken serum and 0.003% reduced nicotinamide adenine dinucleotide, (NADH) (Sigma). The suspected isolates were confirmed as *A. paragallinarum* by PCR according to Chen *et al.*, (1996) as described below.

3.4.2 Detection by PCR

The HP-2 PCR developed by Chen *et al.*, (1996) was performed according to modification by Chen *et al.*, (1998b) on all the bacteriological samples and all the suspected colonies. Briefly, 1 ml of each well-mixed bacteriological clinical sample

was transferred into a 1.5 ml microfuge tube and processed for the PCR. Samples were spun at 8000 x g for 10 minutes in a micro-centrifuge and the supernatant discarded. The pellet was re-suspended in 20 µl of lysis buffer containing 0.1 M Tris-HCL (pH 8.3), 0.5% Tween 20, 0.5% Nonidet 40 and 200 µg/ml Proteinase K (final concentration) and allowed to lyse at 56^{0}C for 1 hour. The samples were then heated at 95^{0}C for 10 minutes in a heated block and held on ice for 10 minutes until later used in the PCR as DNA template. For confirmation of suspected colonies, two suspected colonies were suspended in 20 µl of distilled water in a microfuge tube. This was heated at 95^{0}C for 10 minutes and kept on ice for later use as DNA template. The reaction mixture in both clinical samples and suspected colonies of 25µl consisted of 10 mM Tris-Hcl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$ 0.1% (w/v) gelatin, 200 µM each of dinucleotides; dATP, dGTP, dTTP and dCTP, 0.4 µM each of primer Ni and R1, 1.25 units of *Taq* polymerase and 4 µl of template DNA sample previously prepared and held on ice. The primer sequences were as described (Chen *et al.*, 1996):-

N1 5'-TGA GGG TAG TCT TGC ACG CGA AT-3'

R1 5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'

Amplification was performed using a thermocycler (Biometra, Germany). The reaction conditions consisted of an initial hold at 95^{0}C for five minutes, 25 cycles of denaturation at 94^{0}C for one minute, annealing at 65^{0}C for one minute, and extension at 72^{0}C for two minutes with a final extension of 72^{0}C for 10 minutes and 4^{0}C final hold until analysed. The PCR products were detected by running a 10 µl sample with

6 x loading buffer in 1% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma) for 30 minutes at 100 V constant voltage and visualisation under UV trans-illumination and photo-documentation.

3.4.2.1 Storage of isolates

Confirmed isolates were preserved in storage medium in a suspension consisting of heat inactivated bovine serum with 7.5% glucose supplemented with 0.003% reduced nicotinamide adenine dinucleotide in 1 ml volume screw-cap vials and stored at – 80°C or under liquid nitrogen until further use. For daily use, each strain was maintained on modified Chen's medium as described above.

3.4.3 Detection of antibodies to *A. paragallinarum* for sero-prevalence

The seroprevalence of *A. paragallinarum* infection was determined by analysis of the sera taken from the sampled birds. The antibodies were detected by the treated haemagglutination inhibition (HI) test as described by Blackall and Yamamoto, 1998) using hyaluronidase-treated reference bacterial cells on glutaraldehyde-fixed chicken erythrocytes.

3.4.3.1 Preparation of haemagglutinins

Haemagglutinins were prepared from reference strains of serotypes A (2403), B (1676), and C (Modesto), kindly provided by Dr K. H. Hinz, of the Poultry Clinic in

Hanover, Germany. These strains were propagated on test medium (TM/SN) described by Eaves *et al.* (1989), consisting of 1% Biosate peptone (BBL), 1% NaCl, 0.1% Starch (Sigma), 0.05% glucose, 1.5% Noble agar base (Difco) supplemented with 5% (vol/vol) oleic albumin complex (Sigma), 0.0005% (vol/vol) thiamine (Sigma), 1% (vol/vol) heat inactivated chicken serum, and 0.003% (wt/vol) reduced nicotinamide adenine dinucleotide (NADH) (Sigma).

Two colonies of the reference strains were inoculated into 100 ml of the broth version of the TM/SN medium without the agar and grown overnight in an incubator shaker (Brunswick) running at 200rpm at 37°C. The broth culture was centrifuged at 8,000 x g for 30 minutes in a centrifuge at 4°C. The cells were washed twice with phosphate buffered saline pH 7.0 containing 0.85% NaCl. The cells were re-suspended in equal volume of hyaluronidase (Sigma) solution (50 units/ml) in PBS and incubated in a water bath at 37°C for 2 hours. Finally the cells were re-suspended in PBS containing 0.01% thimerosal (Sigma) (PBS/T) to a concentration of 10^{12} cell per ml by spectrophotometry. The antigens were held at 4°C for three days before use in the haemagglutination inhibition test.

3.4.3.2 Preparation of glutaraldehyde-fixed RBCs

Glutaraldehyde (GA) -fixed chicken red blood cells (RBCs) were prepared according to the method of Bing *et al.*, (1967). Briefly, blood was collected from the wing vein of commercial chickens into Alsever's solution (1 vol: 4 volumes of blood) and RBCs harvested by centrifugation at 400 x g for 10 minutes. The RBCs were washed

three times in 0.15M NaCl. Red blood cells were fixed in 1% glutaraldehyde salt solution [1% GA from a 25% stock (Sigma) diluted with a solution containing 0.15M $NaPO_4$ (pH 8.2) (one volume) and 0.15M NaCl (nine volumes) in distilled water (five volumes)] for 30 minutes at 4°C with occasional mixing. The cells were again washed five times with 0.15M NaCl and then with double distilled water before a 30% stock was made in double distilled water containing 1:10000 (wt:vol) thimerosal (Sigma) and kept aliquoted in 1 ml at 4°C until use.

3.4.3.3 Determination of haemagglutination units

The haemagglutinating activity of the antigens for each of the reference strains was determined against glutaraldehyde-fixed chicken red blood cells by the micro titer method according to Blackall and Yamamoto (1998). Briefly, 25 µl of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.001% gelatin were placed in each well in a U-bottom microtiter plate followed by addition of 25 µl of the bacteria haemagglutinin antigen into first column and mixed well. A doubling dilution was made across the plate by removing 25 µl from the first well into the next well and leaving out the last column as control. Another 25 µl of PBS-BSA-gelatin was added to all wells followed by addition of 25 µl of 1% glutaraldehyde fixed RBCs to all wells including the last column and mixing gently. The plates were allowed to stand at room temperature with a cover. After one hour, the haemagglutination titre was determined as the highest dilution of antigen causing agglutination of the rbcs with the last well showing haemagglutination taken as 1

haemagglutination unit (HA). The titre of the original preparation was calculated accordingly.

3.4.3.4 Haemagglutination inhibition (HI) test

For the demonstration of HI antibodies against *A. paragallinarum*, two-fold serial dilution was made of each serum sample with PBS containing 0.1% bovine serum albumin (BSA) and 0.001% gelatin in 96 well-microtiter plates according to Takagi *et al.*, (1991). To each well, equal amounts of haemagglutinin with 4 haemagglutinating units (HU)/25µl were added and left at room temperature for 20 minutes. Then, 25µl of GA-fixed rbcs were added. After standing for at least 2 hours at room temperature, the result was read as the highest serum dilution that inhibited haemagglutination completely. Each serum sample was tested against the three reference haemagglutinins.

3.5 Characterisation of isolates

The isolates were characterised using several phenotypic and genotypic methods.

3.5.1 Phenotypic characterisation

3.5.1.1 Carbohydrate fermentation and enzyme activity

The isolates were characterised by carbohydrate fermentation patterns of sugars (glucose, fructose, sucrose, mannitol, sorbitol, maltose, rhamnose, arabinose,

trehalose, lactose, dulcitol, salicin, and raffinose); and enzyme activity (oxidase, catalase, urease and β-galactosidase) according to described procedures (Blackall *et al.*, 1990d). The inoculum for the characterisation tests was prepared from pure colonies on TM/SN medium. The carbohydrate fermentation tests were incubated for 48 hours to seven days on blood agar base as the basal medium with 40μg/ml phenol red, 25μg/ml NADH, 1% inactivated horse serum and 2 percent of the carbohydrates. The medium was dispensed in 7-ml slopes in screw-capped tubes. The tubes were incubated with the caps loosened in a candle jar for 24-48 hours at 37°C. Fermentation was indicated by a distinct yellow colour.

Urease activity was determined using Christensen's urea agar supplemented with 25μg/ml NADH and 1% (v/v) heat inactivated horse serum and a positive reaction was taken as a pink-red colour change. The test for β-galactosidase was performed using appropriate enzyme substrate, ortho-nitro-phenyl β-D galactosidase (ONPG) and a positive reaction was taken as a yellow colour change within 24 hours. The catalase test was performed by flooding a glucose fermentation tube that had been incubated for two days in a candle jar for 24–48 hours at 37°C with hydrogen peroxide (30%) and the formation of bubbles within 3 minutes was regarded a positive result. The oxidase reaction was determined using Kovac's reagent (N,N,N,N-tetramethyl-*p*-phenyldiamine dihydrochloride) by dropping a few drops of the reagent onto colonies of the isolates on chocolate agar. A positive reaction was shown by a blue colour within less than 30 seconds.

3.5.1.2 Determination of NAD-dependence

The determination of NAD-dependence of the the isolates was done according to Blackall and Farrah (1985) on TM/S medium (TM/SN medium lacking NADH) using commercial growth factor discs; V (NAD), X (haemin), or VX (both haemin and NAD) from Oxoid. A uniform inoculum from an overnight culture of the isolates was made on the TM/S agar plates followed after 15 minutes with placement of commercial discs V, X or VX and incubated for 24-48 hours a candle jar at 37°C. Absence of growth around the X disc and growth around both VX and V discs was interpreted as the bacteria being NAD-dependent (V-factor dependent), whereas growth around all the three factors was interpreted as NAD-independence. The NAD-dependence was also determined by comparison of growth on blood agar with or without cross-streaking with a V-factor-producing nurse culture of *Staphylococcus hyicus*. Luxuriant growth around the *S. hyicus* cross-streak was intrepreted as being being NAD-dependent, while growth on plates without the nurse culture was interpreted as being NAD-independent.

3.5.1.3 Sensitivity to antimicrobial agents

The sensitivity of the isolates to antimicrobial agents was done by determination of minimimum inhibitory concentrations using E-test strips according to the manufacturers (AB Biodisc Solna, Sweden) on TM/SN medium. Different antibiotic E-test strips were applied on each plate inoculated uniformly by surface spread with the isolates. The plates were incubated at 37°C for 48 hours under microaerophilic

conditions and inhibitory concentration of each antibiotic was read at the point where the elliptical zone of inhibition intersected the E-test strip. The antimicrobial strips used for E-test were tetracycline, streptomycin, neomycin, sulphamethoxazole, ampicillin and chloramphenicol. The strips were first checked with a control *Escherichia coli* (ATCC 25922) strain. The criteria followed for interpreting the antibiotic sensitivity or resistance was as described by Blackall (1988) as shown in Table 2.

Table 2: Criteria for interpreting antibiotic sensitivity and resistance based on minimum inhibitory concentrations (MICs)

Antibiotic	MIC (µg/ml)		
	Sensitive	Intermediate	Resistant
Ampicillin	≤8	>8 - 16<	≥16
Chloramphenicol	≤4	>4 - 16<	≥16
Streptomycin	≤4	>4 - 16<	≥16
Neomycin	≤4	>4 - 16<	≥16
Sulphamethoxazole	≤4	>4 - 16<	≥16
Tetracycline	≤4	>4 - 16<	≥16

Source: Blackall, 1988

3.5.1.4 Serotyping of the isolates

The isolates were sero-typed according to the Page scheme (Page. 1962a) using a haemagglutination inhibition (HI) test described by Blackall *et al.*, (1990a). The isolates were incubated in the broth version of TM/SN medium without agar, for 18 hours in an incubator shaker running at 200 rpm at 37^{0}C. The cells were harvested by centrifugation at 8,000 x g for 30 minutes at 4^{0}C and washed in PBS containing 0.1% (wt/v) thimerosal. Preparation of the haemagglutinins and the rest of the HI procedures were carried out as described before. Each strain was reacted with each specific immune sera to determine the reactivity. Specific immune serum was kindly provided by Pat Blackall, from Primary Industries, Queensland Australia. These included antiserum to serotypes A (Page 083), B (Strain Sprose), and C (H-18).

3.5.2 Genetic characterisation of the isolates

The genetic characteristics of the isolates was determined by two methods namely genotyping by examination of enterobacterial repetitive intergenic consensus (ERIC) sequences and the examination of a selection of clinically relevant antibiotic resistance genes.

3.5.2.1 Genotyping by ERIC-PCR

Isolates were typed at genetic level by the enterobacterial repetitive intergenic consensus sequences polymerase chain reaction (ERIC-PCR) method as described by Versalovic *et al.*, (1991). A modification of a previously described whole cell colony preparation method described by Gunawardana *et al.*, (2000) was used to prepare template DNA for examination by ERIC-PCR. Four colonies of each isolate grown on TM/SN medium were picked and mixed in 20 µl of sterile water in a microfuge tube. This was heated at 95°C for 10 minutes in a heating block. The sample was then centrifuged for 5 min at 12,000 x g and immediately placed on ice. The supernatant was used as source of template DNA for PCR.

The primers used were; ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG- 3') as described by Versalovic *et al.*, (1991)

Thermocycling was carried out in a 25µl reaction mixture containing 10 mM of Tris-HCl (pH 8.4), 50 mM of KCl, 3 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.5 U of Taq DNA polymerase (Amersham), and 3µl of DNA template. The PCR thermocycling consisted of an initial 5-minute denaturation at 94°C, followed by 35 cycles of denaturation for 1 minute at 94° C, annealing for 1 minute at 52°C, and extension for 6 minutes at 74°C and a final extension at 74°C for 6 minutes and the reaction held at 4°C until analysis. The amplified products were electrophoresed in 2% agarose gel at

80 V for 1 hr in 1x TAE buffer containing ethidium bromide (0.5 µg/ml). The products were visualised and photographed under UV light trans-illumination for further analysis of the banding patterns.

3.5.2.2 Detection of antimicrobial resistance genes

PCR was used to detect the presence of antimicrobial resistance genes of clinically relevant antimicrobial agents. The isolates were examined for resistance genes for ampicillin (bla_{TEM} genes), chloramphenicol (*catA1* gene), neomycin (*aphA-2* gene), streptomycin (*strA* and *aadA* genes) sulphamethoxazole (*sul1* and *sul2* genes), and tetracycline (*tet*(A), *tet*(B), *tet*(C) and *tet*(G) genes). The primer sequences used for the detection of the resistance genes were as described by Aarestrup *et al* (2003) as shown in Table 3. The amplification and thermocycling was performed in a 25µl reaction mixture containing 12.5 µl FideliTaq™ PCR Master Mix (USB Corporation, USA), 1µl of 10µM of each primer, 3µl of DNA template, and 7.5 µl of PCR water. The PCR thermocycling consisted of an initial 2-minute denaturation at 94°C, followed by 30 cycles of denaturation for 30 seconds at 94° C, annealing for 30 seconds at 55°C, and extension for 2minutes at 68°C and a final extension at 68°C for 5 minutes and the reaction held at 4°C until analysis. The amplified products (6µl in an equal volume of 6 x loading buffer) were electrophoresed in 1% agarose gel at 70 V for 1 hr in 1x TAE buffer containing ethidium bromide (0.5 µg/ml). The products were visualised and photographed under UV light trans-illumination for further analysis of the bands.

Table 3: Primer sequences used for the detection of resistance genes of clinically important antibiotics

Antibiotic	Primer sequence	Gene
Ampicillin	5'-ATGAGTATTCAACATTTCCG-3'	bla_{TEM}
	5'-AC-CAATGCTTAATCAGTGAG-3'	
Chloramphenicol	5'-CGCCTGATGAATGCTCATCCG-3'	*catA1*
	5'-CCTGCCACTCATCGCAG TAC-3'	
Neomycin	5'-GCTATTCGGCTATGACTGGGC-3'	*aphA-2*
	5'-CCACCATGATATTCGGCAAGC-3'	
Streptomycin	5'-CCAATCGCAGATAGAAGGC-3'	*strA*
	5'-CTTGGTGATAACG-GCAATTC-3'	
	5'-ATCCTTCGGCGCGATTTTG-3'	*aadA*
	5'-GCAGCGCAATGACATTCTTG-3'	
Sulphamethoxazole	5'-CTTCGATGAGAGCCGGCGGC-3'	*sul1*
	5'-GCAAGGCGGAAACCCGC GCC-3'	
	5'-GCGCTCAAGGCAGATGGCATT-3'	*sul2*
	5'-GCGTTTGATACCGGCACCCGT-3'	
Tetracycline	5'-GTAATTCTGAGCACTGTCGC-3'	*tet(A)*
	5'-CTGCCTGGACAACATTGCTT-3'	
	5'-CTCAGTATTCCAAGCCTTTG-3'	*tet(B)*
	5'-ACTCCCCTGAGCTTGAGGGG-3'	
	5'-GGTTGAAGGCTCTCAAGGGC-3'	*tet(C)*
	5'-CCTCTTGCGGGAAT- CGTCC-3'	
	5'-GCAGCGAAAGCGTATTTGCG-3'	*tet(G)*
	5'-TCCGAAAGCTGTCCAAGCAT-3'	

Source: Aarestrup *et al* (2003)

3.6 Evaluation of virulence characteristics of the isolates

3.6.1 Bacterial isolates

The five isolates from this study were evaluated for their virulence characteristics. The isolates were retrieved from storage and grown on TM/SN medium for use in the experiments.

3.6.2 Evaluation of the isolates for serum susceptibility

The strains isolated from the study were evaluated for their growth kinetics in chicken sera as well as that of other related poultry species, namely turkeys and guinea fowls. In addition a reference strain 2403 was included in this experiment for comparison. The *A. paragallinarum* strains were propagated on TM/SN medium.

3.6.2.1 Collection of serum

Healthy chickens, turkeys and guinea fowls of about 10 weeks of age with no prior vaccination history against *A. paragallinarum* and no previous history of infectious coryza were used for collection of serum. The serum was filter sterilised with 200 nm pore filters, pooled and kept at –20°C in aliquots of 1.5 ml until used in the experiments. Absence of infectious coryza antibodies was confirmed prior to the use of the sera by HI test. Normal sera referred to non-heated sera.

3.6.2.2 Measurement of serum resistance

The activity of the bactericidal power of sera against the various strains was tested using a micro method previously described by Diallo and Frost, (2000). The isolates were grown in TM/SN broth. The test sera were used at 90% dilution in TM/SN broth. To each well of a 96-well microtiter plate, 225µl of diluted serum and 25µl of the isolate suspension to a final concentration of 10^5 cfu/ml were added and the mixture incubated in a water bath at 37^{0}C for 6 hours. A portion (25µl) of the sample was taken for viable cell count every hour. The counting was performed on TM/SN agar medium by the Miles and Misra technique (Miles and Misra, 1938).

The growth kinetics were monitored over a six hour incubation period and comparisons made between the different strains, source of sera and heat treatment. Comparison was made between the mean bacterial counts of the of triplicate counts at 3 hours and 6 hours for the different strains by comparison of the survival index as a measure of serum susceptibility at 3 and 6 hours. The survival index was calculated as the average of triplicate counts of bacteria that survived at 3 or 6 hours post incubation divided by the average of the triplicate counts of the bacteria at the start of the incubation. When the index was ≥ 0.5, the isolate was classified as serum-resistant and when it was <0.5 it was classified as serum sensitive.

3.6.2.3 The role of the capsule on serum resistance

The role of the capsule on serum resistance was investigated using a derivative of one of the isolates Apg-01. The wild type was decapsulated using treatment with hyaluronidase to determine the effect of the hyaluronic capsule material on resistance to serum. The isolate was grown in TM/SN containing 9000 units/ml of hyaluronidase (Sigma) for 6 hours. The bacteria were checked for lack of the capsule by negative staining with Indian Ink. The derivative was tested for serum resistance in serum containing 9000 units/ml of hyaluronidase. Bacterial counts were done and comparisons made with the wild type as above.

3.6.3 Evaluation of the pathogenicity of the isolates

The five strains were used in testing for the pathogenicity and compared with the reference strain 2403. Unvaccinated commercial layer chickens were obtained from flocks without previous history of coryza and used in the study. Free-range local chickens were hatched and bred at the Faculty of Veterinary Medicine, Makerere University's experimental unit. All the birds to be used in the experiment were screened for absence of exposure to infectious coryza by the HI test. Birds were housed in animal houses on coffee husks and were de-wormed and given coccidiostats two weeks prior to the experiment. The birds were kept with food and water *ad libitum*.

3.6.3.1 Testing for pathogenicity of the isolates

The pathogenicity of the isolates were determined according to described procedures (Lin *et al.*, 1996). In the first experiment, seventy commercial layer chickens, 10-weeks old, raised as indicated above, were randomly allocated into seven groups of ten using a table of random numbers and each group housed in a separate experimental unit. Five groups were inoculated into the infra-orbital sinus with 0.2 ml of the broth culture containing at least 10^8 cfu with the field isolates, one isolate for each group. The sixth group was similarly inoculated with the reference strain 2403. A seventh group was inoculated with sterile medium as a negative control. The chickens in all the groups were examined for clinical signs by the same observer on all odd days post inoculation for a 20 days period post inoculation when the experiment was terminated. The signs were evaluated numerically according to Bragg (2002b) as follows: 0= no clinical signs; 1=for mild (slight nasal discharge), 2= moderate (slight facial swelling and nasal discharge), and 3= severe (severe facial swelling with lacrimation and anorexia). Re-isolation of *A. paragallinarum* organisms and confirmation by PCR were done at each day of sampling to ascertain the cause of the coryza. The experiment was repeated with different age groups; 4 weeks, 10 weeks, and 21 weeks old layer commercial chickens. In the third experiment, ten local chickens of 10 weeks of age experimentally infected and compared with the commercial chickens of the same age.

3.7 Investigation of persistence of *A. paragallinarum* in the host and environment

The persistence of *A. paragallinarum* in both the host and the environment were investigated. Experimental infections were initiated using one of the representative strains as described above in 10-week-old chickens. Ten commercial layer chickens were inoculated as described above. Nasal swabs were taken for culture and PCR on daily basis until one week after all the birds were negative by either method. A group of unchallenged sentinel un-infected commercial chickens were introduced in the same unit one week after the inoculated chickens had all turned negative by both culture and PCR to see if they would pick up the infection. The monitoring continued weekly until 60 days when the experiment was terminated. Ten random samples of environmental samples (25 ml of water) were taken at the same time as the host samples were taken to compare the re-isolation and PCR detection in the various samples. Comparison was made of the recovery of the organisms between environmental samples and host samples as well as between the sentinel chickens and recovered chickens. A control group consisted of chickens inoculated with only medium.

3.8 Susceptibility of turkeys and guinea fowls to infection with *A. paragallinarum*

The ability of turkeys and guinea fowls to be colonised by and transmit *A. paragallinarum* to chickens was investigated. Turkeys and guinea fowls were maintained at Makerere University Faculty of Veterinary Medicine poultry facility

under observation and ensured they were negative for antibodies to infectious coryza. The birds were allowed to lay and hatch chicks, which were used in the experiments. At ten weeks they were randomly allocated into groups as described below for the experiment and were given food and water *ad libitum* and kept in clean and well ventilated experimental units.

3.8.1 Susceptibility of turkeys and guinea fowls to *A. paragallinarum* infection

Ten-week-old turkeys were randomly allocated into three groups of 15 birds each using random numbers and housed in separate experimental units. In group one, each turkey was inoculated in the infraorbital sinus with 0.2 ml containing 10^8 cfu of *A. paragallinarum*. Fifteen un-inoculated sentinel chickens were immediately introduced into the same unit as tracer birds to assess the potential of the turkeys to transmit infection to the chickens. In group two, the turkeys were not inoculated and instead fifteen 10-week-old commercial layer chickens similarly inoculated were immediately introduced to see if the un-inoculated turkeys would pick up infection from the chickens. The third group consisted of similar numbers of turkeys and commercial layer chickens both inoculated with only media as the control group. For the five-day experimental period, three chickens and three turkeys were picked randomly and sacrificed daily. Clinical signs were noted in all the birds before sampling. Culture and PCR was performed on the sinus swabs for detection of *A. paragallinarum* on each of those days.

The same experimental design was used to determine the potential of guinea fowls to transmit *A. paragallinarum* to chickens or pick infection from chickens and similarly treated.

3.9 Evaluation of the efficacy of a commercial coryza vaccine

A commercial vaccine (Nobilis Coryza[R], Intervet International) currently used in Uganda was used. This vaccine contains inactivated *A. paragallinarum* strain 083 (serotype A), strain Spross (serotype B) and strain H-18 (serotype C). Sixty ten-week old commercial layer chickens were vaccinated according to the vaccine manufacturer's instructions and boosted 4 weeks later by injection with 0.25 ml of the vaccine in the breast muscle. A group of 10 chickens was used for challenge with each isolate and one group with the reference strain 2403. Another 20 chickens were not vaccinated and were used as control groups, ten of which were not challenged and the other challenged with the reference strain 2403. All the vaccinated chickens were challenged with the strains, 7 days after the last booster dose with 0.2 mls of broth containing at least 10^8 cfu intrasinus. Birds showing presence of clinical signs in each group were recorded at 1, 3, 7 and 14 days following challenge. On day seven post-challenge, birds were sampled for culture and PCR. The experiment was terminated at day 14 post challenge and all the birds scarificed and sinus swabs taken for PCR and culture. Antibody levels were determined at weeks 3, 5 and 7 by HI test following the first vaccination.

3.10 Data handling and analysis

The data collected was entered into Microsoft Excel for data handling and later imported into GraphPad Prism® version 3.02 for Windows. GraphPad Software, San Diego California USA, for statistical analyses to determine differences between the various variables and parameters investigated.

For the occurrence of antibodies against Apg, the proportion of positives was calculated as the number of positives (percentage) over the total number of samples examined.

The pathogenicity data was analysed basing on a scoring system described by Bragg, (2002b). The total clinical signs score for each group for each day was calculated. The mean daily clinical signs score was calculated by dividing the sum of the daily clinical signs score of all the chickens within a group by the total number of chickens in the group. Differences in the overall mean clinical sign scores (disease score) between the isolates were analyzed by one-way analysis of variance (ANOVA). Graphical presentation of the mean scores was made using Microsoft Excel for Windows 2000.

The results of serum resistance were analysed using a general linear model (GLM) procedure for repeated measures analysis of variance between the various isolates, source of sera, sera treatment, hyaluronidase treatment of the isolates, and heat treatment of the sera over time. Tukeys multiple comparisons was only performed

when the p value was less than the alpha value to determine which groups differed. Graphical presentation of the growth kinetics of the various compared groups over time was made using Microsoft Excel for Windows 2000.

Comparison was also made between the survival of the isolates at 3 hours and 6 hours for the different strains and in different sera by comparison of the survival indices. The survival index was calculated as the average of triplicate counts of bacteria that survived at 3 or 6 hours post incubation divided by the average of the triplicate counts of the bacteria at the start of the incubation.

The susceptibility of gallinaceous birds and their potential for transmission of Apg was assessed by the number of birds showing clinical signs or positive on culture and PCR as described above over the total number examined at different times.

In the vaccination and challenge experiment, the antibody titres data was recorded as reciprocals of the highest dilution that caused haemagglutination inhibition, which were then logarithmically transformed, by $\log_2$ and all the analyses done on the transformed data. The HI titre ($\log_2$) for each group was calculated as geometric mean titres (GMT). Protection cut-off was taken as HI titre $\log_2$ of 5. Proportions of birds showing signs in comparison to the non-vaccinated group was made. An alpha level of 0.05 was used in all the statistical analyses for testing significant differences.

CHAPTER 4

4.0 RESULTS

4.1 Occurrence of *A. paragallinarum*

Seven hundred and ten bacteriological samples were examined for presence of *A. paragallinarum*. Six hundred and fourty two were from apparently healthy birds and sixty eight were from birds with suspicious clinical signs.

4.1.1 Occurence of *A.paragallinarum* from farms/households with healthy birds

From the farms or households where birds (both turkeys and chickens) were not showing suspicious clinical signs, no positive samples were found either by PCR or through isolation. The samples from the northern region which were taken from birds in markets in Kampala were also negative both by PCR and culture methods. There was no isolate recovered from turkeys, which were sampled in the study areas nor was any of them positive by culture.

4.1.2 Occurence of *A.paragallinarum* farms with birds showing suspicious clinical signs

Samples were obtained from sixty-eight chickens on five farms, which had chickens showing clinical signs suspicious of infectious coryza of facial swelling and nasal discharge. Farms 1, 2, 3 and 4 were from Kampala and Farm 5 was from Mpigi both in the central region of Uganda. Five isolates were recovered from two farms (Farm 2

and Farm 4) out of the five commercial chicken flocks (Table 4). Two isolates (Apg-01 and Apg-02) were recovered from Farm 2, while three isolates (Apg-03, Apg-04, and Apg-05) were from Farm 4. The same numbers of PCR postive samples were obtained on Farm 4 where no treatment had been given before sampling, while on Farm 2 where treatment had been given before sampling, more PCR samples were obtained. All the samples, which were positive by culture, were also positive by PCR. No isolates were recovered from Farms 1, 3, and 5 where similar signs had been reported although PCR positive samples were obtained from Farm 1 where the birds had been treated seven days earlier. Farm 3 where treatment had been done 10 days earlier no isolates were recovered and all the samples were negative for PCR.

4.1.3 Culture and PCR results of positive samples

The colonies on Chen's modified medium were grayish and glistening of about 1-2mm in size after 48 hours growth. After 48 hours of culture in a candle jar at 37°C colonies on chocolate agar were round pinpoint in size and greyish. The organisms demonstrated satellitism on blood agar cross-inoculated with *Staphylococcus hyicus*. They were Gram-negative, pleomorphic coccobacilli under Gram stain. All the isolates were confirmed as *A. paragallinarum* by PCR showing a single band of about 0.5 kb by the species-specific PCR corresponding to the band amplified from the reference strains (Fig.1).

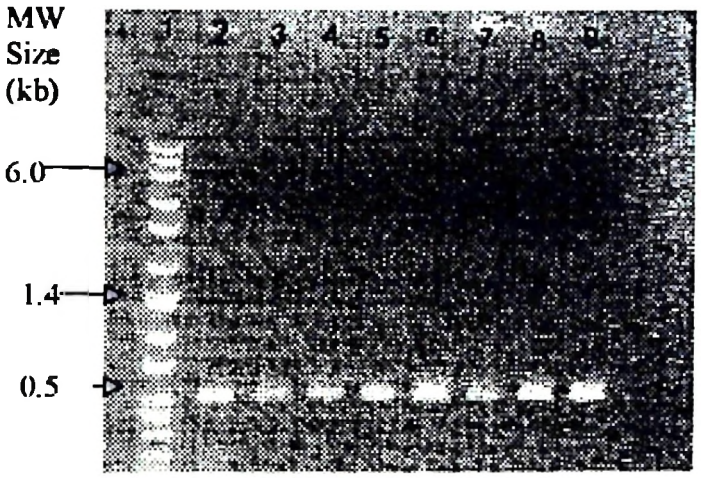

Figure 1: Confirmation of *Avibacterium paragallinarum* isolated from chickens in Uganda by PCR

Lane 1; Direct Load ™ Wide Range DNA Marker, Lanes 2, 3, 4, 5, 6, are strains Apg-01, Apg-02, Apg-03, Apg-4, Apg-05 respectively, isolated from outbreaks of infectious coryza in chickens in Uganda; Lanes 7,8,and 9 are reference strains 243, 1676, and Modesto respectively

Table 4: Recovery of *A. paragallinarum* from cases of infectious coryza on commercial chicken farms

Source of samples	Area	Flock type	Age (months)	Coryza Vaccination	Coryza signs	Coryza treatment	Time sampled	Culture[a] +ve	PCR[a] +ve
Farm 1	Kampala	parent	24	None	typical	Tetracycline 7days earlier	September 2001	0/14	5/14
Farm 2	Kampala	layer	8	None	typical	Tetracycline 3days earlier	October 2001	2/14	3/14
Farm 3	Kampala	broiler	1.5	None	suspicious	Penicillin and streptomycin 10 days earlier	October 2002	0/13	0/13
Farm 4	Kampala	layer	10	None	typical	Not yet	September 2002	3/14	3/14
Farm 5	Mpigi	layer	24	None	suspicious	Tetracycline 21 days earlier	June 2004	0/13	0/13

[a] Number positive/total number of chickens sampled

4.2 Occurence of antibodies against *A. paragallinarum*

The overall seropositivity of antibodies to all the three Page's serotypes demonstrated in this study is shown in Table 5. The overall occurrence of anti-Apg antibodies was 40.5% and the seropositivity to serotype A, B and C was 18%, 0.5% and 22% respectively. Antibodies to all the three Page's serotypes A, B and C were demonstrated in free-range chickens but only serotype A and C antibodies were demonstrated in commercial chickens.

No antibodies were demonstrated in turkeys. Both serotypes A and C were demonstrated in the western and eastern parts of the country. Serotype B antibodies were only observed in samples from the northern region (which were sampled from the markets in Kampala) and had a seropositivity of 0.5 %. Turkeys did not show antibodies to any serotype. Some chickens in free-range poultry had antibodies to more than one serotype suggesting they could have been exposed to more than one serotype.

Table 5: Overall occurrence of HI antibodies in poultry against *A. paragallinarum* serotypes

Region	Type	Level	Seropisitivity to the various serotypes (%)[a]			
			Serotype A	Serotype B	Serotype C	Total*
Western	Commercial	Farm	1 (1)	0 (0)	2 (2)	10 (30)
		Chicken	4 (4)	0 (0)	12 (12)	100(16)
	Local	Household	7 (58)	0 (0)	3 (25)	12 (67)
		Chicken	24 (24)	0 (0)	14 (14)	98 (38)
Eastern	Commercial	Farm	4 (40)	0 (0)	1 (10)	10 (50)
		Chicken	25 (25)	0 (0)	8 (8)	100(33)
	Local	Household	6 (38)	0 (0)	3 (19)	16 (57)
		Chicken	31 (28)	0 (0)	19 (17)	112(41[b])
		Turkeys	0 (0)	0 (0)	0 (0)	114 (0)
North	Local	Household	NA	NA	NA	NA
		Chicken	46 (39)	4 (0.3)	51 (43)	118(70[b])
Central	Commercial	Farms	0 (0	0(0)	4 (80)	5 (80)
		Chicken	0(0)	0(0)	55 (80.1)	68 (80)
Overall seropositivity for all birds sampled in the study			18%	0.5%	22%	40.5%

[a] Number of positives with percentages shown in brackets.
[b] Some chickens had antibodies to more than one serotype.
* Total number of farms or households or birds from which samples were taken with percent of those positive shown in brackets.
NA= Not available,

Only antibodies to serotype C were demonstrated from the serum samples taken from four farms (Farm 1, 2, 3 and 4) where cases were reported and no antibodies were demonstrated on Farm 5 where infectious coryza had been suspected (Table 6).

Table 6: Seropositivity for HI serotypes against *A. paragallinarum* on farms where cases were reported from the central region

Area	Farm	Seropositivity of HI serotypes (%)[a]		
		Serotype A	Serotype B	Serotype C
Kampala	1	0/14 (0)	0/14(0)	14/14 (100)
Kampala	2	0/14 (0)	0/14(0)	14/14 (100)
Kampala	3	0/13 (0)	0/14(0)	13/13 (100)
Kampala	4	0/14 (0)	0/14(0)	14/14 (100)
Mpigi	5	0/13 (0)	0/14(0)	0/13 (0)
Overall occurrence		0%	0%	81%

[a] Number of positives/total number tested with percentage in brackets.

4.3 Phenotypic characteristics of the isolates

4.3.1 Biochemical characteristics of the isolates

The biochemical characteristics and serotypes of the isolates are shown in Table 6. All the five isolates belonged to Page serotype C. The isolates were all catalase negative, urease negative, oxidase positive and β-galactosidase positive and all were NAD-dependent. They produced acid from glucose, sucrose, mannitol, fructose, maltose and sorbitol except Apg-03, which did not produce acid from sorbitol. They all did not produce acid from rhamnose, lactose, dulcitol, trehalose, raffinose, arabinose and salicin.

Table 7: Biochemical characteristics and serotypes of *A. paragallinarum* isolated from chickens in Uganda

Property	*A. paragallinarum* strains					
	Apg-01	Apg-02	Apg-03	Apg-04	Apg-05	2403
Serotype	C	C	C	C	C	A
NAD-dependence	yes	yes	yes	yes	yes	yes
Oxidase	+	+	+	+	+	+
β-galactosidase	+	+	+	+	+	+
Urease	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Sugar fermentations						
Glucose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-
Sorbitol	+	+	-	+	+	+
Lactose	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Fructose	+	+	+	+	+	+
Salicin	-	-	-	-	-	-

4.3.2. Antimicrobial susceptibility of the isolates

The isolates were tested for susceptibility to clinically relevant antimicrobial agents. The minimum inhibitory concentrations of the antimicrobial agents against the various isolates are shown in Table 7. Antimicrobial resistance was demonstrated in some of the isolates. The five isolates could be divided into two types according to their resistance patterns, which matched their epidemiological background. Isolates Apg-01 and Apg-02 which were isolated from Farm 2 belonged together but only differ in tetracycline resistance, while the other three isolates Apg-03, Apg-04 and Apg-05 isolated from Farm 4 showed similar multidrug resistance patterns and differed from Apg-01 and Apg-02. The three isolates from Farm 4 showed multidrug resistance. They were resistant to ampicillin, streptomycin, sulphamethoxazole and tetracycline but sensitive to chloramphenicol and neomycin. The two isolates from Farm 2 were sensitive to all the antibiotics except isolate Apg-01, which was resistant to tetracycline. The corresponding resistance genes detected in isolates Apg-03, Apg-04 and Apg-04 in the present study are shown in Table 8.

Table 8: Minimum inhibitory concentrations (MICs) of six antimicrobial drugs tested against *A. paragallinarum* strains isolated in Uganda

Antibiotic	MIC (µg/ml) for the different isolates				
	Apg-01	Apg-02	Apg-03	Apg-04	Apg-05
Ampicillin	0.5 (S)	1 (S)	16 (R)	32 (R)	64 (R)
Chloramphenicol	2 (S)	1 (S)	2 (S)	1 (S)	0.5 (S)
Streptomycin	0.25 (S)	0.5 (S)	32 (R)	32 (R)	16 (R)
Neomycin	1 (S)	2 (S)	2 (S)	4 (S)	4 (S)
Sulphamethoxazole	2 (S)	4 (S)	64 (R)	64 (R)	32 (R)
Tetracycline	32 (R)	2 (S)	>64 (R)	>64 (R)	64 (R)

S = susceptible; R = resistant

4.3.3 Serotypes of the isolates

All the isolates recovered from this study belonged to Page's serotype C (Table 7).

4.4 Genetic characteristics of the isolates

4.4.1 Genetic characteristics by ERIC-PCR

The genetic characterisation of the isolates was performed by examination of the enterobacterial repetitive intergenic consensus (ERIC) sequence variations. The ERIC-PCR genotyping did not reveal any differences among the isolates. All the isolates showed similar banding patterns as shown in Figure 2.

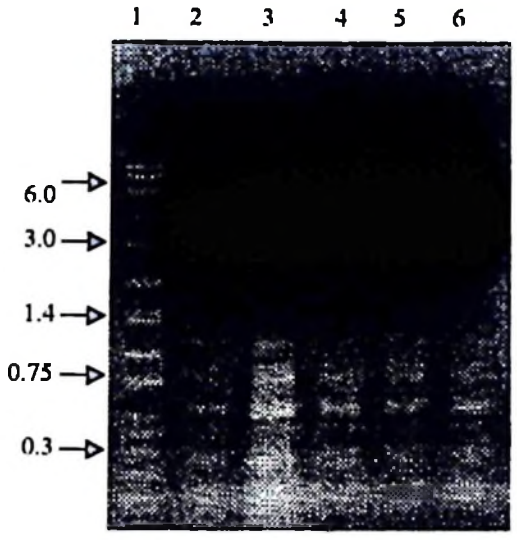

Figure 2: Genotyping of *A. paragallinarum* isolates from outbreaks of infectious coryza in Uganda by ERIC-PCR

Lane 1: Direct Load ^{TM} Wide Range DNA Marker; Lane: 2, 3, 4, 5, and 6 are *A. paragallinarum* strains Apg-01, Apg-02, Apg-03, Apg-04 Apg-05 respectively isolated from two different outbreaks of infectious coryza in Kampala.

4.4.2 Resistance genes in the isolates

All the isolates were screened for selected resistance genes. Isolates Apg-01 and Apg-02 were negative for genes tested in the present study. Resistance genes were detected only in the three isolates namely; Apg-03, Apg-04, and Apg-05, which were isolated from the same farm (Farm 4) and their resistance correlated with the phenotypic resistance observed as shown in Table 7. The resistance genes detected for tetracycline and sulphamethoxazole are shown in Figure 3. All the three isolates Apg-03, Apg-04 and Apg-05 carried *tet*(C) and *tet*(A) genes for tetracycline resistance and also carried *sul*2 gene for sulphamethoxazole resistance. No *tet*(B) and *tet*(G) genes for tetracycline resistance and *sul1* gene for sulphamethoxazole resistance were demonstrated in any of the isolates.

Resistance genes for ampicillin and streptomycin detected are shown in Figure 4. Three isolates; Apg-03, Apg-04, and Apg-05; carried *bla*TEM genes for ampicillin resistance which were not demonstrated in isolates Apg-01 and Apg-02. The *strA* gene for streptomycin resistance was demonstrated in only isolates Apg-03 and Apg-04. None of the streptomycin resistance genes screened for in the present study were demonstrated in isolate Apg-05, which had shown phenotypic resistance to streptomycin as shown in Table 7. The *aadA* gene for streptomycin resistance was not demonstrated in any of the isolates. No isolate was found with *aphA*-2 gene for neomycin resistance and *catA*1 gene for chloramphenicol resistance. A summary of the genes detected in the various isolates is shown in Table 8.

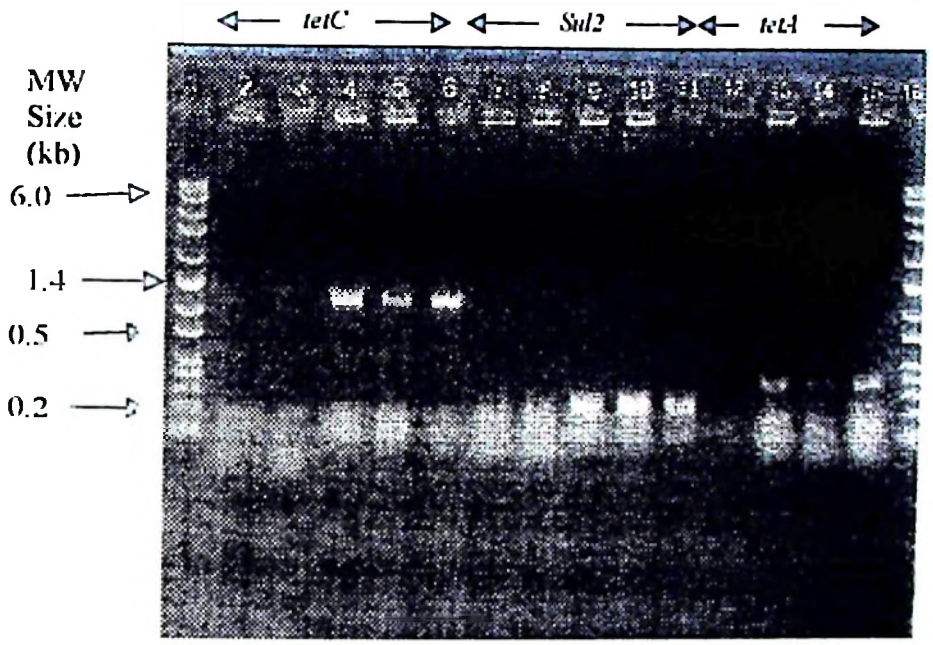

Figure 3: Detection of tetracycline (*tetC* and *tetA*) and sulphamethoxazole (*sul2*) resistance genes among *A. paragallinarum* strains isolated from Uganda

Lanes: 1 and 16: Direct Load ™ Wide Range DNA Marker; Lane: 2,3,4,5 and 6 correspond to isolates Apg-01, Apg-02, Apg-03, Apg-04 and Apg-05 tested for *tetC* genes; lanes: 7,8,9,10 and 11 are the same isolates in the same order tested for *sul2* gene while lanes 12,13,14 and 15 are isolate Apg-01, Apg-03, Apg-04 and Apg-05 tested for *tetA* gene. Isolates Apg-03, Apg-04 and Apg-05 are positive for *tetC*, *sul2* and *tetA* with corresponsing band sizses of about 1200, 200, 400 bp respectively

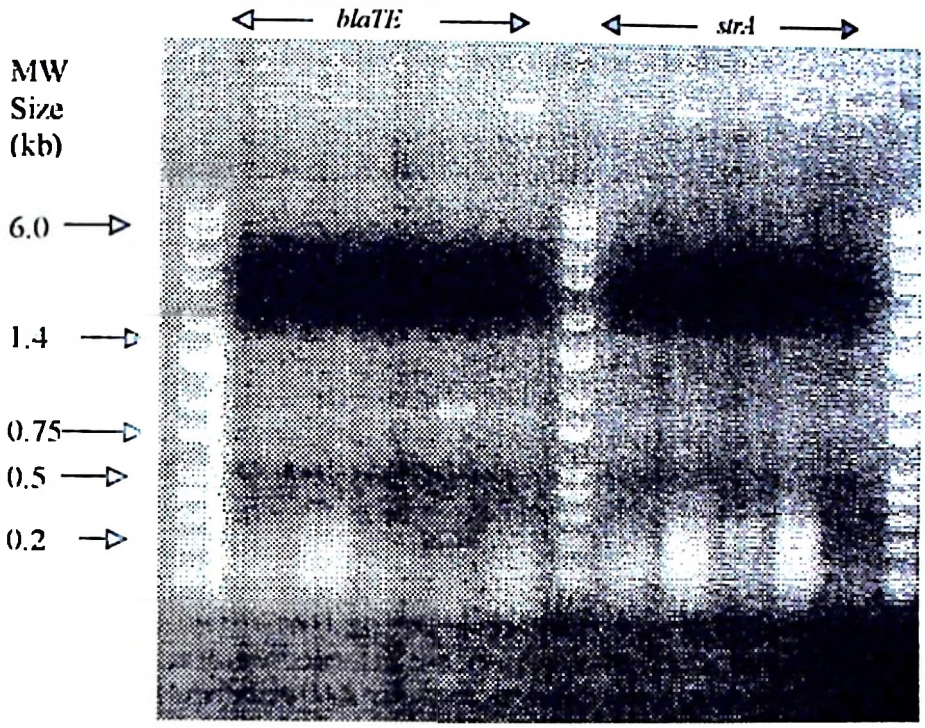

Figure 4: Detection of ampicillin (*blaTEM*) and streptomycin (*strA*) resistance genes in *A. paragallinarum* strains isolated from Uganda

Lanes 1, 7, and 13: Direct Load ™ Wide Range DNA Marker, Lane 2,3,4,5 and 6 correspond to isolates Apg-01, Apg-02, Apg-03, Apg-04 and Apg-05 tested for *blaTEM* gene, while lanes: 8,9,10,11 and 12 are the same isolates in the same order tested for *strA* gene. Isolate Apg-03, Apg-04 and Apg-05 show a band of about 850 bp for *blaTEM* genes while isolate Apg-03 and Apg-04 show a band of about 250 bp for *strA* gene

Table 9: Occurrence of selected resistance genes in *A. paragallinarum* isolates from infectious coryza outbreaks in Uganda

Antibiotic	Gene	Isolates				
		Apg-01	Apg-02	Apg-03	Apg-04	Apg-05
Ampicillin	bla_{TEM}	-	-	+	+	+
Chloramphenicol	*catA1*	-	-	-	-	-
Neomycin	*aphA-2*	-	-	-	-	-
Streptomycin	*strA*	-	-	+	+	-
	aadA1	-	-	-	-	-
Sulphamethoxazole	*sul1*	-	-	-	-	-
	sul2	-	-	+	+	+
Tetracycline	*tet(A)*	-	-	+	+	+
	tet(B)	-	-	-	-	-
	tet(C)	-	-	+	+	+
	tet(G)	-	-	-	-	-

- = Not detected, + = detected

4.5 Virulence characteristics of the isolates

4.5.1 Susceptibility of the isolates to serum

4.5.1.1 Comparison of the susceptibility of the different isolates in normal chicken serum

The growth kinetics of the different isolates and the reference strain in normal chicken serum over the six hours of incubation are shown in Figure 5. The graph shows an increase in the bacterial counts over time in all the isolates. The hourly increase in the bacterial counts did not differ significantly among the isolates ($p = 0.971$, $R^2=0.0234$).

The isolates were further compared by their survival indices in normal chicken sera after 3 and 6 hours of incubation. All the isolates were resistant by 3 hours after incubation and even much so at 6 hours (Table 9). The isolate that was treated with hyaluronidase (Apg-01A) was susceptible both at 3 hours and 6 hours of incubation.

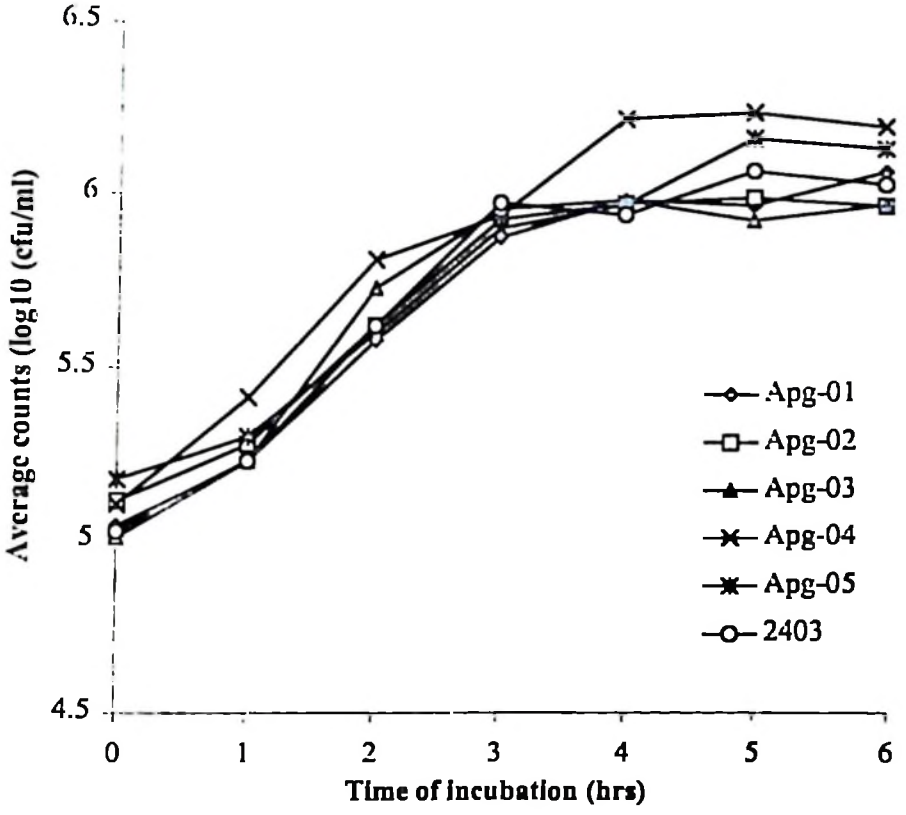

Figure 5: Comparison of the growth kinetics of *A. paragallinarum* isolates from infectious coryza in normal chicken sera. (Average of triplicate counts were plotted)

Table10: Susceptibility of *A. paragallinarum* isolates in normal chicken sera after 3 and 6 hours of incubation

Isolate	Incubation for 3 hours		Incubation for 6 hours	
	survival index[a]	susceptibility[b]	survival index[a]	susceptibility[b]
2403	4.0	R	10.4	R
Apg-01	3.5	R	10.9	R
Apg-02	3.2	R	7.3	R
Apg-03	5.3	R	9.4	R
Apg-04	3.3	R	5.1	R
Apg-05	2.7	R	9.3	R
Apg-01A	0.2	S	0.1	S

[a] Survival index (si) calculated as average of triplicate counts at 3 or 6 hours divided by average triplicate counts at initial count

[b] Susceptibility; R = resistant ($si \geq 0.5$), S = sensitive ($si < 0.5$)

Apg-01A is a hyaluronidase treated variant of Apg-01

4.5.1.2 Comparison of susceptibility of *A. paragallinarum* in sera from different species of poultry

Comparison of the growth kinetics of *A. paragallinarum* in sera from commercial chicken, local chicken, turkeys and guinea fowls is shown in Figure 6. Table 10 shows a summary of Tukey's multiple comparisons between the susceptibilities of the bacteria in sera of different poultry species showing which sera differed from each other. The bacteria were more susceptible in turkey serum ($P < 0.05$) and guineafowl serum ($P < 0.05$) than commercial and local chicken.

There was, however, no significant difference in the susceptibility of the bacteria in commercial chicken serum and local chicken serum ($P > 0.05$) neither was there a significant difference between susceptibility in turkey serum and guineafowl ($P > 0.05$).

When the sera from all the poultry species was heat treated for 30 minutes at 56^{0}C, to inactivate complement, the bacteria were equally resistant in all the sera, ($p > 0.05$, R^2=0.0370).

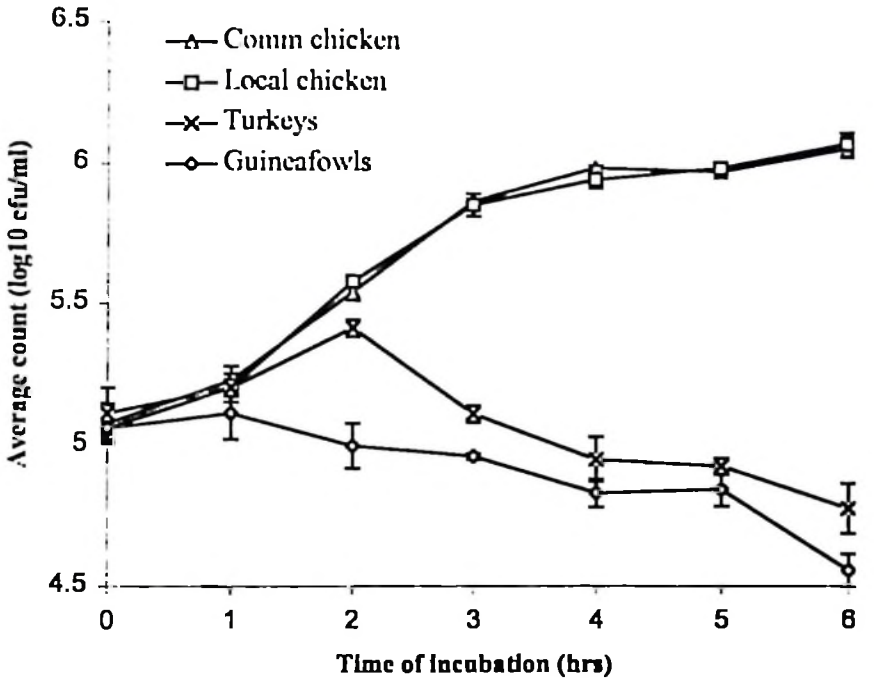

Figure 6: Comparison of growth kinetics of *A. paragallinarum* in normal sera from different poultry species, (Average of triplicate counts ± SEM are plotted).

Table 11: Tukey's multiple comparisons of the mean differences in susceptibility of *A. paragallinarum* in normal sera of different poultry species

Different sera compared	Mean difference (log_{10} cfu/ml)	P value	95% CI of diff
Commercial chicken vs local chicken	0.004	P > 0.05	-0.462 to 0.470
Commercial chicken vs turkeys	0.606	P < 0.01*	0.140 to 1.072
Commercial chicken vs guinea fowls	0.768	P < 0.001*	0.302 to 1.234
Local chicken vs turkeys	0.602	P < 0.01*	0.136 to 1.068
Local chicken vs guinea fowls	0.764	P < 0.001*	0.298 to 1.230
Turkeys vs guinea fowls	0.162	P > 0.05	-0.304 to 0.628

* Significantly different at the indicated level

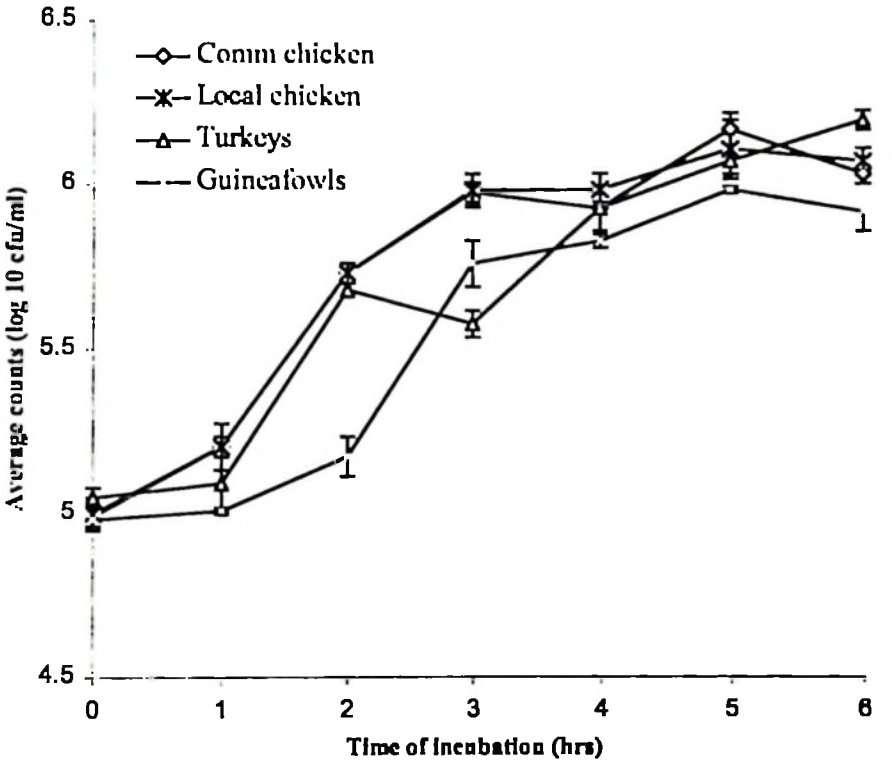

Figure 7: Comparison of growth kinetics of *A. paragallinarum* in heat-treated sera from different poultry species, (Average of triplicate counts ± SEM are plotted)

Comparison was also made of the survival index of *A. paragallinarum* in sera of the different poultry species at 3 and 6 hours of incubation both with and without heat treatment. The results of the survival indices are shown in Table 11. The results show that the bacteria were still resistant in guineafowl and turkey sera by 3 hours but became sensitive by 6 hours of incubation. After heat treatment, the bacteria were resistant to all the sera from all species at both 3 and 6 hours of incubation.

Table 12: Comparison of susceptibility of *A. paragallinarum* Apg-01 isolate in sera from different poultry with and without heat treatment.

Host sera	Incubation for 3 hours		Incubation for 6 hours	
	Survival index[a]	susceptibility[b]	survival index[a]	susceptibility[b]
Layer chicken[A]	3.1	R	9.6	R
Local chicken[A]	3.3	R	10.4	R
Turkeys[A]	1.2	R	0.4	S
Guinea fowl[A]	0.9	R	0.3	S
Layer chicken[B]	5.5	R	11.2	R
Local chicken[B]	5.4	R	12	R
Turkey[B]	4.3	R	14.3	R
Guinea fowl[B]	1.6	R	8.8	R

[a] Survival index (si) calculated as average of triplicate counts at 3 or 6 hours divided by average triplicate counts at initial count

[b] Susceptibility; R = resistant ($si \geq 0.5$), S = sensitive ($si < 0.5$)

[A] Normal sera (non-heated sera)

[B] Heated sera (sera heated at 56°C for 30 minutes to inactivate the complement)

4.5.1.3 Effect of the capsule on *A. paragallinarum* susceptibility to serum

The effect of hyaluronidase treatment on the susceptibility of *A. paragallinarum* is shown in Figure 8. The hyaluronidase removed the resistance effect of the bacteria to normal chicken serum unlike its wild type, which was resistant. The decapsulated variant was significantly more susceptible than the capsulated wild type ($p < 0.05$)

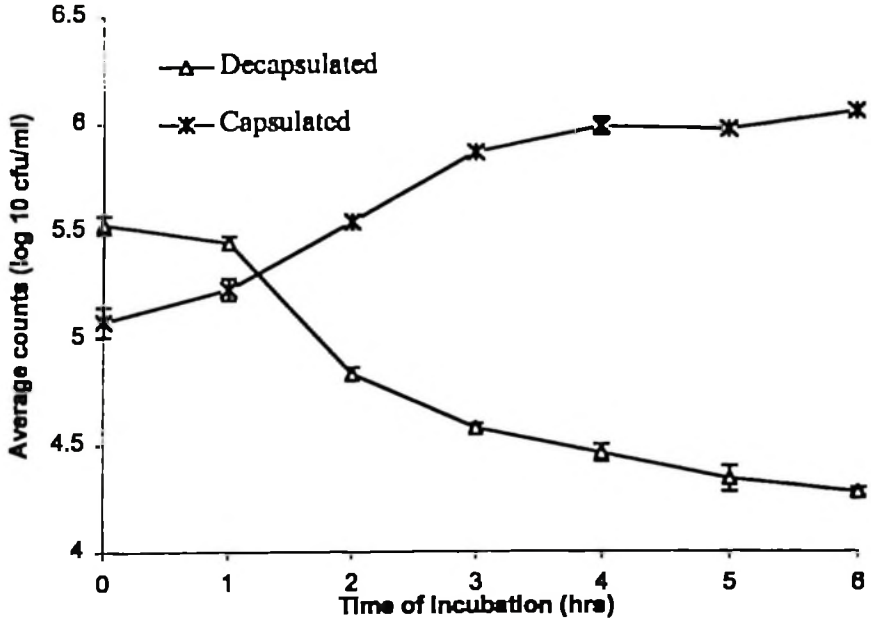

Figure 8: Comparison of growth kinetics of a wild type *A. paragallinarum* isolate and its variant decapsulated by hyaluronidase treatment in normal chicken serum, (average of triplicate counts ± SEM are plotted)

4.5.2 Pathogenicity of the isolates

4.5.2.1 Comparison of the virulence of the isolates in layer commercial chickens

The pathogenicity of the various isolates from the field outbreaks were compared for their pathogenicity in commercial layer chickens using disease scores for a 20 day period. Figure 9 shows a graphical representation of the mean disease scores of 10 chickens in each group for the field isolates and one reference strain. The mean disease scores were compared by one-way analysis of variance and the various isolates did not show any significant difference in their pathogenicity to chickens at 10 weeks of age ($p > 0.05$, R^2=0.006).

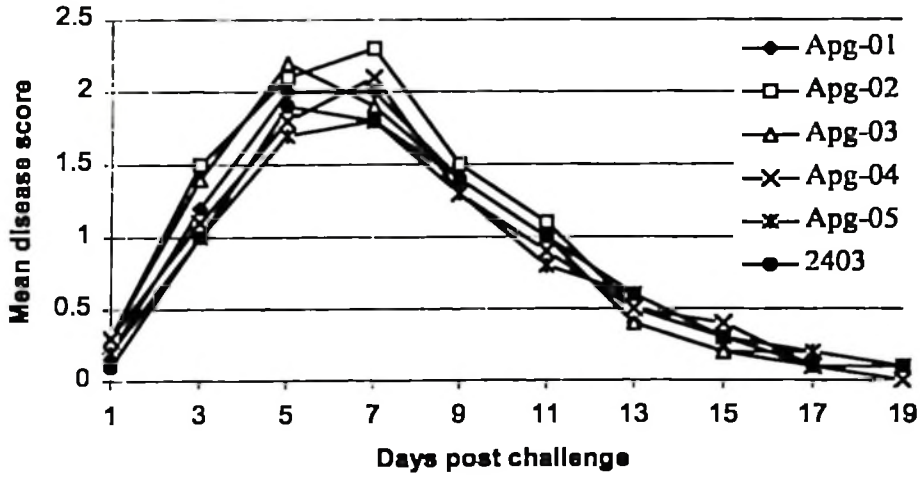

Figure 9: Comparison of the pathogenicity of the various strains of *A. paragallinarum* isolated from outbreaks of infectious coryza in Uganda.

4.5.2.2 Comparison of susceptibility of local and commercial layer chickens to *A. paragallinarum*

The susceptibility of local chickens was compared to commercial layers using disease scores as described earlier. Figure 10 shows the graphical representation of the mean disease scores of 10 chickens in each group for a 20-day period of observation following challenge with isolate Apg-01. It was observed that the disease scores were consistently lower in local chickens than in commercial chickens. However statistical analysis showed that there was no significant difference in the susceptibility between local chickens and commercial layers ($p > 0.05$, R^2=0.013).

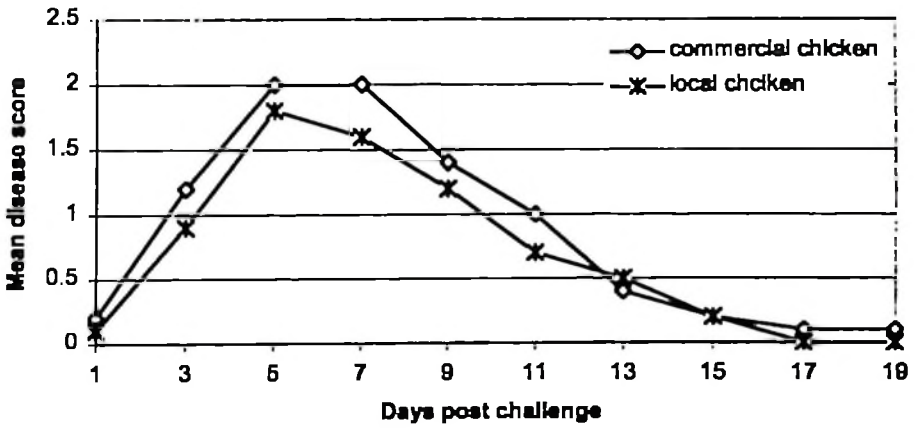

Figure 10: Comparison of pathogenicity of *A. paragallinarum* in commercial layer chickens and local chickens

4.5.2.3 Comparison of susceptibility of the different age-groups of chicken to *A. paragallinarum*

The susceptibility of the different age groups was compared. Figure 11 shows a graphical representation of the mean disease scores of 10 chickens in the different age groups; 4 weeks, 10 weeks and 21 weeks. The graph shows that 21-weeks old chickens have consistently higher mean disease scores than the rest of the age groups. Statistical comparison of the mean disease scores showed that the differences were not significant ($p > 0.05$, $R^2=0.063$).

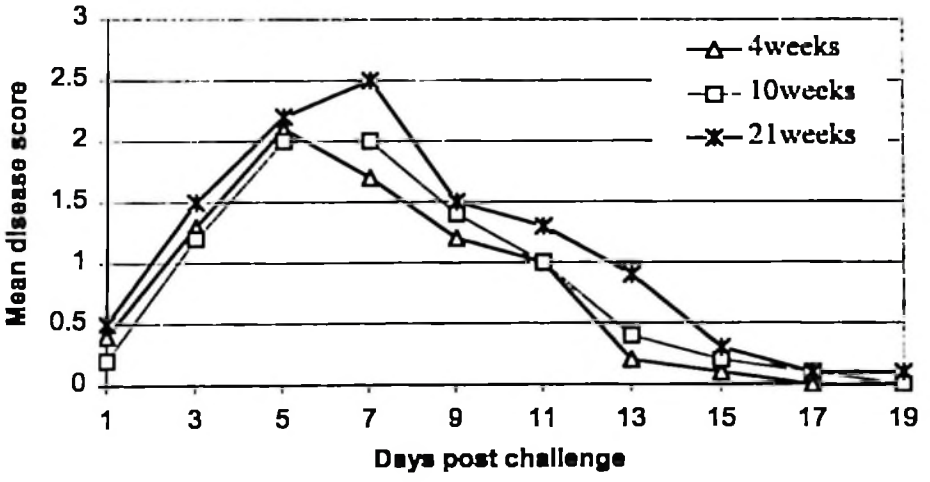

Figure 11: Comparison of pathogenicity of *A. paragallinarum* in different age groups of chickens.

4.6 Persistence of *A. paragallinarum* in the host and environment

None of the chickens showed clinical signs by day 21 post challenge (Table 12) and all were negative by culture and PCR and remained so till 60 days when the experiment was terminated. Sentinel chickens introduced at day 21 also remained negative throughout the experiment. The proportion of positives by PCR; calculated as the total number of positive samples by either method in the 20-day observation period divided by the total number of samples examined in the 20-day observation period; was significantly higher than culture for both host and environmental samples ($p < 0.05$). In the host samples, all the birds were negative by culture by day 13 post-inoculation while they wer all negative by day 16 by PCR. In the environmental samples the duration for detection of the organisms was shorter. Water samples were all negative by culture by day seven post-inoculation, while the samples turned all negative by day 10.

Table 13: Persistence of *A. paragallinarum* in the host and the environment as detected by culture and PCR

Day p.i	Host culture (n=10)	Host PCR (n=10)	Water culture (n=10)	Water PCR (n=10)
1	8	10	5	8
2	10	10	10	10
3	10	10	10	10
4	10	10	10	10
5	9	10	10	10
6	8	10	7	10
7	6	9	5	8
8	5	9	0	4
9	2	8	0	4
10	2	5	0	2
11	2	5	0	0
12	2	4	0	0
13	1	4	0	0
14	0	4	0	0
15	0	1	0	0
16	0	1	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
Total	*75/200	*110/200	*37/200	*66/200

*Total number of positives over the total number of samples analysed over the twenty-day period of observation

4.7 Susceptibility and potential transmission of *A. paragallinarum* by gallinaceous birds

The potential role of gallinaceous birds for transmission of *A. paragallinarum* was investigated in experimental infections. By 24 hours post challenge 80% of the turkeys showed evidence of unilateral swelling of the eyelid and the sinus samples were positive for culture in two of the three sampled birds. All the sinus swab samples were positive by PCR (Table 13). No signs however were evident by day four and no sinus sample of the three turkeys sacrificed was positive although one was still positive by PCR. By day five, all the birds had no evidence of any clinical signs, and none was positive by culture or PCR. Throughout this period none of the unchallenged sentinel chickens showed any evidence of any signs, neither was any of them positive by culture and PCR.

In group two where challenged chickens were housed together with turkeys, all the chickens showed clinical signs by day two and all the sinus samples were positive by culture and PCR over the five day period, but none of the turkeys showed any evidence of clinical signs nor was any of the sinus samples taken from them positive by culture or PCR. The control groups remained negative throughout the experiment.

Table 6: Susceptibility and transmission of *A. paragallinarum* from inoculated turkeys to unchallenged chickens and *vice versa*

Type of bird	Day post challenge	Clinical signs	Culture	PCR
Challenged turkeys[1]	1	12/15	2/3	3/3
	2	10/12	3/3	3/3
	3	3/9	1/3	2/3
	4	0/6	0/3	1/3
	5	0/3	0/3	0/3
Unchallenged chickens[1]	1	0/15	0/3	0/3
	2	0/12	0/3	0/3
	3	0/9	0/3	0/3
	4	0/6	0/3	0/3
	5	0/3	0/3	0/3
Challenged chickens[2]	1	12/15	3/3	3/3
	2	12/12	3/3	3/3
	3	9/9	3/3	3/3
	4	6/6	2/3	3/3
	5	3/3	2/3	3/3
Unchallenged turkeys[2]	1	0/15	0/3	0/3
	2	0/12	0/3	0/3
	3	0/9	0/3	0/3
	4	0/6	0/3	0/3
	5	0/3	0/3	0/3

[1]Group 1: Challenged turkeys with unchallenged chickens
[2]Group 2: Challenged chickens with unchallenged turkeys

Table 15: Susceptibility and transmission of *A. paragallinarum* from inoculated guinea fowls to unchallenged chickens and *vice versa*

Type of bird	Day post challenge	Clinical signs	Culture	PCR
Challenged guinea fowls[1]	1	10/15	2/3	3/3
	2	9/12	2/3	3/3
	3	3/9	0/3	1/3
	4	0/6	0/3	0/3
	5	0/3	0/3	0/3
Unchallenged chickens[1]	1	0/15	0/3	0/3
	2	0/12	0/3	0/3
	3	0/9	0/3	0/3
	4	0/6	0/3	0/3
	5	0/3	0/3	0/3
Challenged chickens[2]	1	14/15	3/3	3/3
	2	12/12	3/3	3/3
	3	9/9	3/3	3/3
	4	5/6	3/3	3/3
	5	3/3	2/3	3/3
Unchallenged guinea fowls[2]	1	0/15	0/3	0/3
	2	0/12	0/3	0/3
	3	0/9	0/3	0/3
	4	0/6	0/3	0/3
	5	0/3	0/3	0/3

[1]Group 1: Challenged with unchallenged chickens
[2]Group 2: Challenged chickens with unchallenged guinea fowls

4.8 Efficacy of a coryza vaccine against challenge with locally isolated *A. paragallinarum* strains from Uganda

The commercial vaccine induced protective antibodies among the chickens by 5 weeks post vaccination (HI titre $\log_2 > 5$) (Table 15). The vaccinated chickens were protected against challenge by the five isolates to the extent that they only showed mild transient clinical signs of short duration. By day seven post-challenge only one chicken in one group (Apg-04-challenged) still showed mild clinical signs. All the chickens in the other groups were no longer showing clinical signs.

Seven days after challenge, no *A. paragallinarum* was isolated from all the birds but one Apg-04-challenged bird, which was still showing mild signs, was positive by PCR and the others were all negative. Birds were all negative by both PCR and culture by 14 days when the experiment was terminated and none was showing clinical signs.

Table 16: Antibody responses to a commercial coryza vaccine and protection against challenge with locally isolated *A. paragallinarum* strains from Uganda

Challenge strain	HI antibody titres (GMT ± SE) post vaccination			Clinical signs post challenge [A]		
	Week 3	Week 5[B]	Week 7	Day 1	Day 3	Day 7
Apg-01	2.8±0.4	5.6±0.2	7.2±0.4	2/5	1/5	0/5
Apg-02	2.8±0.4	5.6±0.5	8.0±0.7	3/5	2/5	0/5
Apg-03	3.2±0.4	5.4±0.5	7.6±0.6	2/5	2/5	0/5
Apg-04	3.4±0.2	6.2±0.4	9.0±0.4	2/5	3/5	1/5
Apg-05	2.6±0.5	5.8±0.4	7.6±0.7	2/5	2/5	0/5
2403	3.2±0.2	6.2±0.2	8.2±0.6	1/5	2/5	0/5
Non-vaccinated	<5	<5	<5	4/5	5/5	4/5

[A] Number of chickens showing signs/number challenged
[B] Challenge was done at end of week 5 following first vaccination
GMT; Geometric means of the titres (log_2); SE; standard error of the mean

CHAPTER 5

5.0 DISCUSSION

A. paragallinarum was isolated from chickens showing signs of coryza in Uganda. This confirms previous reports of the disease, observed in chickens that were based on clinical and pathological examinations (Ojok, 1993). This is the first time the bacterium has been isolated in Uganda. Systematic random sampling in healthy birds did not result in isolation of *A. paragallinarum* bacteria. Isolation of the agent was only possible during purposive sampling on farms where cases were reported. Although previous reports were suggestive of infectious coryza, no systematic studies had been conducted to isolate the causative agent. Post-mortem records at the Pathology department, Makerere University, only show eight cases since 1990 (Makerere University, Veterinary Pathology; unpublished data). Clinical signs combined with pathological lesions are not pathognomonic and cannot be relied upon.

Clinically, chickens with signs suggestive of infectious coryza may also be due to other organisms such as *Ornithobacterium rhinotracheale*, (ORT) (Amonsin, *et al.*, 1997). Reports of isolation of ORT from chickens showing signs of coryza have become frequent (Bragg *et al.*, 1997). When infectious coryza is associated with other infectious agents, the typical clinical signs and lesions are no longer straightforward and this frustrates accurate diagnosis of the disease. In the present study, cases were reported on five farms basing on the clinical signs. However, it was possible to isolate *A. paragallinarum* from only two of the farms. Even on farms where PCR positive

samples were obtained, not all the samples were positive by culture. Failure rates in isolation of the bacterium have been reported as being due to overgrowth with other contaminating organisms and because Apg is fastidious and does not survive for a long time outside the host (Blackall, 1999). The isolation rates in many countries have been indeed few. In Taiwan, Lin *et al.*, (1996) only managed to get two isolates from broiler and breeder chickens, while in China just over ten could be recovered over a period of 7 years (Chen *et al.*, 1993). Other countries have reported more isolates but over a long time of deliberate surveillance (Reid and Blackall 1984; Horner *et al.*, 1992).

Page (1962b) demonstrated that the organisms couldn't survive in drinking water for more than four hours. Chen *et al.*, (1998b) demonstrated that traditional culture failed to detect the organisms after storage for 3 days regardless of storage medium or storage temperature even when stored at low temperatures of –20°C. PCR was more sensitive and could detect the organisms after storage for 14 days and some up to 180 days when stored in glycerol PBS medium at –20°C. Indeed the isolation rate from samples in the present study was poor even in samples which were positive by PCR. PCR detected more positive samples than culture was able to do in agreement with Chen *et al.*, (1998b). With availability of PCR, it may offer a better and more accurate alternative for the diagnosis of this very fastidious organism.

Routine isolation and biochemical characterisation requires the availability of specialised complex media, which are often expensive (Blackall and Yamamoto, 1998). *A. paragallinarum* are also relatively slow-growing and difficult to obtain in

pure cultures. In field investigations where nasal samples are used, Apg is frequently overgrown by related organisms such as *Avibacterium volantium*, *Avibacterium avium*, or *Avibacterium gallinarum*, which are part of the normal flora in chicken upper respiratory system and less nutritionally demanding (Blackall *et al.*, 1997).

In the present study, all the isolates obtained belonged to serotype C by the Page serotyping scheme. The occurrence of the different serotypes varies around the world although most countries have reported the occurrence of the three serotypes. All the three serotypes have been reported in Argentina (Terzolo *et al.*, 1993), USA (Page, 1962a), Philippines (Nagaoka *et al.*, 1994), South Africa (Bragg *et al.*, 1996) Brazil (Blackall *et al.*, 1994) and Mexico (Fernandez *et al.*, 2000). Serotype A and C have been reported in Australia (Thornton and Blackall 1984, Blackall and Eaves, 1988), Japan (Kume *et al.*, 1978), Malaysia (Zaini and Iritani, 1992) and Indonesia (Tagaki *et al.*, 1991). Serotype A and B have been reported in Germany (Hinz, 1976). In China serotypes A and B have been described (Chen *et al.*, 1993; Zhang *et al.*, 2003). Description of the bacterium has also been done in several other countries including India (Srinivasa *et al.*, 1989), and Morocco (Mouahid *et al.*, 1989). The isolation of serotype C from chickens in Uganda correlates with other studies that have been carried out in Africa where serotype C has been isolated in Zimbabwe and South Africa (Bragg, 2002c). However it cannot be concluded from the present study that serotype C is the only serotype occurring in Uganda. Moreover, antibodies to other serotypes have been demonstrated in the present study.

The occurrence of antibodies to all the serotypes demonstrated in the present study indicated that all the serotypes occur in Uganda although the prevalence of antibodies

to serotype B was negligible. Reports of the occurrence of all the serotypes has been made in South Africa and other countries, it is therefore possible that all the serotypes occur in Uganda in spite of the fact that in this study only serotype C was isolated. It is also possible that serotype C is more prevalent than the others.

Although the isolation of the agent in many African countries has not been reported, there is reason to believe that the agent exists just like it had not been isolated in Uganda previously. Published reports show that in Africa the bacterium has only been isolated in South Africa (Verschoor *et al.*, 1989; Horner *et al.*, 1992, Bragg *et al.*, 1997), Zimbabwe (Bragg, 2002c) and Morocco (Mouahid *et al.*, 1989). Uganda is the fourth African country from which *A. paragallinarum* has been isolated and described. Difficulties associated with isolation of the agent and the cost may be responsible for the little work that has been reported on this organism in many developing countries due to lack of resources to buy the expensive culture requirements. Indeed even in the present study these difficulties were encountered and on some farms where PCR showed positive cases, and birds were showing typical signs, it was still not possible to make any isolations. It is also possible that the disease is easy to treat, or does not cause a lot of economic losses compared to other diseases. These could also partly account for the limited interest in isolating the organism in many African countries.

The biochemical characteristics of the strains isolated in the present study were typical of *A. paragallinarum* as described by other authors (Blackall *et al.*, 2005). There were no variations in their biochemical characteristics except one isolate (Apg-03) that did

not ferment sorbitol. Biotyping has been used in different studies including *A. paragallinarum* and found to be able to differentiate isolates that are epidemiologically unrelated and group those that are related into the same biotype. Blackall *et al.*, (1989) used biotyping to differentiate between their isolates and were able to group them into three biotypes, although more than 80% belonged to one biotype. In studies involving a few isolates like in the present study, this may not offer much discrimination among isolates. A combination of different phenotypic methods such as biotyping and susceptibility testing, however, has been observed to offer better discrimination.

On the basis of antimicrobial resistance patterns, the isolates from the present study could be differentiated and grouped according to their epidemiological background. Two of the isolates (Apg-01 and Apg-02), which were isolated from the same farm, were sensitive to all the antibiotics except one (Apg-02), which was resistant to tetracycline. On the other hand the three isolates, which were isolated from another farm, were all multidrug resistant to similar drugs (tetracycline, streptomycin, ampicillin and sulphamethoxazole). Whereas the carbohydrate fermentation failed to differentiate the isolates, the drug resistance typing clearly differentiated the strains from the two farms into two groups.

Blackall *et al* (1989) reported that a combination of serotyping, carbohydrate fermentation and antimicrobial resistance typing provide a suitable approach for epidemiological studies of infectious coryza. In another study Blackall *et al* (1990d) reported that antimicrobial biotyping was more useful in differentiating

epidemiologically unrelated strains than carbohydrate fermentation. They reported that antimicrobial types were found to compare favourably with genotyping methods such as restriction enzyme analysis (REA). The present study agrees with those observations of Blackall *et al.*, (1989) about antimicrobial resistance patterns being more useful in differentiating epidemiologically unrelated strains where carbohydrate fermentations patterns failed to do so. Other workers (Soriano *et al.*, 2001), however, have been able to ascribe their isolates into groups basing on biochemical variations among their isolates that closely corresponded with antimicrobial resistance patterns.

The strains isolated from the present study were all NAD-dependent. Until the early 1990s, all the isolates of *A. paragallinarum* were known to be NAD-dependent (Blackall, 1999). There are now increasing reports of NAD-independent isolates of *A. paragallinarum*. These were first reported in South Africa by Horner *et al.*, (1992), but have also been recently described in Mexico (Garcia *et al.*, 2004). Originally most of the NAD-independent isolates belonged to serotype A, but recently those belonging to other serotypes have been described (Bragg, 2004; Garcia *et al.*, 2004).

As discussed later, experimentally infected turkeys and guineafowl only showed clinical signs of short duration and those in contact with infected chickens did not pick up infection neither were any organisms isolated from them nor were they detected by PCR. Whereas turkeys have been reported to be refractory to the disease (Blackall and Yamamoto 1998), there had been no systematic investigations of the susceptibility of these species reported in literature. Studies regarding susceptibility of guineafowl to *A. paragallinarum* are not available either. The isolation of Apg from only two Japanese quail out of many showing coryza signs (Reece *et al.*, 1981)

strengthens the argument that IC is a disease of chickens and other birds are refractory to the disease (Blackall *et al.*, 1997).

It has become difficult to identify with precision organisms reported in previous studies as causing infectious coryza and especially so in other poultry species. This is because there have been more and more NAD-independent isolates being reported and other new organisms causing similar clinical signs such as *Ornithobacterium rhinotracheale* (Amonsin *et al.*, 1997). Further reclassification of former avian haemophili has also made the status of previous studies involving avian haemophili uncertain. It would be interesting to repeat such experimental infections in other poultry species using reference strains of the newly defined species of *A. paragallinarum* in other poultry related to chickens in which reports of infectious coryza have been made. Turkeys are particularly susceptible to *O. rhinotracheale,* which has been only described recently (Vandamme *et al* 1994).

It has been reported that the occurrence of most respiratory diseases vary considerably depending on several factors including overcrowding. Overcrowding is not a serious phenomenon in most free-range poultry systems in Uganda where households keep a few chickens (Byarugaba *et al.*, 2002). In the present investigations, clinical disease was not encountered in the free-range local poultry and no isolates were recovered from them. Experimental infections conducted in the present study and elsewhere though show that local chickens are susceptible (Lin *et al.*, 1996). Failure to isolate any organisms from free-range local poultry did not mean that they are not susceptible just like it was not possible to isolate any Apg from healthy commercial chickens. Experimental infections demonstrated that the Ugandan free-range local chickens are

equally susceptible to Apg as commercial chickens (this study). Infectious coryza has been reported to be more frequent where there is multi-aged birds kept on the same farms (Blackall, 1999). Free-range poultry systems in Uganda typically keep multi-aged birds and often mixed species (Byarugaba *et al.*, 2002). However, many factors, which are not well understood, seem to be required to set off outbreaks. Free-range local poultry in Uganda are typically kept in small flocks of 5-20 birds; less crowded and therefore may be less prone to disease of intensification.

Bacterial antimicrobial resistance in both the medical and agricultural fields has become a serious problem in developing countries just like elsewhere in the world (Byarugaba, 2005). In the present study, all the isolates were sensitive to chloramphenicol and neomycin. The two isolates (Apg-01 and Apg-02) recovered from the same farm were also sensitive to the rest of the drugs tetsted namely; ampicillin, streptomycin, and sulphamethoxazole except isolate Apg-01, which was resistant to tetracycline. The three other isolates (Apg-03, Apg-04 and Apg-05) from another farm showed multidrug resistance to tetracycline, ampicillin, streptomycin and sulphamethoxazole. Resistance in *A. paragallinarum* has been reported in earlier studies. Blackall (1988) examined a number of isolates and reported varying patterns of resistance with multidrug resistance patterns to tetracycline, neomycin and streptomycin comparable to the present study.

Increasing trends of resistance in different bacteria have been reported over the years in many countries including Uganda (Byarugaba and Nakavuma, 2005). Antibiotic resistant strains of bacteria are an increasing threat to animal and human health. In the

present study the multidrug resistant isolates were obtained from a farm, where birds had not yet undergone treatment, which meant that the birds were infected with an organism already resistant to the various drugs possibly from previous infections. This poses a danger for spread of such resistant bacteria and poses a challenge in treatment. Treatment failures have been reported in other infectious coryza outbreaks in various places (Lu *et al.*, 1983; Reece and Coloe. 1985; Verma *et al.*, 1985; Blackall, 1988; Blackall *et al.*, 1989; Takahashi *et al.*, 1990; Prasad *et al.*, 1999). Relapses after treatment may also occur thus creating and sustaining carrier status within flocks of drug resistant strains.

Basing on the antimicrobial resistance patterns, the strains could be grouped into two groups that corresponded with the epidemiological background of the strains. This is in agreement with other studies (Blackall *et al.*, 1989), which have found antimicrobial resistance to be a relatively good epidemiological marker in infectious coryza studies comparable to some genetic typing methods such as restriction enzyme analysis. This might be explained by the different drug use patterns that different farmers might be using on their farms that may result in generating resistant reservoirs on their farms with particular resistance patterns.

In the present study, drug use on the farms was different. Farms 1,2 and 5 had used tetracycline, while Farm 3 had used a combination of penicillin and streptomycin and Farm 4 reported having not used any drug in since the present signs were observed. Previous records of drug use prior to these particular cases were not available. Indeed it was on Farm 1 where an isolate resistanct to tetracycline was isolated while multi

drug resistant isolates were receovered from Farm 4, which had not yet used any drug. This therefore may mean that these birds were infected with already resistant bacteria from previous infections or due to treatment from other infections. It is now well established that antimicrobial use in the major cause of development of resistance in bacteria (Byarugaba, 2004). Commercial poultry farmers are known to use drugs frequently and this could resulted in selection of resistant bacteria that exhibited resistance in the present study.

Genetic characterisation of the isolates from the present study by enterobacterial repetitive intergenic consensus (ERIC) sequences polymerase chain reaction did not reveal any differences among the isolates. Similar reports of clonality of *A. paragallinarum* have been reported using ERIC-PCR by Soriano *et al* (2004a) among isolates from the same region in Mexico although differences were however noted between the isolates from other countries. Other genotyping methods that have been used to type *A. paragallinarum* have had varying levels of differentiation among strains. Using ribotyping and restriction enzyme analysis, the NAD-independent isolates from South Africa were reported to be clonal (Miflin *et al.*, 1995). Comparison of these NAD-independent isolates revealed however that they were different from the NAD-dependent isolates from same country and elsewhere in the world suggesting that the South African NAD-independent isolates were clonal.

Restriction enzyme analysis (REA) has been used with different enzymes being able to reveal differences among isolates, which were comparable to antimicrobial resistance in differentiating between the isolates (Blackall *et al.*, 1990d). Different

enzymes result in differences in banding patterns and extent of differentiation. Blackall and Yamamoto (1989) also attempted to type *A. paragallinarum* using whole cell protein. All the isolates showed similar profiles and could not be differentiated on that basis. Using outer membrane proteins (OMPs) also failed to differentiate between epidemiologically unrelated *A. paragallinarum* isolates (Blackall *et al.*, 1990e). These studies involving the use of whole cell proteins or outer membrane proteins have shown that *A. paragallinarum* are homogeneous with regard to protein profiles and have not been useful tools in differentiating between unrelated isolates of Apg.

Although there is no gold standard typing method available today, more powerful methods such as pulsed field gel electrophoresis (PFGE) are thought to give better differention among isolates. However, the expensive equipment required for PFGE often precludes its frequent use in many countries. Sequencing the whole genome and comparison of sequence variation among isolates or strains of course would be the ideal, but it is even more expensive and impractical and possibly not always necessary.

Whereas typing using antimicrobial resistance patterns has been found to be comparable to some genotyping methods such as REA (Blackall, *et al.*, 1990d) in differentiating among strains of Apg, and phenotypic resistance of *A paragallinarum* for a number of important antibiotics described, no information is available in published literature about the mechanisms of resistance in *A. paragallinarum*. Resistance mechanisms have been identified and described for almost all known antimicrobials currently available for clinical use (Sundsfjord *et al.*, 2004).

In the present study, the isolates were screened for selected resistance genes for clinically relevant antibiotics. The study demonstrated for the first time, resistance genes for sulphamethoxazole, tetracycline, streptomycin and ampicillin resistance in isolates of *A. paragallinarum*.

The *sul-2* gene for resistance against sulphamethoxazole was demonstrated in three isolates from the same farm while the *sul-1* gene was not demonstrated in any of the isolates. Sulphonamide resistance in gram-negative bacilli arises from the acquisition of either of the two genes *sul1* and *sul2*, encoding forms of dihydropteroate synthase that are not inhibited by the drug (Enne *et al.*, 2001). The *sul-1* gene is normally found linked to other resistance genes in class 1 integrons, while *sul-2* is said to be usually located on small non-conjugative plasmids or large transmissible multi-resistance plasmids. Plasmids in *A. paragallinarum* were not found in isolates that were found resistant phenotypically to several antibiotics (Blackall, 1988). It is possible that these resistance genes in *A. paragallinarum* are chromosomally encoded.

In the present study, isolates, which exhibited *sul2* resistance gene, were found with more than one other resistance genes in agreement with the observations made by Enne *et al* (2002). Chromosomally encoded *sul-2* gene has been reported in *Pasteurella* and *Mannheimia* associated with other resistance genes; *cmr* and *strA* for chloramphenicol and streptomycin resistance respectively (Kehrenberg and Schwarz, 2001). Genetic linkage of sulphonamide resistance to other resistances is thought to be important for maintenance of sulphonamide resistance even in absence of sulphonamide usage (Enne *et al.*, 2002).

Tetracycline is one of the most commonly used antibiotics in Uganda and resistance to this drug has been demonstrated both in human and animal bacterial isolates (Byarugaba, 2005; Byarugaba and Nakavuma, 2005). Phenotypic resistance to tetracycline was demonstrated among four of the five *A. paragallinarum* strains isolated in the present study. A limited number of bacteria acquire resistance to tetracycline by mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, change the regulation of innate efflux systems, or alter the 16S rRNA (Chopra and Roberts, 2001). The most frequent types of *tet* genes encoding these resistance mechanisms belong to classes A, B, C, D, and G (Roberts, 1996). In the present study, screening was done for *tet* genes of classes A, B, C, and G which are common among Gram-negative bacteria and often encode for efflux mechanism of resistance to tetracycline.

Both *tet(*C) and *tet(A)* genes were demonstrated in three isolates from the same farm. This is the first time that these resistance genes are reported in *A. paragallinarum.* Several different *tet* genes have been described as conferring resistance to tetracyclines in many bacteria. Many of these genes are associated with mobile plasmids or transposons and can be distinguished from each other using molecular methods. Both *tet(*A) and *tet(*C) genes have been demonstrated in clinical isolates of different Gram-negative bacteria (Zhang and Chen, 1991; Aminov *et al.*, 2002; Lanz *et al.*, 2003; Hartman *et al.*, 2003) just as these two genes were demonstrated in the present study.

The *tet*(B) gene represents the predominant gene among *Enterobacteriaceae* (Marshall *et al.*, 1983; Martinez-Salazar *et al.*, 1986) and has been reported to be widely distributed among other families of gram-negative bacteria (Levy, 1988). It has been found in members of the family *Pasteurellaceae*, namely, isolates of *Haemophilus influenzae*, *Haemophilus ducreyi*, and *Haemophilus parainfluenzae* involved in infections of humans (Marshall *et al.*,1983; Levy *et al.*, 1984; Heuer *et al.*, 1987) and has also been recently demonstrated in *Haemophilus parasuis* for the first time in this organism on a plasmid from pig isolates (Lancashire *et al.*, 2005). This gene was not demonstrated in all the isolates of *A. paragallinarum* examined in the present study.

The *tet*(G) resistance gene was not demonstrated in any of the isolates from the present study. This is in contrast to reports of this gene in related genera of bacteria. *Pasteurella multocida* has been shown to carry *tet*(G) gene associated with *sul-2* for sulphonamide resistant dihydropteroate synthase, and *catB2* encoding chloramphenicol acetyltransferase on plasmids (Wu *et al.*, 2003). Although *sul-2* gene was demonstrated in the present study, no *cat* gene was demonstrated either.

The *strA* and *strB* genes are highly prevalent in *Salmonella* strains (Pezella *et al.*, 2004) and have recently been reported in *Pasteurella* and *Mannheimia* (Kehrenberg and Schwarz, 2001). In the present study *strA* was demonstrated in two isolates of the three, which showed phenotypic resistance to streptomycin. Aminoglycoside resistance is frequently associated with genes borne on integrons and are diffused in gram-negative bacteria. These integrons have frequently been associated with the

widely distributed transposon Tn*21* (Liebert *et al.*,1999). The Tn*21*-associated integrons often carry the *aadA1* gene cassette, known to confer resistance to streptomycin (Liebert *et al.*, 1999). In the present study *aadA1* gene was not demonstrated in any of the isolates either, just like the *aphA*-2 gene for neomycin resistance. The absence of both *aphA*-2 and the *aadA* genes, which are associated with the Tn21 transposon, might imply absence of this transposon in these isolates.

The present study did not detect *aphA*-2 gene for neomycin resistance among all the isolates screened. The *aph* genes are the most prevalent resistance genotypes in neomycin (Gomez-Lus 1998). The *aphA*-2 belongs to the major AAC-related resistance determinant capable of acetylating a variety of aminoglycoside antibiotics. Resistance that arises by mutation of the 16S RNA gene are rare in bacteria that have multiple copies of rRNA genes (Dessen *et al.*, 2001). It is therefore possible that the lack of demonstration of resistance to neomycin in strains isolated from the present study could be due to this reason. These genes have been described as being part of transposon Tn*5393* and are frequently located on plasmids.

Chloramphenicol resistance has been well described in *Salmonella* since it was considered as the drug of choice to treat salmonellosis in human and veterinary medicine over a long period of time, which has led to the selection of resistant strains. The resistance to chloramphenicol is known to be mediated by the plasmid-located enzymes called chloramphenicol acetyltransferases (CAT) (Cannon *et al.*, 1990), or by the nonenzymatic chloramphenicol resistance gene *cmlA*, that encodes an efflux pump (Dorman and Foster, 1982). The CAT enzymes encoded by the *cat* family of

genes are widespread in Gram-negative bacteria and are sub-categorised into *catA* and *catB* groups (White *et al.*, 2000). Resistance gene *catA1*, for chloramphenicol resistance was not demonstrated in any of the isolates in the present study. However similar genes have been reported in related genera in the family *Pasteurellaceae*. Kehrenberg and Schwarz (2001) reported the occurrence and integration of *catA-3* gene in the non-coding spacer between *sul2* and *strA* on structurally different plasmids, but also on the chromosome of *Pasteurella* and *Mannheimia*, which underlines their mobility. Although both *sul2* and *strA* genes were demonstrated in isolates from the present study no *cat* genes were detected.

β-lactam antibiotics, a vast group of molecules that includes penicillins and cephalosporins, are used extensively in Uganda and resistant strains have been reported frequently in bacteria isolates of animal origin (Byarugaba and Nakavuma, 2005). The β–lactam resistant bacteria produce an enzyme β–lactamase, which enzymatically cleaves the four membered β–lactam ring, rendering the antibiotic inactive. In the present study, *blaTEM* genes, which are part of the genes that encode for β–lactamases, were demonstrated in three isolates. β-lactamases represent the main mechanism of bacterial resistance to β-lactam antibiotics.

Wu *et al.*, (2003) found a plasmid carrying a *blaP1* encoding a beta-lactamase for resistance to ampicillin in *Pasteurella* associated with *aadA1* gene encoding an aminoglycoside adenylyltransferase that confers resistance to streptomycin. This association of several antibiotic resistance genes has been reported in many bacteria

associated with integrons. There is therefore a possibility most of these genes demonstrated in the present study might be associated with integrons.

Indeed just like the phenotypic antimicrobial resistance was the only method that differentiated the isolates on their epidemiological basis in agreement with Blackall *et al* (1989), the occurrence of selected genes was also able to differentiate the epidemiologically unrelated strains into different groups. The occurrence of the resistance genes in most isolates correlated with the phenotypic resistance observed in the present investigations. No resistance genes screened for in the present study, were detected in the two isolates (Apg-01 and Apg-02) isolated from the same farm although one of them (Apg-01) had shown phenotypic resistance to tetracycline. It is possible resistance mechanism that were not investigated in this study were responsible for the resistance in this isolate.

Today, the emergence of bacterial strains which display resistance to a variety of drugs (multiple drug resistant; MDR) is a major cause of failure of treatment of infections worldwide. The major factor responsible for development of resistance is the exposure of the organisms to antibiotics. Inappropriate use such as use of sub-therapeutic doses, which is common in developing countries (Byarugaba, 2004), is the major reason for development of antimicrobial resistance.

The present study has reported occurrence of multidrug resistance and demonstrated for the first time genes responsible for the observed phenotypic resistance. The study has demonstrated almost similar resistance genes reported by Ridley and Threlfall

(1998) in MDR *Salmonella enterica* serotype Typhimurium phage type DT104 with the exception of the absence of the chloramphenicol genes. Similar multidrug resistance mechanisms are becoming frequently reported in related genera of the family *Pasteurellaceae* (Kehrenberg *et al.*, 2003; Kehrenberg and Schwarz, 2001). High-level multidrug resistance is normally associated with mobile genetic elements (plasmids, transposons or integrons) that encode specific resistance genes (Hall, 1997).

Kehrenberg and Schwarz (2001) have previously showed that *sul-2* and *strA* genes are widely distributed among epidemiologically unrelated isolates of the genera *Pasteurella* and *Mannheimia* occuring in clusters of sulphonamide, chloramphenicol and streptomycin resistance genes (*sul2-catA3-strA)*, as well as *sul2-strA* or *strA-sul2* clusters alone on structurally different plasmids, but also on the chromosome of *Pasteurella* and *Mannheimia* which underlines their mobility. In the present study both *sul2* and *strA* were detected in two isolates suggesting they could be occurring in similar clusters as was reported in *Pasteurella multocida*.

The similarity of multidrug resistance genes observed in the present study with those of related pathogens gives a suggestion for potential exchange of these genes in the respiratory system. This phenomenon has been well demonstrated in bacteria that inhabit the gut (Courvalin, 1994). Further extensive studies to elucidate the molecular basis of their mechanisms of resistance and potential for spread to other organisms is crucial to curtail further spread and containment of possible treatment failures.

Several workers have reported on the pathogenicity and virulence of *A. paragallinarum* from different countries as an important aspect in characterising the isolates and their role in causation of infectious coryza (Page, 1962a; Reid and Blackall, 1984; Sawata *et al.*, 1985a; Yamaguchi *et al.*, 1990; Lin *et al.*, 1996; Bragg, 2002a; Soriano *et al.*, 2004b). The virulence of the field isolates in the present study was evaluated by examination of the ability to survive in serum and ability to cause disease in experimental infections.

The present investigations confirmed that the *A. paragallinarum* isolates from Uganda are pathogenic to chickens in agreement with other reports of isolates from elsewhere (Page, 1962a; Reid and Blackall, 1984; Sawata *et al.*, 1985b;Yamaguchi *et al.*, 1990; Lin *et al.*, 1996; Bragg, 2002a; Soriano *et al.*, 2004b). Using disease scores described by Bragg (2002a), there was no significant variation in the virulence between the isolates. Similarity in virulence of *A. paragallinarum* isolates has also been reported by other workers (Reid and Blackall, 1984) in agreement with the findings of the present study. It was also shown that the virulence of the NAD-independent isolates belonging to serogroup C and serogroup A were similar to each other (Bragg, 2002b). These South African NAD-independent isolates had previously been shown to be clonal by various typing methods and therefore it was not surprising that even their virulence did not differ (Miflin *et al.*, 1995). The strains isolated from the present study had similar genotype by ERIC-PCR too.

However, differences in virulence of Apg isolates have been reported by other workers. Bragg (2002b) demonstrated that the South African serotype C of the NAD-

dependent type were more virulent than the serotype A and B. Differences in virulence were also noted among serotype C strains with serotype C-3 being more virulent (Bragg 2002b, Bragg, 2005). It was observed that transformation of the wild type NAD-dependent serotype C-3 to a NAD-independent variant also decreased its virulence (Taole *et al.*, 2002). Similar differences in virulence of the same Page's serotype classification have also been reported by Soriano *et al.*, (2004b) among serotype C strains. A large number of isolates and more detailed characterization would be required to discern differences among strains from Uganda involving other serotypes as well.

Variations in virulence have also been observed in different variants of similar serotypes and between encapsulated and non-encapsulated strains of *A. paragallinarum*. Sawata and Kume (1983) showed that variants, which were encapsulated, were highly virulent and those, which were slightly encapsulated, were moderately virulent, while the non-encapsulated were avirulent. Virulence was therefore well correlated with the amount of capsule substance containing hyaluronic acid. In the present study all the isolates were all capsulated in agreement with Sawata and Kume's (1983) observation about the capsulated types.

Differences in susceptibility to Apg of different ages have been alluded to in literature (Thitisak, *et al.*, 1988; Blackall and Yamamoto, 1998). In the present investigation where comparison was made of three age groups of 4, 10 and 21 weeks, no differences were observed using disease scores described by Bragg (2002a). Thitisak *et al* (1988) reported more deaths in free-range chickens and speculated that infectious

coryza was the second most important disease next to salmonellosis. The validity of their observations are questionable since they did not confirm IC by isolation of the organism and the clinical picture of infectious coryza can easily be confused with other organsimsms that cause similar signs such as *O. rhinotracheale*. However it may be true that certain stressors such as physiological changes and energy requirement at different ages may be necessary for these variations in age susceptibility that were not investigated in the present study.

Most *A. paragallinarum* virulence studies have been evaluated in commercial specific-pathogen-free (SPF) chickens. However in Uganda, like many other African countries, over 80% of the poultry production is dominated by the free-range indigenous local chickens which play an important role in the social and economic lives of many people in those countries (Kitalyi, 1996). These free-range poultry have been confirmed as an important tool for poverty alleviation in hardcore poor households elsewhere (Alam, 1997). Despite this potential, limited studies have been done on important limitations to their full production capacity. In the present study, the susceptibility of the indigenous free-range local chickens to *A. paragallinarum* were assessed and compared with commercial layers.

The findings indicated that the isolates obtained in the present study were equally pathogenic to the indigenous free-range chickens just like experimentally infected commercial layers. Similar studies were conducted by Lin *et al.*, (1996) using a hybrid line to Taiwanese local chickens, and found they were susceptible to Apg. Zaini and Kanameda (1991) using indigenous chickens in Malaysia, found that the

chickens were susceptible, developed acute clinical signs but were of short duration unlike the findings of the present investigations. These differences could be explained by the differences in the strains of *A. parugallinarum* used in the different studies although Zaini and Kanameda (1991) never reported the virulence of their isolates to commercial chickens for comparison. The differences could also be in the natural disease resistance of the breeds of chickens used. Such differences in disease resistance between different ecotypes of indigenous free-range local chickens and commercial chickens have been reported for some diseases (Msoffe *et al.*, 2002).

The most valid method to determine the virulence of an infectious agent would be to determine the pathogenicity of the organism in the definitive host. However, such tests are frequently both costly and time consuming as well as causing unnecessary pain or death of the hosts and may not augur well with animal welfare issues (Goldenberg, 2004). Alternative means of evaluation of the virulence of organisms to the host has become a serious issue in many countries.

In an attempt to seek such valid alternatives for evaluation of the virulence of Apg, the present study compared the susceptibility of the isolates in serum of different poultry species and with their pathogenicity in those species and found them to be positively correlated. Several studies have used the *in vitro* measurement of serum resistance of different pathogens as indicators of *in vivo* virulence especially for septicaemia conditions (Snipes and Hirsh, 1986; Muhairwa *et al.*, 2002; Nolan *et al.*, 2003) and demonstrated good correlation between serum resistance and pathogenicity of isolates.

Morishita *et al.*, (1990) developed a model which they used to predict the virulence of the isolates of *Pasteurella multocida* strains based on serum susceptibility.

Because *A. paragallinarum* does not cause septicaemic conditions, the possibility of using serum susceptibility as a predictor for virulence of *A. paragallinarum* in definitive hosts has not received much attention. Yet control of infectious coryza is still hampered by lack of easily identifiable *in vitro* markers for virulent strains. Resistance to serum complement is a widespread trait of virulent pathogens (Taylor, 1983) suggesting that bacterial factors promoting survival in serum may be useful in discriminating between virulent and avirulent isolates. Such distinguishing factors would prove useful in diagnostic protocols or as targets in future assessment of the virulence of strains without animal experiments as recommended by several alternatives to use of animals in experimentation activists (Goldenberg, 2004).

In the present investigations, all the field isolates were equally resistant in normal chicken serum by 3 hours and 6 hours. Furthermore, differences were not observed between the behaviour of the isolates in commercial and indigenous free-range local chickens in agreement with earlier observations in the present study on the pathogenicity of the isolates between indigenous free-range local chickens and commercial layers. On the contrary, the isolates were resistant in normal turkey and guineafowl serum at 3 hours but sensitive by 6 hours. This observation correlated with the observations in experimental infections in the present investigations where chickens were susceptible but turkeys and guinea fowls were less susceptible only showing mild signs of short duration. These findings are in agreement with Sawata *et*

al., (1984b) observations of resistance to bactericidal power of normal chicken serum for encapsulated *A. paragallinarum* strains.

Several factors are known to enable gram-negative bacteria to survive host defence mechanisms. These include the composition and presence of polysaccharide capsules, the lipopolysaccharide structure, the outer membrane proteins and several others (Saylers and Whitt, 2002). The capsule of *A. paragallinarum* has been associated with colonisation (Sawata *et al.*, 1985b) but its role thereafter in the development of lesions associated with coryza is still controversial (Sawata and Kume 1983). There is evidence however, that highly encapsulated organisms are responsible for the gross coryza lesions in chickens with the degree of virulence being well correlated with the amount of capsulation (Sawata *et al.*, 1985b) and that non-encapsulated organisms are avirulent in chickens. It was suggested that clinical signs are related to a certain substance in the capsule or a substance that is specifically produced by encapsulated *A. paragallinarum* during proliferation (Sawata and Kume, 1983).

The present study investigated the role of the capsule in serum resistance. A hyaluronidase treated variant was susceptible in normal chicken serum unlike its wild type, which was serum resistant. These observations are in agreement with previous observations by Sawata *et al.*, (1984b) and concur with similar studies with some strains of *Pasteurella multocida*, which have demonstrated similar role of the hyaluronic capsule in serum resistance (Diallo and Frost, 2000). It has also been demonstrated in other studies that the capsule is related to the resistance of *A. paragallinarum* against chicken bactericidal activity based on differences in the

recovery of both encapsulated and non-encapsulated organisms when inoculated into chickens (Sawata *et al.*, 1985a).

The capsule has been shown to protect outer membranes from the deposition of terminal products of the complement cascade (the membrane attack complex) (Taylor, 1983). The role of the capsule in protection against complement action was demonstrated in the present investigation in *A. paragallinarum* behaviour in turkey and guinefowl sera. It was noted that when the complement activity was removed from the sera of both turkeys and guinea fowls by heating for 30 minutes at 56^{0}C, the isolate became resistant in the heated serum. This has also been demonstrated in other studies involving *Pasteurella multocida* in chicken serum (Diallo and Frost, 2000). However it is also possible that some other factors that are heat sensitive may be involved in this process in addition to complement.

These data suggest that serum susceptibility might be a subject for further investigation as a predictor of virulence even in non-septicaemic conditions like infectious coryza but requires extensive evaluation involving a wider collection of well-characterised virulent and non-virulent strains of Apg.

One of the vexing riddles about infectious coryza is its ability to persist in chickens despite efficient assault by the chicken immune defences (Blackall *et al.*, 1997). Within 24 hours of infection clinical symptoms including swelling, nasal discharges, anorexia, lacrimation and sometimes diarrhoea are evident. However by 21 days post infection, the host immune defences have cleared the infection and the pathological

lesions have been repaired (Blackall *et al.*, 1997). Despite the repairs, some organisms seem to persist and can be transmitted to other susceptible chickens in later episodes. The persistence of *A. paragallinarum* in chickens after recovery has not been deeply studied. The present study attempted to use PCR, which is more sensitive than culture to evaluate the persistence of *A. paragallinarum* in the hosts. It was still not possible to demonstrate the organisms beyond 21 days post infection and sentinel chickens kept together with recovered chickens remained negative by both PCR and culture. This was in agreement with many other earlier reports (Blackall *et al.*, 1997) but did not offer solutions to the understanding of persistence of *A. paragallinarum*.

Based on limited molecular epidemiological markers, studies (Blackall *et al.*, 1990d) have demonstrated that chicken farms can be repeatedly infected with a single strain or clone of *A. paragallinarum*. Blackall *et al* (1990d) speculated that re-infection was due to a chronic situation in which cases re-appeared at intervals but they were unable to localise the origin of the infection. Infected replacement stocks were also suspected to be responsible for transmission of *A. paragallinarum* but this was not based on persistence studies in these recovered birds. Yamamoto and Somersett (1964) were able to demonstrate carrier status in recovered healthy chickens following experimental infections and recovery from infection, but this phenomen has not been authoritatively reported elsewhere.

Re-occurrence of infection is influenced by a number of factors including stress, such as production status, overcrowding, bad weather, and other infections (Page, 1962b). It has not been established how the organisms persists in the body of chickens

following recovery from initial infection and for how long the carrier birds are able to transmit the infection. Other birds seem not to play an important role in maintaining the organisms in nature. Systematic studies involving sparrows proved that they were unable to transmit infection (Yamamoto and Clark, 1966). Thus the theory of migratory birds was also put in doubt. Besides, the organisms cannot survive in the environment beyond 4 hours (Page, 1962b).

The investigation of the carrier status of *A. paragallinarum* has been limited by lack of sensitive methods to ascertain that carrier birds harbour the organisms and are the source of infections in subsequent outbreaks. In other related pathogens, Roman *et al.*, (2004) have suggested that human patients are colonized by hyper mutable strains of *Haemophilus influenzae* and suggested that long-term clonal persistence was associated with better-adapted clones that could persist for long periods of time. This theory requires thorough investigations by systematic following up infections on farms where periodic outbreaks are reported. Isolation and study of the persitent Apg isolates using more powerful tools such as DNA microarray techniques or *in vivo* expression technologies may enable the understanding of how persistence occurs in this paricular bacterium and thus facilitate design of more effective control strategies.

It is now known that many virulence genes are expressed as a result of stress factors such as temperature, starvation, host immune assaults, limitation of iron and others (Boyce *et al.*, 2002). They are only expressed when the pathogen needs them to enhance its survival (Saylers and Whitt, 2000). This gene switch may facilitate bacteria to adapt to other or same host environment without being eliminated by the

host enabling their persistence in healthy or chronic carriers that serve as a source of infection to other susceptible chcikens.

The situation has been made more complex by recovery of Apg in other remote areas. Sandoval *et al.*, (1994) recovered *A. paragallinarum* from the heart, liver, ovary, intra-abdominal abscesses, eye contents and also from purulent contents of tarsal tenosynovitis in complicated cases. Many outbreaks of infectious coryza are often complicated by a range of different bacterial and viral disease agents such as *Mycoplasma, E.coli, Salmonella* and *Pasteurella* and other respiratory infections (Verma *et al.*, 1985; Sandoval *et al.*, 1994) which result in the occurrence of *A. paragallinarum* elsewhere in the chickens other than the sinuses. It could be possible that these organisms stay elsewhere in other tissues and cause infection when the birds are stressed.

The role of gallinaceous birds in transmission of infectious coryza has also been suspected especially in free-range systems where they flock together. Previous reports indicated the isolation of *A. paragallinarum* from the Japanese quail (Reece *et al.*, 1981). The present study investigated the susceptibility and possible role of turkeys and guinea fowls in the transmission of *A. paragallinarum*. It was demonstrated that both turkeys and guinea fowls are mildly susceptible only showing mild signs of unilateral swelling and of short duration. No transmission was demonstrated either from infected chickens or from infected turkeys to in-contact chickens. Similar studies involving sparrows (Blackall *et al.*, 1997) failed to transmit infection to in-contact

chickens or pick up infection from infected in-contact chickens in agreement with the findings of the present study.

Since the designation of *A. paragallinarum* as a separate genus to differentiate it from other avian haemophili that require both X and V factors by Biberstein and White (1969) no report of isolation of *A. paragallinarum* has been made in other birds other than chickens. Thus it appears that currently *A. paragallinarum* causes infectious coryza in chickens and the role of other birds in its epidemiology is yet to be demonstrated.

The ideal control strategy for most infectious diseases is the use of vaccines. Infectious coryza has not been an exception in this strategic control of infectious diseases. This field has attracted much attention in the last 40 years to curtail the losses caused by the disease. Commercial bacterins are widely available for use as vaccines for IC (Blackall, 1995). However outbreaks of infectious coryza have been reported in vaccinated flocks in different countries, indicating that new serotype(s) or variants of *A. paragallinarum* may have evolved.

The ability of the commercially available infectious coryza vaccine used in Uganda to give protection against the local strains was investigated in the present study. Following vaccination and challenge with the different locally isolated field strains from outbreaks, chickens only showed mild clinical signs and of very short duration. Several field isolates have been made from vaccinated flocks with sevre clinical signs in other countries such as Argentina (Sandoval *et al.*, 1994), and Zimbabwe (Bragg,

2002c). Examination of these isolates indicated that apart from one serotype C-3 strain reported in South Africa and Zimbabwe, all were serotype B (Jacobs *et al.*, 2003). Using a trivalent vaccine in which the serotype B was included all birds were protected against challenge with all the variant isolates (Jacobs *et al.*, 2003).

Vaccination trials however, indicated that although vaccination results in high protective antibody titers, the high virulence of serovar C-3 isolates often result in expression of clinical signs thus explaining the large number of so-called vaccination failures that are reported in South Africa (Bragg, 2005). It is therefore important to periodically examine the efficacy of available vaccines against locally isolated strains in various countries in order to effectively control the disease by vaccination.

The present study demonstrated antibodies to different serotypes in the country suggesting that all the serotypes of *A. paragallinarum* exist although only serotype C was isolated. More extensive studies might be able to isolate more serotypes and or variants that can be used to authoritatively evaluate the efficacy of the currently used vaccines in the country. Recent reports have indicated that some South African NAD-independent strains evade the immune responses mounted by current vaccines based on the NAD-dependent strains (Bragg, 2004). This calls for further action and constant monitoring and application of an appropriate vaccination strategy based on efficacy studies against locally isolated strains.

CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The present study has confirmed the occurrence of *Avibacterium paragallinarum* and the presence of infectious coryza in Uganda. This is the first report of isolation and characterisation of *A. paragallinarum* in Uganda. The demonstration of antibodies to all the serotypes suggests that all the serotypes exist although only serotype C was isolated from two farms in this study. Characterisation of the isolates revealed that the isolates are epidemiologically different basing on both phenotypic antibiotic resistance patterns and demonstration of resistance genes in the isolates. These differences between the isolates could not be detected by other phenotypic and genotypic methods employed in this study implying that use of different methods to discount relatedness of isolates is particularly important.

The present study is the first report to demonstrate some of the genetic mechanisms involved in phenotypic expression of antibiotic resistance in *A. paragallinarum*. Resistance genes to streptomycin (*strA*), ampicillin (*blaTEM*), tetracycline (*tetC* and *tetA*) and sulphamethoxazole (*sul2*) were demonstrated in the isolates from Uganda. Occurrence of multiple genes similar to what has been reported in other Gram-negative bacteria was demonstrated. The occurrence of these genes in relation to other respiratory pathogens such as *Pasteurella* and *Mannheimia* raises important questions for the potential spread of these genes on mobile genetic elements leading to treatment failures in different respiratory diseases.

The present investigations have demonstrated that the isolates were equally pathogenic to both commercial layers and indigenous free-range local chickens. Although infectious coryza is not a systemic disease, the correlation between the behaviour of the isolates from this study and other reports in literature seem to indicate that serum resistance could be a subject for further investigation as a model to predict pathogenicity of isolates, eliminating the need for using animals to test for pathogenicity of isolates.

Systematic investigations of the present study have revealed that turkeys and guinea fowls are susceptible to *A. paragallinarum* infection showing mild transient signs. However it was noted that the infection was of short duration and they seem not to play a role in transmission of infection to the definitive hosts. This is because they could not pick infection from experimentally infected chickens and neither could chickens pick infection from experimentally inoculated turkeys and guinea fowls. Therefore their role in the epidemiology of infectious coryza may be very limited.

In the present study, the carrier status of chickens after recovery from experimental infection was not demonstrated using PCR, which is a superior technique than culture in detection of Apg infection. The PCR, however, detected more positive samples both in the environment and the host and for a longer period.

The vaccination and challenge trials performed in the present study demonstrated that the current trivalent vaccine used in the country was protective against the strains that were isolated from the present study. Because vaccine failures have been reported

elsewhere due to differences in strain virulence and serotype variants. it is important that more and frequent evaluation of the efficacy of such commercial vaccines be periodically done against local isolates.

6.2 RECOMMENDATIONS

This study has confirmed the occurrence of *A. paragallinarum* in Uganda. However only a few isolates were recovered which only represented one serotype. It is therefore important that more extensive studies be conducted targeting outbreaks to isolate a wide collection of strains that can enable more elaborate analysis of the various serotypes, subtypes and their variants. This would be important in further evaluation of the currently used vaccines in the country following reports of vaccine failures in other countries. This would require follow ups even on farms where vaccinations have been done following reports of isolation of variants in vaccinated flocks elsewhere which can evade the immune responses mounted by the used vaccines.

The demonstration of the occurrence of multiple drug resistance genotypes against the commonly used drugs is a cause for worry especially with reports of similar patterns in other respiratory avian pathogens. It is therefore recommended that further investigations be carried out not only among *A. paragallinarum* strains but other respiratory and non-respiratory pathogens to exlore further the resistance mechanisms and to study the potential of exchange of these genes among these pathogens. In order to curtail development of further resistance, therefore, understanding these resistance mechanisms in these pathogens is essential.

The persistence of *A. paragallinarum* needs further re-examination especially in the field where IC is often complicated by other infections. A combination of PCR and other methods such as immunohistochemistry and *in-situ* hybridisation may be able to unravel the persistence of the organism in chickens on these farms. Examination of these organism during the course of infection and the persistent ones using methodologies such *in vivo* expression technology, signature tagged mutagenesis and DNA microarrays may shed some light on how the organism survive the host defence mechanisms. This will enable understanding further the virulence mechanisms of these organisms and facilitate design of more effective control strategies of the disease.

7.0 REFERENCES

Aarestrup, F. M., Seyfarth, A. M., Emborg, H. D., Pedersen, K., Hendriksen, R. S. and Bager, F., (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrobial Agents and Chemotherapy* 45: 2054-2059.

Aarestrup, F. M., Lertworapreecha, M., Evans, M. C., Bangtrakulnonth, A. Chalermchaikit, T., Hendriksen, R. J. and Wegener, H. C. (2003). Antimicrobial susceptibility and occurrence of resistance genes among *Salmonella enterica* serovar Weltevreden from different countries. *Journal of Antimicrobial Chemotherapy* 52:715-718

Alam, J. (1997). Impact of smallholder livestock development project in some selected areas of rural Bangladesh. *Livestock for Rural Development* 9 (3) [http://www.cipav.org.co/lrrd/lrrd9/3/bang932.htm] site visited on 5/11/2005

Aminov, R. I., Chee-Sanford, J. C., Garrigues, N., Teferedegne, B., Krapac, I. J., White, B. A., and Mackie, R. I. (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Applied Environmental Microbiology* 68(4): 1786 - 1793.

Amonsin, A., Welleah, J. H., Li, L. L., Vandamme, P., Lindeman, C., Edman, M., Robinson, R. A and Kapur, V. (1997). Molecular epidemiology of *Ornithobacterium rhinotracheale*. *Journal of Clinical Microbiology* 35(11): 2894-8

Andrews, S. C. (1998). Iron storage in bacteria. *Advances in Microbial Physiology* 40:281-351

Angelichio, M. J. and Camilli, A. (2002). *In Vivo* Expression Technology. *Infection and Immunity* 70: 6518-6523

Badger, J. L., Wass, C. A., Weissman, S. J. and Kim, K. S. (2000). Application of signature-tagged mutagenesis for identification of *Escherichia coli* K1

genes that contribute to invasion of human brain microvascular endothelial cells. *Infection and Immunity* 68:5056-5061

Baeza, I., Giono, S., Santiago, J. C., Martinez, F., Aquino, C., and Wong, C. (1987). Membrane protein profiles of different species of *Haemophilus*. *Revista Latinoamericana de Microbiologia* 29: 157-163.

Beach, J. R. (1920). The diagnosis, therapeutic and prophylaxis of chicken pox(contagious epithelioma) of fowls. *Journal of American Veterinary Medical Association* 58: 301-302

Beachy, E. D. (1981). Bacterial Adherence: Adhesin-receptor Interactions Mediating the Attachment of Bacteria to Mucosal Surfaces. *Journal of Infectious Diseases* 143: 325-345.

Biberstein, E. L. and White, D. C. (1969). A proposal for the establishment of a new *Haemophilus* species. *Journal of Medical Microbiology* 2: 75-78.

Bing, D., Weyland, G. M. and Stavisky, A. B. (1967). Haemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. *PSEBM* 124: 1166-1170.

Bisgaard, M. (1995). Taxonomy of the family *Pasteurellaceae* Pohl 1981. In: *Haemophilus, Actinobacillus and Pasteurealla*, Edited by Donachie W. Plenum Press, NewYork pp 1-7.

Blackall, P. J. (1988). Antimicrobial drug resistance and the occurrence of plasmids in *Haemophilus paragallinarum*. *Avian Diseases* 32 (4): 742 - 747.

Blackall, P. J. (1995). Vaccines against infectious coryza. *World's Poultry Science Journal* 51: 17-26.

Blackall, P. J. (1999). Infectious coryza: overview of the disease and new diagnostic options. *Clinical Microbiology Reviews* 12: 627-632.

Blackall, P. J. and Eaves, L. E. (1988). Serological classification of Australian and South African isolates of *Haemophilus paragallinarum*. *Australian Veterinary Journal* 65(11): 362 - 363.

Blackall, P. J. and Farrah, J. G. (1985). An evaluation of commercial discs for the determination of the growth factor requirements of avian haemophili. *Veterinary Microbiology* 10(2): 125 - 131.

Blackall, P. J. and Yamamoto, R. (1989). Whole-cell protein profiles of *Haemophilus paragallinarum* as detected by polyacrylamide gel electrophoresis. *Avian Diseases* 33: 168-173.

Blackall, P.J. and Yamamoto, R. (1998). Infectious Coryza. In: *A Laboratory Manual for the Isolation and Identification of Avian Pathogens.* Edited by Swayne, D.E., Glisson, J.R., Jackwood, M.W., Person, J. E., and Reed, W.M. The American Association of Avian Pathologists, Pennsylvania pp. 29-34.

Blackall, P. J., Eaves, L. E., and Rogers, D. G. (1989). Biotyping of *Haemophilus paragallinarum* isolates using hemagglutinin serotyping, carbohydrate fermentation patterns, and antimicrobial drug resistance patterns. *Avian Diseases* 33: 491-496.

Blackall, P. J., Eaves, L. E., and Aus, G. (1990a). Serotyping of *Haemophilus paragallinarum* by the Page scheme: comparison of the use of agglutination and hemagglutination-inhibition tests. *Avian Diseases* 34: 643-645.

Blackall, P. J., Yamaguchi, T., Iritani, Y., and Rogers, D. G. (1990b). Evaluation of two monoclonal antibodies for serotyping *Haemophilus paragallinarum*. *Avian Diseases* 34: 861 - 864.

Blackall, P. J., Eaves, L. E., and Rogers, D. G. (1990c). Proposal of a new serovar and altered nomenclature for *Haemophilus paragallinarum* in the Kume hemagglutinin scheme. *Journal of Clinical Microbiology* 28(6): 1185 - 1187.

Blackall, P. J., Morrow, C. J., McInnes, A., Eaves, L. E., and Rogers, D. G. (1990d). Epidemiologic studies on infectious coryza outbreaks in northern New South Wales, Australia, using serotyping, biotyping, and chromosomal DNA restriction endonuclease analysis. *Avian Diseases* 34: 267 - 276.

Blackall, P. J., Rogers, D. G., and Yamamoto, R. (1990e). Outer-membrane proteins of *Haemophilus paragallinarum*. *Avian Diseases* 34: 871 - 877.

Blackall, P. J., Zheng, Y. Z., Yamaguchi, T., Iritani, Y., and Rogers, D. G. (1991a). Evaluation of a panel of monoclonal antibodies in the subtyping of *Haemophilus paragallinarum*. *Avian Diseases* 35: 955 - 959.

Blackall, P. J., Eaves, L. E., and Morrow, C. J. (1991b). Comparison *of Haemophilus paragallinarum* isolates by restriction endonuclease analysis of chromosomal DNA. *Veterinary Microbiology* 27: 39-47.

Blackall, P. J., Silva, E. N., Yamaguchi, T., and Iritani, Y. (1994). Characterization of isolates of avian haemophili from Brazil. *Avian Diseases* 38: 269-274.

Blackall, P.J., Matsumoto, M. and Yamamoto, R. (1997). Infectious Coryza. In: *Poultry Diseases* Edited by Calnek R. W. Mosby-Wolfe, London pp. 179-190.

Blackall, P. J., Christensen, H., Beckenham, T., Blackall, L. L., and Bisgaard, M. (2005). Reclassification of *Pasteurella gallinarum, [Haemophilus] paragallinarum, Pasteurella avium* and *Pasteurella volantium* as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 55(Pt 1): 353 - 362.

Bowles, R., Blackall, P. J., Terzolo, H. R. and Sandoval, V. E. (1993). An assessment of the genetic diversity of Australian and overseas isolates of *Haemophilus paragallinarum* by multilocus enzyme electrophoresis. In: *Proceedings of the 10th World Veterinary Poultry Association Congress,* August 16–19, 1993.Sydney, Queensland, Australia. p. 148.

Boyce, J. D., Chung, J. Y., and Adler, B. (2000a). Genetic organisation of the capsule biosynthetic locus of *Pasteurella multocida* M1404 (B:2). *Veterinary Microbiology* 72(1-2): 121 - 134.

Boyce, J. D., Chung, J. Y., and Adler, B. (2000b). *Pasteurella multocida* capsule: composition, function and genetics. *Journal of Biotechnology* 83(1-2): 153 - 160.

Boyce, J. D., Wilkie, I., Harper, M., Paustian, M. L., Kapur V. and Adler, B. (2002). Genomic-scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. *Infection and Immunity* 70:6871-6879.

Boyce, J. D., Wilkie, I., Harper, M., Paustian, M. L., Kapur, V. and Adler, B. (2004). Genomic-scale analysis of *Pasteurella multocida* gene expression during growth within liver tissue of chickens with fowl cholera. *Microbes and Infection* 9(3):290-298

Boyle, R. and Finlay, T. (2003). Bacterial pathogenesis: exploiting cellular adherence. *Current Opinion in Cell Biology*, 15:633-639.

Bragg, R. R. (2002a). Virulence of South African isolates of *Haemophilus paragallinarum*. Part 2: naturally occurring NAD-independent field isolates. *Onderstepoort Journal of Veterinary Research* 69(2): 171 - 175.

Bragg, R. R. (2002b). Virulence of South African isolates of *Haemophilus paragallinarum*. Part 1: NAD-dependent field isolates. *Onderstepoort Journal of Veterinary Research* 69(2): 163 - 169.

Bragg, R. R. (2002c). Isolation of serovar C-3 *Haemophilus paragallinarum* from Zimbabwe: A further indication of the need for the production of vaccines against infectious coryza containing local isolates of *H. paragallinarum*. *Onderstepoort Journal of Veterinary Research* 69(2): 129 - 132.

Bragg, R. R. (2004). Evidence of possible evasion of protective immunity by NAD-independent isolates of *Haemophilus paragallinarum* in poultry. *Onderstepoort Journal of Veterinary Research* 71(1): 53 - 58.

Bragg, R. R. (2005). Effects of differences in virulence of different serovars of *Haemophilus paragallinarum* on perceived vaccine efficacy. *Onderstepoort Journal of Veterinary Research* 72(1): 1 - 6.

Bragg, R. R., Coetzee, L., and Verschoor, J. A. (1993). Plasmid-encoded NAD independence in some South African isolates of *Haemophilus paragallinarum*. *Onderstepoort Journal of Veterinary Research* 60(2): 147 - 152.

Bragg, R. R., Coetzee, L., and Verschoor, J. A. (1996). Changes in the incidences of the different serovars of *Haemophilus paragallinarum* in South Africa: a possible explanation for vaccination failures. *Onderstepoort Journal of Veterinary Research* 63: 217-226.

Bragg, R. R., Greyling, J. M., and Verschoor, J. A. (1997). Isolation and identification of NAD-independent bacteria from chickens with symptoms of infectious coryza. *Avian Pathology* 26: 595-606.

Bragg, R. R., Jansen, V. R., Van Heerden, E., and Albertyn, J. (2004). The testing and modification of a commercially available transport medium for the transportation of pure cultures of *Haemophilus paragallinarum* for serotyping. *Onderstepoort Journal of Veterinary Research* 71(2): 93 - 98.

Brown, G. M. (1962). The biosynthesis of folic acid. II. Inhibition by sulfonamides. *Journal of Biology and Chemistry* 237:536-540

Butaye, P., Cloeckaert, A., and Schwarz, S. (2003). Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. *International Journal of Antimicrobial Agents* 22(3): 205 - 210.

Buys, S. B. (1971). Therapeutic effect of certain drugs against *Haemophilus gallinarum* infection. Final Report. *Agricultural Research, Pretoria* 1970: 164.

Buys, S. B. (1972). *Haemophilus* coryza: therapy with selected drugs. *Journal of the South African Veterinary Association* 43: 383-389.

Byarugaba, D. K. (2003). Prudent use and containment of antimicrobial resistance in developing countries: In: *OIE International Standards on Antimicrobial Resistance.* World Organisation for Animal Health (OIE), Paris, 601-3.

Byarugaba, D. K. (2004). A view on antmicrobial resistance in developing countries and responsible risk factors. *International Journal of Antimicrobial Agents* 24(2) 105-110.

Byarugaba, D. K. (2005). Antimicrobial resistance and its containment in developing countries In: *Antibiotic Policies: Theory and Practice* Edited by Ian Gould and van der Meer, Springer, NewYork, ISBN: 0-306-48500-1 pp 617-646.

Byarugaba, D. K. and Nakavuma J. (2005). Trends of antimicrobial resistance in bacteria isolates from mastitis in Uganda. In: *Proceedings of the 4th IDF International Mastitis Conference,* 12th –15th June 2005 Maastrcht, The Netherlands.

Byarugaba, D. K., Olsen, J. E. and Katunguka –Rwakishaya, E., (2002). Production, management and marketing dynamics of rural free-range poultry in Uganda. In : *Proceedings of the 2nd electronic International Network for Family Poultry Development (INFPD) on the The Bangladesh Model and Other Experiences in Family Poultry Development*, 2002 [http://www.fao.org/ag/againfo/subjects/en/infpd/documents/econf_bang/add_paper9.html] site visited 5/11/2005

Cannon, M., Harford, S., Davies, J. A. (1990). A comparative study on the inhibitory actions of chloramphenicol, thiamphenicol and some fluorinatedderivatives. *Journal of Antimicrobial Chemotherapy* 26: 307–317.

Casadevall, A. and Pirofski, L. A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infection and Immunity* 67(8): 3703-13.

Chart, H., Stevenson, P. and Griffiths, E. (1988). Iron-regulated outer-membrane proteins of Escherichia coli strains associated with enteric or extraintestinal diseases of man and animals. *Journal of General Microbiology* 134 (Pt 6): 1549-1559.

Chen, X., Miflin, J. K., Zhang, P., and Blackall, P. J. (1996). Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. *Avian Diseases* 40(2): 398 - 407.

Chen, X., Chen, Q., Zhang, P., Feng, W., and Blackall, P. J. (1998a). Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. *Avian Pathology* 27: 296-300.

Chen, X., Song, C., Gong, Y., and Blackall, P. J. (1998b). Further studies on the use of a polymerase chain reaction test for the diagnosis of infectious coryza. *Avian Pathology* 27: 618-624.

Chen, X., Zhang, P., Blackall, P. J., and Feng, W. (1993). Characterization of *Haemophilus paragallinarum* isolates from China. *Avian Diseases* 37: 574-576.

Chopra, I. and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* 65(2): 232 - 260.

Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R., and Olsen, J. E. (2003a). Genetic relationships among avian isolates classified as *Pasteurella haemolytica*, '*Actinobacillus salpingitidis*' or *Pasteurella anatis* with proposal of *Gallibacterium anatis* gen. nov., comb. nov. and description of additional genomospecies within *Gallibacterium* gen. nov. *International Journal of Sytematic Bacteriology* 53(Pt 1): 275 - 287.

Christensen, H., Foster, G., Christensen, J. P., Pennycott, T., Olsen, J. E., and Bisgaard, M. (2003b). Phylogenetic analysis by 16S rDNA gene sequence comparison of avian taxa of Bisgaard and characterization and description of two new taxa of *Pasteurellaceae*. *Journal of Applied Microbiology* 95(2): 354 - 363.

Christensen, H., Bisgaard, M., Aalback, B., and Olsen, J. E. (2004a). Reclassification of Bisgaard taxon 33, with proposal of *Volucribacter psittacicida* gen. nov., sp. nov. and *Volucribacter amazonae* sp. nov. as new members of the *Pasteurellaceae*. *International Journal of Sytematic Bacteriology* 54(Pt 3): 813 - 818.

Christensen, H., Kuhnert, P., Olsen, J. E., and Bisgaard, M. (2004b). Comparative phylogenies of the housekeeping genes *atpD*, *infB* and *rpoB* and the 16S rRNA gene within the *Pasteurellaceae*. *International Journal of Sytematic Bacteriology* 54(Pt 5): 1601 - 1609.

Confer, A.W., McCraw, R. D., Durham, J.A., Morton, R. J. and Panciera, R. J. (1995). Serum antibody responses of cattle to iron-regulated outer membrane proteins of *Pasteurella haemolytica* A1. *Veterinary Immunology and Immunopathology* 47(1): 101-110.

Courvalin, P. (1994). Transfer of resistance genes between Gram-positive and Gram negative bacteria. *Antimicrobial Agents and Chemotherapy* 38(7):1447-1451.

De Blieck, L. (1932). A haemoglobinophilic bacterium as the cause of contagious catarrh of the fowl (*coryza infectiosa gallinarum*). *Veterinary Journal* 88: 9-13.

De Ley, J., Mannheim, W., Mutters, R., Piechulla, K., Tytgat, R., Segers, P., Bisgaard, M., Frederiksen, W., Hinz, K. H., and Vanhoucke, M. (1990). Inter- and intrafamilial similarities of rRNA cistrons of the *Pasteurellaceae*. *International Journal of Sytematic Bacteriology* 40(2): 126 - 137.

De Smidt, O., Albertyn, J., Bragg, R. R., and Van Heerden, E. (2004). Genetic organisation of the capsule transport gene region from *Haemophilus paragallinarum*. *Onderstepoort Journal of Veterinary Research* 71(2): 139 - 152.

Delaplane, J. P., Erwin, L. E., and Stuart, H. O (1934). A haemophilic bacillus as the cause of an infectious rhinitis (coryza) of fowls. *Research Institute of Agricultural Experimental Station Bulletin*, 244.

Dessen, A., Di Guilmi, A. M., Vernet, T. and Dideberg, O. (2001). Molecular mechanisms of antibiotic resistance in gram-positive pathogens. *Current Drug Targets for Infectious Diseases* 1(1):63-77

Dewhirst, F. E., Paster, B. J., Olsen, I., and Fraser, G. J. (1992). Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. *Journal of Bacteriology* 174(6): 2002 - 2013.

Dewhirst, F. E., Paster, B. J., Olsen, I. and Fraser, G. J. (1993). Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. *Zentralblatt fur Bakteriologie* 279: 35–44.

Dho-Moulin, M. and Fairbrother, J. M. (1999). Avian pathogenic *Escherichia coli* (APEC). *Veterinary Research* (2-3):299-316.

Diallo, I. S. and Frost, A. J. (2000). Survival of avian strains of *Pasteurella multocida* in chicken serum. *Veterinary Microbiology* 72(1-2): 153 - 161.

Dorman, C. J. and Foster, T. J. (1982). Non-enzymatic chloramphenicol resistance determinants specified by plasmids R26 and R55-1 in *Escherichia coli* K-

12 do not confer high-level resistance to fluorinated analogues. *Antimicrobial Agents and Chemotherapy* 22: 912–914.

Droual, R., Bickford, A. A., Charlton, B. R., Cooper, G. L., and Channing, S. E. (1990). Infectious coryza in meat chickens in the San Joaquin Valley of California. *Avian Diseases* 34(4): 1009 - 1016.

Eaves, L. E., Rogers, D. G., and Blackall, P. J. (1989). Comparison of hemagglutinin and agglutinin schemes for the serological classification of *Haemophilus paragallinarum* and proposal of a new hemagglutinin serovar. *Journal of Clinical Microbiology* 27(7): 1510 - 1513.

Eisen, M. B, Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Science USA* 95:14863–14868.

Eliot, C. P. and Lewis, M. R. (1934). A haemophilic bacterium as the cause of infectious coryza in the fowl. *Journal of American Veterinary Medical Association* 84: 878-888.

Ellner, P. D., Fink, D. J., Neu, H. C. and Parry, M. F. (1987). Epidemiologic factors affecting antimicrobial resistance of common bacterial isolates. *Journal of Clinical Microbiology* 25(9): 1668–1674

Enne, V. I., Livermore, D. M., Stephens, P. and Hall, L. M. C. (2001). Persistence of sulfonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *Lancet* 357:1325-1328

Enne, V. I., King, A., Livermore, D. M., and Hall, L. M. (2002). Sulfonamide resistance in *Haemophilus influenzae* mediated by acquisition of *sul2* or a short insertion in chromosomal *folP*. *Antimicrobial Agents and Chemotherapy* 46(6): 1934 - 1939.

Escolar, L., Pérez-Martín, J. and de Lorenzo, V. (1998). Coordinated Repression *In Vitro* of the Divergent fepA-fes Promoters of *Escherichia coli* by the Iron Uptake Regulation (Fur) Protein. *Journal of Bacteriology* 180: 2579-2582

FAO (1996). *World Livestock production systems: current status and trends,* Edited by Sere, C., Steinfield, H and Groenewold, J. FAO Animal Production and Health Paper No. 127. Rome

Fedde, M. R. (1998). Relationship of structure and function of the avian respiratory system to disease susceptibility. *Poultry Science* 77: 1130-1138.

Fernandez, R. P., Garcia-Delgado, G. A., Ochoa, P. and Soriano, V. E. (2000). Characterisation of *Haemophilus paragallinarum* isolates from Mexico. *Avian Pathology* 29: 473–476.

Finlay, B. B. and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews* 16: 136-169.

Fluit, A. C., Visser, M. R. and Schmitz, F. J. (2001). Molecular detection of antimicrobial resistance. *Clinical Microbiological Reviews* 14(4):836-71,

Garcia, A. J., Angulo, E., Blackall, P. J., and Ortiz, A. M. (2004). The presence of nicotinamide adenine dinucleotide-independent *Haemophilus paragallinarum* in Mexico. *Avian Diseseas* 48(2): 425 - 429.

Goering, R. V. (1993). Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. *Infection Control and Hospital Epidemiology* 14: 595-600.

Goldenberg, A. M. (2004). Animals and alternatives: Societal expectations and scientific need. *Alternatives to Laboratory Animals* 32(6): 545-551.

Gomez-Lus, R. (1998). Evolution of bacterial resistance to antibiotics during the last three decades. *International Microbiology* 1(4): 279-284.

Gonzales, A. E., Glisson, J. R., and Jackwood, M. W. (1996). Transposon mutagenesis of *Haemophilus paragallinarum* with Tn916. *Veterinary Microbiology* 48(3-4): 283 - 291.

Grimont, F. and Grimont, P. A. (1986). Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annals of the Institute Pasteur Microbiology* 137B: 165-175.

Gueye, E. F. (1998). Village egg and fowl meat production in Africa. *World's Poultry Science Journal* 54: 73-86.

Gunarwardana, G, Townsend, K. M, and Frost, A. J. (2000). Molecular characterisation of avian *Pasteurella multocida* isolates from Australia and Vietnam by REP-PCR and PFGE. *Veterinary Microbiology* 72: 25-33.

Gyles, C. L., and Theon, C. O. (Eds.). (1993). *Pathogenesis of Bacterial Infections in Animals*. Iowa State University Press, Ames, IA. pp 324.

Hall, R. M. (1997). Mobile gene cassettes and integrons: moving antibiotic resistance genes in Gram-negative bacteria. *Ciba Foundation Symposium* 207: 192–205

Harper, M., Boyce, J. D., Wilkie, I. W., and Adler, B. (2003). Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. *Infection and Immunity* 71(9): 5440 - 5446.

Hartman, A. B., Essiet, I. I., Isenbarger, D. W., and Lindler, L. E. (2003). Epidemiology of tetracycline resistance determinants in *Shigella* spp. and enteroinvasive *Escherichia coli:* characterization and dissemination of tet(A)-1. *Journal of Clinical Microbiology* 41(3): 1023 - 1032.

Hartmann, L., Schroder, W., and Lubke, Becker A. (1996). A comparative study of the major outer membrane proteins of the avian haemophili and *Pasteurella gallinarum*. *Zentralblatt fur Bakteriologie* 284: 47-51.

Heuer, C., Hickman, R. K., Curiale, M. S., Hillen, W., and Levy, S. B. (1987). Constitutive expression of tetracycline resistance mediated by a Tn10-like element in *Haemophilus parainfluenzae* results from a mutation in the repressor gene. *Journal Bacteriology* 169(3): 990 - 994.

Hinz, K. H. (1976). Differentiation of *Haemophilus* strains isolated from fowls. IV. Studies on the dissociation of *Haemophilus paragallinarum*. *Avian Pathology* 5: 51-66.

Hinz, K. H. (1980). Heat-stable antigenic determinants of *Haemophilus paragallinarum*. *Zentralbl VeterinarmedB* 27(8): 668-676.

Horner, R. F., Bishop, G. C. and Haw C. (1992). An upper respiratory disease of commercial chickens resembling infectious coryza, but caused by a V-factor independent bacterium. *Avian Pathology* 21: 421-427

ICSP, (2005). International Committee on Systematics of Prokaryotes (ICSP); Taxa covered by the ICSP Subcommittee on the Taxonomy of *Pasteurellaceae* -

November 2005 http://www.the-icsp.org/taxa/Pasteurellaceaelist.htm. Site visited 5/11/2005

Ieven M. and Goossens, H. (1997). Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. *Clinical Microbiological Reviews* 10(2):242-56.

Ikeda, J. S. and Hirsh, D. C (1988). Antigenically related iron-regulated outer membrane proteins produced by different somatic serotypes of *Pasteurella multocida*. *Infection and Immunity* 56: 2499-2502.

Iritani, Y., Iwaki, S., and Yamaguchi, T. (1980). Properties of heat-stable antigen in the culture supernate of *Haemophilus paragallinarum*. *Nippon Juigak Zasshi* 42(6): 635 - 641.

Iritani, Y., Sugimori, G. and Katagiri. K. (1977). Serologic response to *Haemophilus gallinarum* in artificially infected and vaccinated chickens. *Avian Diseases* 21:1-8

Iritani, Y., Yamaguchi, T., Katagiri, K., and Arita, H. (1981). Hemagglutination inhibition of *Haemophilus paragallinarum* type 1 hemagglutinin by lipopolysaccharide. *American Journal of Veterinary Research* 42(4): 689 - 690.

Jacobs, A. A., van den, B. K., and Malo, A. (2003). Efficacy of a new tetravalent coryza vaccine against emerging variant type B strains. *Avian Pathology* 32(3): 265 - 269.

Jonas, D., Spitzmuller, B., Weist, K., Ruden, H. and Daschner, F. D. (2003). Comparison of PCR-based methods for typing *Escherichia coli*. *Clinical Microbiology and Infection* 9(8): 823-31.

Kagan, B. L., Selsted, M. E., Ganz, T. and Lehrer. R. I. (1990). Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proceedings of the National Academy of Science USA* 87:210-214.

Kehrenberg, C. and Schwarz, S. (2001). Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin and chloramphenicol in bacteria of

the genera *Pasteurella and Mannheimia. FEMS Microbiology Letters* 205(2): 283 - 290.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J. L., Chaslus-Dancla, E., and Schwarz, S. (2001). Antimicrobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis. *Veterinary Research* 32(3-4): 323 - 339.

Kehrenberg, C., Tham, N. T., and Schwarz, S. (2003). New plasmid-borne antibiotic resistance gene cluster in *Pasteurella multocida*. *Antimicrobial Agents and Chemotherapy* 47(9): 2978 - 2980.

Kellam, P. (2000). Host-pathogen studies in the post-genomic era. *Genome Biology* 1(2): 1009.1–1009.4.

Kerr, K. G. (1994). The rap on REP-PCR-based typing systems. *Reviews in Medical Microbiology* 5:233-244.

Kerr, J. R. (1999). Cell adhesion molecules in the pathogenesis of and host defence against microbial infection. *Molecular Pathology* 4(2): 220-230.

Khan, M., Chen, X. and Blackall, P. J. (1998). Differentiation of *Haemophilus paragallinarum* isolates using ERIC-PCR analysis. In: *Proceedings of the 47th Western Poultry Diseases Conference*, March 8–10, 1998. Sacramento, CA. pp. 8–9.

Kitalyi, A. J. (1996). Socio-economic aspects of village chicken production in Africa: the role of women, children and non-governmental organisations. *Proceedings of the 20th World's Poultry Congress*. World's Poutry Association.New Delhi, India, 1, 35-45.

Korczak, B., Christensen, H., Emler, S., Frey, J., and Kuhnert, P. (2004). Phylogeny of the family *Pasteurellaceae* based on rpoB sequences. *International Journal of Sytematic Bacteriology* 54(Pt 4): 1393 - 1399.

Kubiet M, Ramphal R, Weber A, Smith A. (2000). Pilus-mediated adherence of *Haemophilus influenzae* to human respiratory mucins. *Infection and Immunity* 68(6):3362-7.

Kume, K. and Sawata, A. (1984). Immunologic properties of variants dissociated from serotype 1 *Haemophilus paragallinarum* strains. *Nippon Juigaku Zasshi* 46(1): 49 - 56.

Kume, K., Sawata, A., and Nakase, Y. (1978). *Haemophilus* infections in chickens. I. Characterization of *Haemophilus paragallinarum* isolated from chickens affected with coryza. *Nippon Juigaku Zasshi* 40(1): 65 - 73.

Kume, K., Sawata, A., and Nakase, Y. (1980a). *Haemophilus* infections in chickens. 3. Immunogenicity of serotypes 1 and 2 strains of *Haemophilus paragallinarum*. *Nippon Juigaku Zasshi* 42(6): 673 - 680.

Kume, K., Sawata, A., and Nakase, Y. (1980b). Relationship between protective activity and antigen structure of *Haemophilus paragallinarum* serotypes 1 and 2. *American Journal of Veterinary Research* 41(1): 97 - 100.

Kume, K., Sawata, A., Nakai, T., and Matsumoto, M. (1983). Serological classification of *Haemophilus paragallinarum* with a hemagglutinin system. *Journal of Clinical Microbiology* 17: 958-964.

Kume, K., Sawata, A., and Nakai, T. (1984). Clearance of the challenge organisms from the upper respiratory tract of chickens injected with an inactivated *Haemophilus paragallinarum* vaccine. *Nippon Juigaku Zasshi* 46: 843-850.

Lancashire, J. F., Terry, T. D., Blackall, P. J., and Jennings, M. P. (2005). Plasmid-encoded *Tet*B tetracycline resistance in *Haemophilus parasuis*. *Antimicrobial Agents and Chemotherapy* 49(5): 1927 - 1931.

Lanz, R., Kuhnert, P., and Boerlin, P. (2003). Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Veterinary Microbiology* 91(1): 73 - 84.

Leflon-Guibout, V., Heym, B., and Nicolas-Chanoine, M. (2000). Updated sequence information and proposed nomenclature for *bla(TEM)* genes and their promoters. *Antimicrobial Agents and Chemotherapy* 44(11): 3232-4

Levy, S. B. (1988). Tetracycline resistance determinants are widespread. *ASM News* 54:418-421.

Liebert, C. A., Hall, R. M. and Summers, A. O. (1999). Transposon Tn21, flagship of the floating genome. *Microbiology and Molecular Biology Reviews* 63(3): 507-522.

Lin, J. A., Shyu, C. L., Yamaguchi, T., and Takagi, M. (1996). Characterization and pathogenicity of *Haemophilus paragallinarum* serotype C in local chickens of Taiwan. *Journal of Veterinary Medical Science* 58(10): 1007 - 1009.

LiPuma, J. J., Richman, H. & Stull, T. L. (1990). Haemocin, the bacteriocin produced by *Haemophilus influenzae*: species distribution and role in colonization. *Infection and Immunity* 58, 1600–1605

Litwin, C. M. and Calderwood, S. B. (1993). Role of iron in regulation of virulence genes. *Clinical Microbiology Reviews* 6(2): 137-149.

Lu, Y. S., Lin, D. F., Tsai, H. J., Tsai, K. S., Lee, Y. L., and Lee, T. (1983). Drug sensitivity test of *Haemophilus paragallinarum* isolated in Taiwan. *Taiwan Journal of Veterinary Medicine and Animal Husbandry* 41: 73-76

Lublin, A., Mechani, S., Malkinson, M., and Weisman, Y. (1993). Efficacy of norfloxacin nicotinate treatment of broiler breeders against *Haemophilus paragallinarum*. *Avian Diseases* 37(3): 673 - 679.

Lupski, J. R., Weinstock, G. M. (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal of Bactriology* 74(14):4525–4529.

Mahan, M. J., J. M. Slauch, and J. J. Mekalanos. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* 259:686-688

Marshall, B. M., Tachibana, C. and Levy S. B.. (1983). Frequency of tetracycline resistance determinant classes among lactose-fermenting coliforms. *Antimicrobial Agents and Chemotherapy* 24:835-840

Martin, W. S., Meek. A. H. and Willeberg, P (1987). *Veterinary Epidemiology: Principles and Methods,* Iowa State University Press, Iowa 420pp.

Martinez-Salazar, J. M., Alvarez, G. and Gomez-Eichelmann, M. C. (1986). Frequency of four classes of tetracycline resistance determinants in *Salmonella* and *Shigella* spp. clinical isolates. *Antimicrobial Agents and Chemotherapy* 30:630-631

Miflin, J. K., Horner, R. F., Blackall, P. J., Chen, X., Bishop, G. C., Morrow, C. J., Yamaguchi, T., and Iritani, Y. (1995). Phenotypic and molecular

characterization of V-factor (NAD)-independent *Haemophilus paragallinarum*. *Avian Diseases* 39(2): 304 - 308.

Miflin, J. K., Chen, X., and Blackall, P. J. (1997). Molecular characterization of isolates of *Haemophilus paragallinarum* from China by ribotyping. *Avian Pathology* 26: 119-127.

Miflin, J. K., Chen, X., Bragg, R. R., Welgemoed, J. M., Greyling, J. M., Horner, R. F., and Blackall, P. J. (1999). Confirmation that PCR can be used to identify NAD-dependent and NAD-independent *Haemophilus paragallinarum* isolates. *Onderstepoort Journal of Veterinary Research* 66(1): 55 - 57.

Miles, A. A. and Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *Journalof Hygiene* 38:732-49.

Minga, U. M. and Nkini, R. P (1986). Diseases as a major constraint to chicken production in Tanzania, In: *Proceedings of Tanzanian Society of Animal Production Scientific Conference* 10: 120-135.

Morishita, T. Y., Snipes, K. P., and Carpenter, T. E. (1990). Serum resistance as an indicator of virulence of Pasteurella multocida for turkeys. *Avian Diseases* 34(4): 888 - 892.

Mouahid, M., Bouzoubaa, K., and Zouagui, Z. (1989). Chicken infectious coryza in Morocco: epidemiological study and pathogenicity trials. *Actes de l'Institut Agronomique et Veterinaire Hassan II* 9: 11-16.

Mouahid, M., Bisgaard, M., Morley, A. J., Mutters, R., and Mannheim, W. (1992a). Occurrence of V-factor (NAD) independent strains of *Haemophilus paragallinarum*. *Veterinary Microbioliology* 31: 363-368.

Mouahid, M., Hinz, K. H., Engelhard, E., Mutters, R., and Mannheim, W. (1992b). Characterization of *Haemophilus paragallinarum* by analysis of whole cell carbohydrates, fatty acids and phospholipids. *Avian Pathology* 21: 127-136.

Msoffe, P. L. M., Mtambo, M. M. A., Minga, U. M., Gwakisa, P. S., Mdegela, R. H. and Olsen, J. E. (2002). Productivity and Natural Disease Resistance Potential of Free-ranging Local Chicken Ecotypes in Tanzania. *Livestock*

Research for Rural Development 14 (3) [http://www.cipav.org.co/lrrd/lrrd14/3/msof143.htm] site visted 10/9/2005

Muhairwa, A. P., Christensen, J. P., and Bisgaard, M. (2002). Serum resistance of *Pasteurella multocida* in avian and porcine sera, and comparative virulence investigations of selected serum-sensitive and resistant strains in chickens. *Avian Pathology* 31(2): 183 - 191.

Munar, F. U., Molina, H. A., Torres, M. I. P., Castro, L. V., Landicho, E. F., Maala, C. P., Mateo, A. A. B., and Vilacorte, E. Z. V. (1992). Determination of the protein profile and antigenic components of local *Haemophilus paragallinarum* (*A. paragallinarum*). *Proceedings 8th Congress of the Federation of Asian Veterinary Associations, 21 25 November 1992, The Manila Hotel Manila, Philippines* 627-633

Mutters, R., Piechulla, K., Hinz, K. H., and Mannheim, W. (1985). *Pasteurela avium* (Hinz and Kunjara1977) comb. nov. and *Pasteurella volantium* sp. nov. *International Journal of Systematic Bacteriology* 35: 5-9.

Nagaoka, K., Mayo, A. de, Takagi, M., Ohta, S., and De Mayo, A. (1994). Characterization of *Haemophilus paragallinarum* isolated in the Philippines. *Journal of Veterinary Medicine Series* 56: 1017-1019.

Nair, N. S. and Bhattacharya, A. R. (1980). Ayurvedic treatment for poultry disease. I. Efficiency of septilin for treatment of bacterial roup [*Haemophilus gallinarum*]. *Poultry Adviser* 13: 59-61.

Nakamura, K., Hosoe, T., Shirai, J., Sawata, A., Tanimura, N., and Maeda, M. (1993). Lesions and immunoperoxidase localisation of *Haemophilus paragallinarum* in chickens with infectious coryza. *Veterinary Record* 132: 557-558.

Namgoong, S., An, S. H., Kim, K. S., Mo, I. P., and Park, K. S. (1982). Studies on *Haemophilus* infection in chickens. *Research Reports of the Office of Rural Development, Korea Livestock and Veterinary* 24.

Nikolovski, J., Nikolovski, Z., Damnjanovic, N., Jovanovic, G., Glavinic, L, and Kaluderovic, V. (1997). Treatment of poultry diseases with streptomycin powder. *Zivinarstvo* 32: 71-72.

Nolan, L. K., Horne, S. M., Giddings, C. W., Foley, S. L., Johnson, T. J., Lynne, A. M., and Skyberg, J. (2003). Resistance to serum complement, iss, and virulence of avian *Escherichia coli*. *Veterinary Research Communications* 27(2): 101 - 110.

Ogunnariwo, J. A. and Schryvers, A. B. (1992). Correlation between the ability of *Haemophilus paragallinarum* to acquire ovotransferrin-bound iron and the expression of ovotransferrin-specific receptors. *Avian Diseases* 36(3): 655 - 663.

Ojok, L. (1993). Diseases as an important factor affecting increased poultry production in Uganda. *Der Tropen Zeitschurft fur die Landwirtschaft in den Tropen und Subtropen* 94: 37-44.

Olive, D. M. and Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology* 37: 1661-1669.

Olsen, I., Dewhirst, F. E., Paster, B. J. and Busse, H.-J. (2004). Family *Pasteurellaceae*. In: *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2. Edited by G. R. Garrity: New York: Springer (in press).

Owen, R. J., Costas, M., and Dawson, C. (1989) Application of different chromosomal DNA restriction digest fingerprints to specific and subspecific identification of Campylobacter isolates. *Journal of Clinical Microbiology* 27: 2338-2343.

Page, L. A. (1962a). *Haemophilus* infections in chickens. 1. Characterisation of 12 *Haemophilus* isolates recovered from diseased chickens. *American Journal of Veterinary Research* 23: 85-95.

Page, L. A. (1962b). *Haemophilus* infection in chickens. III. Factors in intraflock transmission of infectious coryza and its chemical and antibiotic therapeusis. *Avian Diseases* 6: 211-225.

Parry, M. F. (1989). Epidemiology and mechanisms of antimicrobial resistance. *American Journal of Infection and Control* 17(5):286–294

Paustian, M.L., May, B.J., and Kapur, V. (2001). *Pasteurella multocida* gene expression in response to iron limitation. *Infection and Immunity* 69:4109-4115

Pezzella, C., Ricci, A., DiGiannatale, E., Luzzi, I., and Carattoli, A. (2004). Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. *Antimicrobial Agents and Chemotherapy* 48(3): 903 - 908.

Prado, M. E., Dabo, S. M. and Confer, A. W. (2005). Immunogenicity of iron-regulated outer membrane proteins of *Pasteurella multocida* A:3 in cattle: molecular characterization of the immunodominant heme acquisition system receptor (HasR) protein. *Veterinary Microbiology* 105(3-4):269-80

Prasad, V., Murthy, K. K., Murthy, P. R., Vakani, Prasad, Krishna, Murthy K., and Radhakrishna, Murthy P. (1999). Antibiotic sensitivity studies on *Haemophillus paragallinarum* isolated from chickens. *Indian Veterinary Journal* 76: 253-254.

Quintiliani, R., and Courvalin, P. (1995). Mechanisms of resistance to antimicrobial agents, In: *Manual of clinical microbiology* Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. R. Tenover, and R. H. Yolken, 6th ed. ASM Press, Washington, D.C. p. 1308-1326.

Raggi, L. G., Young, D. C., and Sharma, J. M. (1967). Synergism between avian infectious bronchitis virus and *Haemophilus gallinarum*. *Avian Diseases* 11: 308-321.

Reece, R. L., Barr, D. A., and Owen, A. C. (1981). The isolation of *Haemophilus paragallinarum* from Japanese quail. *Australian Veterinry Journal* 57(7): 350 - 351.

Reece, R. L. and Coloe, P. J. (1985). The resistance to anti-microbial agents of bacteria isolated from pathological conditions of birds in Victoria, 1978 to 1983. *Australian Veterinary Journal* 62(11): 379 - 381.

Reid, G. G. and Blackall, P. J. (1984). Pathogenicity of Australian isolates of *Haemophilus paragallinarum* and *Haemophilus avium* in chickens. *Veterinary Microbiology* 9(1): 77 - 82.

Relman, D. A. (2002). Genome-wide responses of a pathogenic bacterium to its host *Journal of Clinical Investigations* 110:1071-1073.

Ridley, A. and Threlfall, E. J. (1998). Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella typhimurium* DT104. *Microbial Drug Resistance* 4: 113–118.

Roberts, M. C. (1996). Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Reviews* 19(1): 1 - 24.

Roman, F., Canton, R., Perez-Vazquez, M., Baquero, F., and Campos, J. (2004). Dynamics of long-term colonization of respiratory tract by *Haemophilus influenzae* in cystic fibrosis patients shows a marked increase in hypermutable strains. *Journal of Clinical Microbiology* 42(4): 1450 - 1459.

Rose, M. E. and Orlans, E (1962). Fowl antibody: IV. The estimation of haemolytic fowl complement. *Immunology* 5:642-8.

Saiki, R., K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G.T., Erlich, H.,A, Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science.* 20:230:1350-4.

Sandoval, V. E., Terzolo, H. R., and Blackall, P. J. (1994). Complicated infectious coryza outbreaks in Argentina. *Avian Diseases* 38: 672-678.

Sawata, A. and Kume, K. (1983). Relationships between virulence and morphological or serological properties of variants dissociated from serotype 1 *Haemophilus paragallinarum* strains. *Journal of Clinical Microbiology* 18(1): 49 - 55.

Sawata, A., Kume, K., and Nakase, Y. (1979). Antigenic structure and relationship between serotypes 1 and 2 of *Haemophilus paragallinarum*. *American Journal of Veterinary Research* 40: 1450-1453.

Sawata, A., Kume, K., and Nakase, Y. (1980). Biologic and serologic relationships between Page's and Sawata's serotypes of Haemophilus paragallinarum. *American Journal of Veterinary Research* 41(11): 1901 - 1904.

Sawata, A., Kume, K., and Nakase, Y. (1982). Hemagglutinin of *Haemophilus paragallinarum* serotype 2 organisms: occurrence and immunologic properties of hemagglutinin. *American Journal of Veterinary Research* 43(7): 1311 - 1314.

Sawata, A., Kume, K., and Nakai, T. (1984a). Relationship between anticapsular antibody and protective activity of a capsular antigen of *Haemophilus paragallinarum*. *Nippon Juigaku Zasshi* 46(4): 475 - 486.

Sawata, A., Kume, K., and Nakai, T. (1984b). Susceptibility *of Haemophilus paragallinarum* to bactericidal activity of normal and immune chicken sera. *Nippon Juigaku Zasshi* 46: 805-813.

Sawata, A., Kume, K., and Nakai, T. (1984c). Serologic typing of *Haemophilus paragallinarum* based on serum bactericidal reactions. *Nippon Juigaku Zasshi* 46: 909-912.

Sawata, A., Nakai, T., Kume, K., Yoshikawa, H., and Yoshikawa, T. (1985a). Intranasal inoculation of chickens with encapsulated or nonencapsulated variants of *Haemophilus paragallinarum*: electron microscopic evaluation of the nasal mucosa. *American Journal of Veterinary Research* 46: 2346-2353.

Sawata, A., Nakai, T., Kume, K., Yoshikawa, H., and Yoshikawa, T. (1985b). Lesions induced in the respiratory tract of chickens by encapsulated or nonencapsulated variants of *Haemophilus paragallinarum*. *American Journal of Veterinary Research* 46(5): 1185 - 1191.

Saylers, A. and Whitt. D. (Eds) (2002). *Bacterial Pathogenesis: A Molecular Approach*.ASM Press. Washington, DC 256pp

Schmitz, F. J., and Fluit. A. C. (1999). Mechanisms of resistance. In: *Infectious Diseases*. Edited by D. Armstrong, and S. Cohen; Mosby, Ltd., London, p. 7.2.1-7.2.14

Scola, B. (1992). The role of Ugandan women in poultry production. *In: Proceedings 19th World's Poultry Congress* Amsterdam, the Netherlands World Poutry Association, 2: 701-705.

Shaw, K. J., Rather, P. N., Have, R. S., Miller, G. M. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside modifying enzymes. *Microbiology Reviews* 57: 138-163

Sneath, P. H. and Stevens M. (1985). A numerical taxonomic study of *Actinobacillus, Pasteurella* and *Yersinia. Journal of General Microbiology* 131(10): 2711–2738.

Snipes, K. P. and Hirsh, D. C. (1986). Association of complement sensitivity with virulence of *Pasteurella multocida* isolated from turkeys. *Avian Diseases* 30: 500-504.

Sonaiya, E. B. (1990). The context and prospects for development of smallholder poultry production in Africa. In: *Proceedings CTA International Seminar on Smallholder Rural Poultry Production*, Thessaloniki, Greece Oct. 9-13 Vol.1 35-52

Sorensen, T. B. (Ed) (1999). *Poultry as a Tool in Poverty Eradication and Promotion of Gender Equality:* Proceedings of a Workshop March 22-26, 1999 Tune Landboskole, Denmark, 185pp

Soriano, V. E., Blackall, P. J., Dabo, S. M., Tellez, G., Garcia-Delgado, G. A. and Fernandez, R. P. (2001). Serotyping of *Haemophilus paragallinarum* isolates from Mexico by the Kume hemagglutinin scheme. *Avian Diseases* 45(3): 680-3.

Soriano, V. E., Tellez, G., Hargis, B. M., Newberry, L., Salgado-Miranda, C., and Vazquez, J. C. (2004a). Typing of *Haemophilus paragallinarum* strains by using enterobacterial repetitive intergenic consensus-based polymerase chain reaction. *Avian Diseases* 48(4): 890 - 895.

Soriano, V. E., Longinos, G. M., Fernandez, R. P., Velasquez, Q. E., Ciprian, C. A., Salazar-Garcia, F., and Blackall, P. J. (2004b). Virulence of the nine serovar reference strains of *Haemophilus paragallinarum*. *Avian Diseases* 48(4): 886 - 889.

Srinivasa, C. S., Reddy, P. K., and Aruna, D. (1989). Incidence of poultry diseases in Kurnool District (A.P.). *Poultry Adviser* 22: 45-48.

Srithar, A., Shoba, K., Saminathan, P., Chandran, N. D. J., Dorairajan, N., and Venugopalan, A. T. (1997). Pattern of mortality in commercial layer farms at Namakkal. *Indian Veterinary Journal* 74: 996-997.

Sugiarto, H. and Yu, P. L. (2004). Avian antimicrobial peptides: the defense role of beta-defensins. *Biochemical and Biophysical Research Communications* 323(3): 721-7.

Sundsfjord, A., Simonsen, G. S., Haldorsen, B. C., Haaheim, H. Hjelmevoll, S. O., Littauer, P. and Dahl, K. H. (2004). Genetic methods for detection of antimicrobial resistance. *APMIS.* 112(11-12): 815-37.

Takagi, M., Takahashi, T., Hirayama, N., Hirayama, N., Istiananingsi, N., Mariana, S., Zarkasie, K., Sumadi, Ogata, M., and Ohta, S. (1991). Survey of infectious coryza of chickens in Indonesia. *Journal of Veterinary Medical Science* 53: 637-642.

Takahashi, I., Yoshida, T., Honma, Y., and Saito, E. (1990). Comparison of the susceptibility of *Haemophilus paragallinarum* to ofloxacin and other existing antimicrobial agents. *Journal of the Japan Veterinary Medical Association* 43: 187-191.

Tang, Y. W., Procop, G. W. and Persing, D. H.(1997). Molecular diagnosis of infectious diseases. *Clinical Chemistry* 43:2021–2038

Taole, M., Albertyn, J., Van Heerden, E., and Bragg, R. R. (2002). Virulence of South African isolates of *Haemophilus paragallinarum*. Part 3: experimentally produced NAD-independent isolate. *Onderstepoort Journal of Veterinary Research* 69(3): 189 - 196.

Taylor, P. W. (1983). Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. *Microbiological Reviews* 47(1):46-83

Terry, T. D., Zalucki, Y. M., Walsh, S. L., Blackall, P. J., and Jennings, M. P. (2003). Genetic analysis of a plasmid encoding haemocin production in *Haemophilus paragallinarum*. *Microbiology* 149(Pt 11): 3177 - 3184.

Terzolo, H. R., Paolicchi, F. A., Sandoval, V. E., Blackall, P. J., Yamaguchi, T., and Iritani, Y. (1993). Characterization of isolates of *Haemophilus paragallinarum* from Argentina. *Avian Diseases* 37(2): 310 - 314.

Thitisak, W. Janvirisayopak O. Morris R. S. Srihakim S. and Kruedener R. V. (1988). Causes of death found in an epidemiological study of native chickens in Thai Villages. *Acta Veterinaria Scandinavica* 84: 200-202.

Thomson, C. J. (1993). Trimethoprim and brodimoprim resistance of gram-positive and gram-negative bacteria. *Journal of Chemotherapy* 5(6): 458–464

Thornton, A. M. and Blackall, P. J. (1984). Serological classification of Australian isolates of *Haemophilus paragallinarum*. *Australian Veterinary Journal* 61(8): 251 - 253.

Towner, K. J. and Cockayne, A. (1993). *Molecular Methods for Microbial Identification and Typing*. Chapman & Hall, London.156 pp.

Uchida, K., Takayama, K., Furuya, T., and Yano, Y. (1990). An outbreak of respiratory disease associated with infectious coryza and *Mycoplasma gallisepticum* infection in commercial broilers. *Journal of the Japan Veterinary Medical Association* 43: 29-32.

Ueda, S., Nagasawa, Y., Suzuki, T., and Tajima, M. (1982). Adhesion of *Haemophilus paragallinarum* to cultured chicken cells. *Microbiology and Immunology* 26(11): 1007 - 1016.

Vandamme, P., Segers. P., Vancanneyt, M., Van Hove, K., Mutters, R., Hommez, J., Dewhirst, F., Paster, B., Kersters, K., Falsen, E., Devriese, L.A., Bisgaard, M, Hinz, K.H. and Mannheim, W. (1994) *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from the avian respiratory tract. *International Journal of Systematic Bacteriology* 44: 24-37.

Verma, R., Kumar, A., Chandiramani, N. K. and Kulshreshtha, R. C. (1985). Coryza in chickens associated with mixed infections. *Indian Journal of Poultry Science* 20: 68-70.

Versalovic, J., Koeuth, T. and Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Reseaserch* 19:6823–6831.

Verschoor, J. A., Coetzee, L., and Visser, L. (1989). Monoclonal antibody characterization of two field strains of *Haemophilus paragallinarum* isolated from vaccinated layer hens. *Avian Diseases* 33(2): 219 - 225.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature* 406: 775-781

White, D. G., Hudson, C., Maurer, J. J., Ayers, S., Zhao, S., Lee, M. D., Bolton, L., Foley, T., and Sherwood, J. (2000). Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. *Journal of Clinical Microbiology* 38(12): 4593 - 4598.

Wilson, R. T., Traore, A., Kuit, H. G. and Slingerland, M. (1987). Chick mortality in scavenging village chickens in Sri Lanka. *Tropical Animal Health and Production* 19: 229-236.

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews* 51(2):221–271.

Woese, C. R., Stackebrandt, E., Macke, T. J. and Fox, G. E. (1985). A phylogenetic definition of the major eubacterial taxa. *Systematic Applied Microbiology* 6:143–151.

Wu, J. R., Shieh, H. K., Shien, J. H., Gong, S. R., and Chang, P. C. (2003). Molecular characterization of plasmids with antimicrobial resistant genes in avian isolates of *Pasteurella multocida*. *Avian Diseases* 47(4): 1384 - 1392.

Yamada, S. (1981). Susceptibility of ducks to *Salmonella pullorum* and *Haemophilus paragallinarum*. *Journal of the Japan Veterinary Medical Association* 34: 477-480.

Yamaguchi, T., Iritani, Y. and Hayashi. Y. (1989). Hemagglutinating activity and immunological properties of *Haemophilus paragallinarum* field isolates in Japan. *Avian Diseases* 33: 511-515

Yamaguchi, T., Kato, K., Takigami, S., Iritani, Y., and Hayashi, Y. (1990). Serological classification of Japanese isolates of *Haemophilus paragallinarum* using two serovar-specific monoclonal antibodies. *Avian Diseases* 34(2): 364 - 368.

Yamaguchi, T., Blackall, P. J., Takigami, S., Iritani, Y. and Hayashi. Y. (1991). Immunogenicity of *Haemophilus paragallinarum* serovar B strains. *Avian Diseases* 35:965-968

Yamaguchi, T., Kobayashi, M., Masaki, S., and Iritani, Y. (1993). Isolation and characterization of a *Haemophilus paragallinarum* mutant that lacks a hemagglutinating antigen. *Avian Diseases* 37: 970-976.

Yamamoto, R. and Clark, G. T. (1966). Intra- and inter-flock transmission of *Haemophilus gallinarum*. *American Journal of Veterinary Research* 27: 1419-1425.

Yamamoto, R. and Somersett, D. T. (1964). Antibody response in chickens to infection with *Haemophilus gallinarum*. *Avian Diseases* 8: 441-453.

Zaini, M. Z. and Iritani, Y. (1992). Serotyping of *Haemophilus paragallinarum* isolated in Malaysia. *Journal of Veterinary Medical Science* 54: 363-365.

Zaini, M. Z. and Kanameda, M. (1991). Susceptibility of the indigenous domestic fowl (*Gallus gallus*) to experimental infection with *Haemophilus paragallinarum*. *Journal of Veterinary Malaysia* 3(1):21-24.

Zander, D. V., Bermudez. A. J. and Mallison E. T. (1997). Principles of disease prevention, diagnosis and control. In: *Poultry Diseases* Edited by Calnek R. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. Mosby-Wolfe, London pp. 179-190.

Zhang, Y., Chen, M. (1991). Use of DNA techniques to detect tetracycline resistance genes in clinical strains. *Wei Sheng Wu Xue Bao* 31(1): 36 - 40.

Zhang, P., Blackall, P. J., Yamaguchi, T., and Iritani, Y. (1999). A monoclonal antibody-blocking enzyme-linked immunosorbent assay for the detection of serovar-specific antibodies to *Haemophilus paragallinarum*. *Avian Diseases* 43(1): 75 - 82.

Zhang, P. J., Miao, M., Sun, H., Gong, Y., and Blackall, P. J. (2003). Infectious coryza due to *Haemophilus paragallinarum* serovar B in China. *Australian Veterinary Journal* 81(1-2): 96 - 97.

Zhao, C., Nguyen, T., Liu, L., Sacco, R. E., Brogden, K. A., and Lehrer, R. I. (2001). Gallinacin-3, an inducible epithelial beta-defensin in the chicken. *Infection and Immunity* 69(4): 2684 - 2691.

8.0 APPENDIX

Map of Uganda showing areas where samples were obtained