

MOLECULAR IDENTIFICATION AND ANTIBIOGRAM PROFILING OF  
*AVIBACTERIUM PARAGALLINARUM* AND CO-PATHOGENIC BACTERIA  
ASSOCIATED WITH RESPIRATORY INFECTIONS IN CHICKENS AT GROOT  
AUB SETTLEMENT, KHOMAS REGION

A MINI THESIS SUBMITTED IN PARTIAL FUFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN  
MICROBIOLOGY

OF

THE UNIVERSITY OF NAMIBIA

BY

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JUNE 2021

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## **Abstract**

This study was undertaken to isolate, characterize and profile *Avibacterium paragallinarum* and some co-pathogenic bacteria for antibiotic sensitivity. *Avibacterium paragallinarum* is the etiologic agent of infectious coryza, a highly infectious respiratory disease of chickens. This disease is of economic importance because it is known to cause economic losses in the poultry industry through slow growth in young birds and declined egg production in layers. Symptoms of the disease include watery eyes, nasal and ocular discharges, sticky eyes (closed eyes), facial swelling and a reduction in egg production. Twenty (20) swab samples were collected from chickens displaying disease symptoms in the Groot Aub area (45 km south of Windhoek). The swabs were inoculated on blood and chocolate agar plates for isolation of the bacteria, followed by DNA extraction and amplification by polymerase chain reaction (PCR), and ultimately identification of the bacteria by sequencing analysis. For antibiogram profiling, morphologically distinct, lone standing colonies from the axenic cultures were subjected to antibiotic sensitivity testing using the disc diffusion method. In total, samples were collected from 20 chickens, 20% of which were solely positive for *Avibacterium paragallinarum*, 20% had *Avibacterium paragallinarum* simultaneously occurring with other co-pathogenic bacteria, whereas the co-pathogenic bacteria were isolated from 60% of chickens. Among some of the identified co-pathogenic bacteria were *Escherichia coli*, *Pasteurella multocida* and *Staphylococcus chromogenes*. The findings suggest that infectious coryza symptoms are not just specific to *Avibacterium paragallinarum*, but infection of chickens by other bacteria such *E. coli*, *Pasteurella multocida* and *Staphylococcus* species also result in the same symptoms being displayed. Antibiotic sensitivity tests

showed that the isolated *Avibacterium paragallinarum* field strains were susceptible to tetracycline and ampicillin, but resistant to gentamycin, ciprofloxacin and trimethoprim sulfamethoxazole. On the contrary, all isolated co-pathogenic bacteria were resistant to tetracycline and sensitive to trimethoprim sulfamethoxazole, ampicillin and ciprofloxacin. These results indicate that *Avibacterium paragallinarum* is more likely to be isolated in acute cases. Therefore, for better disease control and prevention, isolation and confirmation is of utmost importance so that right control measures are applied.

**Keywords:** *Avibacterium paragallinarum*, co-pathogenic bacteria, infectious coryza, chickens

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## List of abbreviations

μl	:	Microliter
B-ELISA	:	Blocking Enzyme-Linked Immunosorbent Assay
bp	:	base pair
CO <sub>2</sub>	:	Carbon Dioxide
CAP	:	Colistin and Aztreama
CPGS	:	Center for Postgraduate Studies
CVL	:	Central Veterinary Laboratory
DNA	:	Deoxyribonucleic Acid
DVS	:	Directorate of Veterinary Services
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-Linked Immunosorbent Assay
HA	:	Hemagglutinating Antigen
HI	:	Hemagglutination Inhibition
HMT <sub>p20</sub>	:	Histone Methyltransferases Protein 20
HPG2-PCR	:	<i>Haemophilus paragallinarum</i> 2- polymerase chain reaction
IBV	:	Infectious Bronchitis Virus
L	:	Liter

lbs	:	Pound
MAWLR	:	Ministry of Agriculture, Water and Land Reform
MEGA	:	Molecular Evolutionary Genetics Analysis
mm	:	Millimeter
NAD	:	Nicotinamide Adenine Dinucleotide
NCBI	:	National Center for Biotechnology Information
nm	:	Nano mole
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction
rpm	:	Rotation per minute
SXT	:	Sulfamethoxazole Trimethoprim
TAE	:	Tris-acetate-EDTA
UNAM	:	University of Namibia
UREC	:	University of Namibia Research and Ethics Committee

## **Acknowledgements**

With high appreciation the author is thankful to the Almighty God who made this research possible by giving the author wisdom, ideas, courage and strength to conduct the research and thesis activities successfully for the degree of Master of Science in Microbiology.

The author would like to express her gratitude with profound respect to the research supervisor Dr Timothy Sibanda, Department of Biological science, Faculty of Science, UNAM for his kindness, valuable instructions, scholastic guidance, educative suggestions, productive criticism and constant motivation throughout the course of this research work and without untiring help and his assistance in preparing the thesis manuscript.

With heartfelt gratitude the author wishes to express her appreciation to her research co-supervisor Dr Charles Ntahoshikira, Faculty of Pathobiology, School of Veterinary Science, UNAM for his immensely kind contribution, inspirations, crucial corrections, constructive criticism and positive instructions throughout the research period and in the preparation of this manuscript.

The author would like to express her thankfulness with sincere appreciation and deep respect to Dr Jean Damascène Uzabakiriho, the course Coordinator, for his valuable advices, corrections and encouragement he offered towards the research work.

In addition, the author wishes to extend her immense acknowledgment to Dr Siegfried Khaiseb, Dr Juliet Kabajani, Dr Kornelia Kandjumbwa, Mr Gotlieb Aikukutu, Mr Eino Iiyambo, Mr Linus Antonand Mrs Georgina Ngaisuie, from the directorate of Diagnosis

and Research services at Central Veterinary Laboratory in Windhoek for giving their encouragement, technical assistance and most importantly for availing the laboratory equipment and facilities for the research work to be conducted successfully at the Central Veterinary Laboratory.

The author also wants to express her deep gratitude and appreciation to all the 2019 intake MSc in Microbiology students of the Department of Biological science, Faculty of Science, University of Namibia and Mr Augustinus Mbang, the technologist in the Department for their inspiration and cooperation during the course of the research.

The author is delighted to express her joyful acknowledgements to the well-wishers specially Dr Rauna Athingo and Dr Naindji Haindongo, for their kind and thoughtful advices and endless assistance throughout the whole research period.

Finally, the author gratefully recognizes her family specially her husband Mr. Jonas Shihungileni and children Milton, Beata, Martin and Helena for their immense support, sacrifices and understanding during this research work.

## **Dedications**

I dedicate this thesis to my husband Jonas and my children Beata, Milton, Martine and Helena for their loyal support, trust, inspiration and encouragements.

## **Declaration**

I, Hellena Vaino, hereby declare that this is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institutions.

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## **Chapter I: Introduction**

### **1.1. Background of the study**

Agriculture has been referred to as the backbone of the Namibian economy with a 7% contribution to GDP in the past 7 years (!Gawaxab, 2020). There is no doubt that the livestock sector, inclusive of the poultry industry, is crucial to Namibia's national Gross Domestic Product (GDP). Namibia's broiler industry has performed exceptionally well, contributing 0.71% to Namibia's overall Gross Domestic Product in 2017 (Matthys, 2018). At local and industrial levels, the poultry industry is particularly significant in the sense that it is the major source of protein-rich, white meat in households (Ekunwe *et al.*, 2010).

The poultry sector is the swiftest developing area among other animal production activities, offering a chance to feed the rapidly growing national population and providing income to poor subsistence farmers (Gurmessa, 2019). The continued growth of the poultry business is grounded on the effectiveness of poultry in converting vegetable protein into animal protein, and the engaging quality and acceptability of poultry meat and eggs to numerous individuals (Gurmessa, 2019; Ekunwe *et al.*, 2010). Poultry rearing has various advantages over other animal sectors, including quick returns on investment, spreading income throughout the year, exceptional return compared to input and expenditure cost such as feed, medicines (Lal *et al.*, 2021).

Poultry farmers also require less land, enjoy flexibility whether they are small part time farmers or large commercial farmers, and can significantly mechanize their operations with high output per unit time (Gurmessa, 2019).

Guèye (2000) argues that keeping poultry has been practiced in remote communities of many African countries, and about 85% of rural families in Sub-Saharan Africa keep a few types of poultry such that in some countries' villages, the ratio of chickens to humans is about 1:5. Moreover, in many African rural communities, poultry are commonly raised in free-range and/or backyard systems (Asem-bansah, 2012). Rural poultry are reared for various reasons including providing the rural population with cheap and readily harvestable meat and eggs, as well as selling chickens for cash or using them in barter trade (Guèye, 2000; Asem-bansah, 2012). Furthermore, poultry have a symbolic importance within the context of many social activities such as offering them at special banquets for recognized family visitors, blessings; cocks are used as morning alarm timers for the residents as well as for religious functions like offerings to the gods (Guèye, 2000).

In Namibia, many households in the Northern Communal Areas (NCAs) raise indigenous chickens for income purposes, as well as for food security as a source of high quality protein through consumption of meat and eggs (Masaire *et al.*, 2018). Similarly, Dereja and Hailemichael (2017) also indicated that chicken production plays a significant role in the improvement of the livelihoods of rapidly expanding inhabitants of developing nations. Many poor individuals in these nations, chickens are the only type of production they can afford to keep, which are moderately risk free (Dereja and Hailemichael, 2017). Moreover, in comparison with exotic chicken breeds, indigenous chickens adapt well to rural settings, and require least input, thus they are easily managed by even underprivileged individuals including women and children (Petrus, 2011; Dereja and Hailemichael, 2017).

However, the contribution of poultry production to the economy is limited by factors such as low input of feeding, poor management, commonness and wide distribution of infectious and non-infectious diseases exacerbated by poor veterinary services and poor breeding practices (Gurmessa, 2019). According to Masaire *et al.* (2018) stressing factors like New castle disease, infectious coryza, ectoparasites and endoparasites are some of the major limitations that hinder the production of indigenous chickens in developing countries. Thus, among the constraints that affect the poultry industry are, poultry diseases that include emerging and reemerging diseases can affect the upper respiratory tract of birds, and this may result in high mortality, international trade ban, condemnation at slaughtering facilities and low production of meat and eggs, which ultimately have adverse economic consequences on the producers (Ali *et al.*, 2013; Gurmessa, 2019).

Multiple infections of the respiratory tract caused by various pathogens often simultaneously occur and present similar symptoms resulting in improper diagnosis (Nabeel Muhammad and Sreedevi, 2015). One of these important respiratory diseases that affect poultry is infectious coryza, an acute to chronic respiratory disease of chickens and other avian species caused by a bacterium, *Avibacterium paragallinarum*, previously known as *Haemophilus paragallinarum* (Ali *et al.*, 2013; Muhammad *et al.*, 2016; Han *et al.*, 2016). Infectious coryza affects the upper respiratory tract organs such as the nasal passages, infra orbital and paranasal sinuses. The disease is commonly referred to as coryza, cold, roup, snot or contagious catarrh (Rajurkar, Roy and Yadav, 2009; Dereja and Hailemichael, 2017). It is characterized by nasal discharge, swelling of the face and wattle, sneezing, sinusitis, inappetance, sleepy chickens and labored breath

(Muhammad *et al.*, 2016). The severity and manifestation of the infection depends on factors such as age, breed, poor housing, co-morbidities which includes other infectious diseases such as mycoplasmosis, fowl pox, infectious bronchitis and pasteurellosis (Han *et al.*, 2016).

Importantly, infectious coryza has a worldwide economic significance, affecting both broiler and layer flocks (Anjaneya *et al.*, 2014). The disease causes reduced feed and water intake in chickens and these results in poor growth performance and a 10-40% decrease in egg production in layers, as well as increased culling rates in broilers, contributing to economic losses (Nabeel Muhammad and Sreedevi, 2015). Infectious coryza has been reported in countries such as Japan, China, India, Indonesia and Uganda among others (Byarugaba *et al.*, 2007b; Bragg *et al.*, 1996; Dereja and Hailemichael, 2017). Thus, it has been causing serious economic losses in the poultry industry worldwide. In developing countries, the presence of different pathogens and predisposing factors linked to poor farming practices and management exacerbate the situation (Han *et al.*, 2016).

In Africa, the isolation of *A. paragallinarum* has been reported in South Africa, Morocco, Zimbabwe and Uganda (Byarugaba *et al.*, 2007a). However, other countries have reported the disease based on clinical symptoms, though the causative agent has not been isolated (Byarugaba *et al.*, 2007a). Infected and recovered birds act as reservoirs of infection in flocks and spread the infection when they come in direct or indirect contact with susceptible chickens making the disease endemic in an area (Nabeel Muhammad and Sreedevi, 2019).

In order to mitigate the economic losses associated with this disease, it is necessary to implement early, rapid and accurate diagnosis so that early effective treatment and control measures are instituted to curb the disease (Nabeel Muhammad and Sreedevi, 2015). As much as definitive diagnosis of a disease is vital, infectious coryza in developing countries has been tentatively diagnosed on the basis of clinical symptoms and post mortem examination of dead chickens (Bastos *et al.*, 2021). Few attempts are done to isolate the etiological microorganism (Wahyuni *et al.*, 2018).

Nonetheless, there are factors like mixed respiratory infections, incidence of Nicotinamide Adenine Dinucleotide (NAD) independent strains, overgrowth of fast growing bacteria which are masking the growth of *A. paragallinarum*, the requirement of special culture media, and the existence of different serovars that make the confirmatory diagnosis by conventional culturing method difficult and taunting (Nabeel Muhammad and Sreedevi, 2015). Therefore, nucleic acid based techniques are recommended as the best methods in the diagnosis of infectious coryza (Nabeel Muhammad and Sreedevi, 2015; Bastos *et al.*, 2021). Thus, infectious coryza can be diagnosed either by isolation and identification using conventional culturing method, the hemagglutination inhibition (HI) test for serovars and by Polymerase Chain Reaction (PCR) (Han *et al.*, 2016). The identification of *A. paragallinarum* by PCR, however, is the easiest, rapid and highly sensitive method as compared to conventional culturing methods that require special conditions and reagents (Rajurkar *et al.*, 2009; Bastos *et al.*, 2021)

Complicated infectious coryza is when the severity and duration of the disease is increased and prolonged due to the presence of other infectious agents such as

*Mycoplasma gallisepticum*, *Pasteurella gallinarum*, *E. coli*, *Pasteurella multocida*, and *Gallibacterium anatis*, among others that lead to the failure of treatment, resulting in increased mortality and condemned birds (Cigoy *et al.*, 2016; Clothier *et al.*, 2019). Due to mixed infections by different pathogens occurring simultaneously, the detection of *A. paragallinarum* and concurrent diseases by culture is often hindered by various factors including slow growth rate of this bacterium, the need for specialized media and growth conditions, and minimal reactivity to standard biochemical tests (Clothier *et al.*, 2019). Eventually, the delays in sample processing, diagnostic procedure and previous antimicrobial treatment have been shown to prevent the recovery of the causative agent (Bastos *et al.*, 2021). Therefore, the present study aimed to isolate and identify *Avibacterium paragallinarum* and co-pathogenic bacteria associated with respiratory infections in chickens, as well as to determine the antibiogram profiles of *Avibacterium paragallinarum* and co-pathogenic bacteria associated with respiratory infections in chickens.

## **1.2. Statement of the problem**

Infectious coryza is one of the diseases that seasonally occur in many small scale farms, causing low egg production and death of chickens (Masaire *et al.*, 2018; Nabeel Muhammad and Sreedevi, 2015). With little or no help from the Government, farmers are reluctant to take up laboratory diagnostic costs on themselves; hence, the disease goes unreported as it is not properly diagnosed or investigated (Gurmessa, 2019). As a result, improper treatment and control measures will lead to poor production yield and the spreading of the disease (Kapena *et al.*, 2020). In Namibia, as in other developing countries, infectious coryza has been tentatively diagnosed based on clinical symptoms

without any laboratory confirmation (Byarugaba *et al.*, 2007a). To the best of the author's knowledge, no experimental study on the isolation and identification of the causative bacteria has been performed in Namibia thus far. Equally important, Dungu *et al.* (2009) argues that some of the commercial vaccines used in South Africa do not confer protection to chickens against infectious coryza caused by NAD independent strain. Therefore, it is of paramount importance to isolate and identify the serovar which causes the infection in specific regions before vaccination is implemented (Deshmukh *et al.*, 2015). The overall objective of this study was to isolate presumptive *Avibacterium paragallinarum* and co-pathogenic bacterial strains from swab samples followed by molecular identification and confirmation of the same. Finally, the antibiogram profiles of the *Avibacterium paragallinarum* and co-pathogenic bacteria were determined.

### **1.3. Objectives**

Specific objectives of this study were to:

- a) Isolate *Avibacterium paragallinarum* and co-pathogenic bacteria from chickens presenting symptoms of infectious coryza in Groot Aub (Windhoek rural constituency).
- b) Determine antibiogram profiles of the identified *Avibacterium paragallinarum* and co-pathogenic bacterial isolates using the disc diffusion method.
- c) Identify the isolated bacteria using sequencing analysis.

### **1.4. Significance of the study**

The study will provide useful information to researchers, laboratory diagnosticians, field veterinarians and animal medicinal suppliers that can help in the identification of the

causative agents, as well as in the design or prescription of appropriate antibiotics for the treatment of infectious coryza and concurrent diseases.

### **1.5. Limitations of the study**

The number of samples was limited to 20 chickens that manifested respiratory symptoms of infectious coryza disease that were reported at Windhoek veterinary office. The study only focused on disease cases reported from Groot Aub settlement (Khomas Region), and unreported cases elsewhere were not assessed. The Covid 19 lockdown and regulations have contributed for failure to include control groups in the study.

### **1.6. Delimitation of the study**

The results obtained in the study could not be related to the other results obtained from similar studies from elsewhere due to different localities and climate conditions. The study only focused on samples collected from 20 chickens that manifested infectious coryza disease symptoms, chickens displayed other disease symptoms from same farm were not studied. The study only focused on disease cases reported from Groot Aub settlement, Khomas Region of Namibia.

## Chapter II: Literature review

### 2.1. Introduction

The poultry respiratory system is composed of, lungs, air sacs, syrinx, trachea, larynx, nasal cavity and the nostrils (Butcher *et al.*, 2018). Respiratory tract diseases are the significant component of diseases affecting poultry and cause heavy economic losses in the poultry industry worldwide as compared to diseases affecting other organs (Glisson, 1998; Blackall and Soriano- Vargas, 2020). In many cases, respiratory diseases observed in a flock may be a component of multisystem infections or it may be the predominant infection involving a specific organ (Glisson, 1998). Diseases like fowl pox and Newcastle disease affect multiple tracts, while diseases such as infectious coryza and infectious laryngotracheitis are limited to the respiratory tract in their acute phases (Glisson, 1998). Furthermore, simultaneous and concurrent infections exacerbate the severity of the disease (Elghazaly, Sedeek and Khalil, 2017).

Respiratory diseases in poultry can be caused by various pathogens like viruses, fungi and bacteria (Samy and Naguib, 2018). In the field its uncommon that chicken are confronted with single etiological agent, but rather by a combination of various disease agents (Nabeel Muhammad and Sreedevi, 2015). Factors such as environmental conditions, genetics, nutrition; management and vaccinations play a significant role in the initiation and outcome of respiratory diseases (Blackall, 1999). Some of the common pathogenic bacteria that cause respiratory infections are like *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycoplasma gallisepticum*, *Bordetella avium Avibacterium paragallinarum* and *Pasteurella multocida* among others (Glisson, 1998; Gopala *et al.*, 2008; Samy and Naguib, 2018).

In addition, *Escherichia coli* is considered one of the most serious bacteria affecting lower respiratory tract of poultry that can cause about 20% mortality in severe cases (Elghazaly, Sedeek and Khalil, 2017). *Pseudomonas aeruginosa* mainly isolated from the upper respiratory tract of poultry, causes rhinitis, sinusitis and laryngitis (Elghazaly *et al.*, 2017). *Staphylococcus aureus* is considered to be part of normal flora of the chicken, is found on the skin, feathers and in the respiratory and intestinal tracts (Elghazaly *et al.*, 2017).

The bacterium *Avibacterium paragallinarum* previously known as *Haemophilus paragallinarum* causes infectious coryza that affects the upper respiratory tract of poultry (Bragg *et al.*, 1996). There is limited published data on the isolation and identification of *Avibacterium paragallinarum* and co-pathogenic bacteria in Namibia, as the diagnosis is usually based on history and clinical picture of the disease.

## **2.2. Economic Significance**

Infectious coryza is a disease of worldwide economic significance, affecting both broiler and layer flocks (Anjaneya *et al.*, 2014). The disease has been reported globally and it poses a substantial poultry health and economic risk, as the disease causes poor growth in young birds and significant drop in egg production (10-40%) in layers (Anjaneya *et al.*, 2014). The disease also results into the condemnation of carcasses at slaughter houses due to pathological lesions of the respiratory tract in broilers (Byarugaba *et al.*, 2007a; Williams and Fulton, 2019). Infectious coryza occurs simultaneously with other infections such as fowl cholera and this may cause economic losses in poultry industry due to high mortality, low production reported in layers, turkeys and broilers (Huberman and Terzolo, 2016).

## 2.3. Pathobiology and Epizootiology

### 2.3.1. Causative agent

Infectious coryza is a severe highly contagious respiratory disease of chicken caused by *Avibacterium paragallinarum* previously known as *Haemophilus paragallinarum* (Bautista, 2019). The bacterium is a Gram negative non-motile coccobacillus, belonging to the family *Pasteurellaceae* (Muhammad *et al.*, 2016). Members of this family are pleomorphic organisms that are able to reduce nitrates and ferment carbohydrates (Dereja and Hailemichael, 2017).

*Avibacterium paragallinarum* (*Haemophilus paragallinarum*) belongs to the genus *Haemophilus*, in the family *Pasteurellaceae*, along with two other species of veterinary importance, namely *Haemophilus parasuis* and *Histophilus somni* (Yeneneh and Lulie, 2019). *Haemophilus* is a greek name that means blood loving simply because, the bacteria of this genus require certain growth factors referred to as X (Haemin) and V (Nicotinamide Adenine Dinucleotide (NAD) factors respectively that are released when the red blood cells break up as a result of heating or hemolysis (Yeneneh and Lulie, 2019). *Haemophilus* species are opportunistic pathogens of the upper respiratory tract and genital tracts in humans and animals. *Haemophilus somni* is found in the respiratory tract of cattle, *Haemophilus parasuis* inhabits the nasopharynx of healthy pigs, whereas *Haemophilus paragallinarum* is found in the respiratory tract of sick and recovered birds from infectious coryza (Yeneneh and Lulie, 2019).

These species are susceptible to desiccation; thus, they are unlikely to survive for a long period of time out of their host (Yeneneh and Lulie, 2019).

Clinically, infectious coryza in layer chickens is commonly portrayed by intense respiratory signs, inflammation of the face and nasal discharges, followed by decreased egg production, with other clinical signs when the disease is intricated by the presence of other pathogens (Welchman *et al.*, 2010).

Based on the Polyacrylamide gel electrophoresis (PAGE) scheme, *Avibacterium paragallinarum* is classified into three serovars, A, B and C, with different distribution of Page serovars between countries (Soriano *et al.*, 2001). These three serovars have been obtained from chickens all over the world including China, Taiwan, the United States, Mexico, and South Africa (Charoenvisal *et al.*, 2017; Sakamoto *et al.*, 2013; Soriano *et al.*, 2001).

In the same way, Kume serotyping scheme also divides the bacteria into 3 (three) significant serogroups: I, II, and III. PAGE serovars can be classified by the HI test to correlate with the Kume serogroups and as a result, PAGE serovars A, B, and C supplement the revised Kume serogroups I, II, and III, respectively (Charoenvisal *et al.*, 2017). Currently, nine (9) Kume serovars have been classified: A-1, A-2, A-3, A-4, B-1, C1, C-2, C-3, and C-4 (Soriano *et al.*, 2001). Importantly, 3 PAGE serovars are particularly different from one another, as the antibodies from one serovar cannot shield chickens from 2 other different serovars, while they can give security against the Kume serovars that is found in similar PAGE group (Charoenvisal *et al.*, 2017). For instance, a bivalent vaccine, which contains *Avibacterium paragallinarum* serovar A and C, cannot ensure chicken protection against serovar B-1, yet can secure the chickens with A-1, A-2, A-3, A-4, C-1, C-2, C-3, and C-4 infection (Charoenvisal *et al.*, 2017).

### **2.3.2. Incidence and Distribution**

The occurrence of infectious coryza is predisposed by factors such as rearing chickens of multiple ages, overcrowding, seasons, virulence of the bacteria as well as the compromised immune system from concurrent infections (Byarugaba *et al.*, 2007b). The causative bacterium *Avibacterium paragallinarum*, is endemic in many areas, but may also appear anywhere where chickens are raised as it can spread and be transmitted by so many routes. In endemic areas, the disease is maintained by chronically diseased birds or recovered birds as they act as reservoirs of the infection in a flock (Dwivedi *et al.*, 2018).

### **2.3.3. Natural hosts and species affected**

Infectious coryza is a disease of chickens; hence, reports of the disease in quail and pheasants most likely depict a similar disease that is caused by a different causative agent (Soriano-Vargas, 2021). Actually, Ali *et al.* (2013) stipulate that there have been reports of infectious coryza in different bird species other than chicken, though necessary interpretation about these reports is needed.

Infectious coryza primarily affects chickens, although it has been reported in pheasants, guinea fowl and Japanese quails (Crispo *et al.*, 2018; Byarugaba *et al.*, 2007a). Based on the studies done by Wahyuni *et al.* (2018), authors are in agreement, that quails are susceptible to *Avibacterium paragallinarum*, and in Indonesia there is an increase in cases of snout in quails, although it is commonly reported in layers and broilers. Other poultry species such as ducks, turkeys, and other wild avian species appear to be resistance to the disease. Infectious coryza is commonly found in chickens and has no public health significance (Rajurkar *et al.*, 2009).

#### **2.3.4. Age of host commonly affected**

Infectious coryza occurs at any age, though mature birds are generally more at risk and layers are often affected at the peak of laying phase (Soriano-Vargas, 2021). The disease is common in pullets aged 8-12 weeks; when opportunities allow, it affects even very young broiler chicks of 3 to 12 weeks of age (Deshmukh *et al.*, 2015). As an example, in developing countries, the disease is often seen in very young chicks, as young as 3 weeks old and this is more likely due to poor biosecurity, poor environmental conditions, and stress from other diseases (Soriano-Vargas, 2021; Deshmukh *et al.*, 2015). In addition, young birds are also prone to infectious coryza due to weak and undeveloped immune system; many cases are observed following stressful events such as transportation, relocation of layers from pullet house to laying hen house as well as during the cold season (Williams and Fulton, 2019).

#### **2.3.5. Carriers and Transmission**

Birds can become carriers without displaying any symptoms of the disease, making infectious coryza very hard to control on farms which do not apply an “all in, all out” program (Williams and Fulton, 2019). Infectious coryza can be spread through aerosols, direct contact with clinically infected and carrier birds, contaminated drinking water, feed and through contaminated clothing, people and equipment (Dereja and Hailemichael, 2017). According to Bautista (2019), although the disease spreads commonly through direct contact between birds, through respiratory airborne droplets and or by ingesting contaminated feed and water, poor biosecurity, harsh environmental conditions and cold weather along with co-infection with other diseases are the predisposing factors for the transmission and onset of the disease. Moreover, birds that

recovered from the disease become carriers of the bacteria and occasionally shed them under stress conditions (Butcher *et al.*, 2018). Therefore, when introduced in a naïve flock, they will introduce the disease into the susceptible flock if it is not vaccinated (Bautista, 2019).

### **2.3.6. Virulence factors, Pathogenicity and Incubation period**

According to Byarugaba *et al.*( 2007b), , little is known about the virulence factors of *Avibacterium paragallinarum* even though few investigations have been carried out to study the association between the features of the bacterium and the virulence of the organisms causing the respiratory tract infections. Significantly, the capsule of *Avibacterium paragallinarum* is one of the features responsible for its virulence; hence the encapsulated bacteria are demonstrated to be more virulent as compared to the non-encapsulated (Vargas and Terzolo, 2004). The capsule has been linked to the colonization of the ciliated epithelial cells of the hosts' nasal mucosa; however, its role in causing the lesions associated with coryza is still debatable (Deshmukh *et al.*, 2015; Soriano-Vargas and Terzolo, 2004). Most importantly, it is understood that it is the capsule which facilitates the bacterial adherence to the surface of the chicken's nasal mucosa (Byarugaba *et al.*, 2007b). Apart from the capsule, the hemagglutinating antigens are also assumed to play a paramount role in the process of adherence (Byarugaba *et al.*, 2007b; Deshmukh *et al.*, 2015). In addition, based on the information revealed by Xu *et al.* (2019), it has been demonstrated that the hemagglutinin antigen (HA) assumes an important function in the pathogenicity and immunogenicity of *Avibacterium paragallinarum*.

Bacterial adherence to epithelial cells is viewed as the initial step of disease of the mucosal surfaces (Soriano-Vargas and Terzolo, 2004). Adhesins are structures that mediate attachment to the corresponding cell structures, the receptors. Both *in vivo* and *in vitro* positive attachment of *Avibacterium paragallinarum* to chicken tracheal epithelial cells have been demonstrated (Soriano-Vargas and Terzolo, 2004). In most cases lesions in chickens are caused by encapsulated bacteria, the level of which connects with the quantity of the capsule, while non-encapsulated organisms are believed to be avirulent; the capsule is involved in resistance of *Avibacterium paragallinarum* against chicken serum bactericidal activity (Byarugaba *et al.*, 2007b).

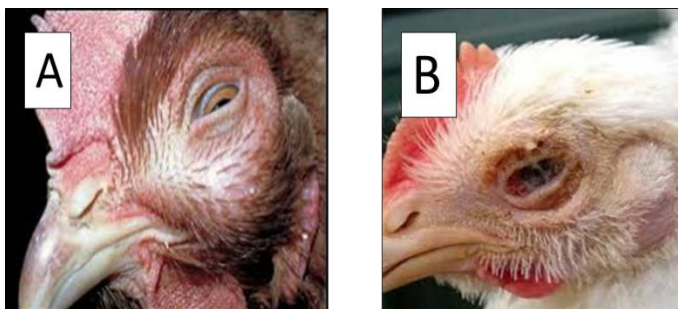
Infectious coryza has abrupt onset that infection occurs when susceptible chickens are exposed by contact to infected chickens are likely to exhibit signs of the disease within 24-72 hours (Soriano-Vargas and Terzolo, 2004). The duration of the disease usually runs for 2-3 weeks (Huberman and Terzolo, 2016). However, under field conditions, the duration could be possibly longer in the presence of stressing factors such as concurrent infection such as mycoplasmosis, and pasteurellosis (Soriano-Vargas, 2021)

### **2.3.7. Clinical signs**

Infectious coryza is manifested by swelling of the face and wattles, lacrimation, foul smelling nasal discharges, sneezing, coughing and decreased feed intake as well as reduced egg production (Bautista, 2019). At the beginning of the infection, chickens are observed to have serous mucus discharges running from the nostrils and the eyes (Soriano-Vargas, 2021), that lately transform into caseous cream white flake-like exudative deposits found around the nostrils as well as in the eyes resulting in sticky closure of the birds' eyes (Deshmukh *et al.*, 2015). As the disease becomes chronic other

pathogens may be involved, the sinus exudate may become consolidated and turn yellowish (Soriano-Vargas, 2021).

So, most dead birds exhibit swollen head syndrome due to the pressure resulting from these caseous cream-white chunks of exudates found either unilateral or bilateral in the infra orbital or supra orbital sinuses (Deshmukh *et al.*, 2015). Firstly, the bacteria colonize the upper respiratory tract, infecting the sinuses, the nostril and then transcend down trachea, air sacs and in severe cases it causes pneumonia in lungs (Crispo *et al.*, 2018). Furthermore, the bacteria infect the reproductive organs such as the ovary and salpinx in older and egg laying chickens, thus contributing to poor egg quality and reduced egg production (Deshmukh *et al.*, 2015). Simultaneous, occurrence with respiratory pathogens like *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Pasteurella spp*, *Escherichia coli* and infectious bronchitis virus (IBV), in addition to various stress factors, can equally fuel the infection (Clothier *et al.*, 2019). Respiratory symptoms of infectious coryza last for a few weeks to months if complicated by other pathogens, and this will have a big negative impact in poultry industry (Ali *et al.*, 2013). Some clinical signs of infectious coryza are illustrated in **Figure 2.1**.



Adopted from Cigoy, Huberman and Terzolo (2016).

**Figure 2.1:** A) Chicken with facial swelling. B) Blindness as sequelae of infectious coryza

### **2.3.8. Morbidity and mortality**

The disease is usually acute but highly contagious with high morbidity of up to 60-100% and a mortality rate ranging from 1 to 15% (Byarugaba *et al.*, 2007a). The morbidity is usually high, with mortality relatively low except in circumstances when there are other co-infectious agents such as coronavirus (causing infectious bronchitis), *Mycoplasma gallisepticum* and *E. coli* (Cigoy *et al.*, 2016) In addition, the disease affects all age groups of chicken, especially when there are predisposing factors like stress, cold season, and runs its course in about two weeks in the absence of those stressing factors that might prolong it (Byarugaba *et al.*, 2007a). Recovered birds become carriers and remain healthy; they are able to infect new susceptible birds that are introduced into the farm, and thus, multi-age farms are not recommended (Cigoy *et al.*, 2016).

#### **2.4.11.1. Gross and histopathological lesions**

Different lesions may include conjunctivitis, tracheitis, bronchitis, and air sacculitis, especially if different microorganisms are included (Soriano-Vargas, 2021). Microscopically, the affected respiratory organs and tissues shows acanthosis, congestion of blood vessels, hyperplasia of mucous glandular cells, hyperplasia of nasal sinus, parakeratosis and pneumonic lesion of lung (Ali *et al.*, 2013; Akter *et al.*, 2014). Soriano-Vargas (2021) mentioned that, the affected respiratory organs are edematous with invasion of heterotrophies, macrophages and mast cells.

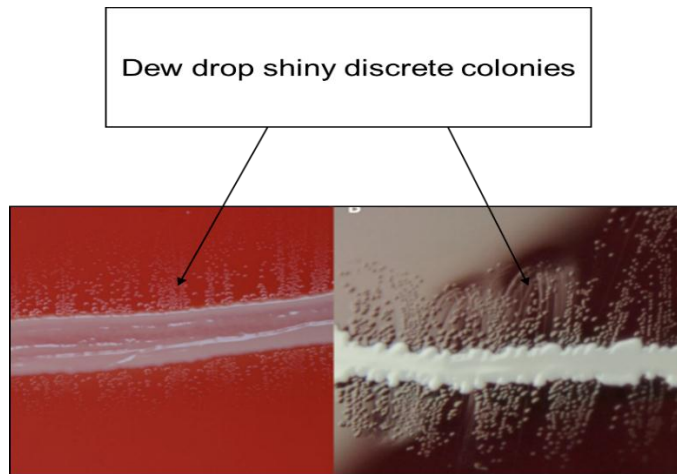
## **2.4. Diagnosis**

Although in many cases infectious coryza is tentatively diagnosed symptomatically, there are various laboratory methods utilized for the diagnosis and confirmation of the disease. These are the direct isolation by culture, the hemagglutination inhibition test for serovar A, and molecular technique such as PCR (Han *et al.*, 2016).

### **2. 4. 1. Isolation and identification**

For direct isolation, the bacterium can be isolated from sterile cotton swabs obtained from the infraorbital sinus, trachea, and air sac, though, the pathogen must be isolated during the acute stage, at least during the 1- 7 days of infection (Han *et al.*, 2016). Infectious coryza is simplest diagnosed based on the history of abrupt onset and rapidly spreading of the infection, coupled with the isolated catalase negative bacteria that shows satellitic features (Ali *et al.*, 2013) as it is shown in figure 2.2.

*Avibacterium paragallinarum* is isolated when cultivated under anaerobic conditions with 5-10% CO<sub>2</sub>, however, the traditional candle jar can also be used to provide CO<sub>2</sub> in the case when CO<sub>2</sub> sachets are unavailable (Deshmukh *et al.*, 2015). Additionally, *Avibacterium paragallinarum* is a relatively slow growing organism that can be overgrown by fast growing bacteria in clinical samples (Askari *et al.*, 2013). Thus, the isolation and identification by tradition culture method requires several days to complete (Corney *et al.*, 2008).



Adopted from Soriano- Vargas *et al.* (2013)

**Figure 2.2:** Colony morphology of *Avibacterium paragallinarum*

#### **2.4.1.1. Growth requirements**

*Avibacterium paragallinarum* is a fastidious organism that requires the haemin (factor X) and NAD (factor V) as growth factors (Feberwee *et al.*, 2019). Thus, these bacteria can be grown in 7-10% sheep defibrinated blood agar plate incubated for 24hrs at 37 °C; yet there are some isolates that do not need NAD, and can grow on non-hemolyzed blood agar (Dereja and Hailemichael, 2017). Remarkably, horse blood is considered as an alternative ingredient for the growth of this fastidious organism where sheep blood is not available (Deshmukh *et al.*, 2015). *Avibacterium paragallinarum* grows well on chocolate gar (delivering the X and V factors through heating of the blood) and on blood agar that's streaked with *Staphylococcus* species releasing the V factor by breaking up the blood cells (Yeneneh and Lulie, 2019). Mostly, the heated blood medium is therefore preferred for initial isolation and identification of other pathogens that may be present (Yeneneh and Lulie, 2019). Furthermore, the isolation of *Haemophilus* sp. is enhanced

when chocolate and blood agar plates are incubated for 24hrs to 48hrs in 5-10% CO<sub>2</sub> atmospheric conditions, (Yeneneh and Lulie, 2019).

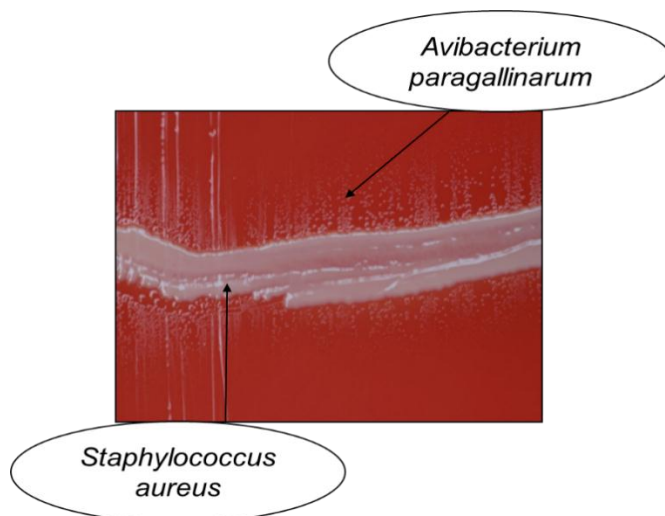
Notably, there are several difficulties that are associated with conventional culturing diagnostic methods such as slow growing characteristic of the bacteria, NAD growth requirements that make the organism easily masked and overgrown by other fast growing members of the haemophilic bacterial family such as *Avibacterium gallinarum*, *Avibacterium avium* and *Avibacterium volantium* (Anjaneya *et al.*, 2014).

According to Clothier, Torain and Reinl (2019), the identification of *Avibacterium paragallinarum* by culture is frequently frustrating both by its demanding characteristics such as slow grow rate, requirement for particular media, growth conditions, insignificant reactivity to standard biochemical and the presence of other bacterial pathogens in samples. In addition, the recovery of the bacteria might also be affected by the delay in test processing as well as by the past antimicrobial treatment (Clothier *et al.*, 2019). Again, since the majority of *Avibacterium paragallinarum* isolates show a prerequisite for nicotinamide adenine dinucleotide (NAD) for development, its isolation and identification method require understanding and utilization of special exceptional media (Askari *et al.*, 2013).

#### **2.4.1.2 Macroscopical and microscopical features**

Based on the study done by Wahyuni *et al.* (2018), it is practically observed that *Avibacterium paragallinarum* colonies on sheep blood agar are circular, transparent, and smooth dewdrops; they appear satellite once cultured perpendicular to the feeder

bacteria such *Staphylococcus aureus* (Figure 2.3). The colonies on blood agar are also tiny dewdrop, mucoid, smooth iridescent which are non-hemolytic (Akter *et al.*, 2014). It is worth to note that satellite colony is mostly observed with NAD dependent serovars that requires the V factor to grow; however, with some *Avibacterium paragallinarum* serovars that are NAD independent could grow without V factor, although the satellite colonies cannot be appreciated (Wahyuni *et al.*, 2018). Thus, when cultured on blood agar with a staphylococcal nurse colony that excretes the V-factor, the satellite colonies appear as dewdrops, growing adjacent to the nurse colony (Soriano-Vargas, 2021).

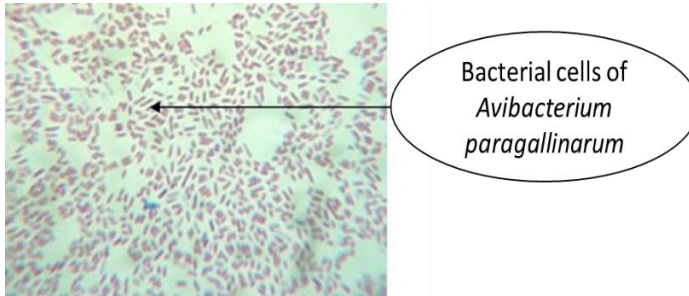


Adopted from Soriano-Vargas *et al.* (2013).

**Figure 2.3:** Diagram showing *Avibacterium paragallinarum* as small white satellitic colonies close to the white colonies of *Staphylococcus aureus* on blood agar plate

When colonies from 24-48hrs cultures are stained with Gram stain, the bacteria appear to be red indicative of being Gram negative and appreciated as short rods or coccobacilli in shape (Figure 2.4) with the length of 1-3 $\mu$ m and width of 0.4-0.8 $\mu$ m (Akter *et al.*, 2016; Wahyuni *et al.*, 2018). The bacterium appears to be polar in single or pairs arrangement (Akter *et al.*, 2014; Blackall and Soriano- Vargas, 2020). These

bacteria have a tendency to form filaments in addition to undergoing degeneration whereby fragments and indefinite forms are observed within 48hrs -60hrs of growing (Akter *et al.*, 2014)).



Source: (Akter *et al.*, 2016).

**Figure 2.4:** Pink rod shape bacterial cells of *Avibacterium paragallinarum* arranged in single or pairs.

#### **2.4.1.4. Biochemical properties**

*Avibacterium paragallinarum* reduces nitrates to nitrites, causes fermentation of glucose without production of gas and it is oxidase and catalase negative (Akter *et al.*, 2014). Besides negative indole production, urea/gelatine hydrolysis are its basic characteristic features (Akter *et al.*, 2014; Deshmukh *et al.*, 2015). Most of non-pathogenic hemophilic bacteria that are present in healthy and diseased chickens are catalase positive, thus catalase test is crucial in order to rule them out (Soriano-Vargas, 2021).

In addition, based on research done by Feberwee *et al.* (2019), failure to ferment galactose and trehalose, and its lack of catalase, clearly distinguish *Avibacterium paragallinarum* from other species of the genus *Avibacterium* (Table 2.1).

**Table 2.1:** Differential tests for avian *Haemophilus* species.

Properties	<i>H. paragallinarum</i>	<i>H. avium</i>	<i>P. avium</i>	<i>P. volantium</i>	<i>Pasteurella species A</i>
<b>Pigment</b>	-	Yellow V	-	Yellow U	-
<b>Catalase</b>	-	+	+	+	+
<b>Growth in aerobic condition</b>	-	+	+	+	+
			Acid from		
<b>Arabinose</b>	-	V	-	-	+
<b>Galactose</b>	-	+	+	+	+
<b>Maltose</b>	+	V	-	+	V
<b>Sorbitol</b>	V	V	-	V	-
<b>Sucrose</b>	V	+	+	+	+
<b>Trehalose</b>	-	+	+	+	+

Susceptibility to chemical and physical agents:

V=variable; + = positive; - =negative.

#### 2.4.2. Molecular Identification (PCR)

To overcome challenges encountered with traditional culturing method, several alternative diagnostic approaches such as Multiplex Polymerase Chain Reaction (PCR), restriction endonuclease analysis, and ribotyping and ‘*hagA*’ gene sequencing that are specific and sensitive have been developed and implemented by developed countries (Anjaneya *et al.*, 2014).

As a matter of fact, PCR can be a substitute to bacteriological culture, because it has the potential to detect low numbers of a target organism in deeply contaminated samples. When applied to diagnosing infectious coryza, the use of PCRs would avoid the problems associated with culture such as slow growing and special medium requirements (Corney *et al.*, 2008). It is for this reason that (Chen *et al.*, 1996) designed two PCRs, designated HPG-1 and HPG-2, for identifying colonies as *Avibacterium*

*paragallinarum* and for detecting them in swabs taken from infected chickens (Chen *et al.*, 1998).

Of little difference, compared to conventional PCR, 5' *Taq* nuclease assay was developed that has several advantages over conventional PCRs specifically its enhanced sensitivity over conventional PCR (Corney *et al.*, 2008). In other words, 5' *Taq* nuclease has less opportunity to release amplified materials into the environment after amplification electrophoresis and hence false positive results due to contamination with amplified material is highly reduced (Corney *et al.*, 2008).

#### **2.4.3. Hemagglutination Inhibition (HI) test**

The hemagglutination inhibition test, which is one of the most generally utilized serological test, is frequently used to identify changes in antibody titers in cases of field infection or vaccination, and is helpful for assessing the prevalence of infectious coryza in certain areas or directing review /epidemiological studies (Han *et al.*, 2016). A range of serological tests for antibodies to *Avibacterium paragallinarum* have been suggested such as monoclonal antibody based blocking enzyme-linked immunosorbent assays (B-ELISAs) for antibodies specific to *Avibacterium paragallinarum* serovar A or C (Miao *et al.*, 2000). The serovar A B-ELISA had a specificity of 99.7% and a sensitivity of 78.7%, while the serovars C B-ELISA had a specificity of 99.8% and a sensitivity of 64.7%, indicating that these B-ELISAs have considerable potential (Miao *et al.*, 2000). While having demonstrated good specificity and acceptable levels of sensitivity, the access to the test is limited due to lack of commercial availability of the monoclonal antibodies that form the heart of the assay as well as the fact that the test is only able to detect the monoclonal antibodies for PAGE serovars A and C (Blackall, 1999). In

addition, some infections may not be detected by these ELISAs because some isolates of *Avibacterium paragallinarum* do not respond to monoclonal antibodies (Blackall, 1999).

## 2.5. Differential diagnosis

Infectious coryza disease must be differentiated from other diseases shown in table 2.2, which cause swelling of the face and wattles such as fowl cholera, mycoplasmosis, laryngotracheitis, Newcastle disease, infectious bronchitis, avian influenza, swollen head syndrome, *Escherichia coli* infection and Vitamin A deficiency , which (Soriano-Vargas, 2021; Butcher *et al.*, 2018).

**Table 2.2:** Some of respiratory diseases with similar symptoms in chickens

Disease	Pathogen	Causative agent	Main symptoms	transmission route
Fowl cholera	Bacteria	<i>Pasteurella multocida</i>	Septicemia in acute stage, head and respiratory lesions in chronic stage. Decreases egg production	Aerosol
Mycoplasma osis	Bacteria	<i>Mycoplasma gallisepticum</i>	Facial swelling, nasal discharge, coughing, foamy eyes	Aerosol
Infectious coryza	Bacteria	<i>Avibacterium paragallinarum</i>	Nasal and ocular discharges, facial swelling and drops in egg production	Feco-oral/ contact and via drinking water
Newcastle	Virus	Paramyxovirus	Decreased egg production, sudden death, greenish runs	Aerosol, droppings and fomites
Avian influenza	Virus	H5N1	Mouth breathing, nasal-ocular discharge. Swelling of the head	Air droplets, fomite, droppings

## **2.6. Treatment**

There are various drugs that have proved to be effective in the symptomatic treatment of the disease despite the fact that it is rarely totally eradicated; therefore, prevention is the only sound method of control (Soriano-Vargas, 2021). Equally, symptomatic treatment is not enough and as a bacteria-caused disease, it should be curable with the aid of antibiotics unless it is an intracellular bacterium. Relapsing infections are more likely to occur when the treatment is discontinued and when the carrier chickens are not eliminated (Dereja and Hailemichael, 2017). Drugs such as erythromycin, ox tetracycline, sulfonamides like sulfadimethoxine or sulfathiazole in feed or water, or erythromycin administered in drinking water can be helpful in reducing the severity of the disease, although none of the therapeutic agents has been found to be bactericidal (Rajurkar, Roy and Yadav, 2009; (Soriano-Vargas, 2021). In any case, immunization, great biosecurity, good farming management, proper disinfection practices, and a decent nutrition program are vital to control diseases and are definitely more effective than the usage of antibiotic agents (Sato and Wakenell, 2020).

## **2.7. Prevention and control**

Enhanced management practices including hygiene, proper housing, flock structure and young chick care etc. are most efficient. Besides, routine activities such as frequent changing of poultry liter, frequent cleaning of feeders and drinkers, and regular decontamination of poultry houses are usually effective measures in the prevention of the disease (Dereja and Hailemichael, 2017). On the other hand, housing measures include ventilation; quarantine of new entries, proper density, and segregation of birds by age is also another remarkable measure that can be applied especially during

outbreaks (Dereja and Hailemichael, 2017). However, the presence of carrier chickens that harbor the bacteria permanently make control and eradication programs difficult, more especially in facilities which do not allow all-in all-out practice (Bautista, 2019). Thus, it is imperative to make sure that, all replacement chickens on endemic farms are quarantined for 3 months, vaccinated regularly and all-in-all-out programs are practiced in order to control and prevent the disease (Dereja and Hailemichael, 2017; Soriano-Vargas, 2021).

The prevention and control of infectious coryza can be accomplished through strict biosecurity, antimicrobial application and relevant vaccinations using commercial inactivated bacteria in aluminum hydroxide gel or mineral oil vaccines against infectious coryza (Charoenvisal *et al.*, 2017). For instance, there are bivalent vaccines such as *Avibacterium paragallinarum* serovars A and C, trivalent vaccines containing serovars A, B, and C. Appropriate vaccines should correspond to the reported and confirmed serovars in an area as there is no guarantee of cross protection between different serovars, even though the hemagglutination inhibition test is used for classified serovars of *Avibacterium paragallinarum*, and the hemagglutination inhibition titer does not signify the level of host protection (Charoenvisal *et al.*, 2017). Thus, it is important to identify the serovars responsible for the infection in the specific area before the vaccination is instituted (Deshmukh *et al.*, 2015).

Previously, inactivated whole cell vaccines of *Avibacterium paragallinarum* were generally utilized for the prevention of infectious coryza, although such vaccines at times caused side effects, like local necrotic lesions and inflammation at the injection site (Sakamoto *et al.*, 2013). In addition, the development of *Avibacterium paragallinarum* vaccine requires media containing nicotinamide adenine dinucleotide

and a few strains require chicken serum for development. Thus, the preparation of such culture media is costly and tiresome (Sakamoto *et al.*, 2013). On the other hand, Sakamoto *et al.* (2013) confirmed that the recombinant fusion peptide derived from HMTp210 protein could be useful for generating effective and safe shots against infectious coryza in chickens.

## **2.8. Concurrent bacterial infections**

### **2.8.1. Fowl cholera**

Fowl cholera is an infectious, bacterial disease that affects domestic and wild birds worldwide (Sander, 2019). Fowl cholera is a respiratory bacterial disease caused by *Pasteurella multocida*, a Gram negative, very short characteristic cocco-bacillus that may form a few long strands of which coccoid forms dissociate free from its ends and are arranged into short chains (Huberman and Terzolo, 2016).

#### **2.8.1.1. Clinical signs**

Finding countless dead birds without previous signs is usually the first sign of fowl cholera disease and in more protracted cases, depression, anorexia, mucoid discharge from the mouth, ruffled feathers, diarrhea, and increased respiratory rate are usually seen. Pneumonia is particularly common in turkeys (Sander, 2019). In chronic fowl cholera, signs and lesions are generally related to localized infections of the sternal bursae, wattles, joints, tendon sheaths, and footpads, which are often swollen because of accumulated fibrino suppurative exudate (Sander, 2019; Glisson, 1998).

### 2.8.1.2. Gross lesions

Pathological lesions such as congested liver, spleen and lungs and/or with purulent or necrotic lesions; free caseous masses in the peritoneum or air sacs; synovial content or caseous material from the interior of affected joints; and indurated caseous or purulent contents within swollen wattles are commonly found in fowl cholera clinical cases (Huberman and Terzolo, 2016).

### 2.8.1.3 Diagnosis

#### 2.8.1.3.1 Isolation and identification by culture

*P. multocida* can be isolated and identified through traditional culture method. It grows well in rich culture media, such as agar base plus 0.1% sterile defibrinated blood of equine or sheep, dextrose starch agar, brain-heart agar or Columbia blood agar whereby plates are incubated for 18 to 24 hours at 37°C (Huberman and Terzolo, 2016). The colonies on blood agar are grey to white in color (Figure 2.5), non-hemolytic smaller than 2-3 mm in diameter (Huberman and Terzolo, 2016). Microscopically, *Pasteurella multocida* bacteria are Gram-negative, small rods, non-motile with a capsule that may exhibit pleomorphism after repeated subculture (Sander, 2019).



**Figure 2.5:** Plate of *Pasteurella multocida* (non-hemolytic colonies) on Blood agar.

#### **2.8.1.3.2 Molecular technique**

PCR has been used for the detection and confirmation of *Pasteurella multocida* in pure and mixed cultures as well as in clinical samples (Sander, 2019). A multiplex PCR which can distinguish between different somatic serotypes and might qualify more efficient vaccine development has been developed (Sander, 2019).

#### **2.8.1.4. Transmission**

Chronically infected birds and asymptomatic carriers are believed to be major sources of infection, whereas wild birds may bring the organism into a poultry flock, though, mammals (including rodents, pigs, dogs, and cats) may also carry the infection (Sander, 2019; Butcher *et al.*, 2018). The disease is transmitted when naïve birds come into contact with excretions from the nose, mouth and conjunctiva from sick birds as well as by contaminated clothes, shoes and other equipment (Sander, 2019; Huberman and Terzolo, 2016).

#### **2.8.1.5. Treatment and prevention**

Different drugs like sulfadimethoxine and tetracycline can be used to reduce the mortality; however, they will not eliminate the disease from a flock and the disease is likely to reoccur when treatment is discontinued (Sander, 2019). Again, the use of these drugs should be used with extra caution due to their potential toxicity in laying hens. Antibiotic sensitivity testing is recommended before any antibiotic treatment is initiated in order to assist in drug selection because of the emergency of multi-resistant strains (Sander, 2019; Glisson, 1998). The eradication of the infection requires

depopulation, followed by thorough cleaning and disinfection of buildings and equipment (Sander, 2019; Glisson, 1998).

### **2.8.2. *Avibacterium gallinarum* and *Avibacterium endocarditis***

#### **2.9.2.1. General overview of *Avibacterium gallinarum* and *Avibacterium volantium***

Apart from *Avibacterium paragallinarum*, the genus *Avibacterium* also contains the species *Avibacterium gallinarum*, once known as *Pasteurella gallinarum* along with 3 other species *Avibacterium avium*, *Avibacterium endocarditidis* and *Avibacterium volantium* and thus no specific syndrome name has been allocated to the disease conditions associated with *Avibacterium. gallinarum* and *Avibacterium endocarditidis*, whereas *Avibacterium volantium* is known to be part of avian respiratory microbiota (Blackall and Soriano- Vargas, 2020; Bisgaard *et al.*, 2007; Blackall, 1999).

#### **2. 9.2.2. Clinical signs and pathological lesions of *Avibacterium gallinarum***

The common signs of the disease caused by *Avibacterium gallinarum* are of acute respiratory disease, coughing, sneezing, peri-orbital swelling, swollen wattles and keratoconjunctivitis (Blackall and Soriano-Vargas, 2020). There is no information on symptoms of the disease caused by *Avibacterium endocarditidis* as it is thought to be an opportunistic pathogen (Blackall and Soriano-Vargas, 2020).

The lesions related to *Avibacterium gallinarum* infections include air sacculitis, conjunctivitis, pericarditis, per hepatitis and sinusitis; however, the only pathological condition that has been associated with *Avibacterium endocarditidis* is valvular endocarditis (Blackall and Soriano-Vargas, 2020).

#### **2.9.2.4. Diagnosis**

The isolation of *Avibacterium gallinarum* and *Avibacterium endocarditidis* is best performed using sheep blood agar plates incubated at 37°C with the plates for *Avibacterium gallinarum* being under a 5–10% carbon dioxide atmosphere (Blackall *et al.*, 2005). *Avibacterium gallinarum* has similar characteristics like those of *Avibacterium paragallinarum* except that its catalase positive (Blackall *et al.*, 2005). It is also a Gram negative non-motile which appears as short rods or coccobacilli 1–3µm in length and 0.4–0.8µm in width, with a tendency to form filaments with extended growth period (Blackall and Soriano- Vargas, 2020). *Avibacterium gallinarum* and *Avibacterium endocarditidis* do not require NAD to grow, and thus they grow on a range of basic media such as blood agar. The colonies of *Avibacterium endocarditidis* on blood agar are non-hemolytic, circular, smooth and entire with a greyish tinge and may reach 1.5mm after 24 hours incubation (Bisgaard *et al.*, 2007).

#### **2.9.2.5. Treatment and control**

Preventive vaccination of poultry and strict biosecurity measures limit the usage of antibiotics (Soriano-Vargas, 2021). Nevertheless, drugs such as florafenicol, trimethoprim-sulfamethoxazole, tetracycline, ampicillin, kanamycin, colistin and enrofloxacin may be used to reduce the mortality in case of disease incidence (Huberman and Terzolo, 2016).

The treatments usually give variable results, depending mainly on the drug used and the virulence of the strain involved in the outbreak; thus, antibiotic resistance testing is necessary to influence the choice of the drugs that are likely to treat the disease in a specific area (Agyare *et al.*, 2019). Irrespective of its therapeutic action, the incorrect use

of antibiotics is of worry, due to the residues in meat and eggs and, on the other hand, by an increase in resistance to antibiotics or chemotherapeutic agents and the danger that these resistance genes can be conveyed to humans (Agyare *et al.*, 2019).

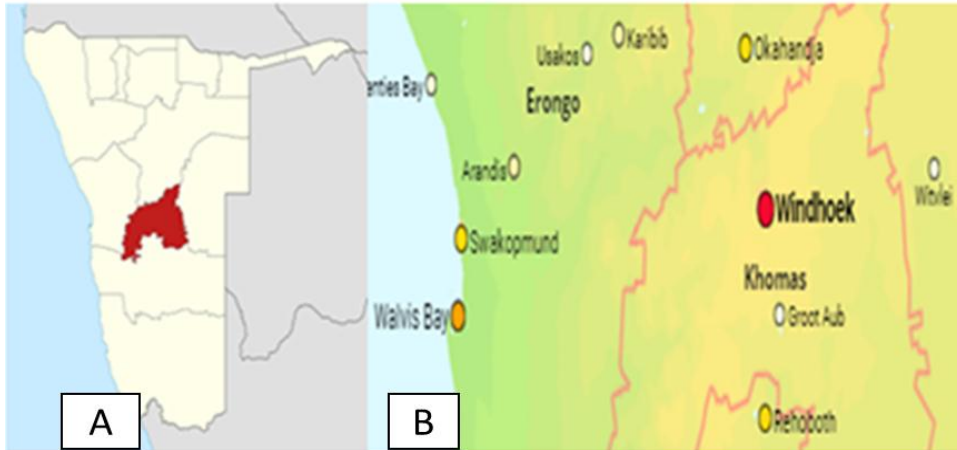
## **Chapter III: Research methods**

### **3.1. Study design**

The study was a cross-sectional experiment to isolate *A. paragallinarum* and copathogenic bacteria from clinically ill chicken in the study area. It was conducted in one poultry farm that reportedly had clinically ill chickens in Groot Aub informal settlement. Sample population was selected using purposive sampling method aiming at sick birds.

### **3.2 Study area**

The study was conducted from the month of June 2020 to October 2020. Swab samples were collected from chickens at Groot Aub informal settlement, transported in nutrient broth, while the laboratory work was carried out at Central Veterinary laboratory, Windhoek, Namibia. Sequencing analysis was carried out at Inqaba Biotechnical industries, Pretoria, South Africa. Groot Aub lies in Khomas region and is located 44 km south of Windhoek. The geographical coordinates of Groot Aub settlement are 22° 56' 0" South, 17° 11' 0" East, with average annual rainfall of about 300 mm.



**Figure 3.1:** A, Namibia map, indicating the Khomas region highlighted in red, where the Groot Aub informal settlement is found. B is showing where Groot Aub lies in Khomas region between Windhoek and Rehoboth.

### 3.3. Study Animals

A total of 20 chickens from 1 farm that exhibited signs of infectious coryza were used in this study. Twenty (20) samples made up of nasal and conjunctival swabs from 20 live chickens were collected for laboratory examination and diagnosis. Chickens were grouped into 2 (two) groups, based on age and disease stage whether acute or chronic stage. Chickens from group A were showing symptoms of acute disease such as gasping, coughing, wheezing, serous eyes and nasal discharges. Chickens classified under group B presented with purulent nasal discharges, blocked nostrils with crusts, swollen faces and unilaterally closed eyes.

### 3.4. Samples collection

The nasal and ocular swabs were collected from 20 chickens. Chickens were identified by breed, sex and age. Samples were identified numerically in chronological order from

number 1 to 20. Each sample tube was labelled properly and information such as sample number, age of the birds, sex of the birds, sample body part (sample type) as well as date of collection was written on the sample collection tubes. Swab samples were collected aseptically, transported and stored in sterile nutrient broth at approximately 4 °C. Samples were transported to the laboratory in ice packs in less than 6 hours after collection.

Samples were collected from suspected infectious coryza cases from Groot Aub informal settlement of Windhoek rural constituency in Khomas Region. Chickens that were clinically sick with symptoms suggestive of infectious coryza were purposefully sampled. The method used for sample collection was adopted from Nabeel Muhammad and Sreedevi (2015). In this study, samples were collected from one (1) small scale poultry farm that keeps all age groups of chickens kept for egg production purposes. The history of vaccination against infectious coryza was incomplete, as some had no updated vaccination status.

The farmer and workers were enlightened about the purpose of the research and a consent letter content was clarified to them before sampling took place. Sampling only commenced after the concerned farmer understood and agreed to participate in the study, in this case offering their chickens to be sampled. Only chickens that exhibited symptoms like lacrimation, catarrhal nasal discharges, gasping and facial swelling suggestive of infectious coryza disease at different stages were included in the study.

### **3.5. Isolation and identification of bacteria**

The isolation procedures used in this study was adopted from Dereja and Hailemichael (2017) with slight modification. For the isolation of the bacterial pathogens, each swab

sample material were inoculated by streaking method on two (2) blood agar media plates and chocolate agar (Appendix 4). One of the blood agar plates and a chocolate agar plate were incubated in an anaerobic jar with anaerobic gas pack at 37 °C for 24 hours. The other blood agar plates were incubated aerobically at 37 °C for 24 hours. After incubation, plates were read under safety cabinet level II, and the growth was recorded followed by sub culturing to obtain pure colonies. Lonely standing colonies from axenic cultures were inoculated into glycerol liquid and stored at -20 °C until further use.

### **3.6. Secondary biochemical testing**

Catalase and oxidase testing were done to identify colonies suspected to be those of *Avibacterium paragallinarum*. These were expected to be negative for both catalase and oxidase test.

#### **3.6.1. Catalase test**

The slide method was used to test for catalase. Using a Pasteur pipette, a loopful of pure colony was added to the slide and a drop of 3% hydrogen peroxide added and blended fast to the colony material on the slide to observe for bubbles formation. Bubbles formation indicates catalase positive, and when no bubbles formed it indicate catalase negative.

#### **3.6.2. Oxidase test**

An oxidase paper was used to test for oxidase. The organism was picked using a sterile loop and a small amount was transferred to the oxidase test paper. The paper was observed for up to 3 minutes to observe for color change from white to dark blue. When

color changes to dark blue within 10 seconds signifies oxidase positive, whereas when there is no change in color within 2 minutes it signifies oxidase negative.

### **3.6.3. Gram staining**

The presumptive colonies of *Avibacterium paragallinarum* and isolated copathogenic bacteria were subjected to gram stain, to observe and appreciate their cell morphology and arrangements. The loopful colonies were blended with a drop of saline on a glass slide, allowed to air dry and then slides were heat fixed. The slides were then flooded with the primary stain (crystal violet), followed by the addition of a mordant (Gram's iodine), rapidly decolorized with acetone and lastly flooded with counterstain (safranin) (Smith and Hussey, 2005). Slides were air dried and viewed under light microscope for cell morphology examination. The *Avibacterium paragallinarum* bacterium is gram negative with short rods arranged in singles or pairs (Figure 2.4).

## **3.7. Bacterial DNA extraction, PCR and gel electrophoresis**

### **3.7.1. Bacterial DNA extraction**

Bacterial DNA was extracted following the manufacturer's protocol with little modifications of the ZYMO Quick DNA Fungal and Bacterial Miniprep extraction Kit from Inqaba Biotech laboratory, South Africa. The modifications on the protocol was done on step 2 where the tissue lyzer was used instead of bead beater. The extracted DNA was subjected to polymerase chain reaction (PCR) analysis, or otherwise stored at -20 °C until further use.

Briefly, a loopful of bacterial cultures was transferred onto freshly prepared nutrient broth and incubated at 37 °C for 18 h. Following this, 1 ml of samples in nutrient broth

was aliquoted into 2 ml Eppendorf tubes. After that, samples were centrifuged at 13,000 rpm for 5 minutes, discarding the supernatant. Two hundred milliliters (200 ml) of PBS was then added to the pellet, to which 750  $\mu$ l of bashing beads buffer was also added. Samples were then homogenized with a tissue lyzer at 4 minutes with turning. The rest of the protocol was as described by the manufacturer. To elute the DNA, tubes were centrifuged at 10,000  $\times$ g for 30 seconds and the collected DNA was stored at 4 °C before used for polymerase chain reaction (PCR).

### 3.7.2. PCR Amplification

The extracted DNA was amplified by PCR aiming the 16S rDNA gene of bacteria. Thus 16S rRNA universal bacterial primers 27F 5'AGAGTTTGATCMTGGCTCAG 3' and 1492R 5'CGTTACCTTGTTACGCTT 3' from Inqaba Biotechnical industries, Pretoria, South Africa were used to amplify 1500 bp fragments from isolates. In this study, fifty microliter (50  $\mu$ l) reaction volumes were prepared and used for each sample (Table 3.1). The premixed master mix used was from New England Biolabs Inc.

**Table 3.1:** PCR reaction mixtures for the amplification of the 16S rRNA gene

Reagent	Volume per reaction $\mu$ l
Nuclease free Water	16
2X Master Mix	25
MgCl*	1
1492R (primer)	2
27F (primer)	2
Template DNA	4
Total reaction volume	50

\*MgCl = magnesium chloride

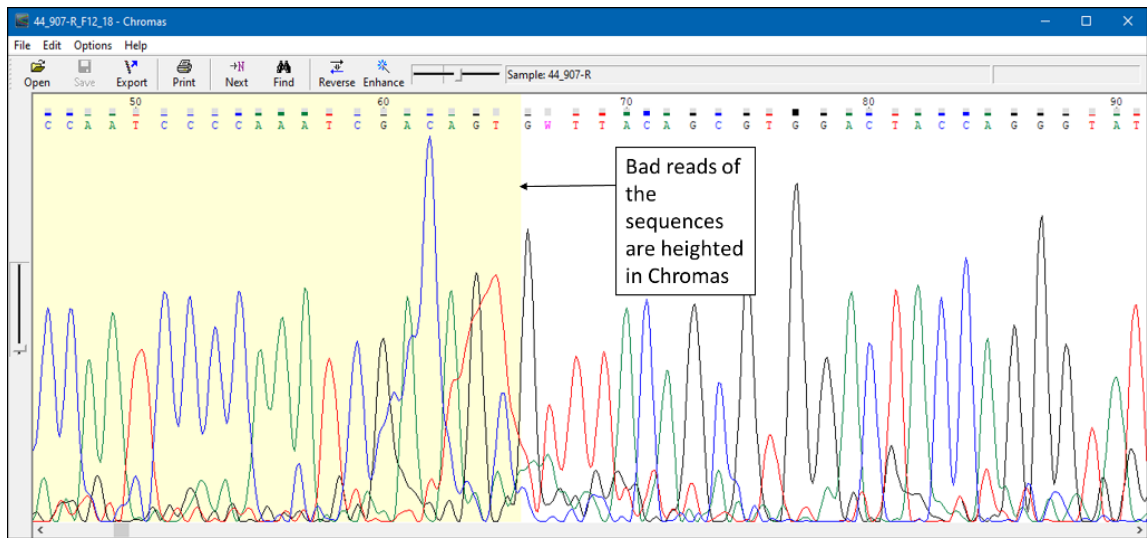
PCR amplification (Nabeel Muhammad and Sreedevi, 2015) was carried out by optimizing initial denaturation at 94 °C for 5 minutes, followed by 30 cycles at 94 °C for

1 minute, annealing at 55 °C for 1 minute, elongation at 72 °C for 1 minute 30 seconds and a final extension at 72 °C for 5 minutes.

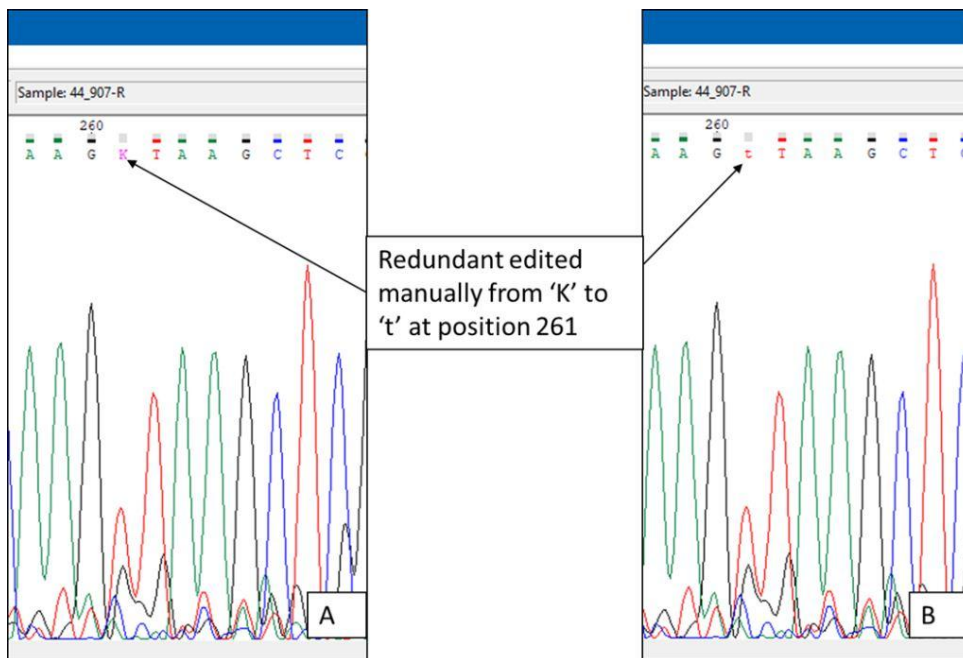
The PCR product was resolved by gel electrophoresis using 1% agarose gel. Five microliter (5 µl) of amplified DNA (PCR products) was mixed with 2 µl of loading dye and the mixture was added to the gel wells. A 1 kb ladder was mixed with loading dye in equal volumes of 2 µl and loaded into the first well labelled molecular marker. The electrophoresis was performed at a voltage of 70 V for 45 minutes. The gel was then transferred to the transilluminator and photographed using a Biometra Bio doc Analyzer and the results were captured and recorded. The used (and photographed) gels were disposed into the hazardous waste bag.

### **3.8. DNA sequencing and sequence editing**

The PCR products were sent to Inqaba Biotechnology (Pretoria, South Africa) for sequencing analysis. Sequences were edited using Chromas software followed by a blast search on the nucleotide database NCBI to identify the closest matches. Analyzed sequences were read and edited using Chromas software (Mbangu, 2020).



**Figure 3.2:** Chromatogram of sequence results. Light yellow part indicates poor reads and white indicate good quality results.



**Figure 3.3:** Chromatogram editing at position 261 where the arrows are pointing. In this case letter K is pink thus it is a redundant and the red peaks under K denote it to be T and written in lower case letters to differentiate between the edited bases from unedited bases

### 3.9. Antibigram analysis

The inoculum for antibiogram analysis was prepared using 18 h old nutrient broth cultures. The microorganism's suspensions were subjected to centrifugation to pellet the microorganisms followed by discarding of the nutrient broth. Sterile normal saline was used to re-suspend the cell pellets whose turbidities were then adjusted to 0.5 McFarland standards using sterile normal saline. Sterile cotton swabs were then used to swab the inoculum onto the surface of Muller Hinton agar as previously described by Bagul and Sivakumar (2016). Selected antimicrobial discs were then evenly distributed onto the surface of the inoculated agar plate using an antibiotic disc dispenser and pressed down with a sterilized forceps to ensure complete contact with the agar surface. Five (5) different antibiotic discs were placed on the 90 mm plates. The plates were covered and left standing in an upright position for about 15 minutes for the antibiotic disc to attach firmly on the agar. Then, the plates were inverted and placed in an incubator set at 37 °C for 24 h. For *Haemophilus* bacteria like *Avibacterium paragallinarum* and *Avibacterium gallinarum*, the plates were put in an anaerobic jar with CO<sub>2</sub> sachets for better growth. After 24 h of incubation, the plates were examined for growth inhibitory zones which were displayed as uniformly circular clear zones around the discs bordered by a lawn growth of bacteria. The diameters of the zones of complete inhibition were measured at the back of the inverted petri plates using a ruler. The sizes of the zones of inhibition were interpreted by referring to the "Zone Diameter interpretative standards and equivalent Minimum Inhibitory Concentration Breakpoints" and Performance Standards for Antimicrobial Susceptibility Testing (Hudzicki, 2009). The organisms were reported as Sensitive (S), Intermediate (I) or Resistant (R) to the tested antibiotics.

### **3.10. Data analysis and taxonomy identification**

All the statistical analysis were carried out on IBM SPSS Statistics software (Version 26). The results of association of the isolated bacteria and clinical signs were analyzed using Chi square. The disc diffusion results of antibiotic sensitivity of isolated bacteria were analyzed with Shapiro –Wilk test to test data for normality, at the significance level of 95% ( $p > 0.05$ ). Kruskal-Wallis non-parametric test was used to test if there were any significant differences in the disc diffusion measurements for different antibiotics and the isolated bacteria.

The query sequences were edited with Chromas lite 20L, and were then copied and pasted to National Center for Biotechnology Information website for identification of the isolates using the basic local alignments search tool (BLASTn). The Similarity percentage was used to search for reference sequences that have the closest match to the query sequence. Thus, the reference sequences that had identical percentage greater than 97% were chosen to be of a bacteria similar to the query sequences (Garibyan and Avashia, 2013).

### **3.11. Research Ethics**

The ethical clearance certificates were obtained from the University of Namibia Research and Ethics Committee (UREC) and the Center for Postgraduate Studies (CPGS), refer to appendix 2. A sample collection authorization letter was obtained from the Directorate of Veterinary Services; in the Ministry of Agriculture, Water and Land Reform, refer to appendix 1. The consent form for this study is attached in the appendix 3. Samples were collected as per standard collection procedures without any drugs used to restrain or anaesthetise chickens.

## Chapter IV: Results

The present study was undertaken to isolate and characterize *Avibacterium paragallinarum* and concurrent bacterial pathogens in chickens that were exhibiting clinical signs suspicious for infectious coryza disease as well as to determine their antibiotic profiles.

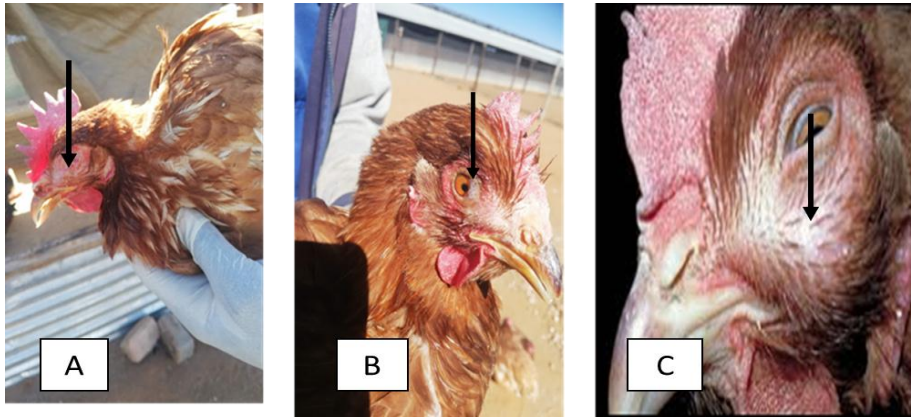
### 4.1. Clinical examination

The figure 4.1 below shows sick chickens exhibiting symptoms suggestive of infectious coryza, while table 4.1 indicates the number of chickens that exhibited clinical symptoms in different stage of clinical presentation. In acute cases, the clinical signs were serous naso-ocular discharge with facial swelling reportedly noticed just 2 days prior to investigation and sampling date. On the other hand, chickens with chronic infection were so lethargic with catarrhal discharges omit foul smell, swollen eyes and some with corneal opacification and blindness resulting from calcification of the cornea. It is worth noting that all target chickens had unilateral facial swelling serve one chicken that had bilateral eyes swelling which was a chronic case according to the caretaker.

**Table 4.1:** Infection based on the age of chicken and disease stage

Age of chicken	Acute cases (watery eyes, lacrimation, gasping) group A	Chronic cases (facial swelling, purulent nasal discharges) Group B
<b>Young</b>	1	0
<b>adult</b>	6	13

Young= 1 week to 2 months old    Adult= 3 months to 9 months old.



**Figure 4.1:** Chickens with signs of infectious coryza, (a) sticky eye, (b) watery eye and (c) facial swelling.

#### 4.2. Bacterial isolation

Chicken were identified with number from 1 to 20, with colonies numbered as 1a, 1b, 1c, 2a, 2b and so forth depending on the number of colonies isolated from the swab sample from each chicken (Table 4.2).

**Table 4.2.** Phenotypic characteristics of colonies isolated from each chicken.

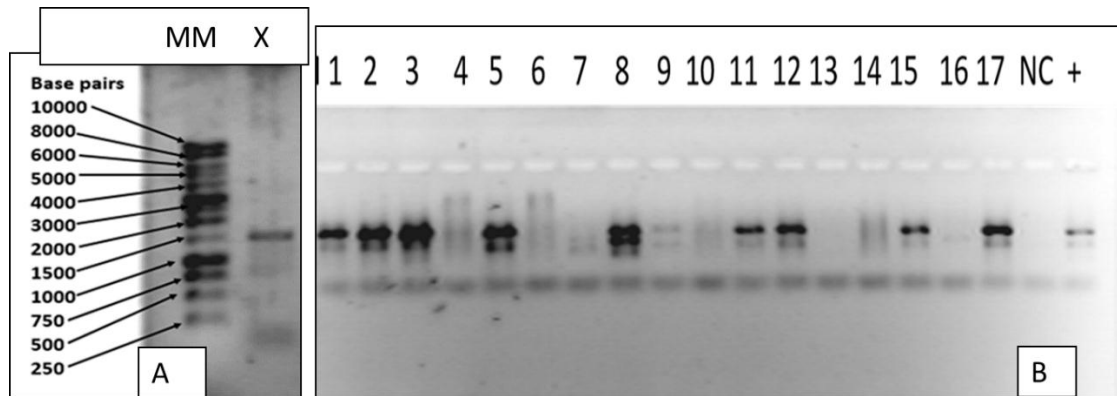
Chicken number	Symptoms	Isolates numbers	Isolate description
1	Watery eyes (lacrimation)	1a 1b	Mucoid ,shiny, raised and medium size White smooth big colonies
2	Gasping Watery	2a 2b 2c	Rough dry grey colonies Mucoid ,shiny, raised and medium size White smooth big colonies
3	Watery eyes	3a 3b	White smooth big colonies Grey mucoid medium size colonies
4	Purulent unilateral nasal discharges	4a 4b	Grey alpha hemolytic tiny colonies White smooth big colonies
5	Facial swelling (swollen eye) Purulent nasal	5a 5b 5c	Mucoid ,shiny, raised and medium size Grey mucoid small colonies White mucoid colonies

Chicken number	Symptoms	Isolates numbers	Isolate description
	discharges		
6	Facial swelling	6a 6b	Clear mucoid colonies Grey tiny alpha tiny colonies
7	Watery eyes	7a 7b 7c	Mucoid ,shiny, raised and medium size Cream medium colonies Grey tiny alpha colonies
8	Watery eyes	8a	Clear small colonies
9	Swollen eye, purulent nasal crusts.	9a 9b	White large colonies Grey mucoid colonies
10	Bilateral facial swelling, congested nostrils	10a 10b	Grey alpha tiny colonies White mucoid colonies
11	Bilateral swollen eyes	11a 11b	White colonies Grey mucoid colonies
12	Facial purulent discharge swelling, nasal	12a	White smooth big colonies
13	Naso-ocular discharges purulent	13a	Mucoid ,shiny, raised and medium size
14	Naso-ocular discharge serous	14a 14b	White colonies Grey alpha hemolytic
15	Naso-ocular discharges serous	15a	Mucoid ,shiny, raised and medium size
16	Naso-ocular discharges Sticky eyes purulent	16a	Clear tiny colonies
17	Naso-ocular discharges purulent	17a	Mucoid ,shiny, raised and medium size
18	Bilateral facial swelling	18a 18b	White big colonies Grey small mucoid colonies
19	Bilateral naso-ocular purulent discharge	19a	Mucoid ,shiny, raised and medium size White smooth big colonies

Chicken number	Symptoms	Isolates numbers	Isolate description
		19b	
20	Bilateral nasal purulent discharges	20a 20b	Grey mucoid medium colonies Grey alpha hemolytic tiny colonies

### 4.3. PCR results, identification and taxonomy of isolates

The expected band size was 1.5 kbp, the expected band size of amplifying 16S rDNA. At least 2 (two) extracted DNA from isolates from each chicken sample were amplified and sent for sequence analysis (Figure 4.2). Thus sample 1a, 1b, 1c were from chicken Number 1, while sample 2a and 2b were from chicken Number 2. It should be noted that more than 1 isolate was isolated from 1 swab from 1 chicken and thus one sample ID number was not represented by one isolate as more isolates were expected from each swab sample.



A) MM= Molecular Ladder (1kb), X=represent how samples were compared to MM. B) Lane 1=1a, lane 2= 1b, Lane 3 =1c3, Lane 5=2a, Lane 6=2b, Lane 7=3a, Lane 8=3b, Lane9= 4a, Lane 11=5a, Lane 12=5b, Lane 14=6a lane 15=7a, Lane 17=8a, Lane NC=Negative Control and Lane +=positive control. Lanes 4, 10 and 13 had no DNA

**Figure 4.2:** Labels of PCR electrophoresed gel documented using Biometra BioDoc Analyzer.

Taxonomy identification revealed that 40% of the chickens were positive for *Avibacterium paragallinarum* and co-pathogenic bacteria were isolated in 80% of chickens including the 20% of chickens that was con-currently isolated with *Avibacterium paragallinarum* (Table 4.3).

**Table 4.3:** Isolates taxonomy using 16s rRNA gene sequences from GenBank database

ID number	Bacteria	Percentage Identity (%)	GenBank accession number
1a	<i>Avibacterium paragallinarum</i>	97.93	GU737687.1
1b	<i>Staphylococcus chromogenes</i>	97.59	NR_036901.1
2a	<i>Escherichia coli</i>	100	<u>CP057194.1</u>
2b	<i>Staphylococcus chromogenes</i>	96.93	JN426805.1
2c	<i>Avibacterium paragallinarum</i>	97.70	MT355918.1
3a	<i>Staphylococcus chromogenes</i>	99.88	CP046028.1
3b	<i>Pasteurella multocida</i>	99.77	CP048792.1
4a	<i>Streptococcus pluranimalium</i>	99.89	CP025536.1
4b	<i>Staphylococcus chromogenes</i>	99.77	CP046028.1

ID number	Bacteria	Percentage Identity (%)	GenBank accession number
5a	<i>Avibacterium paragallinarum</i>	98.14	GU737687.1
5b	<i>Escherichia coli</i>	99.77	CP054345.1
5c	<i>Streptococcus pluranimalium</i>	98.86	MH329623.1
6a	<i>Escherichia coli</i>	99.31	CP051711.1
6b	<i>Streptococcus pluranimalium</i>	99.21	KT943470.1
7a	<i>Escherichia coli</i>	99.88	CP057194.1
7b	<i>Avibacterium paragallinarum</i>	97.78	GU737687.1
7c	<i>Streptococcus pluranimalium</i>	97.62	MH329623.1
8a	<i>Pasteurella multocida</i>	100	CP048792.1
9a	<i>Staphylococcus chromogenes</i>	99.54	CP031470.1
9b	<i>Escherichia coli</i>	99.31	CP051714.1
10a	<i>Streptococcus pluranimalium</i>	97.62	CP022601.1
10b	<i>Staphylococcus chromogenes</i>	99.77	CP031470.1
11a	<i>Staphylococcus chromogenes</i>	99.77	CP046028.1
11b	<i>Escherichia coli</i>	99.88	CP057194.1
12a	<i>Staphylococcus chromogenes</i>	99.54	CP031470.1
13a	<i>Avibacterium paragallinarum</i>	98.25	GU737687.1
14a	<i>Staphylococcus chromogenes</i>	99.77	CP046028.1
15a	<i>Avibacterium paragallinarum</i>	97.23	GU737687.1
16a	<i>Streptococcus pluranimalium</i>	99.88	CP054015.1
17a	<i>Avibacterium paragallinarum</i>	98.38	GU737687.1
18a	<i>Escherichia coli</i>	92.46	KX650759.1
19a	<i>Avibacterium paragallinarum</i>	98.01	MK886552.1
19b	<i>Staphylococcus chromogenes</i>	97.84	KJ783378.1
20a	<i>Pasteurella multocida</i>	100	CP048792.1
20b	<i>Avibacterium paragallinarum</i>	99.66	CP022601.1

All the isolates' sequences were able to be identified using the 16S rRNA, because they yielded the identical percentage of greater than 97%, however there were only 2 sequences that had identical percentage below 97%, and they were identified based on the first hit in this case.

**Table 4.4:** Chickens that had *Avibacterium paragallinarum* isolated concurrently with co-pathogenic bacteria.

Chicken ID	Stage of infection	Clinical signs	Bacterial pathogens isolated
1	Acute	Watery eyes	<i>Staphylococcus chromogenes</i> <i>Avibacterium paragallinarum</i>
2	Acute	Gasping	<i>Escherichia coli</i> , <i>Staphylococcus chromogenes</i> <i>Avibacterium paragallinarum</i>
5	Chronic	Swollen eye, purulent nasal discharges	<i>Escherichia coli</i> <i>Streptococcus pluranimalium</i> <i>Avibacterium paragallinarum</i>
19	Chronic	Nasocular discharges bilateral	<i>Staphylococcus chromogenes</i> <i>Avibacterium paragallinarum</i>

The co-infection with other respiratory pathogens was a common finding, in that about 62.5% of the chickens with infectious coryza were concurrently infected with *Escherichia coli*, *Pasteurella multocida* or *Staphylococcus chromogenes*. The isolation of these bacteria were not limited to a certain stage of infection as most of them were isolated in both infection stages (Table 4.5).

**Table 4.5:** Results of chickens that tested negative for *Avibacterium paragallinarum* and tested positive for co-pathogenic bacteria.

Chicken Id	Stage of infection	Clinical signs	Bacterial pathogen isolated
3	Acute	Watery eyes	<i>Staphylococcus chromogenes</i> , <i>Pasteurella multocida</i>
4	Chronic	Purulent nasal discharges	<i>Streptococcus pluranimalium</i> <i>Staphylococcus chromogenes</i> strain
6	Chronic	Facial swelling with caseous mass	<i>Escherichia coli</i> , <i>Streptococcus pluranimalium</i>
8	Acute	Naso-ocular discharges serous	<i>Pasteurella multocida</i>
9	Acute	Facial swelling, purulent nasal crusts	<i>Escherichia coli</i> , <i>Staphylococcus chromogenes</i>

<b>Chicken Id</b>	<b>Stage of infection</b>	<b>Clinical signs</b>	<b>Bacterial pathogen isolated</b>
10	Acute	Bilateral facial swelling, congested nostrils	<i>Streptococcus pluranimalium</i> , <i>Staphylococcus chromogenes</i>
11	Acute	Facial swelling, serous nasal discharge	<i>Escherichia coli</i> , <i>Streptococcus pluranimalium</i>
12	Chronic	Facial swelling, yellowish nasal discharges	<i>Staphylococcus chromogenes</i> , <i>Escherichia coli</i>
14	Acute	Serous nasal discharges	<i>Staphylococcus chromogenes</i>
16	Chronic	Naso-ocular purulent discharges	<i>Streptococcus pluranimalium</i> , <i>Staphylococcus chromogenes</i> ,
18	Chronic	Bilateral facial swelling	<i>Escherichia coli</i> , <i>Pasteurella multocida</i>
20	Chronic	Naso-ocular purulent discharges	<i>Pasteurella multocida</i> , <i>Streptococcus pluranimalium</i>

The co-pathogenic bacteria were isolated in both chickens with acute and chronic stage of infection. *Staphylococcus chromogenes* and *Pasteurella multocida* were mostly isolated in chickens with acute infection, whereas *Escherichia coli* and *Streptococcus pluranimalium* were mostly isolated from chickens with chronic infection. In acute infection stage, there were few multiple pathogens isolated as compared to the number of bacterial pathogens isolated in chronic infection stage. The frequency of the isolated bacteria and percentage is presented in table 4.6.

**Table 4.6:** Frequency of the isolated bacteria.

<b>Isolated Bacteria</b>	<b>Bacteria frequency</b>	<b>Percentage%</b>
<i>Avibacterium paragallinarum</i>	8	22.8
<i>Escherichia coli</i>	7	20
<i>Pasteurella multocida</i>	3	8.6
<i>Staphylococcus chromogenes</i>	10	28.6
<i>Streptococcus pluranimalium</i>	7	20
Total	35	100

The most isolated pathogenic bacteria were *Staphylococcus chromogenes* with 28.6%, followed by *Avibacterium paragallinarum* 22.8%, *Escherichia coli* 20%, *Streptococcus pluranimalium* with 20%, while the least isolated bacteria was *Pasteurella multocida* with 8.6%. *Pasteurella multocida* was only isolated in 3 chickens were *Avibacterium paragallinarum* was not isolated.

#### **4.4. Antibigram testing**

Identified bacteria were subjected to antimicrobial sensitivity testing used disk diffusion method to test for antibiotic sensitivity. The results of antimicrobial sensitivity testing for the different bacterial isolates are presented in table 4.7 and appendix 5.

**Table 4.7:** Results of antimicrobial sensitivity test

<b>The inhibition zone average and standard errors of the Isolated bacteria</b>												
	<i>S. chromogenes</i>			<i>P. multocida</i>			<i>S. pluranimalium</i>			<i>A. paragallinarum</i>		<i>E. coli</i>
<b>Antibiotics</b>												
Ciprofloxacin	29.33 ± 0.66	S	20.00 ± 0.00	±	S	20.00 ± 0.00	±	S	13.67 ± 0.33	R	24.67 ± 0.33	S
Gentamycin	26.00 ± 0.58	S	24.00 ± 0.58	±	S	16.00 ± 1.00	±	I	0 ± 0.00	R	18.00 ± 0.58	I
Tetracycline	0 ± 0.00	R	0 ± 0.00		R	12.00 ± 0.58		R	21.67 ± 0.33m	S	0 ± 0.00	R
Ampicillin	36.33 ± 0.67	S	25.67 ± 0.41	±	S	20.67 ± 1.33	±	S	23.67 ± 1.34	S	21.00 ± 0.58	S
SXT*	13.67 ± 0.43	R	19.00 ± 0.58	±	I	19.67 ± 0.33		I	0 ± 0.00	R	26.33 ± 0.33	S

R = Resistance I = Intermediate S = Sensitive

\*SXT= Sulfamethoxazole Trimethoprim

All the bacteria isolates were 100% sensitive to ampicillin, as well as to ciprofloxacin, except for *Avibacterium paragallinarum* which was resistant toward ciprofloxacin. *Staphylococcus chromogenes* and *Pasteurella multocida* showed sensitive to gentamycin, whereas *Streptococcus pluranimalium* and *Escherichia coli* had intermediate sensitivity. The *Avibacterium paragallinarum* was 100% resistant to gentamycin and SXT. On the other hand *Avibacterium paragallinarum* was sensitive to tetracycline, whereas the concurrent bacteria were resistant to tetracycline.

#### 4.5. Results of Statistical analysis

The association of the clinical signs and the isolated bacteria was analyzed using Chi square test and a Chi-square value of 0.970 was obtained (Appendix 6). The p-value was greater than 0.05 at 95% confidence level, conclude that the symptoms and isolated bacteria are independent of each other, hence there is no statistical relationship between the isolated bacteria and the symptoms displayed by the chickens. The symptoms that

were displayed in chicken that had *Avibacterium paragallinarum* isolated, were also found in chickens where co-pathogenic bacteria were isolated.

The normal distribution on antimicrobial disc diffusion was first tested with Shapiro Wilk test for which presented the significant value of 0.000 that is less than 0.05 at 95% confidence level. This significant value means the data deviates from the normal distributions. Therefore, the nonparametric Kruskal Wallis test was carried out and the significant value of 0.187 was obtained that signifies the result to be not normally distributed at  $p=0.05$  indicating no differences in inhibition zone measurements (Appendix 7). This articulates that some antibiotics were effective towards certain bacteria whereas some bacteria were resistant against some antibiotics.

## Chapter V: Discussion

Infectious coryza is a disease of economic importance, negatively affecting the poultry industry. In this study, 20 chickens of different ages that showed typical clinical signs of infectious coryza were sampled. For the isolation and molecular characterization of *Avibacterium paragallinarum* and co-pathogenic bacteria, nasal and eyes swab samples from 20 chickens were cultured and isolates were identified by PCR using 16SrRNA universal primers.

The isolated *Avibacterium paragallinarum* that were identified by DNA techniques, grew both on chocolate and blood agar without the NAD supplement, suggesting the isolated bacteria to be NAD independent as suggested by Blackall (1999). To observe and appreciate pure colonies of *Avibacterium paragallinarum*, special media and complex media with costly ingredients such as NAD and chicken serum must be used (Nabeel Muhammad and Sreedevi, 2015; Dwivedi *et al.*, 2018). Therefore, the challenges and intensive work involved in the diagnosis of infectious coryza by conventional culture method and biochemical characterization made the molecular technique like PCR attractive and worthwhile.

However, the research confirms the presence of *Avibacterium paragallinarum* and isolate some of the co-pathogenic bacteria from chicken with respiratory infection in Namibia. There have not been published reports of infectious coryza disease diagnosed in Namibia whether by anamnesis or clinical signs, yet there are different pathogens that produce diseases with similar symptoms. Clinical signs are not confirmatory, neither are pathognomonic diagnostic methods.

Samples collected from chickens which were in the acute stage of infection and were within three days of onset of clinical signs yielded more positivity percentage of *Avibacterium paragallinarum* 83.3% than samples collected from chickens with infection of fewer weeks to months that was 23.1%. Works from previous researchers recommended that prompt and accurate diagnosis of infectious coryza is achieved when fresh samples are collected from the acute stage of the disease (Scholar *et al.*, 2019; Dwivedi *et al.*, 2018). This correlates with the results in this study where there was an 83.3% recovery rate from samples collected from acutely sick chickens against 23.1% recovery rate in chronic cases. Poor recovery may perhaps be as a result of multiple infections caused by fast growing bacteria and other pathogens that could have masked the growth of *Avibacterium paragallinarum*. According to Nabeel Muhammad and Sreedevi (2015) the high recovery of *Avibacterium paragallinarum* bacterium on PCR when swab samples were collected from infra orbital sinuses and less recovery from nasal swabs. In this study, samples were collected from eye conjunctival and this could be attributed to lower recovery of *Avibacterium paragallinarum*.

In the acute group, out of 7 chickens with acute symptoms, *Avibacterium paragallinarum* was isolated in 4 chickens (57.1%), whereas out of 13 chickens with chronic infectious coryza disease, *Avibacterium paragallinarum* was isolated in 4 chickens (30.7%). Co-pathogenic bacteria isolated in these chickens were *Staphylococcus chromogenes*, *Escherichia coli*, *Pasteurella multocida* and *Streptococcus pluranimalium* (Table 4.4). On the other hand, among the 13 chickens with chronic symptoms, *Avibacterium paragallinarum* along with *Streptococcus pluranimalium*, *Staphylococcus chromogenes* and *Escherichia coli*, *Pasteurella*

*multocida* were isolated from 4 chickens. *Pasteurella multocida* was isolated in 3 chickens in which *Avibacterium paragallinarum* was not isolated. Also, transport media was not specific for *Avibacterium paragallinarum*, therefore it could also enhance the growth and survival of other fast growing pathogens. To minimize false negative of both conventional and molecular diagnostic methods, samples must be collected before any antibiotic therapy is initiated, because antibiotics significantly reduce the capacity of both methods (Scholar *et al.*, 2019). In the present study, the student equally made sure that samples were collected from untreated chickens.

The suspected colonies of *Avibacterium paragallinarum* were dew drop tiny transparent, iridescent and non-hemolytic on blood agar. While on chocolate agar, the suspicious colonies of *Avibacterium paragallinarum* bacterium were medium sized, white to grey in color, smooth and round. Microscopically, on Gram stain, *Avibacterium paragallinarum* appeared as red coccobacilli in shape arranged in singular cells or in pairs. Biochemical characterization revealed that *Avibacterium paragallinarum* suspicious colonies were both catalase and oxidase negative. The colony morphology of suspected co-pathogenic bacteria were as follows; *Escherichia coli* = grey mucoid, *Pasteurella multocida* = grey medium sized, *Staphylococcus chromogenes* = white big mucoid colony and *Streptococcus pluranimalium* = grey tiny alpha hemolytic colony (Table 6).

The suspected colonies of the isolated copathogenic bacteria (*Pasteurella multocida*, *Staphylococcus chromogenes*, *Escherichia coli* and *Streptococcus pluranimalium*) were not subjected to catalase and oxidase biochemical test, as they are common bacteria that

are easily isolated and identified by both traditional culture method and by nucleic based methods such as PCR.

The diagnosis of infectious coryza can be complicated by the presence of these co-pathogens and opportunistic pathogens (Dwivedi *et al.*, 2018). The co-pathogenic bacteria that were isolated were *Escherichia coli*, *Staphylococcus chromogenes*, *Streptococcus pluranimalium*, *Pasteurella multocida* and *Corynebacterium*. These bacteria cause primary and secondary infections in chickens such as colibacillosis, streptococcus, staphylococcus and fowl cholera infections respectively. These results were in agreement with those reported by Nabeel Muhammad and Sreedevi (2015) who stated that other pathogens like *Staphylococcus* sp., *Streptococcus* sp., *Corynebacterium* sp., and *Pseudomonas* sp. can complicate the diagnosis of infectious coryza. On the other hand, coinfections may lead to immune suppression that exacerbate chickens to be susceptible to many infections which eventually results into poor weight gain, low production and increased mortality (Clothier *et al.*, 2019).

The observed lack of correlation between the bacteria and the symptoms suggests that these symptoms are not merely found in infections caused by the isolated bacteria. Other pathogen such as viral and fungi pathogen that are known to cause infections with similar symptoms were not investigating (Bagust, n.d; Butcher, Jacob, and Mather, 2018). Also, this could be attributed to the fact that samples were taken from body parts like nostril and eyes that are housing many normal flora and opportunistic bacteria that might have masked the growth of *Avibacterium paragallinarum* and other co-pathogenic bacteria as suggested by Dwivedi *et al.* (2018). This may also lead to the satellite growth of *Avibacterium paragallinarum* not to be appreciated and the bacteria could be missed

giving false negative results (Dwivedi *et al.*, 2018). Additionally, some of the normal flora bacteria may become opportunistic pathogens when the primary causative agent has suppressed the immunity system, hence chickens may become vulnerable to many different infections caused by various pathogens (Wu *et al.*, 2021). The results could be different and reveal the association between the bacteria and the stage of infection if the direct swab samples were analyzed by PCR instead of obtaining colonies from the traditional culturing method that might resulted in some of the bacteria not isolated due to different growth requirements (Dwivedi *et al.*, 2018).

From this study's findings, high positivity percentage of *Avibacterium paragallinarum* and *Pasteurella multocida* were recorded when sample were collected in acute stage, whereas the highest positivity percentage of other isolates like *Streptococcus pluranimalium* and *Escherichia coli* were high in the chronic stage and this suggests the isolates as opportunistic pathogens. The isolation percentage could be improved by using specific transport and culture media (Clothier *et al.*, 2019). This high positivity percentage of *Avibacterium paragallinarum* was achieved with molecular identification as compared to cultural isolation (Phenotypic) and identification method.

The *Avibacterium paragallinarum* isolates were sensitive to ampicillin and tetracycline, this is in agreement with findings of Rajurkar, Roy and Yadav (2009) and those of Mohammad and Sreedevi (2019). In the present study, *Escherichia coli*, *Pasteurella multocida*, *Staphylococcus chromogenes* and *Streptococcus pluranimalium* were found to be resistant to tetracycline. This is correlating to the results of studies by (Kapena *et al.* (2020) and those of Nhung, Chansiripornchai and Carrique-Mas (2017). Furthermore,

the antibiogram patterns displayed by *Escherichia coli* and *Streptococcus pluranimalium* in this study correlate with the observations of (Awad, Ahmed and Abd (2009).

What is worrisome is the 70-100% resistance of *Staphylococcus chromogenes*, *Pasteurella multocida*, *Escherichia coli* and *Streptococcus pluranimalium* to tetracycline and the resistance of *Avibacterium paragallinarum* to SXT. *Avibacterium paragallinarum* and the associated co-pathogenic bacteria were 100% sensitive to ampicillin and ciprofloxacin, 70% sensitive to tetracycline, whereas they had 100% resistance against sulfamethoxazole-trimethoprim. This is supported by study findings carried out by Awad, Ahmed and Abd (2009). On the other hand, co-pathogenic bacteria were resistant to tetracycline. These results were in agreement with the results reported by Wahyuni et al. (2018). The challenge findings of *Escherichia coli* and *Avibacterium paragallinarum* show resistance towards tetracycline, hence control in dispensing and usage of these drugs is necessary as farmers will continue to access and use these antibiotics without diagnosis and laboratory confirmation.

Identification of bacteria on the basis of phenotypic characteristic is regarded less accurate and tedious, as compared to identification performed with molecular methods. However there are also shortcomings when identifying bacteria with 16S rRNA gene. It is believed that the identification of two recently diverged bacteria that share almost the same 16S rDNA sequence would make it difficult to distinguish them by 16S rRNA sequences. Horner and Pesole (2004) are in agreement, that the limitation in using the 16S rRNA sequence is the high number of unvalidated and inaccurate sequences in the database Sharma, Polkade and Shouche, (2015). Nevertheless, the 16S rRNA gene sequences has been used for the study and identification of bacteria taxonomy because it is found in almost every bacteria, there has not been a change in the function of the 16S

rRNA as well as their 1500bp size is enough for informatics purposes (Janda and Abbott, 2007).

Therefore this study applied the taxonomic identification of bacteria based on the 16S rRNA gene criteria which implies that the same bacteria will share the percentage identity of more than 97%. Taxonomy may also be linked to evolutionary studies of organisms and in such occasions molecular chronometer such as 16S rRNA gene can be used in the identification of bacteria (Horner and Pesole, 2004).

## Chapter VI: Conclusion

Overall, this study has been able to isolate and identify the *Avibacterium paragallinarum* and some of the co-pathogenic bacteria associated with respiratory infections in chickens in Namibia. Since the isolation and identification of *Avibacterium paragallinarum* by conventional culture method is laborious, time consuming and requires special techniques, molecular diagnostic method is of utmost importance in diagnosis of infectious coryza. In this study, the isolation by culture and identification by molecular method were used for rapid detection of the bacteria. This study was able to isolate and identify *A. paragallinarum* and co-pathogenic bacteria such as *Escherichia coli*, *Pasteurella multocida*, *Staphylococcus chromogenes*, and *Streptococcus pluranimalium*. In this present study, 20 chickens with typical clinical symptoms of infectious coryza were tested, and *Avibacterium paragallinarum* was isolated from 40% of chickens, whereas co-pathogenic bacteria were isolated in 80.0% of the chickens. Furthermore, clinical symptoms at significance value ( $p > 0.05$ ) indicates that there was no association between the clinical symptoms and the isolated bacteria. This shows that the symptoms found in chickens were neither caused by *Avibacterium paragallinarum* nor by the co-pathogenic bacteria; therefore, it calls for further investigation on infectious coryza cases with emphasis on the possible pathogens responsible for such symptoms. The *Avibacterium paragallinarum* isolates in this study were nicotinamide adenine dinucleotide independent, because they grew on blood agar medium without feeder bacteria.

The study findings also showed that *Avibacterium paragallinarum* has more chances to be isolated and detected when samples are collected in acute stages of infection where

symptoms like watery eyes, gasping and serous nasal discharges are exhibited. Additionally, there were fewer co-pathogenic bacteria identified during the acute stage of infection in comparison to those identified in chickens with chronic infection. Bacteria like *Escherichia coli* and *Pasteurella multocida* were also isolated in acute stage and this implies that there is always simultaneous infection colonizing the respiratory tract of chicken. This suggests that the severity and prolonged duration of the disease were seen in chickens with opportunistic and co-pathogenic bacteria such as *Streptococcus pluranimalium*, *Escherichia coli* and *Pasteurella multocida*.

The determination of the isolates that are possible causes of the infection in the region is vital for the selection of vaccine to be used in the prevention of infectious coryza as well as for the choice of drugs to be used in the treatments and symptoms alleviation. Antibiotic sensitivity testing *in vitro* should be done prior to the treatment to establish the efficacy of the drugs to be used. *Avibacterium paragallinarum* and isolated co-pathogenic bacteria such as *Escherichia coli*, *Pasteurella multocida* and *Staphylococcus chromogenes* were found to be highly resistant to over the count drugs such as tetracycline and sulfamethoxazole-trimethoprim. For better management and control of infectious coryza, there is a continual need for extensive investigation, isolation, rapid molecular characterizations of *Avibacterium paragallinarum* and co-pathogenic bacteria so that prompt strategic plans are implemented in order to combat the disease and prevent future outbreaks. PCR proved to be an accurate and rapid diagnostic tool for confirmatory diagnosis of *Avibacterium paragallinarum* and co-pathogenic bacteria and it was found to be highly sensitive for the screening of field samples as compared to conventional culture method.

## **Chapter VII: Recommendations**

Further investigation on infectious coryza disease and its causative agent is necessary in Namibia. Many studies are needed to investigate the bacterial pathogens that simultaneously infect chicken so that proper control measures are put in place. Additionally, future studies in connection with this research need to work on screening direct field samples by molecular techniques to compare the detection percentage between screening done on isolates and those done on direct field samples. Another helpful study on infectious coryza could be aiming at prevalence of the disease in the country that focus on the seasonal distribution patterns as well as identifying other microorganism that causes other co-infection, rather focused only on bacterial pathogens. Furthermore, serotyping of *Avibacterium paragallinarum* serovars isolated from suspected clinical samples is necessary for proper recommendation on vaccination.

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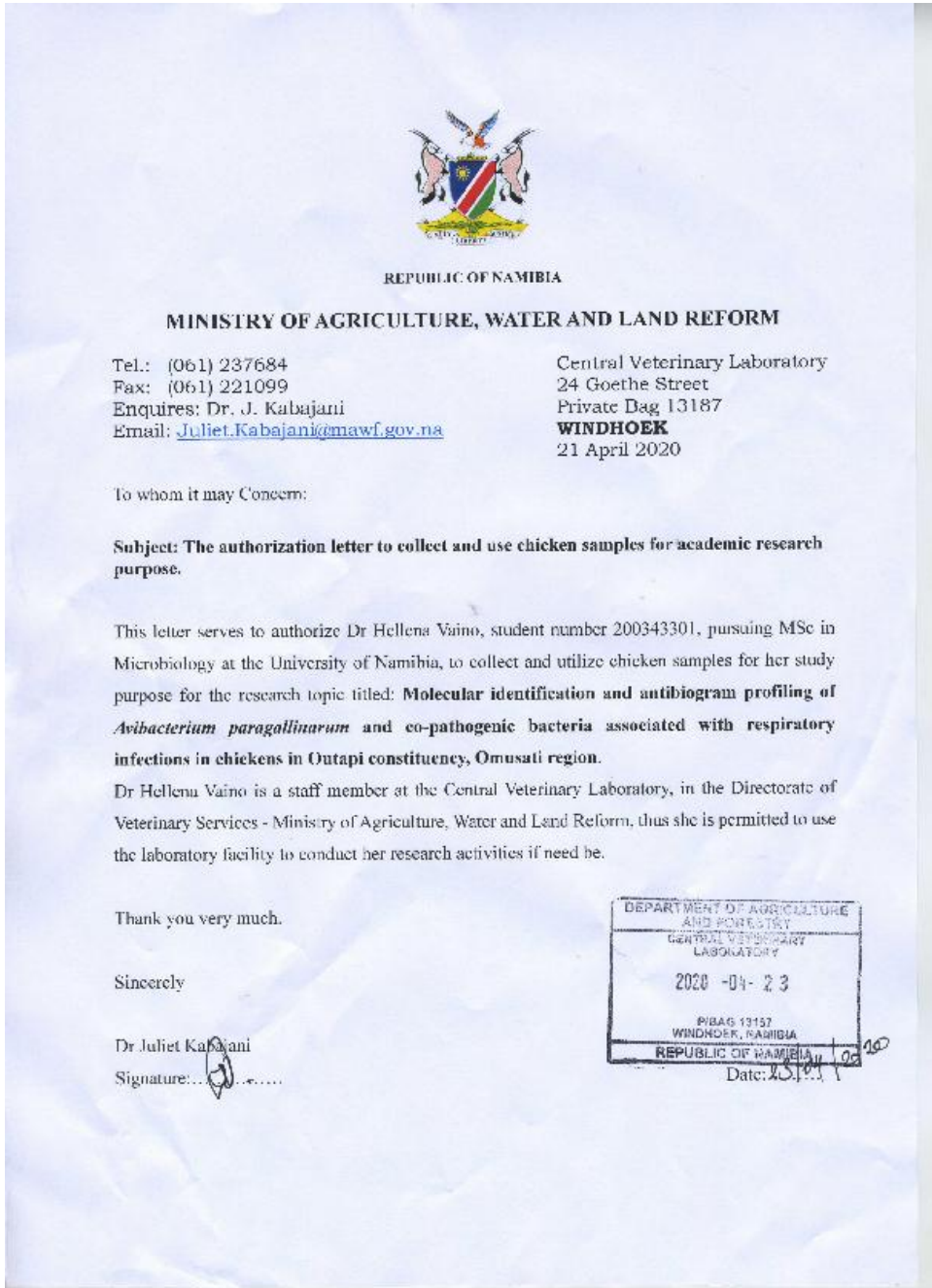
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## Appendices

### Appendix 1: Ministry of Agriculture, Water and Land Reform permission letter



**Appendix 2:** Ethical Clearance Certificate issued by the University of Namibia Research Ethics Committee (UREC).



**ETHICAL CLEARANCE CERTIFICATE**

**Ethical Clearance Reference Number:** AREC/019/2020      **Date:** 05/05/2020

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

**Title of Project:** Molecular identification and antibiogram profiling of *Avibacterium paragallinarum* and co-pathogenic bacteria associated with respiratory infections in chicken in Outapi constituency, Omusati region

**Nature/Level of Project:** Master of Science in Microbiology

**Researcher:** Hellena Vaino

**Student Number:** 200343301

**Faculty:** Faculty of Science

**Supervisor:** Dr. T. Sibanda

**Co-Supervisor:** Dr. C. Ntahoshikira

Take note of the following:


- (a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.
- (b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.
- (c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.
- (d) The UREC retains the right to:
  - (i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
  - (ii) Request for an ethical compliance report at any point during the course of the research.

AREC wishes you the best in your research.

*Dr. Yvonne Hemberger*

Dr. med. vet M.Y. Hemberger  
AREC Chairperson

## Appendix 3: Consent form

<p><b>PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM</b></p> <p style="text-align: right;"><b>ANNEX 5</b></p> <div style="text-align: center;"><p><b>UNAM</b> UNIVERSITY OF NAMIBIA</p></div> <p><b>TITLE OF THE RESEARCH PROJECT:</b> MOLECULAR IDENTIFICATION AND ANTI-BIOGRAM PROFILING OF <i>AVIBACTERIUM PARAGALLINARUM</i> AND CO-PATHOGENIC BACTERIA ASSOCIATED WITH RESPIRATORY INFECTIONS IN CHICKENS IN OUTAPI CONSTITUENCY,</p> <p>OMUSATI REGION</p> <p><b>REFERENCE NUMBER:</b></p> <p><b>PRINCIPAL INVESTIGATOR:</b> Hellena Vaino</p> <p><b>ADDRESS:</b> Erf 5928, Schubert Street 09, Windhoek West</p> <p><b>CONTACT NUMBER:</b> 0811259948</p> <p>You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is <b>entirely voluntary</b> and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.</p> <p>This study has been approved by the Research Ethics Committee at The University of Namibia and will be conducted according to the ethical guidelines and principles of the international Declaration of</p> <hr/> <p>UREC Participant Information Leaflet and Consent Form (ICF VERSION) <span style="float: right;">Page 1</span></p>	<p>Helsinki, South African Guidelines for Good Clinical Practice and Namibian National Research Ethics Guidelines.</p> <p><b>1. What is this research study all about?</b></p> <p>a) Where will the study be conducted; are there other sites; total number of participants to be recruited at your site and altogether.</p> <p>b) Explain in participant friendly language what your project aims to do and why you are doing it?</p> <p>c) Explain all procedures.</p> <p>d) Explain any randomization process that may occur.</p> <p>e) Explain the use of any medication, if applicable.</p> <p><b>2. Why have you been invited to participate?</b></p> <p>a) Explain this question clearly.</p> <p><b>3. What will your responsibilities be?</b></p> <p>a) Explain this question clearly.</p> <p>b) Explain the duration the participant is expected to participate in the study (i.e. 2 hours, 4 days, etc.)</p> <p><b>4. Will you benefit from taking part in this research?</b></p> <p>a) Explain all benefits objectively. If there are no personal benefits then indicate who is likely to benefit from this research e.g. future patients.</p> <p><b>5. Are there in risks involved in your taking part in this research?</b></p> <p>a) Identify any risks objectively.</p> <p><b>6. If you do not agree to take part, what alternatives do you have?</b></p> <p>b) Clearly indicate in broad terms what alternative treatment is available and where it can be accessed, if applicable.</p> <p><b>7. Who will have access to your medical records?</b></p> <p>a) Explain that the information collected will be treated as confidential and protected. If it is used in a publication or thesis, the identity of the participant will remain anonymous. Clearly indicate who will have access to the information.</p> <p><b>8. What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?</b></p> <p>a) Clarify issues related to insurance cover if applicable. If any pharmaceutical agents are involved will compensation be according to ABPI guidelines? (Association of British Pharmaceutical Industry)</p> <hr/> <p>UREC Participant Information Leaflet and Consent Form (ICF VERSION) <span style="float: right;">Page 2</span></p>
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compensation guidelines for research related injury which is regarded as the international gold standard). If yes, please include the details here. If no, then explain what compensation will be available and under what conditions.

**9. Will you be paid to take part in this study and are there any costs involved?**

**10. Is there anything else that you should know or do?**

- a) You should inform your family practitioner or usual doctor that you are taking part in a research study. (Include if applicable)
- b) You should also inform your medical insurance company that you are participating in a research study. (Include if applicable)
- c) You can contact Dr ..... at tel ..... if you have any further queries or encounter any problems.
- d) You can contact the Centre for Research and Publications at +264 061 2063061; [pclaassen@unam.na](mailto:pclaassen@unam.na) if you have any concerns or complaints that have not been adequately addressed by the investigator.
- e) You will receive a copy of this information and consent form for your own records.

**11. Declaration by participant**

By signing below, I Benedictus Kotekeni agree to take part in a research study entitled **(Molecular identification and antibiogram profiling of *Avibacterium paragallinarum* and co-pathogenic bacteria associated with respiratory infections in chickens in Outapi constituency, Omusati ).**

**I declare that:**

- a) I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- b) I have had a chance to ask questions and all my questions have been adequately answered.
- c) I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- d) I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- e) I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place) Groot aub on (date) 27/01/2020.

Ben K Signature of participant  
[Signature] Signature of witness

**12. Declaration by investigator**

I (Helena Vaino) declare that:

- I explained the information in this document to B. Kotekeni
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (If a interpreter is used then the interpreter must sign the declaration below.

Signed at (place) Groot aub on (date) 27/01/2020.

[Signature] Signature of investigator  
[Signature] Signature of witness

**13. Declaration by interpreter**

I (name)/declare that: N/A

## **Appendix 4. Preparation of media used**

### **4.1. Nutrient broth**

The nutrient broth was prepared according to the manufacturer's protocol. To test for sterility, 10 ml aliquots of the broth were poured into test tubes and incubated at 37 °C for 18 h. When satisfied that it was sterile, it was stored at 4 °C in the refrigerator until it was used.

### **4.2. Sheep blood agar**

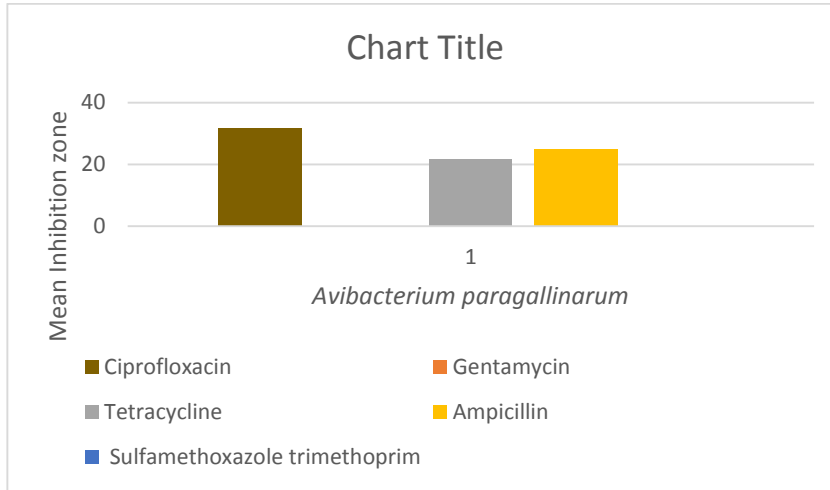
The blood agar media was prepared according to the manufacture's protocol. When the agar solidified, plates from each batch were incubated at 37°C for overnight to test for sterility. When satisfied that it was sterile, it was stored at 4 °C in the refrigerator until it was used.

### **4.3. Chocolate agar**

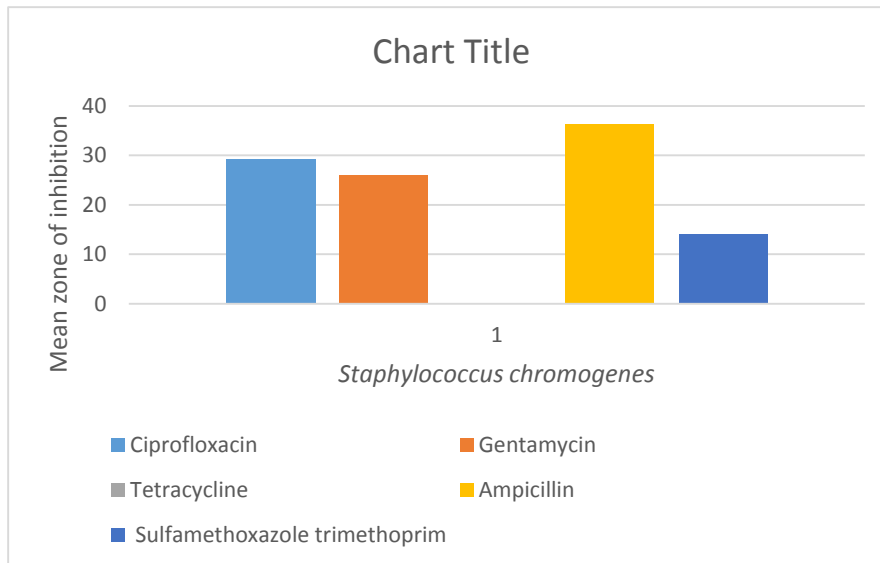
The chocolate agar media was prepared according to the manufacture's protocol. The sterile defibrinated sheep blood was added to the base, and heat-lyzed in the water bath at 80 °C with gentle mixing for about 30minutes to 1 hour until chocolate agar is obtained. The medium was cooled down to 50 °C and then poured into petri dishes and allowed to solidify. Few of the plates from the prepared batch were incubated overnight at 37°C to check for sterility. When satisfied that it was sterile, it was stored at 4 °C in the refrigerator until it was used.

**Appendix 5:** Antibiotic sensitivity test bar charts diagrams

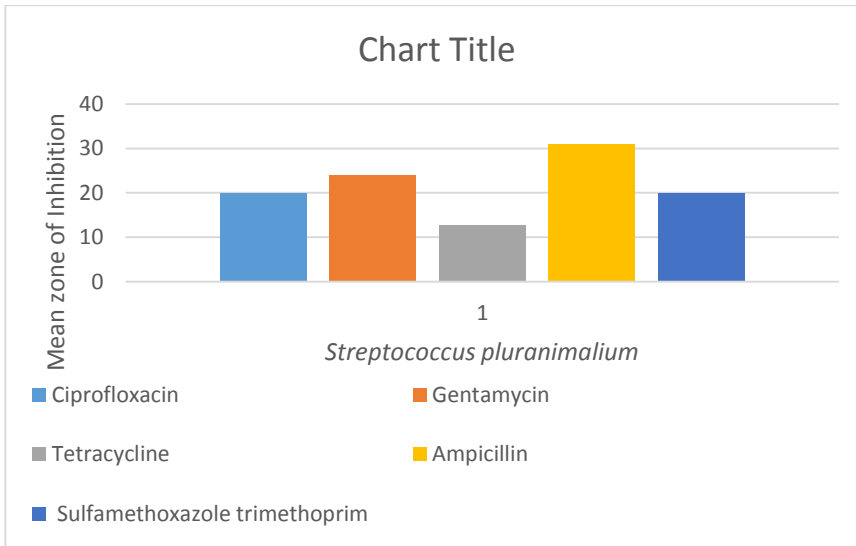
Bar chart for Mean zone of Inhibition for *Avibacterium paragallinarum*



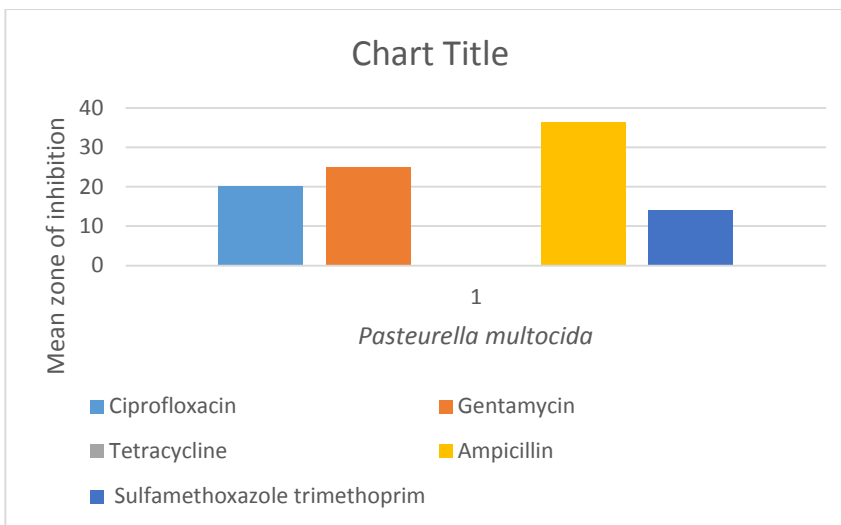
Bar chart for Mean zone of Inhibition for *Staphylococcus chromogenes*



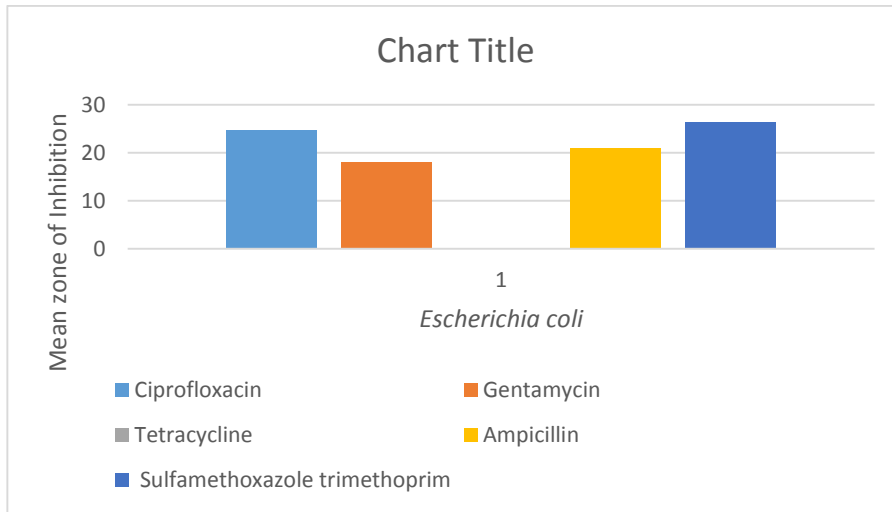
Bar chart for Mean zone of Inhibition for *Streptococcus pluranimalium*



Bar chart for Mean zone of Inhibition for *Pasteurella multocida*



Bar chart for Mean zone of Inhibition for *Escherichia coli*



**Appendix 6:** Association of the isolated bacteria and the symptoms in chickens tested with Chi square.

<b>Chi-Square Tests</b>			
	<b>Value</b>	<b>df</b>	<b>Asymptotic Significance (2-sided)</b>
<b>Pearson Chi-Square</b>	<b>12.754<sup>a</sup></b>	<b>24</b>	<b>.970</b>
<b>Likelihood Ratio</b>	<b>17.536</b>	<b>24</b>	<b>.825</b>
<b>N of Valid Cases</b>	<b>22</b>		
<b>a. 35 cells (100.0%) have expected count less than 5. The minimum expected count is .27.</b>			

**Appendix 7:** Shapiro –Wilk test for testing normality distribution of data.

	<b>Tests of Normality</b>					
	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Inhibition Zone	.150	75	.000	.912	75	.000

a. Lilliefors Significance Correction

Kruskal Wallis test data that are not normal distributed

	<b>Test Statistics<sup>a,b</sup></b>
	Inhibition Zone
Kruskal-Wallis H	6.172
df	4
Asymp. Sig.	.187

a. Kruskal Wallis Test

b. Grouping Variable: Isolated Bacteria