

**UNIVERSITY OF GHANA**

**COLLEGE OF BASIC AND APPLIED SCIENCES**

**FOREST AND WATER- BIRDS AS RESERVOIRS OF SOME  
PATHOGENIC AND ANTIMICROBIAL-RESISTANT BACTERIA**

**BY**

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## DECLARATION

I, Louisa Sawyerr declare that the information presented in this thesis is my own and was generated from the original research undertaken by me in the Department of Animal Biology and Conservation Science under the supervision of Prof. Boniface B. Kayang, Prof. Yaa Ntiamoa-Baidu, and Prof. Erasmus H. Owusu. I certify that this work, to the best of my knowledge, contains no material previously published or written by another person elsewhere. All references to other works have been duly acknowledged.

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## **DEDICATION**

I dedicate this thesis to my mother, Mrs. Cynthia Sawyerr for encouraging me to believe in myself and for supporting me in prayers to complete this thesis and my late dad who did not live to see this day but always wished the best for me.

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## LIST OF ABBREVIATIONS

ACA	-	Ankasa Conservation Area
AMP	-	Ampicillin
APEC	-	Avian Pathogenic <i>E. coli</i>
BLI	-	BirdLife International
CDC	-	Center for Disease Control and Prevention
CIP	-	Ciprofloxacin
CLSI	-	Clinical Laboratory Standards Institute
DAEC	-	Diffuse Adherent <i>E. coli</i>
DNA	-	Deoxyribonucleic Acid
EAggEC	-	Enteroggregative <i>E. coli</i>
EIEC	-	Enteroinvasive <i>E. coli</i>
EPEC	-	Enteropathogenic <i>E. coli</i>
ETEC	-	Enterotoxigenic <i>E. coli</i>
FCG	-	Forestry Commission of Ghana
H <sub>2</sub> S	-	Hydrogen Sulphide
LB	-	Luria Bertani
MDR	-	Multidrug Resistance
OXY	-	Oxytetracycline
PCA	-	Principal Component Analysis
PCR	-	Polymerase Chain Reaction
STR	-	Streptomycin
TSI	-	Triple Sugar Iron
PBS	-	Phosphate Buffered Saline
WHO	-	World Health Organisation

## ABSTRACT

Wild birds, particularly, migratory species can move from one place to the other within a short time. Their ability to fly over distances presents them with the potential to pick and spread pathogens. Despite their potential to acquire and spread pathogens, not many researchers have investigated their gastrointestinal flora. Apparently healthy birds have received little attention when it comes to surveillance for the occurrence of enteric pathogens. Many of the studies that have been conducted have focussed on a few species of birds or were conducted in the event of disease outbreaks with high mortality. In this study, apparently healthy wild birds were investigated for gram-negative enteric bacteria. Gram-negative bacteria were considered because they are the commonest in the environment. Similarly, enteric pathogens were considered because they can be found along the gastrointestinal tract and are mainly obtained from the diet of the bird. The birds were sampled from the Ankasa Conservation Area, Esiam beach and the Densu Delta Ramsar Site. Birds that were sampled from the Ankasa Conservation Area were forest birds and birds from the Densu Delta Ramsar Site and Esiam beach were waterbirds. Overall, 15 gram-negative enterobacteria genera were obtained from 377 cloacal swabs from the sampled birds. The overall prevalence of gram-negative bacteria in the birds was 61.8% (233/377). The prevalence in forest and water- birds were 65.2% (90/138) and 59.8% (143/239) respectively. Common genera of bacteria isolated from the birds were *Escherichia* (22.3%), *Yersinia* (19.4%), *Enterobacter* (12.2%) and *Klebsiella* (11.4%). On the whole, the most frequently isolated genus was *Escherichia*, which was obtained from 20 species of sampled birds. However, in the waterbird samples, the genus *Yersinia* was frequently isolated. The genera *Ochrobactrum* (0.5%), *Morganella* (0.3%), *Achromobacter* (0.3%) and *Alcaligenes* (0.3%) were the least isolated. Though wild birds do not use antimicrobial agents and rarely come into contact with antimicrobials in the

wild, bacteria isolated from 15.5% of the birds were resistant to Colistin by the agar dilution method. The prevalence of Colistin-resistant bacteria in the forest and water- birds were 10% (9/90) and 18.8% (27/143) respectively. Colistin-resistant isolates were recorded in seven forest bird species and six waterbird species. Similarly, the overall prevalence of Ciprofloxacin-resistant bacteria in the birds was 41.7% (97/233). The prevalence in forest and water- birds were 35.6% (32/90) and 41.3% (59/143). Ciprofloxacin-resistant isolates were recorded in 11 forest bird species and nine waterbird species. When the Colistin-resistant isolates (106) were subjected to multidrug resistance tests by the disc diffusion method, the prevalence of gram-negative bacteria resistant to antimicrobial agents tested were Ampicillin (73.6%), Streptomycin (50.9%), Oxytetracycline (52.8%) and Ciprofloxacin (8.5%). Colistin and Ciprofloxacin-resistant isolates were further investigated for plasmid-mediated resistance genes. Plasmid-mediated polymyxin-resistant genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and, *mcr-5* were recorded in bacteria isolated from the birds. The *mcr-3* gene was the most prevalent polymyxin-resistant gene in this study and was recorded in bacterial isolates from both forest and water- birds. The *mcr-1* gene was recorded in bacterial isolate from a forest bird, while *mcr-2*, *mcr-4* and *mcr-5* were recorded in bacterial isolates from waterbirds. Similarly, plasmid-mediated quinolone-resistant genes (*qnrB*, *qnrD*, *qnrS* and *qnrVC*) were recorded in bacteria isolates from sampled birds. The prevalent gene was *qnrVC* and occurred in 9.8% (16/164) of the Ciprofloxacin-resistant isolates. Moreover, bacterial isolates belonging to the gram-negative bacteria genera *Salmonella*, *Escherichia*, *Yersinia* and *Shigella* were examined for the occurrence of the species *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, *Shigella flexneri*, *Shigella dysenteriae*, Enterotoxigenic *E. coli*, and *Salmonella enterica*. These species are known to cause severe diarrhoeal infections in humans. Only enterotoxigenic *E. coli* (ETEC) was detected among

the isolates. The prevalence of ETEC was 38.6% and 62.5% in forest and water- birds respectively. Phylogenetic analysis of all *E. coli* isolates obtained from this study showed that 60.6% of the *E. coli* isolates belonged to virulent phylogenetic groups, while 39.4% belonged to commensal groups. The results obtained from this study suggest that wild birds are reservoirs of enteric bacteria and may serve as sources of infection to humans, domestic animals, and other wild animals. The findings also suggest that wild birds harbour antimicrobial-resistant bacteria that carry plasmid-mediated genes. These genes are reported to have the potential to confer resistance to other bacteria isolates. Evidence from the study shows that enterotoxigenic *E. coli* occurs in the wild birds and these birds may serve as sources of infection to humans. In conclusion, apparently healthy wild birds harbour pathogenic gram-negative bacteria, though they may seem asymptomatic. Therefore, is it important to monitor wild birds for the occurrence of pathogenic bacteria as well as antimicrobial-resistant bacteria. Although prevalence may be low, the potential to spread pathogens is considerable. From a conservation standpoint, the occurrence of antimicrobial-resistant bacteria could hamper conservation efforts as birds carrying resistant bacteria could serve to disperse bacteria between widely separated locations and from hotspots to vulnerable populations.

## **1.0 CHAPTER ONE: GENERAL INTRODUCTION**

### **1.1 Introduction**

The emergence, re-emergence, and resurgence of infectious diseases are increasingly recognised as a high priority public health concern. These high fatality diseases such as Acquired Immune Deficiency Syndrome (AIDS), Ebola Virus Disease (EVD), Lassa Fever, Severe Acute Respiratory Syndrome (SARS) and Avian Influenza have devastated several communities of human populations all over the world. A more recent issue of the challenge posed by infectious diseases is the recent pandemic of the Ebola Virus Disease in 2014 which affected several parts of sub-Saharan Africa, the United States of America, Spain, and Italy.

The origin of most of these infections have been traced to wildlife and Jones *et al.* (2008) estimated that about 61% of emerging infections in humans are zoonotic. Wildlife has also been reported to be the primary reservoirs of 72% of these zoonotic infections (Taylor *et al.*, 2001). For example, the Human Immunodeficiency Virus (HIV) is known to have emerged from two non-human primate reservoirs in Africa (Hahn *et al.*, 2000). Also, fruit bats are reported as reservoirs of many viral zoonoses including SARS and Nipah fever (Li *et al.*, 2005).

Emerging Infectious Diseases (EIDs) exert a heavy toll on public health as well as economies. The World Bank estimates that aside from the mortality and morbidity caused by the pandemic of infectious diseases, it could also cause a decline of up to 5% global GDP (Mackenzie *et al.*, 2013). For example, the cost of SARS in the east and southeast Asia was estimated at USD 18 million with cost per each infected person amounting to about USD 2 million (ADB, 2003).

Though the incidence of infectious diseases in wildlife is reportedly low, Mackenzie *et al.* (2013) strongly believe that the associated risks such as spill-over to humans and domestic animals are high.

Spill-overs from domestic animals to wildlife have been reported in several studies. For example, the spill-over of rabies from domestic dogs devastated populations of the Ethiopian wolf and endangered wild African dog (Gascoyne *et al.*, 1993; Laurenson *et al.*, 1998). Also, the canine distemper virus epidemic decimated the lion population in the Serengeti in 1994. The source of this infection was reported to have originated from domestic dogs (Roelke-Parker *et al.*, 1996). Most infectious disease spill-overs are reported to originate from developing countries, as people in these countries tend to have frequent contact with wildlife (Stewart, 2006).

Infectious diseases involve the interaction between at least two species, the pathogen and the host it infects. In some cases, more than one host is needed and transmission usually involves a vector (Keesing *et al.*, 2006). Against this backdrop, interactions between humans, domestic animals and wildlife may cause pathogens to circulate among these interfaces. Consequently, these interactions may lead to problems in public and animal health. Thus, emerging infectious diseases are not only a problem for human health but also pose a major threat to animal welfare and species conservation (Cunningham *et al.*, 2003).

When a pathogen is restricted to one host species and is transmitted directly between individuals, then the usual public health methods such as public education, vaccination, and quarantine are effective. However, when there are multiple hosts involved and the pathogen is transmitted by a vector, then the regular methods stated above may not remedy

the situation. Some studies have suggested that targeting the ecological interaction between wildlife hosts and zoonotic pathogens may be more effective. In a global effort to mitigate problems that arise from such interactions, the One Health Approach was adopted.

The One Health Approach recognises the connection between the health of animals and humans (Conrad *et al.*, 2009) and promotes the use of transdisciplinary collaborative efforts in addressing health issues, thus, engaging ecologists, epidemiologists, physicians, veterinarians and agricultural scientists in solving health-related issues. Through this collaborative approach several factors have been identified to contribute to transmission of pathogens from wildlife to humans and vice versa. These include, but are not limited to, human population growth, agricultural development, urbanisation, and wildlife trade (Daszak *et al.*, 2000; Aguirre & Tabor, 2008).

While wild animals are known to be agents in the spread of infectious diseases, wild birds have been identified as particularly important vehicles in the transmission process. The mobility (flight) of birds presents them with the potential to spread pathogens. In a review by Tsiodras *et al.* (2008), they identified 58 pathogens for which wild birds may serve as biological reservoirs, mechanical vectors or both. For example, indirect transmissions of zoonotic pathogens from wild birds to humans have been reported for the West Nile Virus (WNV), *Salmonella* spp., *Escherichia coli*, *Chlamydia* spp., *Cryptococcus* spp. and *Mycobacterium* spp. (Tsiodras *et al.*, 2008).

Migratory birds have the ability to travel long distances across continents. It is estimated that about 5 billion birds representing 300 species migrate from Europe to Africa and also from North America to Central America (Gill, 1995). Thus, these birds may serve as reservoirs carrying pathogens across long distances (Grenfell & Dobson, 1995) and have the potential to acquire or spread pathogens along their migration routes (Reed *et al.*, 2003; Foti *et al.*, 2011).

Migration is energetically demanding and stressful and the stress associated with it may cause birds to become more susceptible to pathogens. Acquisition or spread of pathogens may occur usually at feeding or stopover sites where different bird populations or species congregate (Klaassen *et al.*, 2012). Occasionally, there may be stopovers at sites that have not been previously explored and this creates the potential for the spread of pathogens to new or remote areas (Gogu-Bogdan *et al.*, 2014). Moreover, migratory birds themselves may be diseased or may be carriers of infected vectors such as ticks (Abulreesh *et al.*, 2007).

The feeding behaviour of wild birds has been associated with pathogen acquisition. Pathogens that live in the gastrointestinal tract are referred to as enteric pathogens or enteropathogens. For example, when raptors feed on the intestines of their prey, they may become infected with enteropathogens. Also, waterfowls may become infected with enteropathogens by feeding on prey items or straining mud for nutrients.

The use of migratory birds as sentinels of enteropathogens also allows for the study of their role in the spread of Antimicrobial-resistant Strains (AMR) in the environment (Matias *et al.*, 2016). Antimicrobial resistance is defined by Mathis *et al.* (2015) as “resistance of a microorganism to an antimicrobial drug that was originally effective for

the treatment of infections caused by it". Jones *et al.* (2008) suggested that 54% of EIDs were caused by bacteria with a substantial representation by drug-resistant isolates such as vancomycin-resistant *Staphylococcus aureus*. The use of antimicrobial agents is commonplace in humans and poultry keeping, giving rise to selection pressure for resistant bacteria. When wild birds come into contact with these resistant bacteria, they spread it to other wild animals. For instance, multi-drug-resistant bacteria have been isolated from Arctic birds, known to inhabit very remote areas (Sjölund *et al.*, 2008).

Major epizootics may arise from wild bird migration and mobility and these may have devastating consequences on the health of domestic animals, wild animals, and humans. However, only few studies have focussed on investigating pathogens that wild birds may carry and disseminate during migration or mobility. In Africa, pathogen surveillance in wild birds has mainly focussed on waterfowls and these surveys were conducted during disease outbreaks (Fuller *et al.*, 2015). Information on pathogens in apparently healthy wild birds is scanty. This study, therefore, focused on enteric bacteria from apparently healthy forest and water- birds.

## **1.2 Justification**

Wild bird migration and mobility provide a means for the acquisition and spread of pathogens. Wild birds either serve as reservoirs of pathogens or act as vectors of parasites. Given their potential to spread pathogens, they pose serious threats to human and animal health, with implications for species conservation.

Despite their consideration as important vehicles in the spread of pathogens, only a few studies examining their normal gastrointestinal flora have been conducted. A major reason

for the paucity in research is because wild birds are assumed to have a low commercial value compared to domestic birds. The few studies conducted on wild birds come from veterinary studies focussing on disease outbreaks that result in high mortality (Daszak *et al.*, 2000). While these studies provide information on birds dying from infections, little is known about the occurrence of these pathogens in apparently healthy birds. Actually, the role of wild birds as reservoirs of pathogens may be underreported as some individuals may carry pathogens without showing any symptoms.

Wild birds inhabiting protected areas such as forests may become contaminated with human-originating antimicrobial-resistant bacteria. These contaminations may be introduced via waterways, bird migration and people who visit protected areas. Yet, little is known about antimicrobial contamination in birds in protected areas. Shorebirds have the potential to spread pathogens along geographical borders. Although some studies have examined gut microbiota of migrating shorebirds (Grond *et al.*, 2014; Ryu *et al.*, 2014; Risely *et al.*, 2018), these studies have focussed on particular species. The scarcity of information regarding gut microbiota in wild birds and their potential threats to health calls for increased surveillance.

Considering the role of wild birds in the spread of pathogens and the possibility of picking pathogens that may affect their populations as well as the populations of other wild animals, this study was guided by four main research questions:

1. What are the species of gram-negative enterobacteria that occur in forest and water- birds?
2. What are the antimicrobial sensitivity profiles of bacteria isolated from forest and water- birds?

3. Does antimicrobial-resistant bacteria from forest and water- birds harbour plasmid-mediated resistance genes?
4. Do forest and water- birds harbour pathogenic bacteria that are known to pose a threat to humans and livestock?

### **1.3 Aim**

The aim of the study was to investigate the occurrence of enterobacteria in wild birds as well as determine the antimicrobial resistance profiles and occurrence of resistance genes in enterobacteria isolated from wild bird species in the study sites.

#### **1.3.1 Specific Objectives**

Four specific objectives were defined for the study, namely:

1. To isolate and characterise gram-negative enterobacteria from forest and water– birds.
2. To determine antimicrobial sensitivity profiles of bacteria isolated from forest and water- birds to selected antimicrobials.
3. To determine if antimicrobial-resistant bacteria harboured plasmid-mediated resistance genes.
4. To determine genetically the prevalence of selected pathogenic enterobacteria that are known to pose a threat to humans and livestock.

#### **1.4 Organisation of thesis**

This thesis is organised into three main sections. The first section comprises three chapters (One, Two and Three). Chapter one gives a background to the study, particularly providing information on infectious disease emergence and how wild birds may serve as possible reservoirs of infectious disease pathogens and also aid in their spread. Chapter two reviews literature which is of particular interest to this study. These include the role of wild animals and infectious diseases; zoonotic diseases as a major threat to wildlife conservation; wild birds as important vehicles in the spread of pathogens; epizootics of diseases associated with wild birds; bacterial species associated with wild birds; antimicrobial resistance and the occurrence of resistance genes in wild birds; exposure and susceptibility of wild birds to enteric bacteria and antimicrobial-resistant bacteria. Chapter three gives a general overview of the materials and general methods used for the study. The chapter also provides information on ethical considerations obtained as well as the statistical tools employed to analyse data obtained.

Section two of the thesis starts with chapter four and ends with chapter seven. Chapter four presents results on species of gram-negative bacteria isolated from wild birds. Specifically, it highlights the prevalent species of gram-negative bacteria and their prevalence in both forest and water- birds. Chapter five presents results and discussion of the occurrence of antimicrobial-resistant gram-negative bacteria in forest and water- birds while chapter six presents results on the occurrence of antimicrobial-resistant genes. Chapter seven presents findings on molecular investigations on the occurrence of six pathogenic species of gram-negative bacteria that are known to occur in and pose a threat to both livestock and humans.

The third section of the thesis comprises chapters eight and nine. Chapter eight presents a general discussion of the findings from this study and chapter nine draws the conclusions and recommendations for future studies.

## **2.0 CHAPTER TWO: LITERATURE REVIEW**

### **2.1 Introduction**

This chapter reviews wild animals and their diseases especially emerging infectious diseases (Section 2.2) and some zoonotic diseases acting as a major threat to conservation (Section 2.3). In addition, the chapter reviews wild birds as important vehicles in the spread of pathogens (Section 2.4), some epizootics of diseases associated with wild birds (Section 2.5) and bacteria species associated with wild birds (Section 2.6). Lastly, the chapter discusses antimicrobial resistance (Section 2.7), antimicrobial resistance genes (Section 2.8), the exposure of wild birds to bacteria (Section 2.9) and the susceptibility of wild birds to bacteria (Section 2.10).

### **2.2 Wild animals and emerging infectious diseases**

Wild animals are untamed animals living in their natural environments. Hitherto, they rarely came into contact with humans. However, with an increasing overlap of interactions resulting from population increases of both humans and wild animals, each group is forced into the other's habitat. These interactions have led to the occurrence of infections or diseases in reservoirs where they used not to occur. The occurrence of infections in new reservoirs has been referred to as emerging (Melnick *et al.*, 2005).

Emerging Infectious Diseases (EIDs) have been reported as causes of death in free-living animals (Daszak *et al.*, 2000) and are a major threat to wildlife species that are in captive breeding and translocation (Lyles & Dobson, 1993; Cunningham *et al.*, 2003). These diseases which are primarily driven by ecological and socio-economic factors (Binder *et al.*, 1999; Morens *et al.*, 2004) cause substantial burden to public health and economies (Taylor *et al.*, 2001; Woolhouse & Gowtage-Sequeria, 2005). For instance, Southeast Asia

is known to be a hotspot for EIDs, including those with pandemic potential (Coker *et al.*, 2011). Novel viruses causing diseases such as Severe Acute Respiratory Syndrome (SARS), Avian Influenza A (H5N1) have emerged from this geographical region.

The Nipah virus disease emerged from peninsular Malaysia and Singapore between 1998 and 1999 and resulted in a fatality rate of about 40% in humans (Lo & Rota, 2008). Pig farming and animal production were suggested as the major means of transmission to humans, with the majority of human cases being pig farmers (Coker *et al.*, 2011). Infected pigs were suggested to have acquired the virus from the fruit bats, sub-order Megachiroptera (known as the natural host of Nipah virus).

The Severe Acute Respiratory Syndrome which is believed to have originated from Southern China in 2003 was spread by an infected doctor to other parts of the region including Singapore and Vietnam (Peiris *et al.*, 2003). Singapore was severely affected by about 33 deaths compared to the 11 deaths across Southeast Asia (WHO, 2003). Again in 2003, the region experienced an outbreak of another emerging infectious disease, H5N1, which also spread from China (Brahmbhatt, 2006). A survey conducted by Li *et al.*, 2005, found a high seroprevalence of SARS-CoV in bats belonging to the genus *Rhinolophus*. The high seroprevalence and wide distribution of seropositive bats qualify these bats as natural reservoirs (Hudson *et al.*, 2002).

The African continent has also suffered from EIDs, notably Ebola Virus Disease (EVD). This virus causes haemorrhagic fever in infected persons. The first cases of the disease were recorded in Sudan (WHO, 1978) and the Democratic Republic of Congo (DRC) (WHO, 1978) in 1976. Since the first outbreak, the EVD has been recorded in about 17 different countries including the DRC and Sudan (CDC, 2017). The disease first outbreaks

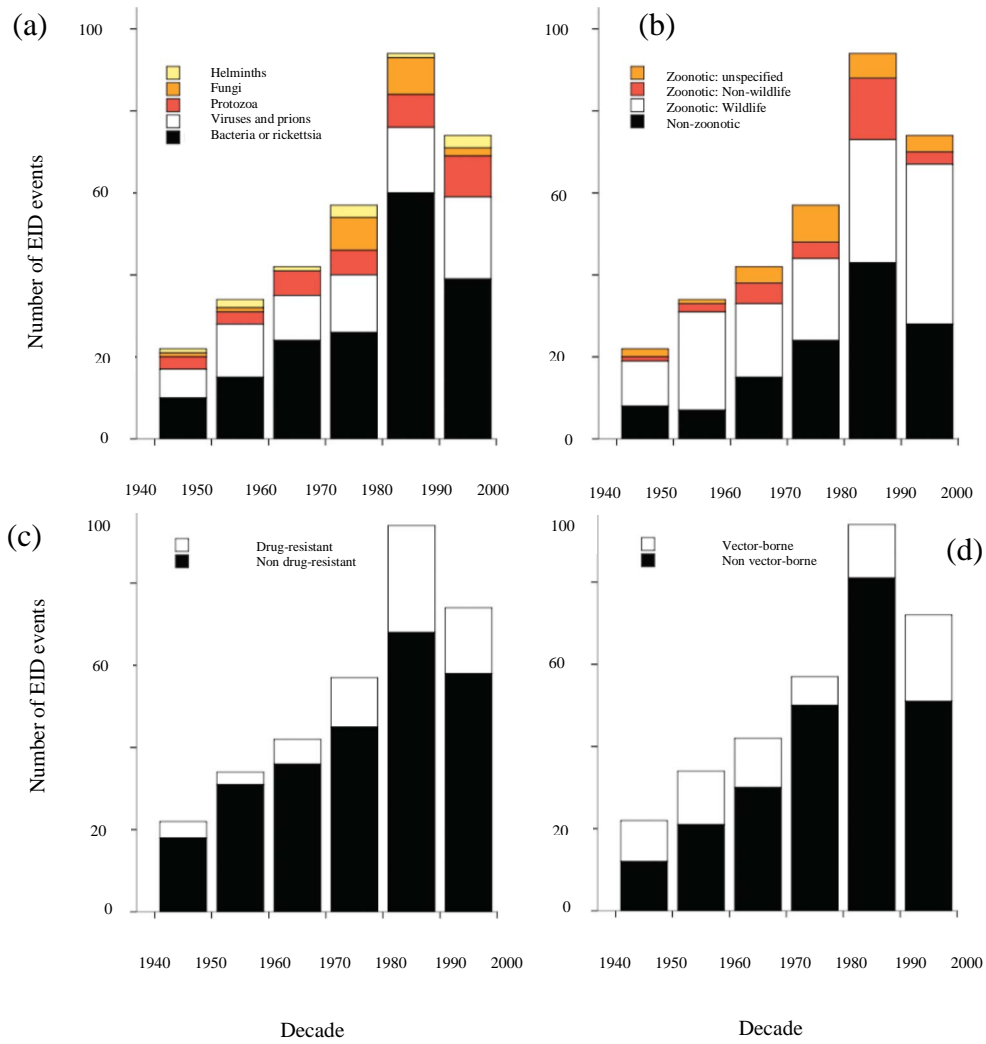
which were originally assumed to have been caused by the same species of the virus were later discovered to have been caused by two different species, *Zaire ebolavirus* and *Sudan ebolavirus*, in the DRC and Sudan respectively (CDC, 2017). In a study conducted by Brooks *et al.* (2004) on 679 bats, 222 birds, 129 small terrestrial vertebrates, immunoglobulin G which is specific for Ebola virus was detected in the serum from three bat species, *Hypsignathus monstrosus*, *Epomops franqueti*, *Myonycteris torquata*. None of the species from the other animals harboured the IgG. Brooks *et al.* (2004) concluded their study by stating that their findings support previous studies that suggest bats are the natural reservoirs of Ebola.

Chikungunya fever, an acute febrile disease caused by the chikungunya virus through the bite of an infected *Aedes* mosquito species, is an EID discovered in Africa. The disease was first recognised in Africa in the 1950s (Robinson, 1955) and is believed to have originated from Central/East Africa (Powers *et al.*, 2000). Urban outbreaks of the disease were recorded in Asia, specifically in Bangkok (Halstead *et al.*, 1970) and India (Padbidri & Gnaneswar, 1979). There were minor outbreaks of the disease from 1973 until 2004 when a major outbreak of the disease occurred in Kenya (Powers & Logue, 2007) causing morbidity to millions of people. Natural reservoirs are monkeys but other reservoirs are buffalos, monkeys, and birds (Kading *et al.*, 2013; Rougeron *et al.*, 2015).

Zika virus was also first identified in the rhesus monkey during yellow fever surveillance in the Zika forest in Uganda in 1947 and later reported in humans in 1952 (Dick *et al.*, 1952). Since then, there have been outbreaks in 2007 in Yap Island (Duffy *et al.*, 2009) and in 2013-2014 there were outbreaks in French Polynesia (Cao-Lormeau *et al.*, 2014) and New Caledonia (Dupont-Rouzeyrol *et al.*, 2015). In 2015, there was an outbreak in Rio de Grande in Brazil.

Continents such as America and Europe have not suffered devastating effects from infectious diseases compared to Asia and Africa.

It is estimated that 60.3% of all EIDs are zoonotic and 71.8% of these zoonotic EIDs are caused by pathogens that originated from wildlife (Jones *et al.*, 2008). The incidence of EIDs originating from wildlife has been increasing, over the decades (Fig. 2.1). For example, the number of EIDs originating from wildlife alone increased significantly from 1990 to 2000 and constituted about 52% of all EIDs (Jones *et al.*, 2008). Thus, EIDs originating from wildlife constitute a significant proportion of all EIDs and also represent a significant threat to global health. Some of these pathogens may cause significant diseases in some wildlife while other wildlife may only serve as reservoirs of these pathogens (Williams *et al.*, 2002).



**Figure 2.1: Number of EID events per decade. Emerging Infectious Disease (EID) events (defined as the temporal origin of an EID, represented by the original case or cluster of cases that represents a disease emerging in the human population) plotted with respect to (a) pathogen type, (b) transmission type, (c) drug resistance and (d) transmission mode (see keys for details) Source (Jones *et al.*, 2008).**

### **2.3 Zoonotic diseases, a major threat to wildlife conservation**

The increase in movement of domestic animals, humans, and wildlife has facilitated the spillover of pathogens between these interfaces. A major reason for the increased overlap of the interfaces between humans, wildlife and domestic animals is population increase (Bradley & Altizer, 2007) causing both humans and wildlife to move into each other's habitats. Consequently, zoonotic infections may spill-over into human, domestic and wild animal populations. As the interfaces continue to increase, pathogens will continue to spill over and possibly spillback (Nugent, 2011).

Generally, wildlife is assumed as a potential health threat to humans and domestic animals. Thus, pathogens from wildlife have been attributed to causing severe diseases in humans and domestic animals. However, wild populations are also faced with the introduction of pathogens from humans and domestic animals which may have adverse consequences on their populations.

Diseases or pathogens that have adversely impacted wildlife populations include Rinderpest, Chytridomycosis, Canine distemper, Elephant Endotheliotropic Herpesvirus, and Anthrax.

#### **2.3.1 Rinderpest**

The rinderpest virus is a member of the order Paramyxovirus and genus Morbillivirus (Dobson, 1995). Rinderpest affects wildlife hosts of the order Artiodactyla (Scott, 1964). The disease is characterised by diarrhoea, ulceration of mucous, nasal lacrimal discharge (Ballard, 1986). The virus caused a pandemic in the 19th century and was accidentally introduced to sub-Saharan African from Asia in 1889 via infected cattle. This extirpated about 90% of the buffalo population in Kenya with similar effects on predator populations

and the local extinctions of the tsetse fly (Daszak *et al.*, 2000). The epidemic in Africa caused high mortality and this confirmed that it was the first time the pathogen had been introduced to the ungulate population in Africa (Plowright & McCulloch, 1967).

### **2.3.2 Chytridiomycosis**

Chytridiomycosis disease is caused by the fungus chytrid. The fungus is a major cause of the decline of amphibian populations worldwide. It is thought to have originated from the release of the African clawed frog into non-native habitats. It has affected about 14 species of frogs in Australia and likely to lead to their extinction (Retallick *et al.*, 2004). Symptoms of the disease include excessive sloughing of skin, anorexia, and lethargy (Kriger *et al.*, 2007). It is known to be solely responsible for almost 90% decline in global amphibian numbers. A report published recently found that chytrid was transferred to different habitats on the feet of migratory birds, particularly, the Canada geese (Hugh-Jones & De Vos, 2002). Also, humans have been found to carry chytrid on field equipment and clothing and so could aid in its transmission.

### **2.3.3 Canine distemper**

This is a viral disease closely related to measles and often mistaken for rabies. The virus belongs to the order Paramyxovirus and is known to cause disease in the families Canidae, Mustelidae, Procyonidae, and Viverridae and may possibly cause disease in three families (Protelidae, Hyanidae, and Felidae) of the order Carnivora (Budd, 1981). Symptoms include ataxia, myoclonus, and seizures (Carpenter *et al.*, 1998). The virus is commonly spread by domestic dogs. It is a frequent cause of death in the African wild dog and lion. It is thought to have been a significant factor in the total eradication of the Tasmanian tiger. Similarly, in the United States it caused the near extinction of the black-footed ferret.

An effective vaccine was developed in 1950. However, the virus persists and remains prevalent worldwide as a result of limited use or lack of vaccination programmes.

#### **2.3.4 Elephant Endotheliotropic Herpesvirus (EEHV)**

The Endotheliotropic Herpesvirus belongs to the family of latent viruses that reoccur throughout the lifespan of an infected individual. Although there are host specific herpes viruses, there are also those that cross host species and in doing so become deadly. Symptoms of disease caused by this virus include reluctance to move, intermittent anorexia, and lethargy. This virus has been identified in both wild and captive Asian and African elephants. It is hypothesized that while each elephant species has specific strains of the virus, the virus lives within individuals of that elephant species with no serious health effects, it is when a particular virus is transmitted to the other elephant species that it almost always results in death (Long *et al.*, 2016). Similarly, a strain of herpes virus known to cause sickness in zebras was recently identified as the cause of death in polar bears in a zoo in Germany. However, in the wild, these two animals (polar bears and zebras) species would never cross paths and so it is possible that these types of infections are strictly held to captive wildlife.

#### **2.3.5 Anthrax**

Anthrax is caused by a lethal bacterium known as *Bacillus anthracis* which leads to sudden death. It attacks all mammals but primarily, herbivores. Symptoms of the disease include excessive sweat, fever, malaise, shortness of breath, chest discomfort, sore throat, vomiting and diarrhoea (Friedlander *et al.*, 1999). The bacterium may live several years under harsh conditions. The origin of the infection may be traced to the fifth and sixth plagues of Egypt (In the Bible) which occurred about 1491 BC (Hugh-Jones & De Vos,

2002). Hence, the probable origin of anthrax was linked to previous Mesopotamia and Northern Africa (Klemm & Klemm, 1959; Kolonin, 1971). However, evidence points to sub-Saharan Africa (Smith *et al.*, 2000). The disease is now widespread and occurs in every continent (Hugh-Jones & De Vos, 2002). Most commonly documented infections occur in areas where domestic livestock and wild herbivores share grazing land via faecal/oral route. While vaccinations for anthrax do exist, and successful treatment with antibiotic therapy has been proven, limited use allows for this disease to persist. Like distemper, anthrax poses a great threat to wildlife and endangered species are of particular concern.

#### **2.4 Wild birds as important vehicles in the spread of pathogens**

Many animals such as mammals, fish, insects, and birds take regular movements each year over long-distance due to seasonal changes in resources and habitats (Altizer *et al.*, 2011). Shorebirds are generally migratory in nature and their migratory flyway span the globe (Piersma & Baker, 2000). High latitude bird species are known to occur in marine and saline habitats during the nonbreeding season while low latitude species are found in freshwater habitats in the nonbreeding season (Piersma, 1997). Birds occurring in marine habitats and associated wetlands feed most likely on crustaceans, shellfish, and worms (van de Kam *et al.*, 1999). The ability of shorebirds to cross geographical borders makes them important vehicles in the spread of epidemic infections or diseases such as botulism and avian cholera (Adams *et al.*, 2003). Several factors have been identified to be involved in the transmission of pathogens by wild birds. The most important ones are seasonality and mobility or migration.

#### **2.4.1 Seasonality**

Seasonality is an important factor that influences the effective transmission of pathogens by migratory birds. Some diseases are known to peak during certain seasons. For example, in the Holarctic, mosquito-borne diseases peak during late summer and early autumn when mosquito species population density peaks (Hubálek, 2004). Although seasonality is known to have an influence where disease vectors are involved, some studies have found seasonality to play a role in non-vector-borne pathogens. For example, influenza A was found to be infectious in the water at low ambient temperatures from late autumn to early spring in the Holarctic (Hubálek, 2004).

#### **2.4.2 Migration**

Animal migrations are mostly thought of as evolutionary adaptations that allow them to track seasonal changes in the availability of resources such as dietary needs (Dusek *et al.*, 2009). Bird mobility and migration, though an important biological process, may present adverse consequences to their health. Thus, both sedentary (resident) and nomadic (migratory) birds are at risk of infections. Sedentary birds have the potential to travel as far as 50 – 100 km while nomadic species travel over very long distances during erratic movement. Migrating birds can cross geographical or continental boundaries and use habitats such as marshes, wetland and other water bodies (UNEP, 2005). Migrating shorebirds can cover a distance of 15,000 miles during annual migrations (Andres *et al.*, 2012). Horizontal transmission of pathogens usually occurs when birds congregate at migratory stopover sites. The carriage of microbial pathogens by wild birds could be done in three ways: Mechanical, biological and as transporters of infected ectoparasites (Hubálek, 2004). Risely *et al.* (2018) reported that active migration is associated with gut microbiota changes in the *Calidris* shorebirds.

#### **2.4.2.1 Mechanical carriers**

Mechanical carriers harbour pathogens, that do not multiply in or on them (Hubálek, 2004). Thus, the pathogen may be located on the bird's body, e.g. fungal spores survive on the feathers of migratory birds for at least 12 days (Warner & French, 1970). The pathogen could also be carried internally but is only viable when it is excreted, e.g. foot and mouth disease virus (FMDV) is speculated to be carried mechanically on free-living birds (Hubálek, 2004). Also, *Cryptosporidium parvum* is suggested to be carried mechanically by waterfowls (Graczyk *et al.*, 1998).

#### **2.4.2.2 Biological carriers**

Pathogens may be carried biologically by birds. Thus, the pathogen multiplies in the avian body (Hubálek, 2004). Such infections could be asymptomatic e.g. Lyme Borreliosis, Campylobacteriosis, Salmonellosis, Yersiniosis, Toxoplasmosis and Influenza A acute e.g. Newcastle disease, Avian cholera, and Duck plague; or chronic e.g. Avian tuberculosis, Avian pox and Aspergillosis (Bengis *et al.*, 2004). Shedding of bacteria agents is prolonged in some bird species and in others (e.g. gulls), shedding of the pathogen is intense and more obvious in younger birds (Tell *et al.*, 2001).

#### **2.4.2.3 Transporters of infected ectoparasites**

Infected ectoparasites may be carried by wild birds from one place to the other especially across geographical borders. Important ectoparasites such as mature ixodid and argasid ticks can be transported across continents (Nuorteva & Hoogstraal, 1963). Fleas have also been reported to be transported over long distances on migrating birds (Schwan *et al.*, 1983). Ticks are able to attach themselves to their hosts for 24-48 hr (Tsiodras *et al.*, 2008). This allows migrating birds enough time to travel hundreds or thousands of miles

before the ticks finish feeding and drop off (Reed *et al.*, 2003), hence, infectious agents may be deposited in a new geographical area. Where the parasite load is small, the number of birds transporting the vectors could play a major role in tick populations (Bengis *et al.*, 2004).

## **2.5 Epizootics of diseases in wild birds**

### **2.5.1 Avian Influenza A virus**

There have been several reports on disease outbreak with avian influenza A virus. These outbreaks have been reported in a wide range of animals including domestic and wild birds, pigs, sea mammals and horses (Reed *et al.*, 2003). The first report of the influenza virus (HPAI H5N3) in wild birds was obtained from Common Terns (*Sterna hirundo*) in South Africa in 1961, killing about 1300 birds (Becker, 1966). Since then positive samples have been obtained in African countries such as Mauritania, Morocco, Niger, Tunisia, Senegal, Mali, Ethiopia and Chad (Gaidet *et al.*, 2007). In 2006, the first case of highly pathogenic avian influenza virus was officially reported to occur in Nigeria (Cattoli *et al.*, 2009). Two other West African countries (Burkina Faso and Cote d'Ivoire) have recorded cases of the virus (Ducatez *et al.*, 2007). The main reservoirs of avian influenza are known to belong to the orders Charadriiformes (sandpipers, terns, surfbirds, gulls) and Anseriformes (swans, duck, and geese) (Swayne & Suarez, 2000). Twelve significant outbreaks of Highly Pathogenic Avian Influenza were observed in chicken since 1959 (Alexander, 2000). The outbreaks occurred in Pennsylvania (1959) (Eckroade *et al.*, 1984), USA (1983-1984) (Garnett, 2003), Mexico and Pakistan (1994 – 1995) (Villareal & Flores, 2003). Since then, HPAI outbreaks caused by H5N1 occurred in gallinaceous poultry in Hong Kong in 2001, 2002, 2003 (Sims *et al.*, 2003). Outbreaks of H7N7 were also reported in the Netherlands, Germany, and Belgium in 2003 (Ellis *et al.*, 2004).

### **2.5.2 Salmonellosis**

Salmonellosis is caused by *Salmonella enterica* ser. *Typhimurium*. Tizard (2004) suggested that Salmonellosis be considered an emerging infectious disease in wild birds because of its increased prevalence since 1964. In the US, *Salmonella enterica* ser. *Typhimurium* was isolated from dead passerines in 1973 (Locke *et al.*, 1973). The same serovar was identified to have caused high mortality in wild birds between 1985 and 2004 in North America (Hall & Saito, 2008). Also, this serovar was isolated and diagnosed as the cause of Salmonellosis in wild birds from Scotland between 1995 and 2008 (Pennycott *et al.*, 2010). In passerines, mortality events occurred in countries such as Norway (Refsum *et al.*, 2003), Sweden (Tauni & Österlund, 2000), United Kingdom (Pennycott *et al.*, 2006), New Zealand (Alley *et al.*, 2002) and Japan (Une *et al.*, 2008).

### **2.5.3 Usutu Viral disease**

Usutu Virus (USUV) is arthropod-borne and belongs to the genus flavivirus which is closely related to important human pathogens including West Nile Virus (WNV), Dengue Virus (DENV), Japanese Encephalitis Virus (JEV), Yellow Fever Virus (YFV) etc. (Poidinger *et al.*, 1996; Kuno *et al.*, 1998). In 2001, USUV killed several blackbirds (*Turdus merula*) and Great Grey Owls (*Strix nebulosi*) in Austria (Weissenböck *et al.*, 2002; Chvala *et al.*, 2004). It was first isolated from mosquitoes in South Africa in 1959 and was named after a river in Swaziland (Woodall, 1964). Prior to the isolation of the virus in Vienna, Austria, the disease was known to occur only in Africa. The virus was also isolated from Barn Swallow in Central Europe (Weissenböck *et al.*, 2002) and was suggested that the virus was probably introduced to the Austrian bird population by swallows or other migrating birds.

## 2.6 Bacterial species associated with wild birds

Bacteria are normal inhabitants of the human and animal bodies. Some species are known to be commensal while others are virulent. Several of these bacteria species have been isolated from wild animals including wild birds. A variety of bird species have been reported to harbour human and avian pathogens. These include terns (Ksoll *et al.*, 2007), gulls (Camarda *et al.*, 2006; Lu *et al.*, 2008), corvids (Keller *et al.*, 2011), geese (Zhou *et al.*, 2004; Lu *et al.*, 2009), raptors (Camarda *et al.*, 2006) and psittacines (Hudson *et al.*, 2000). Bacteria species including *Klebsiella* spp, *Serratia* spp., *Escherichia coli*, *Alcaligenes* spp., *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Kluyvera* spp., and *Citrobacter freundii* have been isolated from birds of the order flaconiformes and strigiformes (Bangert *et al.*, 1988). Similarly, bacteria of the genera *Escherichia*, *Aeromonas*, *Shewanella*, *Proteus*, *Enterococcus*, and *Citrobacter* have been isolated from dead knots, Sanderling and White-rumped sandpiper in Brazil in 1997. Bacteria species such as *Micrococcus* spp. and *Staphylococcus* spp. have been isolated from the intestinal tract of juvenile greater flamingos (Rollin *et al.*, 1983). In a study conducted by Keeler Huffman (2009) at the Delaware Bay, bacteria species such as *Enterobacter sakazakii*, *Chromobacterium* spp., and *Staphylococcus hominis* which had not been previously isolated from bird species were isolated from migrating shorebirds. Another study suggested that bacteria species such as *Pseudomonas* spp., *Enterobacter cloacae*, *Micrococcus* spp., and *Staphylococcus aureus* isolated from all examined species of shorebirds (Ruddy Turnstone, sanderling, red knots, semipalmated sandpipers and dunlins) could be considered part of their normal gastrointestinal flora of these birds (Keeler & Huffman, 2009). Moreover, bacteria species such as *Alcaligenes* spp., *E. sakazakii*, *Kluyvera* spp, *Salmonella* spp., *Serratia* spp., *S. epidermidis*, *S. hominis* isolated could be considered as

transient species or opportunistic species resulting from stress or compromised immune system.

Below are commonly isolated bacteria genera in wild birds:

### **2.6.1 *Campylobacter* spp.**

*Campylobacter* spp. occur in several hosts including mammals (Rosef *et al.*, 1983) and birds (Waldenström *et al.*, 2002). The species *C. coli*, *C. jejuni* and *C. lari* are reported to be pathogenic and cause gastroenteritis in humans (Coker *et al.*, 2002). Pathogenic species have been detected in gulls, sandhill cranes, geese, whistling swans and Canada geese (Waldenström *et al.*, 2007; Lu *et al.*, 2013). Wild birds become infected when they come into contact with food or farm production animals (Pickering *et al.*, 2008). Infected wild birds may function as animal vectors spreading the bacteria via faecal droppings (Benskin *et al.*, 2009). The pathogen is often spread through the faecal-oral route in humans and birds (Keller *et al.*, 2011).

### **2.6.2 *Chlamydia* spp.**

*Chlamydia* spp. belong to the family Chlamydiaceae which has two genera and nine species (Everett *et al.*, 1999). The avian causative agent is referred to as *Chlamydophila psittaci* and is a gram-negative obligate intracellular bacterium (Andersen & Vanrompay, 2000). An infection with *Chlamydophila psittaci* is referred to as psittacosis, ornithosis or chlamydiosis. It is regarded as a list B disease by the World Organisation for Animal Health (OIE) and usually occurs in parakeets, parrots, and humans (Andersen & Vanrompay, 2000). The avian species comprises seven avian serovars (A, B, C, D, E, F, and E/B) and two mammalian serovars (WC and M56). The avian serovars are host-specific; thus, serovars A and B are frequently isolated from pigeons and psittacine birds,

serovar C is often isolated from ducks and geese, serovar D is usually isolated from turkeys, serovar E has a wide host range including humans and serovar F is usually isolated from psittacine birds and turkeys (Harkinezhad *et al.*, 2009). In 1939, *Chlamydophila psittaci* was isolated from two South African racing pigeons (Harkinezhad *et al.*, 2009). Also, they were reported in turkey in the United States in 1980 (Grimes & Wyrick, 1991) and in Europe in 1990 (Ryll *et al.*, 1994).

Currently, *C. psittaci* occurs in about 475 bird species representing 30 bird orders (Kaleta & Taday, 2003). Significant reservoirs of *Chlamydia* spp. include gulls, egrets, wild ducks etc. (Grimes *et al.*, 1979; Brand, 1989). Its occurrence is highest in birds belonging to the family Psittacidae and order Columbiformes (Harkinezhad *et al.*, 2009). In psittacine birds, its occurrence is between 16-81% with a mortality rate of about 50% (Raso *et al.*, 2002; Dovč *et al.*, 2007). Though some strains of chlamydia may not be virulent to wild avian hosts, they may be highly pathogenic to humans and domestic fowls (Grimes *et al.*, 1979).

*Chlamydophila psittaci* is transmitted via contact with faecal matter and nasal discharges (Harkinezhad *et al.*, 2009). It mainly occurs by inhalation or ingestion of contaminated material (Andersen & Franson, 2007). Avian species sharing water sources that are contaminated could be infected with the bacteria (Graczyk *et al.*, 2008). Granivorous birds are at risk of infection when they inhale dust from contaminated barnyard or grain storage sites (Harkinezhad *et al.*, 2009). Predators and scavengers may also become infected when they feed on infected carcasses (Harkinezhad *et al.*, 2009). Species such as pigeons, herons, egrets, and cormorants may transmit causative agents to their young through feeding and regurgitation (Andersen & Franson, 2007). The agent may also be transmitted from bird to bird by blood-sucking ectoparasites including flies, lice, and mites

(Longbottom & Coulter, 2003). In birds such as chickens, parakeets, seagulls, and turkeys, vertical transmission is reported, however, its frequency is low (Wittenbrink *et al.*, 1993). Infection with chlamydiae may cause pneumonia, hepatitis, splenitis, and pericarditis depending on the avian host and chlamydial strain. In psittacine birds, it may cause conjunctivitis, enteritis, and pneumonitis.

Treatment in both humans and birds is by administering tetracycline or doxycycline but preferably tetracycline. However, for pregnant women and children below nine years, erythromycin is administered (Harkinezhad *et al.*, 2009).

### **2.6.3 *Yersinia* spp.**

Yersiniosis is caused by *Yersinia* spp., a gram-negative coccobacillus. The genus *Yersinia* includes three virulent species (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) which are virulent for both animals and humans (Niskanen *et al.*, 2003). *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are both food and waterborne pathogens and widely distributed. These food and waterborne pathogens occur in avian species, however wild birds are an important reservoir of *Y. pseudotuberculosis* (Aleksić *et al.*, 1995). There are a few outbreaks of disease involving these pathogens in developed countries (Kämpfer, 2000). In Japan, several outbreaks have occurred (Inoue *et al.*, 1991). Serious epizootics in wild and domestic birds have been reported in ducks, doves, pigeons, and finches. *Yersinia pseudotuberculosis* is classified into four biotypes with about 20 serovars all of which are pathogenic. On the other hand, only a few serovars of *Yersinia enterocolitica* are pathogenic to humans and animals.

In a study conducted by Niskanen *et al.* (2003) in Sweden on 57 bird species, six different species of Yersinia were isolated, including *Y. kristensenii*, *Y. frederiksenii*, *Y. intermedia*, *Y. pseudotuberculosis*, *Y. rohdei*, *Y. enterocolitica*. The species *Y. enterocolitica* was isolated more frequently (5.6%). Three strains of *Y. pseudotuberculosis* were also isolated from two species of thrushes (Song Thrush and Red Wing) during spring migration. In a previous study by Fukushima *et al.* (1988), the serotypes 1b, 2b, 3 and 4b of *Y. pseudotuberculosis* known to be prevalent in humans were isolated from water sources contaminated with faecal matter of domestic and wild animals, suggesting an epidemiologic link between humans and wild animals. In addition, they found that the frequency of isolation from these wild animals was similar to that of humans. Also, in a French study, *Y. kristensenii* and *Y. enterocolitica* showed occurrences of 5.1% and 12.5% in 272 birds, respectively. Hubbert (1972) reported of isolation of 3 serotypes of *Y. pseudotuberculosis* along the major flyways of migratory birds in the US. In a similar study, 5% of 76 Norwegian birds were reported to harbour *Yersinia* spp. with a greater proportion occurring in non-passerine birds (Kapperud & Olsvik, 1982). Also, a prevalence of 12.8% *Yersinia* spp. was reported in wild birds from Japan (Fukushima & Gomyoda, 1991).

#### **2.6.4 *Escherichia* spp.**

The genus *Escherichia* was represented by a single species *Escherichia coli* until the 1980's when a taxonomic reorganisation was done. The genus now includes four species; *E. hermannii*, *E. vulneris*, *E. fergusonii* and *E. blattae* (Farmer *et al.*, 1985). Currently, there are five species under this genus with a new species *E. albertii* added to the list of species. The dominant species is *E. coli* (Chaudhury *et al.*, 1999).

*Escherichia coli* is a gram-negative, flagellated, rod-shaped, facultative anaerobic bacteria belonging to the family Enterobacteriaceae (Buxton & Fraser, 1977). *E. coli* is a normal inhabitant of the gastrointestinal tract (Selander *et al.*, 1987) mainly found in the lower part of the intestine (Levine, 1984). Most *E. coli* strains are non-pathogenic. Nonetheless, some strains are virulent. These virulent/diarrheagenic strains are often Enteropathogenic (EPEC), Avian pathogenic (APEC), Enterotoxigenic (ETEC), Enteroinvasive (EIEC) Enterohaemorrhagic (EHEC), Enteroaggregative (EAaggEC) and Diffuse adherent (DAEC). Avian pathogenic *E. coli* are considered a significant cause of airsacculitis and sepsis (Cunha *et al.*, 2014).

Shiga toxin-producing *E. coli* carries Shiga toxin 1 or 2 gene (Croxen *et al.*, 2013). Only a few serotypes have been associated with human illnesses though there are over 400 serotypes (Kenny, 2001). Mode of transmission is the faecal-oral route (Croxen *et al.*, 2013). Major reservoirs are ruminants and exposure to their faecal matter is a significant source of human infection (Gyles, 2007).

Enteroinvasive *E. coli* was first described in 1897 by Kyoshi Shiga during an epidemic in Japan which caused a mortality rate greater than 20% in humans (Croxen *et al.*, 2013). Mode of transmission is via the faecal-oral route through contaminated food and water (Harris *et al.*, 1985). It is underrepresented in clinical surveys because it has severe clinical manifestations (Croxen *et al.*, 2013). Clinical symptoms include diarrhoea, abdominal cramps and mucoid bloody stool (Niyogi, 2005).

Enteroaggregative *E. coli* was first identified in 1987 (Nataro *et al.*, 1987). Transmission is through contaminated food and water (Tompkins *et al.*, 1999). Clinical symptoms include nausea, abdominal pain, and vomiting (Tompkins *et al.*, 1999; Weintraub, 2007).

Enterotoxigenic *E. coli* is a major cause of traveller's diarrhoea (Qadri *et al.*, 2005). It has been isolated from both asymptomatic and symptomatic carriers (Gupta *et al.*, 2008). It is distinguished by its ability to produce heat-labile (LT) or heat-stable (ST) enterotoxin (Croxen *et al.*, 2013). Transmission is via the faecal-oral route (Croxen *et al.*, 2013). Clinical symptoms include watery stool, fever, vomiting, nausea and abdominal cramps (Dalton *et al.*, 1999).

Enteropathogenic *E. coli* was identified in the 1940s and 1950s as a cause of infantile diarrhoea (Robins-Browne, 1987). Though initially prevalent in the developed world, in recent times it is much more prevalent in developing countries (Nataro & Kaper, 1998). Enteropathogenic *E. coli* is transmitted via the faecal-oral route (Levine & Edelman, 1984). Symptoms of infection with EPEC include vomiting and fever (Robins-Browne, 1987).

Diffuse adherent *E. coli* attaches itself to cells but not in the classical adherence pattern (Nataro & Kaper, 1998). Mode of transmission is the faecal-route transmission (Croxen *et al.*, 2013). A major symptom is a watery diarrhoea (Croxen *et al.*, 2013).

Phylogenetic analysis has grouped *E. coli* into four main phylogenetic groups, A, B1, B2 and D (Herzer *et al.*, 1990; Picard *et al.*, 1999; Clermont *et al.*, 2000). The virulent or extraintestinal group is B2 and to a lesser extent group D (Picard *et al.*, 1999).

Awadallah *et al.* (2013) reported a prevalence of 48% of *E. coli* from wild birds in Egypt while Rogers (2006) reported a prevalence of 38% in California wild birds. Brittingham *et al.* (1988) reported a prevalence of 1% of *E. coli* in free-living birds from Wisconsin. A prevalence of 18.7% was also reported in a study conducted in Egypt (Hedawy & El-Shorbagy, 2007). Only 5% of 98 apparently healthy granivorous birds harboured *E. coli* in a study conducted by Glünder (1981). Similarly, Fiennes (1982) reported that the gastrointestinal flora of granivorous birds rarely harbour *E. coli*. In a study conducted by Brittingham *et al.* (1988), it was found that *E. coli* was low in both granivorous and omnivorous birds.

#### **2.6.5 *Salmonella* spp.**

*Salmonella* spp. are common enterobacteria found in the intestines of vertebrates, especially mammals and birds (Roelke-Parker *et al.*, 1996). They are commensal organisms but may become virulent when they increase significantly in number and invade their host (Tizard, 2004). They are a significant cause of gastroenteritis in humans worldwide, being the second most reported food-borne pathogen (Silva *et al.*, 2010). The prevalence of *Salmonella* spp. in healthy wild birds is usually low (Goodchild & Tucker, 1968). The prevalence of *Salmonella* spp. from 109 apparently healthy wild birds in Brazil was 2.75% (Matias *et al.*, 2016). *Salmonella* spp. was isolated from 470 carcasses belonging to 26 species of birds and 94% of the cases were reported in small passerine birds with the highest percentage (81%) recorded among bullfinches (Refsum *et al.*, 2002). Salmonellosis in wild birds frequently occurs in birds that feed on the ground or those with an omnivorous or carnivorous feeding habit (Tizard, 2004). Commonly isolated species from the intestines of wild birds is *Salmonella enterica*, serotype typhimurium (Tizard, 2004).

*Salmonella* spp. cause acute illness in passerine birds (Refsum *et al.*, 2003). In Sweden, *Salmonella enterica*, serotype *typhimurium* isolated from passerines is reported to have caused mortality in the birds (Hurvell *et al.*, 1974). Similarly, it caused mortality in the United States (Hudson & Tudor, 1957; Locke *et al.*, 1973) and England (Wilson & MacDonald, 1967). *Salmonella enterica*, serotype *typhimurium* was isolated from migrating gulls (Palmgren *et al.*, 1997). Craven *et al.* (2000) reported a prevalence of 10.6% of *Salmonella* spp. in wild birds while Awadallah *et al.* (2013) reported a prevalence of 10.7% in wild birds in Egypt. Kobayashi *et al.* (2007) reported a prevalence of 5.8% in wild birds from Tokyo bay while Vlahović *et al.* (2004) reported a prevalence of 7.4% in free-living birds in Croatia. Awadallah *et al.* (2013) reported a prevalence of 13% *Salmonella* in doves. Vlahović *et al.* (2004) also reported a prevalence of 14.3% in doves. In Bagdad, Ahmed *et al.* (2011) reported an infection rate of 95% of salmonellae in doves while in Norway and Egypt the prevalences were 0.2% (Refsum *et al.*, 2002) and 8.3% (Effat & Moursi, 2005) respectively.

#### **2.6.6 *Enterobacter* spp.**

*Enterobacter* species are aerobic gram-negative bacilli and are common nosocomial pathogens representing 6% of all hospital-acquired isolates and 11% of pneumonia isolates (Center for Disease Control and Prevention (CDC), 1996). They are ubiquitous in the environment and can survive on the skin and dry surfaces Three major species have been identified *Enterobacter cloacae*, *Enterobacter aerogenes* and *Enterobacter agglomerans* (Villegas & Quinn, 2002).

The first major outbreak of this pathogen was in 1976 leading to septicaemia in 378 patients at 25 hospitals (Maki *et al.*, 1976). Many of the outbreaks resulting from this

pathogen are due to humidifiers (Wang *et al.*, 1991) and contaminated enteral feedings (Simmons *et al.*, 1989).

### **2.6.7 *Klebsiella* spp.**

*Klebsiella* is a gram-negative bacillus of the family Enterobacteriaceae. It is common in nature and usually occurs in surface water, sewage, soil and plants (Matsen *et al.*, 1974; Bagley *et al.*, 1981). It includes several species, some of which are known to cause diseases in animals and humans. *Klebsiella pneumonia* and *Klebsiella oxytoca* are frequently isolated from humans (Podschun & Ullmann, 1998). *Klebsiella pneumonia* isolated from birds is associated with respiratory tract infections (Sandra & Duarte, 1998). Most *Klebsiella* species can be isolated from reptiles, insects, birds, and mammals. Species of *Klebsiella* are only second to *E. coli* as causes of sepsis (Feigin & Cherry, 1998). *Klebsiella variicola* has been isolated from banana plants, sugar cane, and corn.

## **2.7 Antimicrobial resistance**

Antimicrobial agents or antibiotics are drugs used to control bacterial or fungal infections (van den Bogaard, 1997). These drugs were key to controlling infectious bacterial diseases in the 20<sup>th</sup> century. However, some of these antimicrobials have been found to be ineffective in the treatment of microbes over the years (Cohen, 2000; Lederberg *et al.*, 2003). For example, Jones *et al.* (2008) reported a significant increase in EID events caused by drug-resistant microbes over the years. As such antimicrobial resistance has been described as the ability of a microbe to withstand the effects of an antimicrobial.

Antimicrobial resistance (AMR) is a recognised global health problem. The 2014 global antimicrobial resistance surveillance report suggests that high resistance rates were

observed in bacteria frequently associated with community-acquired infections and healthcare (World Health Organisation (WHO), 2014). However, Africa was poorly represented (17%) in terms of participation of member states in antimicrobial surveillance. In addition, the report revealed that *E. coli* showed resistance to a combination of third-generation cephalosporins and fluoroquinolones in some African countries. Furthermore, antimicrobial resistance was reported for *Shigella* spp, *Klebsiella pneumonia*, and *Staphylococcus aureus*.

The overuse of antimicrobial agents in humans and veterinary medicine has been blamed for the increasing emergence of antimicrobial resistance and the dissemination of resistance genes. Thus, environmental pollution by antimicrobials or resistant bacteria through human activities such as antimicrobial use in medicine and agriculture has led to the widespread occurrence of antimicrobial resistance (Martinez, 2009).

Various antimicrobial agents have been widely used on farm animals. These antimicrobial agents serve as Antimicrobial Growth Promoters (AGP) and have been widely used in the poultry industry since the 1950s. Thus, to reduce the costs of production, AGPs are added to feed to promote weight gain (Jukes *et al.*, 1950). In contrast to the therapeutic usages of antimicrobials, AGPs are administered sub-therapeutically at low doses over longer periods and this has been reported to favour the selection of resistant microorganisms (Diarra *et al.*, 2007). The discussion on AGP heightened with the finding that administration of Avoparcin, a glycopeptide AGP, was involved in emerging glycopeptide-resistant bacteria (Howarth & Poulter, 1996). This led to strong recommendations for the use of AGP in the European Union (EU). Similarly, the use of Colistin as an AGP in livestock led to the emergence of plasmid-mediated mechanisms involved with polymyxin resistance (Rhouma *et al.*, 2016). As a result, the World Health

Organisation (WHO), World Organisation for Animal Health (OIE) and Food and Agriculture Organisation (FAO) carried out systematic evaluations on the impact of veterinary antimicrobial resistance on public health (WHO, 2014). In their report, they stated that the misuse and overuse of antimicrobial agents are accelerating the processes of antimicrobial resistance.

Antimicrobial resistance has also been reported in places where the use of antimicrobial agents is rare or usually considered as “pristine environments” such as forests, artic, savannahs, and deserts. Animals that have been commonly examined for antimicrobial resistance include birds, small mammals, and primates that have been examined for the presence of antimicrobial-resistant bacteria (Rwego *et al.*, 2008; Sjölund *et al.*, 2008; Radhouani *et al.*, 2014; Jobbins & Alexander, 2015).

### **2.7.1 Antimicrobial resistance in Ghana**

Ghana, a resource-limited setting, is highly burdened with the incidence of infectious diseases and require antimicrobial therapy to save lives (Lozano *et al.*, 2012). The first-ever AMR surveillance in Ghana was between 2002 and 2003 (Newman *et al.*, 2011). In another surveillance to determine antimicrobial resistance in Ghana, the results showed that most of the isolates were multidrug-resistant and over 80% of them were extended-spectrum beta-lactamases-producing. The bacteria species that showed resistance to the antimicrobial agents included *Escherichia coli*, *Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus* spp. and *Salmonella enterica* serovar Typhi (Opintan *et al.*, 2015). In another study conducted at the Korle-Bu Teaching hospital over a three month period, the overall prevalence of bacteria producing extended-spectrum beta-lactamase was 49.3% (Obeng-Nkrumah *et al.*, 2013). Since Korle-Bu Teaching hospital is a major

referral health facility in Ghana, the results from the study were indicative of the heavy antimicrobial selection pressure in Ghanaian hospitals.

The research conducted by Groß *et al.* (2011) on patients with bacteria bloodstream infections in some rural hospitals in Ghana in the years, 2001, 2002 and 2009 showed that although ciprofloxacin proved to be an effective drug against *S. enterica*, the resistance rate of this quinolone increased from zero in 2001 to 50% in 2009. Namboodiri *et al.* (2011) found that resistance to quinolones, particularly, Ciprofloxacin is common in Ghana and occurs via multiple mechanisms. They also found that quinolone resistant *E. coli* were also resistant to multiple antimicrobials.

### **2.7.2 Antimicrobial resistance in wildlife**

Evidence from most studies has demonstrated the occurrence of AMR bacteria exchanges between humans, wildlife and livestock. These exchanges are mainly due to the growing human population and the fragmentation of natural habitats which have led to frequent contacts (direct or indirect) between the human, livestock, and wildlife interfaces (Arnold *et al.*, 2016).

Although AMR is considered an important threat to the security of global health, the focus of most AMR research was on a clinical setting (Davies & Davies, 2010). Most of the studies focussing on antimicrobial resistance in wildlife did not investigate the whole bacteria community but rather assessed whether a bacterial species could be found in a particular host population (Vittecoq *et al.*, 2016).

Antimicrobial agents and resistant isolates are excreted by patients or livestock and these are dispersed in the environment via sewage effluents pumped into rivers (Graham *et al.*, 2014). Consequently, sewage effluents may runoff into the sea resulting in contamination

of beaches and estuaries (Graham *et al.*, 2014). Human and sea animals that come into contact with this contaminated water could be exposed to AMR (Leonard *et al.*, 2015).

In a review by Vittecoq *et al.* (2016), they found that only three bacterial groups in wildlife were studied in more than 10% of the 210 studies they analysed. These bacterial groups were *E. coli*, *Salmonella* spp. and *Enterococcus* spp. with *E. coli* being the most frequently targeted bacteria in AMR studies. Extended-spectrum beta-lactamase-producing *E. coli* have been frequently found in wildlife (Guenther *et al.*, 2011). *Salmonella enterica* serotype Typhimurium has also been isolated from wild mammals (Caleja *et al.*, 2011) and birds (Čížek *et al.*, 2007). Enterococci have also been frequently studied in wildlife because they act as indicators of antimicrobial resistance in gram-positive bacteria (Vittecoq *et al.*, 2016).

Other bacteria species groups that have been isolated from wildlife include *Campylobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Kluyvera* spp. and *Staphylococcus* spp.

### **2.7.3 Antimicrobial resistance in farm animals**

Farm animals exposed to antimicrobials through antibiotic prophylaxis or via growth promoters may serve as reservoirs (Silbergeld *et al.*, 2008). Direct contact with these reservoirs, may lead to the acquisition or spread of these resistant bacteria (Hammerum & Heuer, 2009). A major issue with the development of antimicrobial resistance is that the same drugs are administered to both humans and animals for prophylaxis and treatment (Aarestrup, 1999). Thus, when resistance occurs in humans, there are chances that it could be transmitted to animals via the food chain.

The intensification of production is a result of increasing demand for livestock production with the possibility of disease outbreak (Rushton, 2015). This may have possibly led to an increase in the use of antimicrobial agents as growth promoters. However, in some countries the use of AGPs has been curtailed (Landers *et al.*, 2012), while in other countries it is poorly controlled.

Results from molecular typing of *E. coli* isolates from pigs showed that isolates were resistant to Cefotaxime and about 65% showed resistance to Ciprofloxacin (Hu *et al.*, 2013). In 2007, a study suggested that resistance to Ciprofloxacin is of great concern since it is a second-generation fluoroquinolone with less than twenty years of FDA approval at that time (Goossens *et al.*, 2007).

A strong correlation has been found between antibiotic use and the isolation of antibiotic-resistant *E. coli* from livestock worldwide (Barton, 2014). Widespread resistance has also been described for antimicrobial classes of tetracycline, aminoglycoside, fluoroquinolone and penicillin (Hu *et al.*, 2013). In Denmark, though the prescription of tetracyclines increased between 2002 and 2008, fluoroquinolones and cephalosporins decreased within the same time period (Vieira *et al.*, 2011). In Canada and the United States, tetracyclines remain the commonly prescribed antimicrobial in animal husbandry (Apley *et al.*, 2012). In Ghana, the Ashanti, Central, and Greater Accra regions constitute about 74% of commercial poultry production (Aning, 1995). The industry faces increasing disease outbreaks and this has made the use of antimicrobials common (Aning, 1995). Some of the antimicrobials/antimicrobial agents are also added to the feed as AGPs (Donkor *et al.*, 2011). The global consumption of antimicrobials in food animal production is projected at 67% between 2010 and 2030; thus, about 95 million kg of antimicrobials will be consumed by 2030 (Van Boeckel *et al.*, 2015).

Although wild birds rarely come into contact with antimicrobials, they may become infected with resistant strains via contact with contaminated water and food (Kozak *et al.*, 2009). Therefore, wild birds could act as reservoirs of resistant bacteria and could also be used as genetic determinants of antimicrobial resistance (Dolejska *et al.*, 2007). For example, *Enterococcus* spp. and *E. coli* isolates from wild species were reported resistant to antimicrobial agents for the first time from Japanese wild birds (Sato *et al.*, 1978).

#### **2.7.4 Antimicrobial classes**

Antimicrobial agents are mainly classified based on their molecular structure and their antimicrobial mechanisms.

##### **2.7.4.1 Aminoglycoside**

Aminoglycoside is a broad-spectrum antimicrobial class used for the treatment of life-threatening infections. In 1944, the first aminoglycoside, Streptomycin, was reported (Schatz & Waksman, 1944). Thereafter, other aminoglycosides such as Kanamycin, Gentamicin, and Tobramycin were introduced. In 1970, the semi-synthetic aminoglycosides such as Dibekacin, Netilmicin, and amikacin were found to have active compounds against strains that had developed resistance to the earlier aforementioned aminoglycosides (Mingeot-Leclercq & Tulkens, 1999). Aminoglycosides are effective in the treatment of clinically important gram-negative bacteria including *Serratia* spp., *Pseudomonas* spp., *Enterobacter* spp., *Morganella* spp., *Citrobacter* spp., *Salmonella* spp., *Acinetobacter* spp. *Proteus* spp, *Klebsiella* spp. *Escherichia coli* as well as some streptococci and *Staphylococcus aureus* (Vakulenko & Mobashery, 2003).

Streptomycin, the first aminoglycoside, was active against *Mycobacterium tuberculosis* (Schatz & Waksman, 1944) and it remains a first-line antimicrobial agent for the treatment of *Mycobacterium tuberculosis* when used in combination with chemotherapy (Gillespie, 2002).

#### **2.7.4.2 Beta-lactam**

Beta-lactams were the first antimicrobial class to be described (Queener, 1986) and they include Penicillins, Cephalosporins, Monobactams, and Carbapenems (Donowitz & Mandell, 1988). All have a beta-lactam ring, which is essential for antimicrobial activity. To date, this class of antimicrobial agents has been classified into four functional groups, ambler classes A, B, C, and D.

Beta-lactams are faced with the most significant threat of resistance. This is because the rapid evolution of beta-lactamases causes every new drug under this class to become obsolete within a short time after the introduction (Drawz & Bonomo, 2010). Beta-lactams are prescribed more than other antimicrobial agents mainly because of their comparatively high effectiveness, low toxicity, and low cost (Wilke *et al.*, 2005). The growing number of beta-lactam antimicrobial agents and the heavy use of these antimicrobial agents have resulted in the selection of multidrug-resistant bacteria or the survival of organisms caused by the production of beta-lactamases (Massova & Mobashery, 1998). Beta-lactamases have been discovered in a number of gram-negative bacteria including *Klebsiella* spp., *Enterobacter* spp., and *P. aeruginosa* (Bonomo *et al.*, 2004).

#### **2.7.4.3 Quinolone**

Quinolones are a class of synthetic antimicrobial agents. There are several antimicrobial agents under this class and they are classified into four generations (Drlica, 1999). The first generation quinolone antimicrobial agents include Nalidixic acid and Cinoxacin and they are effective against gram-negative bacteria but not *Pseudomonas* species (Just, 1993). The second-generation antimicrobial agents are effective against gram-negative bacteria (including *Pseudomonas* species), some gram-positive organisms (including *Staphylococcus aureus* but not *Streptococcus pneumoniae*) and some atypical pathogens (Nichols, 2000). The second-generation antimicrobial agents include Norfloxacin, Lomefloxacin, Enoxacin, Ofloxacin, and Ciprofloxacin, with Ciprofloxacin being the most potent against *P. aeruginosa* (Stein & Ensberg, 1998). The third-generation quinolones including Levofloxacin, Sparfloxacin, Gatifloxacin, Moxifloxacin have similar antimicrobial spectrum as second-generation antimicrobials but in addition, they have expanded gram-positive coverage and expanded activity against atypical pathogens (Abramowicz, 2000). Fourth-generation quinolones have the same antimicrobial spectrum as third-generation agents but have broad anaerobic coverage in addition (Symonds & Nix, 1992). Unlike, the first-generation quinolones, the second, third and fourth contain fluoride and are therefore referred to as fluoroquinolones (Wolfson & Hooper, 1989).

#### **2.7.4.4 Tetracycline**

This class of antimicrobial agents was discovered in the 1940s and was active against several microorganisms including both gram-negative and gram-positive bacteria (Chopra *et al.*, 1992), rickettsiae, chlamydiae, mycoplasmas and protozoan species (Cunha, 1985). Oxytetracycline and Chlortetracycline were the first members of the tetracycline group to be described (Darken *et al.*, 1960). Antimicrobials of this class are inexpensive and are

extensively used in human and animal therapy due to their broad-spectrum activity and relative safety (Curiale *et al.*, 1984). In some countries in the United States, they are added to feed of farm animals at sub-therapeutic levels to act as growth promoters (NRC, 1999). In 1953, the first tetracycline-resistant bacterium, *Shigella dysenteriae* was reported and since then, the occurrence of tetracycline resistance has been reported severally (Chopra & Roberts, 2001).

#### **2.7.4.5 Macrolide**

Macrolides are broad-spectrum antimicrobials used against gram-positive bacteria such as beta-hemolytic streptococci and pneumococci. This class of antimicrobial evolved in 1952 when erythromycin was isolated from the bacterium *Saccharopolyspora erthraea* (Roberts *et al.*, 1999). Generally, gram-negative bacteria are resistant to this class of antimicrobials with the exceptions of *Legionella*, *Campylobacter*, *Bordetella pertussis* and *Helicobacter* species) (Leclercq, 2002). Macrolide compounds have two or more amino or neutral sugars attached to a lactone ring of variable size. They are commercially available as a 14-membered lactone ring (Dirithromycin, Roxithromycin, Clarithromycin, and Erythromycin) or 15-membered lactone ring (Azithromycin). In some countries, there are 16-membered lactone ring macrolides such as Rokitamycin, Josamycin, Spiramycin, Miocamycin, and Midecamycin) (Leclercq, 2002).

The first incidence of resistance to erythromycin was reported in 1956 and was detected in staphylococci (Weisblum, 1995). Resistance to macrolides is by three mechanisms: (1) drug inactivation (2) through target-site modification by methylation or mutation that prevents the binding of the antibiotic to its ribosomal target (3) through efflux of the antibiotic (Leclercq, 2002). Ribosomal methylation is the most widespread resistance

mechanism in macrolides (Leclercq, 2002). The first case of erythromycin-resistant strains in streptococci was reported in the United Kingdom in 1959 and North America in 1967 (Dixon, 1968).

#### **2.7.4.6 Phenicol**

Chloramphenicol was the first antimicrobial agent to be introduced under this class of antimicrobials. It was isolated from *Streptomyces venezuelae* in 1947 and the first natural antimicrobial found to contain a nitro group (Schwarz *et al.*, 2016). Other derivatives of Chloramphenicol include Thiamphenicol and Florfenicol (Dowling, 2013). It is active against a broad spectrum of microorganisms including gram-positive and gram-negative bacteria (Shaw, 1983). However, since the 1960s, several adverse effects of administering this antimicrobial agent have been observed (Schwarz *et al.*, 2004). Hence, it is only used for topical applications. The use of chloramphenicol in food-producing animals was banned in the EU in 1994 and thereafter other countries also banned the use of this antimicrobial in food-producing animals (Schwarz *et al.*, 2016).

#### **2.7.4.7 Polymyxin**

Polymyxins are a class of non-ribosomally positively charged ions, cyclic peptide antimicrobial agents. They were originally found to be produced by the gram-positive bacterium *Paenibacillus polymyxa* (Caniaux *et al.*, 2017). Their antibacterial properties were recognised in the 1940s but their introduction into regular clinical therapies was hampered by their potential nephrotoxicity and neurotoxicity (Brown *et al.*, 1970; Nation & Li, 2009). However, the high incidence of Multidrug Resistance (MDR) to

antimicrobial classes such as beta-lactams, quinolones, aminoglycosides, and tetracyclines has renewed interest in polymyxin (Falagas *et al.*, 2005; Li *et al.*, 2006).

There are five known subtypes of polymyxin (A, B, C, D, E) of which polymyxin B and Colistin (polymyxin E) have been used in the clinical setting (Stansly *et al.*, 1947). Intrinsic resistance to polymyxin has been observed in *Serratia* spp., *Providencia* spp. and *Proteus* spp. (Kwa *et al.*, 2008).

Colistin was discovered in 1949 and is used as a ‘last resort’ antimicrobial agent. Thus, it is used in treating life-threatening conditions like septicaemia (Li *et al.*, 2006). It belongs to the polymyxin class of antimicrobials. The role of Colistin as the “last resort” has necessitated the need for research into monitoring antimicrobial resistance in this polypeptide (Kempf *et al.*, 2016). Two forms of Colistin are commercially available: colistimethate sodium for parenteral use and Colistin sulfate for oral and topical use. However, only Colistin sulfate has been recommended for susceptibility testing (Li *et al.*, 2005; Sun *et al.*, 2009).

Colistin is the most active antimicrobial used in the treatment of gram-negative infections especially *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Jain & Danziger, 2004; Li *et al.*, 2005). In pig and poultry production, Colistin is highly effective against *E. coli* and *Salmonella enterica*, though in recent years there are reports of Colistin resistant *E. coli* strains (Boyen *et al.*, 2010).

In some Asian countries such as Japan, India, Vietnam, and China, Colistin is added to the feed of farm animals as growth promoters (Kempf *et al.*, 2016). In Europe, it is used for the treatment of Enterobacteriaceae infections in sheep, cows, goats, chickens, and

pigs (Catry *et al.*, 2015). In addition, Colistin is widely used to promote growth in livestock (pigs and poultry) in Brazil and has been approved for use by the Food and Drug Administration in the USA (Fernandes *et al.*, 2016).

## **2.8 Antimicrobial resistance genes**

Resistance to antimicrobials can be intrinsic or acquired. Acquired resistance can result from mutation of cellular genes, or acquisition of foreign resistance genes (horizontal gene transfer) or a combination of both (Džidić *et al.*, 2008). Several genes have been identified to be responsible for antimicrobial resistance. These genes can be found in both commensal and pathogenic bacteria (Džidić *et al.*, 2008). The identification of these genes is necessary for verification of resistant phenotypes. Once resistance genes are acquired they are not easily lost but rather additional genes add on to them making them multidrug-resistant and leading to a diminished treatment option (Tauxe *et al.*, 1989; Summers, 2006).

Horizontal gene transfer is the principal mechanism for the spread of antibiotic genes. Three main mechanisms have been identified for this transfer including conjugation, transformation, and transduction (Thomas & Nielsen, 2005). Transformation is the only mechanism under the control of the bacteria, the remaining two are mediated by semi-autonomous temperate phages and conjugative elements (conjugative plasmids are the most significant) respectively (Thomas & Nielsen, 2005).

Plasmid-mediated resistance is the transfer of antibiotic resistance genes via plasmids (Bennett, 2008). These plasmids can be transferred between the same species of bacteria or different species via a mechanism known as conjugation (Thomas & Nielsen, 2005).

Unlike intrinsic resistance that is caused by functional expression or mutation of chromosomal genes, plasmid-borne genes have the potential to transfer resistance (Liu *et al.*, 2016).

Quinolone resistance is often caused by chromosomal mutation. However, plasmid-mediated quinolone resistance has been recently discovered. The gene responsible for this resistance is *qnr*. *QnrA* was the first gene that was detected by Luis Martinez (Martínez-Martínez *et al.*, 1998). *Qnr* proteins are capable of protecting DNA gyrase from quinolones (Jacoby *et al.*, 2009). Another study also isolated a variant of *qnrA* differing from the one detected by Martinez (Castanheira *et al.*, 2007). In a study conducted by Wang *et al.* (2003) they detected *qnr* genes in 11% of *Klebsiella pneumonia* isolates and no *qnr* genes in 38 quinolone-resistant *E. coli* strains tested. Six *qnr* genes, *qnrA*, (Martínez-Martínez *et al.*, 1998), *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC*) have been identified till date.

The mobilized colistin resistance (*mcr*) gene, which confers plasmid resistance colistin, was detected in strains in the 1980s, *mcr-1* was the first gene to be detected in strains from poultry from China (Liu *et al.*, 2016). A major outbreak was reported in 2009 in broilers; however, the percentage increased to about 30% by 2014 (Shen *et al.*, 2016). In places like Europe and Japan the prevalence of Colistin resistant strains recorded was very low (Kempf *et al.*, 2016). The *mcr-1* resistant strains are usually *E. coli* strains and these strains have been reported in North America, Asia, and Europe. In Africa, *mcr-1* positive *E. coli* isolates have been reported to occur in chicken from Algeria and one human isolate from Nigeria (Olaitan *et al.*, 2016). In 2016, *mcr-2* plasmid-mediated Colistin resistant gene determinant was reported in *E. coli* isolates from Belgium (Xavier *et al.*, 2016). In 2017, *mcr-3* plasmid-mediated Colistin resistant gene was reported by Yin *et al.*, 2017. The *mcr-*

4 plasmid-mediated gene was first reported by Carattoli *et al*, 2017 and *mcr-5* plasmid-mediated gene by Borowiak *et al.*, 2017.

## **2.9 Exposure of birds to enteric and antimicrobial-resistant bacteria**

Several factors expose wild birds to enteric bacteria. The feeding ecology of birds has been suggested as the main factor influencing the exposure of wild birds to enteric bacteria (Cornelius, 1969; Williams *et al.*, 1976; Fenlon, 1981). For example, birds of prey such as raptors are exposed to enteric bacteria from the intestines of the prey they feed on. Similarly, scavengers are at risk of acquiring enteric pathogens from carcasses. A study conducted by Luechtefeld *et al.* (1980) suggests that enteropathogens are frequently isolated from waterfowls that strain mud to obtain food or feed on animals while waterfowls that feed solely on vegetables have a low prevalence of enteropathogens. Benskin *et al.* (2009), also suggested that ground-foraging animals may ingest food contaminated by infected bird droppings or may feed on filter-feeding mollusc prey from sewage-contaminated environments.

Bird aggregation/congregation is another factor that has been suggested to expose wild birds to enteric bacteria. Thus, most pathogens may be transmitted in a density-dependent manner (Andersen & Vanrompay, 2000). Birds usually congregate at staging sites during migration to feed, and this may increase the risk of transmission of enteric bacteria to otherwise healthy birds.

Furthermore, wild birds that come into contact with sewage or human refuse are likely to pick enteric bacteria. If birds feeding at sewage outfalls pick up pathogens, they may spread it to other birds in the population. When sewage effluents are present in the habitat

of filter-feeding organisms, pathogens become concentrated in them and thereby, they may serve as a source of infection to predators (birds). Various wading birds such as oystercatchers *Haematopus ostralegus*, which feed mainly on bivalves (Fricker, 1984) and shore-foraging birds feeding on invertebrates (Waldenström *et al.*, 2002) have been reported to harbour a high prevalence of *Campylobacter*. Species feeding in urban habitats face a high risk of human infection. A range of bacterial pathogens including pathogenic *E. coli* such as vero-cytotoxin-producing *E. coli* 0157, *Salmonellae*, *Listerias*, and *Campylobacters* have been isolated from the faeces or cloacae of gulls (*Larus* spp.), lapwings *Vanellus vanellus* and corvids feeding at refuse sites (Quessy & Messier, 1992; Wallace *et al.*, 1997). A comparative study of gull feeding habitats recorded higher prevalence of *Campylobacter* and *Salmonella* species from gulls feeding at rubbish dumps than from coastal- and inland dwelling places (Williams *et al.*, 1976; Glünder & Siegmann, 1989).

## **2.10 Susceptibility of wild birds to enteric and antimicrobial-resistant bacteria**

Several factors may make birds susceptible to infections. Factors such as body size, sex, and age have been suggested to affect susceptibility to bacterial infection (Benskin *et al.*, 2009).

### **2.10.1 Age**

Previous studies have established that birds can become infected with bacteria especially pathogenic ones at all stages even when they are not hatched. Thus, bacteria can infect eggs by penetrating (Cook *et al.*, 2003). When eggs are hatched, they may become infected with bacteria via food from parents and materials from their nests (Lombardo *et al.*, 1996).

Bacteria may also be transmitted sexually through cloacae during the breeding season (Reiber *et al.*, 1995).

### **2.10.2 Body size**

The body size of animals has been suggested to play a role in the acquisition of enteric bacteria. Thus, body size may influence susceptibility to pathogens if the bacterial acquisition occurs predominantly through foraging, as larger individuals should eat more, increasing their exposure to infected food (Zuk & McKean, 1996; Moore & Wilson, 2002).

With regard to foraging, body size may influence susceptibility to bacterial infections. For instance, larger birds feed more and therefore have increased exposure to infected food (Arneberg *et al.*, 1998). A positive correlation between host body size and parasite load has been established for mammals that show sexual dimorphism (Moore & Wilson, 2002). However, a comparative study in birds showed otherwise; thus, the study concluded that host size dimorphism has no effect on the prevalence or intensity of infections (McCurdy *et al.*, 1998).

### **2.10.3 Sex differences**

Sex differences may also play a role in the susceptibility of animals to pathogens. Some studies have suggested that males and females differ in their susceptibility to pathogen (Moore & Wilson, 2002; Robb & Forbes, 2006). In the majority of bird species, males lack an intromittent copulatory organ (Briskie & Montgomerie, 1997), hence sperm transfer occurs through very brief cloacal contact (Sheldon, 1993; Lombardo, 1998). Given the brevity of copulation, males should have relatively little chance of contracting bacteria pathogens from contact with the female cloaca. On the other hand, females have

prolonged exposure to the ejaculate once it enters the reproductive tract and may become infected with any pathogen from the male ejaculate (Lombardo, 1998). This means of pathogen transmission has been documented in domestic fowls (Perek *et al.*, 1969) and in Red-winged blackbirds *Agelaius phoeniceus* (Westneat & Birch Rambo, 2000). Differences in exposure to the bacterial transmission through copulation may render females more susceptible to pathogens than males.

### **3.0 CHAPTER THREE: MATERIALS AND GENERAL METHODS**

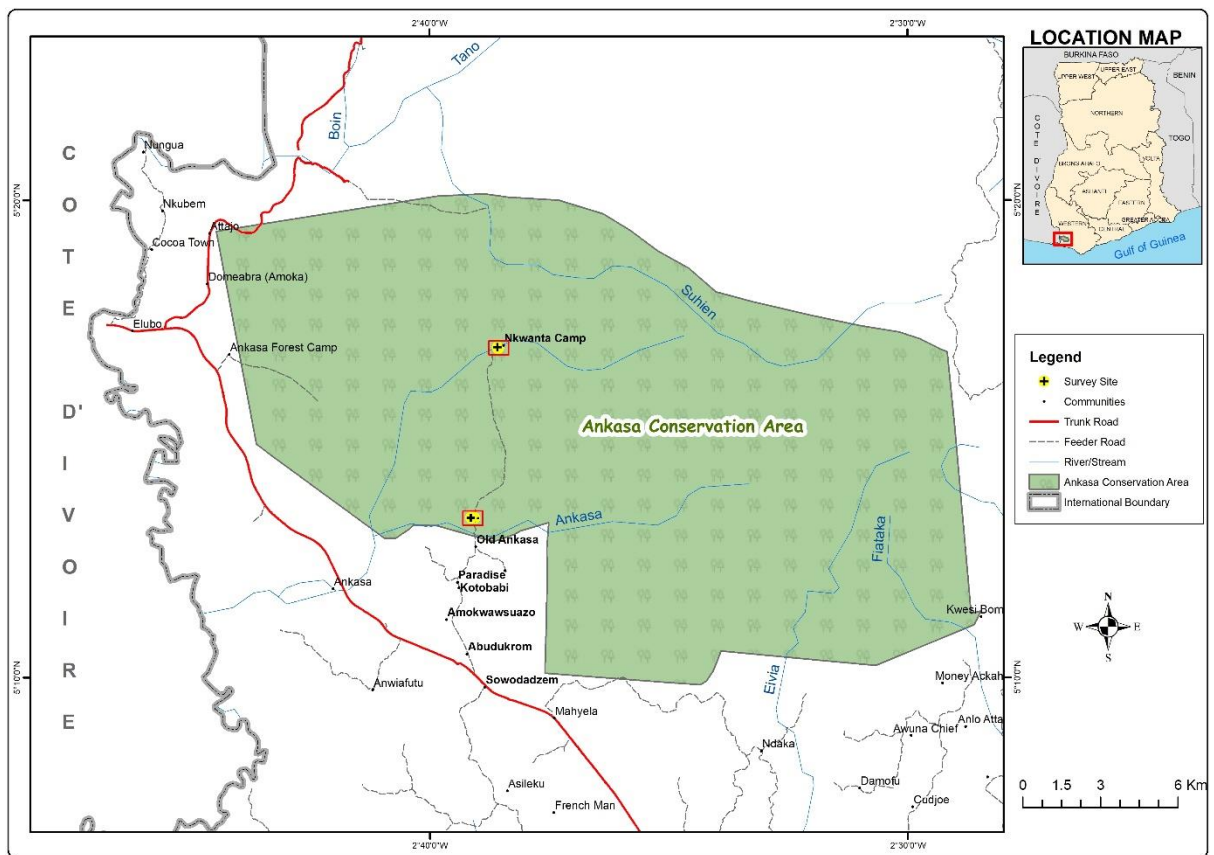
#### **3.1 Study areas**

The study was undertaken in three sites: Esiama beach and Densu Delta Ramsar site for waterbirds and the Ankasa Conservation Area (ACA) for forest birds. The Ankasa Conservation Area was selected because it is a natural environment with relatively little human disturbance. The Esiama beach was selected because it is home to many waterbird species and located in the same region as the Ankasa Conservation Area. The Densu Delta Ramsar site was added to the study areas because only a few species of waterbirds were captured at the Esiama beach during the reconnaissance study. The Densu Delta Ramsar site also harbours several species of waterbirds and was also selected because of its proximity to the University of Ghana and the high probability of trapping different species in this area.

##### **3.1.1 Ankasa Conservation Area (ACA)**

The Ankasa Conservation Area (ACA) is a protected area comprising the Nini Suhien National Park and the Ankasa Resource Reserve. It is located in the Western Region of Ghana (Figure 3.1). It lies within the administrative districts of Jomoro, Ellembelle and Wassa Amenfi West (Damnyag *et al.*, 2013). The site, originally the Ankasa Forest Reserve, was managed for timber production until 1976 when it was gazetted as a wildlife protected area. The Nini Suhien National Park covers an area of 166 km<sup>2</sup> (33%) while the Ankasa Resource Reserve covers 343 km<sup>2</sup> (67%). The Conservation Area is a wet evergreen forest and covers an area of approximately 50,900 ha of land (UICN/PACO, 2010). The site was selectively logged until 1976 but the Nini Suhien part was untouched and remains in an almost pristine state (Hawthorne, 1999). The Ankasa Conservation Area

has a mean annual rainfall of 2000 – 2200 mm (Jachmann, 2008). It is biodiverse and home to several species of plants and animals including 800 vascular plants, 600 butterfly species (UICN/PACO, 2010), 43 mammal species and 10 primate species (Forestry Commission of Ghana (FCG), 2018). Two roads and a powerline run through a larger portion of the Conservation area. The Old Nkwanta village and the Nkwanta camp serve as corridors and secondary forest habitats for non-forest animal species (Rödel *et al.*, 2005). The surrounding communities produce cash crops such as cocoa, rubber and palm plantations (Damnyag *et al.*, 2013). Mistnets for trapping birds in this study area were set around the study sites indicated in yellow on the map (reception area, N°05.28192° W°002.64266° ; about 8 km away from the reception area N°05.22194° W°002.65180° ).



**Figure 3.1: Map of Ankasa Conservation Area showing survey areas**

### 3.1.2 Esiama Beach

The Esiama beach is located in the Western Region of Ghana within the administrative district of Ellembelle (Figure 3.2). It is a 13 km stretch of sandy beach that lies between the Ankobra and Amansure estuaries. This sandy beach is home to migratory waterbirds. The most abundant waterbird found on this sandy beach is the Sanderling *Calidris alba* (Ntiamo-Baidu & Gordon, 1991; Ntiamo-Baidu *et al.*, 2014). The Esiama beach holds an average of 1,200 to 2,850 Sanderlings between the period of September to February annually constituting about 40-70% of the total Sanderling population in Ghana, especially during the non-breeding period (Reneerkens *et al.*, 2009). Ntiamo-Baidu *et al.* (2014) suggest that the site should be given a conservation priority as it holds about 3.5% of the Sanderling population connecting West and South Africa to Greenland. Samples from this study area were collected from a roosting site near the Asenko Village (04.55.11N 02.19.08W).



Figure 3.2: Map of Esiama beach showing survey area

### 3.1.3 Densu Delta Ramsar Site

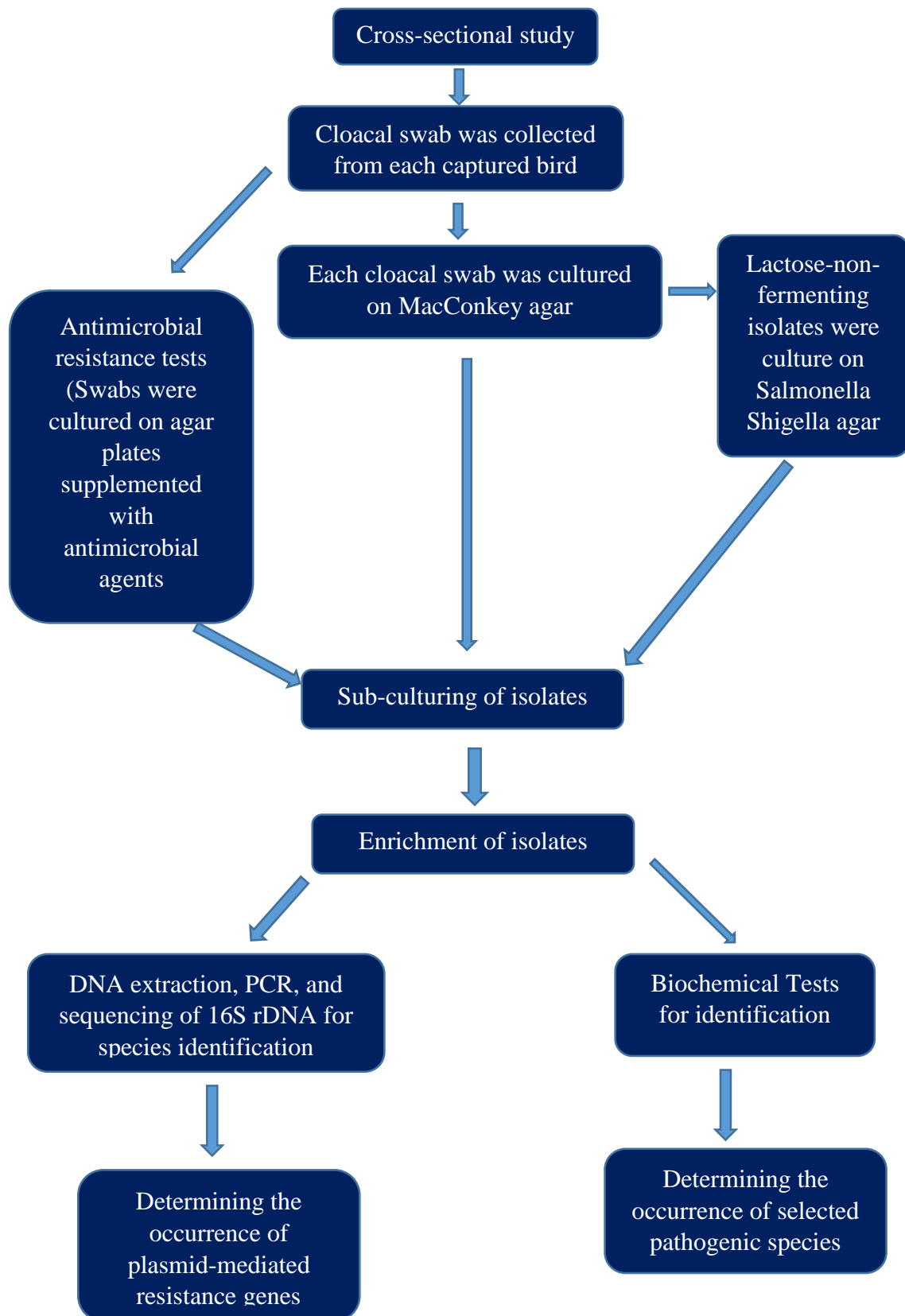
The Densu Delta Ramsar site is one of the five coastal Ramsar sites in Ghana. It is located in the Greater Accra region of Ghana, approximately 11 km southwest of Accra ( $5^{\circ} 31' N$   $0^{\circ} 20' W$ ). The wetland is *ca.* 22 km<sup>2</sup> and situated close to the confluence of the Densu River. It comprises an open lagoon, marsh, sand dunes, saltpans, and scrub. It is an important site for resident and migratory water birds. A greater proportion of this Ramsar site is owned by the Panbros Salt Industry Ltd. Surrounding communities include Aplaku, Tetegbu, Bortianor, and Grefi.



**Figure 3.3: Map of Densu Delta Ramsar Site showing survey areas**

### 3.2 Study Design

The study design is outlined in the chart below



### **3.3 Bird Trapping**

The study employed a repeated cross-sectional sampling approach. Samples were collected between September 2015 and April 2017.

Birds were captured with nylon mist-nets. Mist netting is an effective trapping method for detecting the presence of birds especially understory birds. Though an effective method, it cannot be used to census all forest species as it is strongly biased toward birds in the lower stratum (Remsen Jr & Good, 1996). However, the goal of the study was not to carry out a census of birds.

For the capture of forest birds, about 6-10 mist nets were erected. The nets were 12 – 18 m long and had four pockets/shelves each. The mesh size of the nets used was about 15 mm and the distance between erected nets were approximately 10 m. Using the road running from the reception through the old Nkwanta village as a transect, 3-5 nets were erected on each side of the road. The nets were erected the evening before the first day of capture and remained furled until the following morning (Plate 3.4). Nets were usually erected at two locations: near the Nkwanta camp (about 8 km from the reception) and about 800 m away from the reception. On the day of trapping, the nets were opened between 6-7 am (06h00-07h00) and checked every 30 min until about 4 pm (16h00). On the whole, a total of 20-22 mist net hours were accumulated within 2-3 days. In the event of rains or strong winds, the nets were closed. Captured birds were removed from the nets and each placed in a cotton bag (Plate 3.5). The mouth of the bag was tied leaving a little space for air passage. The captured birds were then transported to the processing area. To minimize handling time, the birds were processed immediately they arrived at the processing area and released within 30 mins from time of capture.

### **3.4 Processing of captured birds**

At the processing area each bag containing a bird was weighed (Plate 3.6). Each bird was removed from the bag and the empty bag was weighed to obtain the actual weight of the bird. Following this, the bird was identified, assessed for health by doing a physical examination for any sign of disease. However, it was difficult to do a complete health screening in the field, hence sampled birds without any discharge from the nostrils, eyes, and mouth were presumed to be healthy. Bird identification was done by an expert from the Ghana Wildlife Society. Also, a bird expert from the Centre for African Wetlands identified the waterbirds. In order to identify recaptures, each bird was ringed (Plate 3.7) according to the Ghana ringing scheme.

For sexually dimorphic species, sex of the bird was recorded. Other information recorded were the ring number, age of the bird and any physical abnormality. The age of the bird was determined using plumage characteristics and the birds were classified as either juvenile or adult.

For the capture of waterbirds, nylon mist nets were set about 2 km apart. A total of 4 nets were usually set taking into consideration the roosting sites of the birds. The nets were about 6-14 m in length and had only two pockets. The nets were set at about 3-4 pm and closed until about 7 pm. Sampling usually started from 7 pm and remained open until 5 am. At Esiama beach, trapping was done at the roosting site near the Asenko village while at the Densu Delta, nets were set on muddy culverts of salt pans at roosting sites. All other procedures remained the same as for forest birds.



**Plate 3.1: Setting of mist nets in the Ankasa Conservation Area (ACA)**



**Plate 3.2: Removal of a trapped bird from the mist net**



**Plate 3.3: Weighing cotton bag containing captured bird**



**Plate 3.4: Collecting faecal swab from a captured bird**



**Plate 3.5: Ringing birds prior to release**

### **3.5 Sample collection**

Cotton-tipped sterile swabs were used to collect faeces from the cloaca of trapped birds (Plate 3.8). Cloacal swabs were collected by inserting a sterile swab into the cloaca and gently rotating the tip against the mucosa. The swabs were placed in 1.5 ml microtubes containing 200  $\mu$ l of Phosphate Buffered Saline solution (PBS) (Plate 3.9). All samples collected were transported on ice packs to the laboratory. Once in the laboratory, samples were stored at  $-20^{\circ}$  C and processed in the Microbiology laboratory, Department of Animal Biology and Conservation Science, within 7 days after sample collection.



**Plate 3.6: Eppendorf tube containing PBS and cloacal swab**

### **3.6 Laboratory examinations**

Samples were cultured on selective and differential agar for gram-negative enterobacteria isolation until pure colonies of isolates were obtained. The isolates were enriched on nutrient agar and subjected to biochemical testing such as Triple Sugar Iron (TSI) and Indole tests.

Samples were later cultured on agar plates containing Colistin or Ciprofloxacin. All Colistin-resistant isolates were further subjected to disc diffusion method to determine whether they were multidrug-resistant to five antimicrobials including Colistin and Ciprofloxacin.

Antimicrobial-resistant isolates were molecularly tested for the occurrence of resistance genes for Colistin and Ciprofloxacin.

All presumptive *Escherichia coli*, *Salmonella* spp., *Yersinia* spp. and *Shigella* spp. were further subjected to molecular tests using specific primers to determine whether they were pathogenic species that are known to affect livestock and humans.

Details of each of the laboratory procedures are described in chapters 4, 5, 6 and 7.

### **3.7 Permissions and Ethical Considerations**

Permission to collect samples in the Ankasa Conservation Area was obtained from the Wildlife Division of the Forestry Commission of Ghana while permission to collect samples from the Densu Delta Ramsar site was obtained from the Panbros Salt Industry Limited. Ethical clearance was obtained from the Ethics Committee of the College of Basic and Applied Sciences, University of Ghana.

### **3.8 Statistical analysis**

To obtain the prevalence of enteric bacteria, antimicrobial-resistant bacteria and antimicrobial-resistant genes in wild birds, the calculation below was used.

$$\text{Prevalence of bacteria genera} = \frac{\text{Number of birds carrying bacteria}}{\text{Total number of birds examined}} \times 100\%$$

To obtain a measure of the body size of birds, the Principal Component Analysis (PCA) was performed on the wing length, tarsus length, total head length, and the bill length measurements. A regression analysis of the body mass and the body size component showed the relationship between the two.

For analysis of the association between variables was determined with the chi-square test of independence or Fisher's exact test were performed where appropriate.

The difference in mean zone of inhibition for each antimicrobial agent was compared between forest and waterbirds using the non-parametric Mann-Whitney U test.

All descriptive analyses were performed using the Microsoft Excel version 2016. Random selection of samples was done with IBM SPSS Statistic Editor version 20.0 and inferential analyses were performed using R statistical software (Version 3.5.1)

## **4.0 CHAPTER FOUR: PREVALENCE OF GRAM NEGATIVE ENTEROBACTERIA ISOLATED FROM FOREST AND WATER– BIRDS**

### **4.1 Introduction**

Bacteria are fundamental components in the bodies of animals and humans. They may occur internally (gastrointestinal tract, respiratory organ or reproductive organ) or externally (skin, feathers, fur, nails, exoskeleton or scales) (Archie & Theis, 2011). Many of the bacteria species that occur in vertebrates are viewed as pathogenic, though only a few of them actually cause diseases (Benskin *et al.*, 2009). Non-disease causing bacteria are known as commensal bacteria and often have a mutually beneficial relationship with their host. For instance, some communities of bacteria help their host to extract energy and nutrients from their food (Ley *et al.*, 2008). Disease-causing bacteria are regarded as pathogenic and most of them are opportunistic pathogens. These opportunistic pathogens usually affect individuals with a compromised immune system. Bacteria found in the gastrointestinal tract are referred to as enterobacteria and are acquired from the diet or feeding behaviour. These types of bacteria are common in animals and humans as both need to feed for survival.

Migratory animals have the potential to disseminate enterobacteria due to their ability to move from one location to the other. This phenomenon is even more pronounced in migratory birds. Thus, migratory birds have the ability to visit different habitats across continents during annual migrations, and some of these habitats are known hotspots for pathogens (Krauss *et al.*, 2010). Shorebirds use staging sites between their origin and destination during migration. These staging sites are used to replenish their energy stores by providing an abundance of food (Pfister *et al.*, 1998). As birds congregate in high

densities at these feeding/staging sites, there is the potential for further spreading of gut microbes due to close contact between individual bird species and via shared resources like water sources. Furthermore, migratory birds arriving at staging sites may be physiologically stressed due to migration, making them vulnerable to infections.

Generally, birds may alter microbial communities in the environment through the deposition of faeces (Grond *et al.*, 2014). However, most researches in the field of health have focussed on humans and other mammal species (Kohl, 2012). Only limited information is available on microbiota of birds despite their many ways of acquiring and disseminating microbes. For example, some birds regurgitate food to their young and this may promote vertical transmission of microbes from the gut of the parent to the offspring. The cloaca of birds serves as both an excretory and gamete transfer organ, providing the possibility of gut microbes to be transmitted sexually, hence gastrointestinal microbiota in birds may be transmitted during copulation.

Though studies on the gastrointestinal microbiota of migratory birds are important for determining what wild birds encounter and carry as they move from one location to the other, not much information is available on them. The few studies carried out focussed mainly on domestic birds.

Given the potential of wild birds to disseminate microbes, especially pathogenic ones, and the profound impact of gastrointestinal microbiota on host condition, it is important to identify microbes that occur in the gut of wild birds and ascertain whether gut microbiota communities vary between bird species. Therefore, the objective of this chapter was to isolate and characterise gram-negative enterobacteria in apparently healthy water and

forest birds. Gram-negative bacteria are the commonest (Weinstein *et al.*, 2005) and also known to be difficult to treat with antimicrobial agents (Fischbach & Walsh, 2009).

## **4.2 Material and Methods**

Birds were trapped from the Ankasa Conservation Area, Densu Delta Ramsar site and Esiamia beach. Cloacal swabs were collected (see Chapter 3 section 3.5 for a detailed description of methods used) and examined for gram-negative enterobacteria.

### **4.2.1 Preparation of agars**

In order to isolate enterobacteria, all samples were grown/cultured on MacConkey agar which is a differential medium (Gordon & FitzGibbon, 1999). A differential medium contains compounds that allow cultured colonies to be visually distinguishable based on their morphology or the colour of colonies. In this case, MacConkey agar was used to distinguish lactose fermenting and non-lactose fermenting bacteria based on the colour of bacteria colonies. Colonies of non-lactose fermenting bacteria were cultured on Salmonella Shigella agar. This agar is a selective medium that supports the growth of *Salmonella* spp. and *Shigella* spp. but inhibits other microorganisms.

MacConkey agar was prepared based on the manufacturer's (Biomark Laboratories, Pune 411 041, India) instructions. For the preparation of a volume of 1000 ml solution, 55 g of MacConkey powder was suspended in 1000 ml of distilled water. The mixture was heated in a microwave to ensure that the powder had dissolved completely and autoclaved at 15 psi, 121° C for 15 mins. After autoclaving, the mixture was allowed to cool to about 50° C

and poured into petri dishes. The petri dishes containing agar solution were allowed to solidify and allowed to dry.

Salmonella Shigella agar was prepared using the manufacturer's (Biomark Laboratories, Pune 411 041, India) instructions. For the preparation of a volume of 1000 ml solution, 63 g of the Salmonella Shigella agar powder was suspended in 1000 ml of distilled water. The mixture was boiled in a microwave to ensure that all the powder had completely dissolved. The mixture was not autoclaved in order for it not to lose its selectivity. After boiling, the mixture was allowed to cool to about 50° C and poured into petri dishes. The agars were allowed to solidify and allowed to dry.

#### **4.2.2 Growth/Culturing on agar plates**

Primary cultures were prepared by streaking cloacal swabs on agar plates (Plate 4.1). The plates were incubated overnight or between 18 to 24 hrs at 37° C. After incubation, the morphological characteristics of bacteria growth for each agar plate were recorded. At most four morphologically different colonies were selected from each culture. Each of the four colonies was streaked on one-quarter of a new agar plate. The plates were incubated overnight or between 18 to 24 hrs at 37° C.



**Plate 4.1: Bacteria culturing process. A cloacal swab carefully being removed from the eppendorf tube containing PBS and ready to be streaked on a labeled MacConkey agar plate.**

#### **4.2.3 Growth of pure colonies of bacteria on nutrient agar**

Nutrient agar was used to grow pure colonies of bacteria. For the preparation of a 1000 ml solution, 28 g of nutrient agar powder (Biomark Laboratories, Pune 411 041, India) was suspended in 1000 ml distilled water. The solution was heated in a microwave to ensure that the powder had dissolved and autoclaved at 15 psi, 121° C for 15 mins. After autoclaving, the solution was allowed to cool to about 50° C and poured into petri dishes. The agars were allowed to solidify and allowed to dry. Each nutrient agar plate was divided into four and each quarter was streaked with a pure culture of bacteria. The plates were incubated overnight or between 14 to 18 hrs at 37° C.

#### **4.2.4 Biochemical tests**

Biochemical tests are tests performed as a routine for bacteria identification and characterization (Holding & Collee, 1971). Two biochemical tests were performed, namely Triple Sugar Iron and the Indole tests. Isolates that could not be identified by the biochemical tests were sequenced for bacteria identification.

##### **4.2.4.1 Triple Sugar Iron (TSI) Test**

This procedure includes the observation of glucose, sucrose and lactose fermentation and the production of gas and hydrogen sulphide (H<sub>2</sub>S). For the preparation of a 1000 ml solution, 65 g of TSI powder (Biomark Laboratories, Pune 411 041, India) was suspended in 1000 ml of distilled water. The solution was heated in a microwave to ensure that the powder had dissolved completely and distributed into test tubes. The test tubes containing TSI solution were autoclaved at 15 psi, 121° C for 15 mins. Following this, the media in the tubes were allowed to set in a sloping form with a butt about 1-inch long. The slant of each media was streaked with a pure culture of bacteria, stabbed to the butt and incubated between 18 to 24 hrs at 37° C.

##### **4.2.4.2 Indole Test**

The Indole Test is used to ascertain the ability of a microbe to degrade the amino acid tryptophan resulting in the production of indole. To perform the Indole Test, Luria Bertani (LB) broth was prepared using Bactotrypton, Yeast extract, and NaCl. For every 1000 ml of LB broth, 10 g of bactotrypton, 5 g Yeast extract and 10 g of NaCl were added to 1000 ml distilled water. The mixture was stirred with a magnetic stirrer until fully dissolved. The solution was placed in an autoclave at 15 psi, 121 ° C for 15 mins and then cooled to room temperature before use. About 1 ml of LB broth was added to a test tube and inoculated with a strain of bacteria. The solution was incubated overnight (18-24 hrs) after which two drops of Kovac's reagent were added and the change in colour was recorded.

A red band on top of the solution was recorded as positive, while a yellow band on top was negative.

#### **4.2.5 Statistical Analysis**

A Fisher's exact test or the chi-square test was used to analyse differences in prevalences of bacteria genera. The analyses were computed with the R statistical software (version 3.5.1). Prevalence of a bacteria genera in the wild birds was calculated with the formula below:

$$\text{Prevalence of bacteria genera} = \frac{\text{Number of birds carrying bacteria}}{\text{Total number of birds examined}} \times 100\%$$

The number of examined individuals for each bird species varied considerably. To be able to compare prevalence among groups, 10 individuals were randomly selected from each of the bird species using IBM SPSS statistic editor version 20.0.

The weights of the birds were used as a measure of their body sizes.

### **4.3 Results**

#### **4.3.1 Bird species captured**

A total of 377 cloacal swabs were obtained from birds captured from the three study sites. These included 160 waterbirds from the Esiama beach (Table 4.1), 79 from the Densu Delta Ramsar site (Table 4.2) and 138 forest birds from the Ankasa Conservation Area (Table 4.3). The captured birds represented 36 species of birds belonging to four orders and 17 families. These included eight species from the Esiama beach, belonging to three families (Scolopacidae, Charadriidae, and Haematopodidae) and 10 species from the Densu Delta Ramsar site, belonging to five families (Laridae, Scolopidae, Ardeidae,

Alcedinidae, Charadriidae). Hereafter, birds from the Esiama beach and Densu Delta will be referred to as waterbirds. Twenty species of birds belonging to two orders (Passeriformes and Coraciiformes) and nine families (Pycnonotidae, Muscicapidae, Alcedinidae, Nectariniidae, Stenostiridae, Scotocercidae, Ploceidae, Turdidae, Platysteiridae) were captured from the Ankasa Conservation Area. Bird names follow the Field Guide to Birds of Ghana (Borrow & Demey, 2013).

**Table 4.1: Species of birds captured from the Esiama beach**

Bird species	Family	Number of birds	Weight of birds (range)
Bar-tailed Godwit <i>Limosa lapponica</i>	Scolopacidae	1	233
Common Sandpiper <i>Actitis hypoleucos</i>	Scolopacidae	1	49
Grey Plover <i>Pluvialis squatarola</i>	Charadriidae	2	142-207
African Oystercatcher <i>Haematopus moquini</i>	Haematopodidae	3	442-680
Common Ringed Plover <i>Charadrius hiaticula</i>	Charadriidae	2	44
Ruddy Turnstone <i>Arenaria interpres</i>	Scolopacidae	2	84-104
Sanderling <i>Calidris alba</i>	Scolopacidae	143	40-76
Whimbrel <i>Numenius phaeopus</i>	Scolopacidae	6	302-546
Total: 8	3	160	

**NB: No juvenile birds were captured for all species, except for the Sanderlings that had 44 juveniles and 99 adults**

**Table 4.2: Species of birds captured from the Densu Delta Ramsar site**

<b>Bird species</b>	<b>Family</b>	<b>Number of birds</b>	<b>Weight of birds (g) (range)</b>
Black Tern <i>Chlidonias niger</i>	Laridae	40	45-124
Common Sandpiper <i>Actitis hypoleucos</i>	Scolopacidae	2	35-47
Common Tern <i>Sterna hirundo</i>	Laridae	17	58-133
Green Shank <i>Tringa nebularia</i>	Scolopacidae	1	159
Green-backed Heron <i>Butorides straita</i>	Ardeidae	1	183
Malachite Kingfisher <i>Corythronis cristatus</i>	Alcedinidae	2	12
Pied Kingfisher <i>Ceryle rudis</i>	Alcedinidae	8	60-79
Common Ringed Plover <i>Charadrius hiaticula</i>	Charadriidae	1	46
Roseate Tern <i>Sterna dougalli</i>	Laridae	6	105-140
Wood Sandpiper <i>Tringa glareola</i>	Scolopacidae	1	116
Total: 10	4	79	

**NB: No juvenile birds were captured for all species, except for the Black Tern that had 1 juvenile and 39 adults**

**Only the Pied Kingfisher were sexually dimorphic and comprised 5 males and 2 females**

**Table 4.3: Species of birds captured from the Ankasa Conservation Area (ACA)**

Bird species	Family	Number of birds	Weight (g) (range)
African Pygmy Kingfisher <i>Ispidina picta</i>	Alcedinidae	2	10-15
Blue-billed Malimbe <i>Malimbus nitens</i>	Ploceidae	1	40
Brown-chested Alethe <i>Chamaetylas poliocephala</i>	Muscicapidae	9	31-36
Chestnut Wattle Eye <i>Dyaphorophya castanea</i>	Platysteiridae	1	14
Dusky Crested Flycatcher <i>Elminia nigromitrata</i>	Stenostiridae	1	10
Finsch's Flycatcher Thrush <i>Stizorhina finschi</i>	Turdidae	1	40
Forest Robin <i>Stiphornis erythrothorax</i>	Muscicapidae	10	15-25
Green Hylia <i>Hylia prasina</i>	Scolocercidae	4	10-15
Green-tailed Bristlebill <i>Bleda eximius</i>	Pycnonotidae	6	40-51
Grey-headed Bristlebill <i>Bleda canicapillus</i>	Pycnonotidae	1	41
Icterine Greenbul <i>Phyllastrephus icterinus</i>	Pycnonotidae	5	15-35
Little Greenbul <i>Eurillas virens</i>	Pycnonotidae	11	25-35
Olive Sunbird <i>Cyanomitra olivacea</i>	Nectariniidae	19	5-15
Red-tailed Bristlebill <i>Bleda syndactylus</i>	Pycnonotidae	4	39-50
Speckled Tinkerbird <i>Pogoniulus scolopaceus</i>	Pycnonotidae	1	15
Western Bearded Greenbul <i>Criniger barbatus</i>	Pycnonotidae	4	30-44
White-tailed Alethe <i>Alethe diademata</i>	Muscicapidae	5	15-33
White-bellied Kingfisher <i>Corythornis leucogaster</i>	Alcedinidae	2	15-20
White-tailed Ant Thrush <i>Neocossyphus poensis</i>	Turdidae	3	60-70
Yellow-whiskered Greenbul <i>Eurillas latirostris</i>	Pycnonotidae	47	10-35
Total: 20		138	

**NB: Only Olive Sunbirds were sexually dimorphic and comprised 10 males and 9 females.**

**All birds were identified as adults except for the following species: Yellow-whiskered Greenbul, 17 juveniles and 30 adults; White-tailed Alethe, 1 juvenile and 4 adults; Western Bearded Greenbul, 1 juvenile and 3 adults; Red-tailed Bristlebill, 2 juveniles and 2 adults; Olive Sunbird, 9 juveniles and 10 adults; Little Greenbul, 4 juveniles and 7 adults; Icterine Greenbul, 2 juveniles and 3 adults**



**Plate 4.2: Some species of forest birds captured in this study**

Chestnut  
Wattle Eye



Source: [hbw.com/ibc](http://hbw.com/ibc)

Dusky  
Crested  
Flycatcher



Source: [hbw.com/ibc](http://hbw.com/ibc)

Finsch's  
Flycatcher  
Thrush



Source: [hbw.com/ibc](http://hbw.com/ibc)

Green Hylia



Source: [hbw.com/ibc](http://hbw.com/ibc)

Grey-headed  
Bristlebill



Source: [hbw.com/ibc](http://hbw.com/ibc)

Icterine Greenbul



Source: [hbw.com/ibc](http://hbw.com/ibc)

Red-  
tailed  
Bristlebill



Source: [www.hbw.com](http://www.hbw.com)

Western  
Bearded  
Greenbul

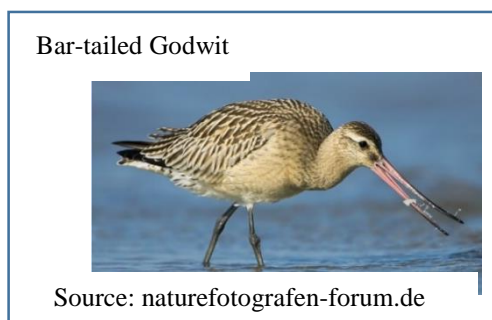
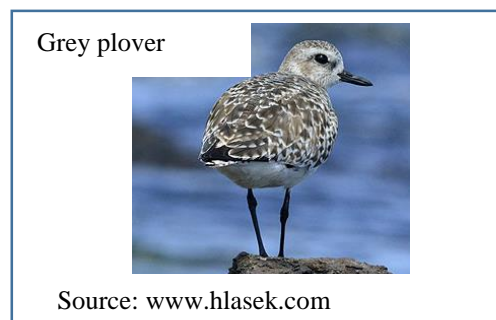


Source: [www.hbw.com](http://www.hbw.com)

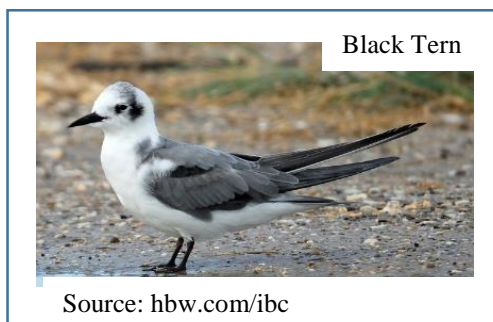
**Plate 4.2 contd.: Species of forest birds captured in this study (Source: The Internet IBC bird collection, [hbw.com/ibc](http://hbw.com/ibc))**



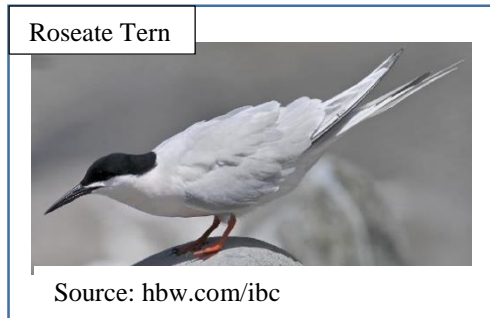
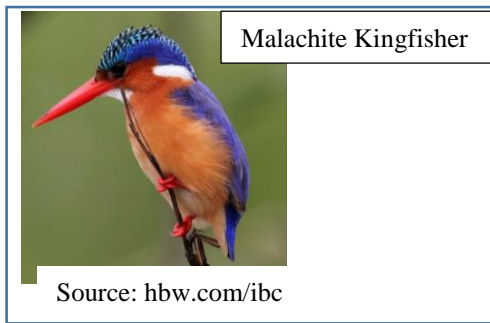
**Plate 4.2 contd.: Species of forest birds captured in this study (Source: The Internet IBC bird collection, [hbw.com/ibc](http://hbw.com/ibc))**



**Plate 4.3: Species of waterbirds captured in this study (Source: The Internet IBC bird collection, [hbw.com/ibc](http://hbw.com/ibc))**



**Plate 4.3 contd.: Species of waterbirds captured in this study (Source: The Internet IBC bird collection, [hbw.com/ibc](http://hbw.com/ibc))**



**Plate 4.3 contd.: Species of waterbirds captured in this study (Source: The Internet IBC bird collection, hbw.com/ibc)**

#### **4.3.2 Gram-negative enterobacteria recovered from sampled birds**

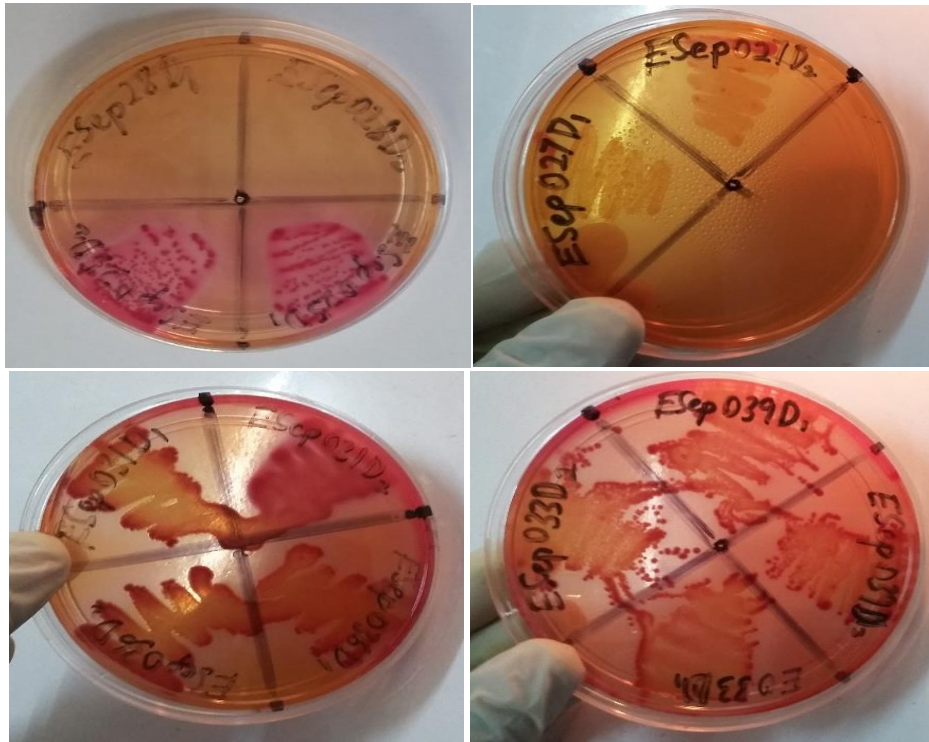
The results from bacteria culturing showed morphological growth for gram-negative enterobacteria (Plate 4.4). Four Hundred and sixty-five bacterial isolates, representing 15 gram-negative bacteria genera were isolated from the 377 cloacal swabs collected from both waterbirds and forest birds. The bacterial genera isolated were *Shigella*, *Yersinia*, *Salmonella*, *Alcaligenes*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Morganella*, *Ochrobactrum*, *Proteus*, *Providencia*, *Pseudomonas*, *Serratia*, and *Achromobacter*.

The most frequently isolated genera were *Escherichia* (isolated from 22.3% of cloacal swabs), *Yersinia* (19.4%), *Enterobacter* (12.2%), and *Klebsiella* (11.4%) (Figure 4.1). Comparatively, the occurrence of the genus *Escherichia* was high regardless of the type of bird species and was recorded in 20 bird species. In addition, the genus *Escherichia*

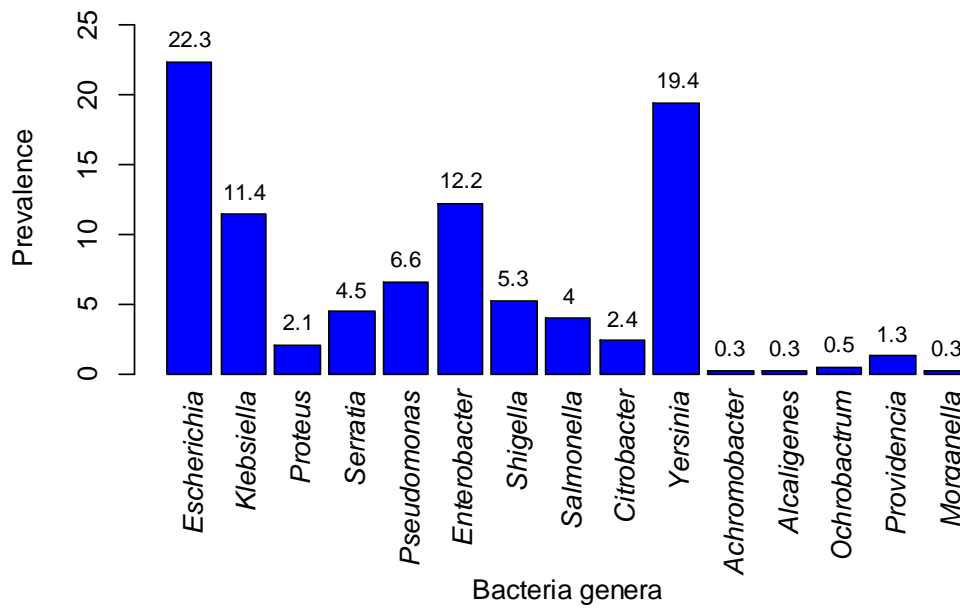
had the highest proportion (30.5%) of all isolates obtained (Table 4.4). The genera *Ochrobactrum*, *Morganella*, *Achromobacter* and *Alcaligenes* had the lowest occurrences, 0.5% (2/377), 0.3% (1/377), 0.3% (1/377) and 0.3% (1/377) respectively.

Overall, gram-negative enterobacteria occurred in 61.8% (233/377) of the birds. In the bird species where 10 or more individuals were sampled, the prevalence of gram-negative bacteria varied between 51.0% and 72.7%, with the highest occurrence recorded in the Little Greenbul. For some species of birds such as the Blue-billed Malimbe, Dusky-crested Flycatcher and the Green Shank, though only one individual was captured for each, they were found to harbour at least one genus of gram-negative enterobacteria.

The prevalence of two genera of gram-negative enterobacteria co-existing in an individual bird was 16.71% (63/377) and was observed in 19 bird species (Table 4.5). The coexistence of *Enterobacter* with *Yersinia* (14.5%), *Escherichia* with *Klebsiella* (14.5%), *Yersinia* with *Escherichia* (11.3%) were more frequently encountered. Furthermore, the occurrence of three or more genera of gram-negative enterobacteria co-existing in an individual bird was 4.8% (18/377) (Table 4.6) and occurred in eight bird species.



**Plate 4.4:** Agar plates showing morphological representations of some cultured bacteria



**Figure 4.1:** Prevalences of gram-negative bacteria genera isolated from both forest and water- birds

**Table 4.4: Proportion of isolates for each bacteria genus**

<b>Bacteria genera</b>	<b>Number of isolates</b>	<b>Proportions of all bacteria genera (%)</b>
<i>Shigella</i>	25	5.4%
<i>Yersinia</i>	95	20.4%
<i>Salmonella</i>	17	3.7%
<i>Alcaligenes</i>	1	0.2%
<i>Citrobacter</i>	9	1.9%
<i>Enterobacter</i>	58	12.5%
<i>Klebsiella</i>	51	11.0%
<i>Morganella</i>	1	0.2%
<i>Onchrobactrum</i>	2	0.4%
<i>Proteus</i>	8	1.7%
<i>Providencia</i>	5	1.1%
<i>Pseudomonas</i>	31	6.7%
<i>Serratia</i>	19	4.1%
<i>Achromobacter</i>	1	0.2%
<i>Escherichia</i>	142	30.5%
<b>Total</b>	<b>465</b>	<b>100.0%</b>

**Table 4.5: Prevalences of two co-existing bacteria genera in the wild bird species**

<b>Bird species</b>	<b>No. of birds examined (N)</b>	<b>No. of birds with 2 co-existing bacteria (n)</b>	<b>Prevalence (n/N*100%)</b>
Bar-tailed Godwit	1	0	0
Common Sandpiper	3	1	33.3
Grey Plover	2	0	0
African Oystercatcher	3	1	33.3
Common Ringed Plover	3	0	0
Ruddy Turnstone	2	0	0
Sanderling	143	13	9.1
Whimbrel	6	2	33.3
Black Tern	40	12	30
Common Tern	17	4	23.5
Green Shank	1	0	0
Green-backed Heron	2	0	0
Malachite Kingfisher	2	0	0
Pied Kingfisher	8	1	12.5
Roseate Tern	6	1	16.67
Wood Sandpiper	1	0	0
African Pygmy Kingfisher	2	1	50
Blue-billed Malimbe	1	0	0
Brown-chested Alethe	9	2	22.22
Chestnut Wattle Eye	1	0	0
Dusky Crested Flycatcher	1	1	100
Finsch's flycatcher thrush	1	0	0
Forest Robin	10	4	40
Green Hylia	4	2	50
Green-tailed Bristlebill	1	1	100
Grey-headed Bristlebill	6	0	0
Icterine Greenbul	5	2	40
Little Greenbul	11	5	45.5
Olive Sunbird	19	4	21.1
Red-tailed Bristlebill	4	1	25
Speckled Tinkerbird	1	0	0
Western Bearded Greenbul	4	0	0
White-tailed Alethe	5	0	0
White-bellied Kingfisher	2	0	0
White-tailed Ant Thrush	3	0	0
Yellow-whiskered Greenbul	47	5	10.64
<b>Total</b>	<b>377</b>	<b>63</b>	<b>16.71 (63/377)</b>

**Table 4.6: Prevalences of three or more bacteria genera in an individual bird in each bird species**

<b>Bird species</b>	<b>No. of birds examined (N)</b>	<b>No. of birds harbouring 3 or more bacteria genera(n)</b>	<b>Prevalence (n/N*100%)</b>
Bar-tailed Godwit	1	0	0
Common Sandpiper	3	0	0
Grey Plover	2	0	0
African Oystercatcher	3	0	0
Common Ringed Plover	3	0	0
Ruddy Turnstone	2	0	0
Sanderling	143	6	4.2
Whimbrel	6	0	0
Black Tern	40	6	15
Common Tern	17	0	0
Green Shank	1	0	0
Green-backed Heron	2	0	0
Malachite Kingfisher	2	0	0
Pied Kingfisher	8	1	12.5
Roseate Tern	6	0	0
Wood Sandpiper	1	0	0
African Pygmy Kingfisher	2	0	0
Blue-billed Malimbe	1	0	0
Brown-chested Alethe	9	1	11.1
Chestnut Wattle Eye	1	0	0
Dusky Crested Flycatcher	1	0	0
Finsch's flycatcher thrush	1	0	0
Forest Robin	10	1	10
Green Hylia	4	0	0
Green-tailed Bristlebill	1	0	0
Grey-headed Bristlebill	6	0	0
Icterine Greenbul	5	0	0
Little Greenbul	11	0	0
Olive Sunbird	19	1	5.3
Red-tailed Bristlebill	4	0	0
Speckled Tinkerbird	1	0	0
Western Bearded Greenbul	4	0	0
White-tailed Alethe	5	0	0
White-bellied Kingfisher	2	0	0
White-tailed Ant Thrush	3	1	33.3
Yellow-whiskered Greenbul	47	1	2.1
<b>Total</b>	<b>377</b>	<b>18</b>	<b>4.78</b>

#### 4.3.3 Occurrence of gram-negative bacteria in forest birds

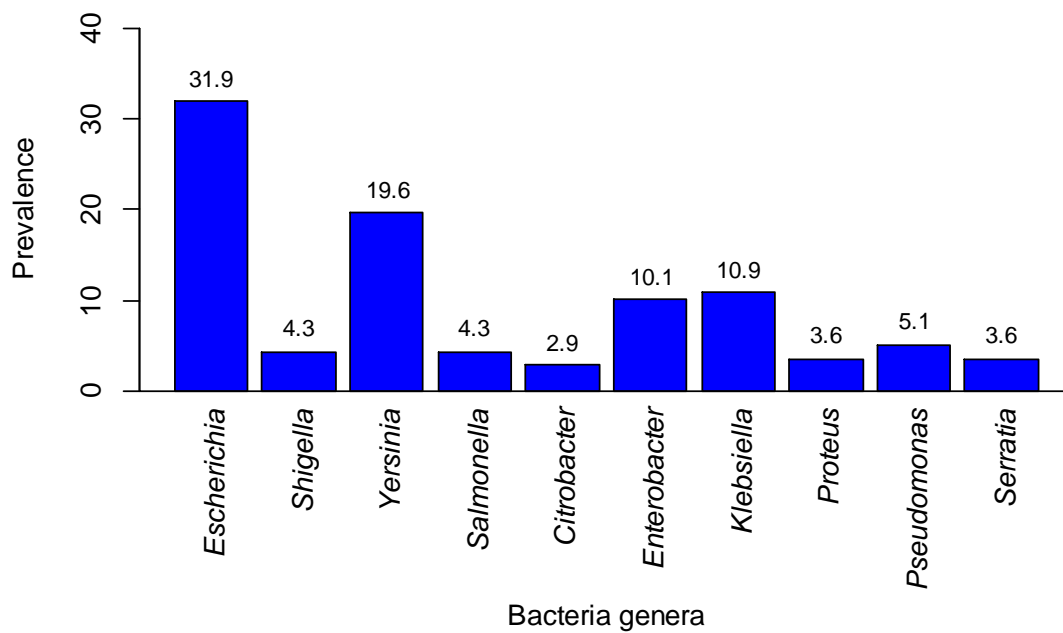
One Hundred and Ninety-five isolates belonging to 10 genera of gram-negative bacteria were obtained from the 138 sampled forest birds. Bacteria genera isolated from forest birds included *Shigella*, *Yersinia*, *Salmonella*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Serratia* and *Escherichia* (Table 4.7).

Gram-negative enterobacteria occurred in 65.2% (90/138) of forest birds comprising 17 bird species (Table 4.8). There were no significant differences between the prevalence of gram-negative bacteria genera in the common bird species (bird species with 10 or more individuals) according to the Fisher's exact test ( $p < 0.05$ ) except for the Yellow-whiskered Greenbul where the occurrence of the genera *Escherichia* and *Yersinia* were significantly higher than the occurrence of the genera *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella* and *Shigella*.

On the whole, the genus *Escherichia* occurred in 31.9% (44/138) of forest bird species (Figure 4.2), constituting about 45.4% of all isolates obtained from forest birds (Table 4.7). The second most prevalent genus was *Yersinia* (19.6%), constituting 18.6% of all isolates and was recorded in 10 species of forest birds. *Citrobacter* had the lowest prevalence (2.9%) comprising only 4 isolates.

Regardless of the type of bird species, four bacteria genera had a prevalence of 10% and above. These were *Yersinia*, *Escherichia*, *Enterobacter* and *Klebsiella* (Table 4.9). All four genera were recorded in the Forest Robin, Olive Sunbird, and Yellow-whiskered Greenbul. Of the four genera, *Escherichia* occurred more in the Brown-chested Alethe (88.9%), with eight out of the nine individuals harbouring this genus of bacteria. Similarly, cloacal swabs of five out of six individuals (88.3%) of the Green-tailed Bristlebill

harboured *Escherichia*. For the genus *Yersinia*, the highest occurrence (60%) was recorded in the Icterine Greenbul and the White-tailed Alethe, with three out of five individuals each testing positive. For *Enterobacter* and *Klebsiella*, the highest prevalence (50% and 33.3%) were observed in the White-bellied Kingfisher and White-tailed Anthrush, respectively. It was also observed that for bird species in which only one individual was captured, the prevalence of bacteria genera was 100%. Such is the case of *Escherichia* and *Yersinia* in the Dusky-crested Flycatcher and Blue-billed Malimbe respectively.



**Figure 4.2: Prevalences of bacteria genera in forest birds**

**Table 4.7: Proportion of each gram-negative bacteria genus isolated from forest birds**

<b>Bacteria genera</b>	<b>Number of isolates</b>	<b>Proportions of all bacteria genera (%)</b>
<i>Shigella</i>	8	4.1%
<i>Yersinia</i>	36	18.6%
<i>Salmonella</i>	8	4.1%
<i>Citrobacter</i>	4	2.1%
<i>Escherichia</i>	88	45.4%
<i>Enterobacter</i>	16	8.2%
<i>Klebsiella</i>	16	8.2%
<i>Proteus</i>	5	2.6%
<i>Pseudomonas</i>	8	4.1%
<i>Serratia</i>	5	2.6%
<b>Total</b>	<b>194</b>	<b>100%</b>

**Table 4.8: Prevalences of gram-negative bacteria in forest birds**

<b>Bird species</b>	<b>No. of birds examined (N)</b>	<b>No. of birds carrying bacteria (n)</b>	<b>Prevalence (n/N*100%)</b>
African Pygmy Kingfisher	2	1	50
Blue-billed Malimbe	1	1	100
Brown-chested Alethe	9	9	100
Chestnut Wattle Eye	1	0	0
Dusky Crested Flycatcher	1	1	0
Finsch's Flycatcher Thrush	1	0	0
Forest Robin	10	6	60
Green Hylia	4	4	100
Green-tailed Bristlebill	6	5	83.3
Grey-headed Bristlebill	1	1	100
Icterine Greenbul	5	3	60
Little Greenbul	11	8	72.7
Olive Sunbird	19	10	52.6
Red-tailed Bristlebill	4	3	75
Speckled Tinkerbird	1	0	0
Western Bearded Greenbul	4	1	25
White-tailed Alethe	5	5	100
White-bellied Kingfisher	2	1	50
White-tailed Ant Thrush	3	3	100
Yellow-whiskered Greenbul	47	28	59.6
<b>Total</b>	<b>138</b>	<b>90</b>	<b>65.2</b>

**Table 4.9: Prevalences of most the frequently isolated bacteria genera among forest bird species**

Bird species	No. of birds examined	Prevalence (Number of birds harbouring bacteria)			
		<i>Escherichia</i>	<i>Yersinia</i>	<i>Enterobacter</i>	<i>Klebsiella</i>
African Pygmy Kingfisher	2	50% (1)	50% (1)	0	0
Brown-chested Alethe	9	88% (8)	0	22.2% (2)	11.1%(1)
Dusky-crested Flycatcher	1	100% (1)	0	0	0
Forest Robin	10	40% (4)	20% (2)	20% (2)	20% (2)
Green Hylia	4	75% (3)	50% (2/4)	0	16.7% (1)
Green-tailed Bristlebill	6	83.3% (5)	0	0	0
Icterine Greenbul	5	20% (1)	60% (3)	0	20% (1)
Olive Sunbird	19	15.8% (3)	26.3% (5)	10.5% (2)	10.5% (2)
Red-tailed Bristlebill	4	75% (3)	0	0	25% (1)
White-tailed Anthrush	3	66.7% (2)	0	0	0
White-tailed Alethe	5	40% (2)	60% (3)	0	0
Yellow-whiskered Greenbul	47	23.4% (11)	21.2% (10)	12.8%(6)	2.1% (1)
Blue-billed Malimbe	1	0	100% (1)	0	0
White-tailed Anthrush	3	0	0	33.3% (1)	33.3% (1)
White-bellied Kingfisher	2	0	0	50% (1)	0

#### **4.3.4 Association between selected bird species and prevalence of *Escherichia* in forest birds**

Ten individuals each of the species shown in Table 4.10 were randomly selected for analysis of the association between type of bird species and prevalence of the genus *Escherichia*. The Forest Robin had only 10 individuals, hence all 10 were included in the analysis. For the analysis of the association between age and prevalence, 10 each of adults and juvenile Yellow-whiskered Greenbuls were randomly selected. To find the association between weight and the prevalence of the genus *Escherichia*, the weight of Yellow-whiskered Greenbuls were grouped into two: A (less or equal to 25 g) and B (above 25 g). Fifteen individuals were randomly selected from each size class.

After a random selection of 10 individuals each of forest bird species, the prevalence of *Escherichia* was 40% in Forest Robin, 10% in Olive Sunbird and 20% in Yellow-whiskered Greenbul. The difference in the prevalence of *Escherichia* among the bird species was not statistically significant (Fisher exact test value = 2.400, df = 2, p = 0.430).

To determine the association between age and prevalence of *Escherichia*, only the Yellow-whiskered Greenbul was used for this analysis. The prevalence was 0% for adults and 10% for juveniles after random selection. None of the adults harboured *Escherichia* after random selection.

The association between size classes and prevalence of *Escherichia* was determined using 15 individuals each of the size classes stated in Table 4.10. The prevalence in classes A and B were 30% and 40% respectively. The results showed no statistical significance ( $\chi^2 = 0.668$ , df = 1, p=0.666) in prevalence of *Escherichia* between the size classes.

**Table 4.10: Forest bird species used in the analyses of associations**

Bird species	Number of samples collected	Number of samples randomly selected
Between species		
Forest Robin	10	10
Olive Sunbird	19	10
Yellow-whiskered Greenbul	47	10
Age		
Adult	33	10
Juvenile	14	10
Size classes		
≤ 25	19	15
> 25	28	15

#### 4.3.5 Occurrence of gram-negative bacteria in waterbirds

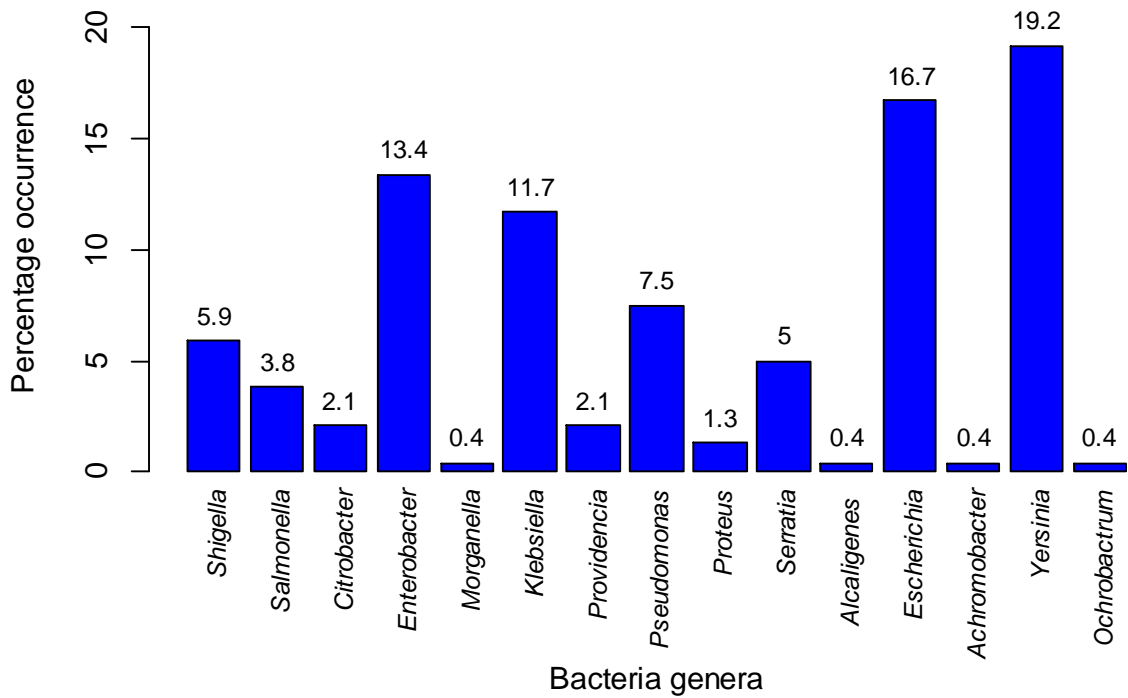
Two hundred and seventy isolates belonging to 15 genera of bacteria were obtained from the 239 sampled waterbirds (Table 4.11). The bacteria genera were *Shigella*, *Salmonella*, *Citrobacter*, *Enterobacter*, *Morganella*, *Klebsiella*, *Providencia*, *Pseudomonas*, *Proteus*, *Serratia*, *Alcaligenes*, *Escherichia*, *Achromobacter*, *Yersinia*, and *Ochrobactrum*.

The prevalence of gram-negative bacteria was 59.8% (143/239) (Table 4.12) and was obtained from 13 species of waterbirds (Figure 4.3). The prevalence of the genera *Yersinia*, *Escherichia*, *Enterobacter*, and *Klebsiella* were significantly more than the other genera that occurred in the Sanderling. Similarly, *Yersinia*, *Escherichia* and *Enterobacter* were significantly more than the other genera isolated from the Black Tern. On the other hand, the difference in prevalence between gram-negative genera isolated from the Common tern was not significant (Fisher's test  $p > 0.05$ ).

Overall, *Yersinia* occurred more frequently in waterbirds (19.2%; 46/239) and recorded the highest number of isolates (59 isolates), constituting 21.9% of all isolates obtained

from waterbirds (Table 4.11). The second most prevalent genus was *Escherichia* with 16.7% (40/239) prevalence in waterbirds. It constituted 20.0% (54 isolates) of all isolates. The difference in prevalence of the genera *Yersinia* and *Escherichia* was not statistically significant ( $\chi^2=0.4186$ ,  $df=1$ ,  $p\text{-value}=0.5176$ ). *Achromobacter*, *Acaligenes*, *Morganella* were the least isolated genera, each with a prevalence of 0.4%.

The genera *Yersinia*, *Escherichia*, *Enterobacter*, and *Klebsiella* recorded an overall prevalence of 10% and above (Table 4.13). All four genera were recorded in the Black Tern, Pied Kingfisher, and Sanderling. The genus *Escherichia* occurred more in the Common Sandpiper (66.7%), with two out of the three individuals harbouring this genus. Similarly, one out of two individuals (50%) of the Ruddy Turnstone harboured *Escherichia*. For the genus *Yersinia*, the highest prevalence (50%) was recorded in the Grey Plover and Malachite Kingfisher with one out of two individuals each showing positive results. For *Enterobacter* and *Klebsiella*, the highest prevalence of 100% each were observed in the Green Shank and Grey Plover. The 100% prevalence of *Enterobacter* in the Green Shank was because the only individual captured harboured this bacteria genus.



**Figure 4.3: Prevalences of each bacteria genera in waterbirds**

**Table 4.11: Proportion of each gram-negative bacteria genus isolated from waterbirds**

Bacteria genera	Number of isolates	Proportion of each bacteria genus (%)
<i>Shigella</i>	16	5.9%
<i>Salmonella</i>	9	3.3%
<i>Citrobacter</i>	5	1.9%
<i>Enterobacter</i>	42	15.6%
<i>Morganella</i>	1	0.4%
<i>Klebsiella</i>	35	13.0%
<i>Providencia</i>	5	1.9%
<i>Pseudomonas</i>	23	8.5%
<i>Proteus</i>	3	1.1%
<i>Serratia</i>	14	5.2%
<i>Alcaligenes</i>	1	0.4%
<i>Escherichia</i>	54	20.0%
<i>Achromobacter</i>	1	0.4%
<i>Yersinia</i>	59	21.9%
<i>Ochrobactrum</i>	2	0.7%
Total	270	100.0%

**Table 4.12: Prevalence of gram-negative bacteria in each waterbird species**

<b>Bird species</b>	<b>No. of birds examined (N)</b>	<b>No. of birds carrying bacteria (n)</b>	<b>Prevalence (n/N*100%)</b>
Bar-tailed Godwit	1	0	0
Common Sandpiper	3	2	66.7
Grey Plover	2	2	100
African Oystercatcher	3	1	33.3
Common Ringed Plover	3	2	66.7
Ruddy Turnstone	2	1	50
Sanderling	143	73	51.0
Whimbrel	6	4	66.7
Black Tern	40	36	90
Common Tern	17	12	70.6
Green Shank	1	1	100
Green-backed Heron	1	0	0
Malachite Kingfisher	2	1	50
Pied Kingfisher	8	4	50
Roseate Tern	6	4	66.6
Wood Sandpiper	1	0	0
<b>Total</b>	<b>239</b>	<b>143</b>	<b>59.8</b>

**Table 4.13: Prevalences of the most frequently isolated bacteria genera among waterbird species**

Bird species	No. of birds examined	Prevalence (Number positive)			
		<i>Escherichia</i>	<i>Yersinia</i>	<i>Enterobacter</i>	<i>Klebsiella</i>
Black Tern	40	25% (10)	42.5% (17)	20% (8)	12.5% (5)
Bar-tailed Godwit	1	0	0	0	0
Common Ringed Plover	3	33.3% (1)	0	0	0
Common Sandpiper	3	66.7% (2)	0	0	0
Common Tern	17	5.9% (1)	0	0	5.9% (1)
Green Shank	1	0	0	100% (1)	0
Green-backed Heron	1	0	0	0	0
Grey Plover	2	0	50% (1)	0	100% (2)
Malachite Kingfisher	2	0	50% (1)	0	0
African Oystercatcher	3	0	0	33.3% (1)	33.3% (1)
Pied Kingfisher	8	12.5% (1)	12.5% (1)	25% (2)	25% (2)
Roseate Tern	6	0	33.3% (2)	33.3% (2)	0
Ruddy Turnstone	2	50% (1)	0	0	0
Sanderling	143	15.4% (22)	12.6% (18)	11.9% (17)	11.2% (16)
Whimbrel	6	33.3% (2)	33.3% (2)	16.7% (1)	0
Wood Sandpiper	1	0	0	0	0
Total (236)	23	16.7	19.2	13.4	11.7

#### 4.3.6 Association between bird species and prevalence of *Escherichia* in waterbirds

For waterbirds, ten individuals each of the species shown in Table 4.14 were randomly selected for analysis of the association between bird species and prevalence of *Escherichia*. For the analysis of the association between age and prevalence, 10 each of adults and juvenile Sanderlings were randomly selected. To find the association between weight and the prevalence of *Escherichia*, the weights of Sanderlings were grouped into three, A, B and C. Fifteen individuals were randomly selected from each size class.

The prevalence of *Escherichia* in waterbirds after random selection of 10 individuals from each species of Black tern, Common tern and Sanderling were 20%, 10%, and 10% respectively. The prevalence rates were not statistically significant (Fishers exact test value = 0.717, df = 2, p = 1.0)

**Table 4.14: Waterbird species used in the analyses of associations**

<b>Bird species</b>	<b>Number of samples collected</b>	<b>Number of samples randomly selected</b>
Between species		
Black tern	40	10
Common tern	17	10
Sanderling	143	10
Age		
Adult	91	40
Juvenile	51	40
Size classes		
A (40 - 49)	53	15
B (50 - 59)	66	15
C (60 - 69)	24	15

#### 4.4 Discussion

In this chapter, the occurrence of gram-negative enterobacteria in forest and waterbirds was investigated. The findings clearly show that both forest and water-birds were colonised by gram-negative enterobacteria. Fifteen different genera of gram-negative bacteria were recorded in this study and were isolated from 61.8% of birds sampled. The number of genera and the high occurrence of gram-negative bacteria supports the claim by Kruse *et al.* (2004) that wild birds are susceptible to colonisation by various bacteria species. The prevalence, however, was low in this study compared to the 78.9% reported by Matias *et al.* (2016) from illegally traded wild birds in Brazil.

The commonly isolated genus, *Escherichia*, was obtained from 20 bird species (8 waterbird species and 12 forest bird species). The species *Escherichia coli* under the genus *Escherichia* is considered a normal inhabitant of the gut flora of avian species (Glünder & Siegmann, 1989). Therefore, the high prevalence of this genus in the present study suggests that most of the isolates belonging to this genus could possibly be *E. coli*. While *Escherichia* was the frequently occurring genus (31.9%) in forest birds, its occurrence in waterbirds was second to *Yersinia* (i.e. *Escherichia* 16.7%, *Yersinia* 19.2%). Comparatively, the occurrence of the genus *Escherichia* in forest birds was almost twice as high as that of waterbirds. Previous studies on wild birds by Awadallah *et al.* (2013) in Egypt and Rogers (2006) in California reported occurrences of *E. coli* to be 48% and 38% respectively. These are higher than the prevalence (22.3%) reported in this study. The high prevalence in the previous studies could possibly be due to the bird species sampled or their proximity to human surroundings. Awadallah *et al.* (2013) sampled bird species including cattle egrets, sparrows, and quails inhabiting human surroundings and also noted that variations in occurrences could be attributed to type of bird species, habitat and feeding habit. Rogers (2006) also sampled birds including sparrows, blackbirds, and

cowbirds on agricultural farms (indicating human influence). It should also be noted that the studies by Awadallah *et al.* (2013) and Rogers (2006) involved the collection of both faecal and cloacal samples while in this study only cloacal swabs were collected. As the probability of isolating bacteria from faecal samples is higher than from a cloacal swab (Sarker *et al.*, 2012), their studies recorded higher occurrences than this study. Cloacal swabs, however, ensures no contamination from the environment.

Fallacara *et al.* (2004) reported a prevalence of *E. coli* of 67% in waterfowls in a zoological setting. Other studies have shown that carnivorous or omnivorous birds had a higher prevalence of *E. coli* (Steele *et al.*, 2005) while Fiennes (1982) and Brittingham *et al.* (1988) reported that the occurrence of *E. coli* in granivorous birds was very low.

The genus *Yersinia*, which was the second commonest bacteria genus recorded in this study and their prevalence were 19.6% and 19.2% in forest and water– birds respectively. Niskanen *et al.* (2003) reported an occurrence of 13% of *Yersinia spp.* in migratory birds in Sweden. This was relatively lower than what was observed in this study. Another study reported a prevalence of 12.4% in Japanese wild birds (Fukushima & Gomyoda, 1991). Also, Rogers (2006) reported an occurrence of *Yersinia spp.* in 12% of wild birds associated with agricultural fields. Comparatively, the occurrence of the genus *Yersinia* in the present study was higher.

The genera *Klebsiella* and *Enterobacter* had relatively similar occurrences (11.4% and 12.2%, respectively) in this study. The occurrence of these genera has been reported also in other studies. For example, *Klebsiella pneumoniae* and *Klebsiella oxytoca* were reported to occur in wild birds from Brazil. Species of this genus have been reported as second to *E. coli* in causing sepsis in humans (Feigin & Cherry, 1998). For the genus

*Enterobacter* an occurrence of 16% was reported in wild birds associated with agriculture (Rogers, 2006).

The genera *Salmonella*, *Serratia*, *Pseudomonas*, *Shigella*, *Proteus* and *Citrobacter* occurred rarely in the samples investigated in this study. For example, the prevalence of *Salmonella* in water and forest birds were 3.8% and 4.3%, respectively. In a study conducted by Konicek *et al.* (2016) on wild birds in Austria and the Czech Republic they recorded a prevalence of *Salmonella* to be 2.2%.

In the present study, most of the bacteria genera that were recorded in waterbirds were also recorded in forest birds. However, five genera (*Ochrobactrum*, *Alcaligenes*, *Achromobacter*, *Morganella*, and *Providencia*) were found only in the waterbird samples suggesting that these species probably occur only in waterbirds. In a similar study along the Western Atlantic migratory corridor, the prevalence of bacteria genera such as *Pseudomonas*, *Enterococcus*, *Escherichia*, *Proteus*, *Shewanella*, *Aeromonas* and *Citrobacter* were recorded in Knots, Sanderlings and White-rumped Sandpipers found dead in southern Brazil in 1997 (Buehler *et al.*, 2010).

Several species of wild birds have been found to harbour pathogenic strains of both human and avian pathogens. These include geese (Zhou *et al.*, 2004; Lu *et al.*, 2009), raptor (Camarda *et al.*, 2006), songbirds (Keller *et al.*, 2011), psittacines (Hudson *et al.*, 2000), shorebirds (Ksoll *et al.*, 2007), gulls (Camarda *et al.*, 2006; Lu *et al.*, 2008) and terns (Ksoll *et al.*, 2007).

While there are few studies conducted on the occurrence of enterobacteria in forest birds, several studies have been conducted on shorebirds. For example, a study conducted by Ryu *et al.* (2014) in Delaware isolated a total of 27 bacteria genera from three species of shorebirds (Ruddy Turnstone, Semipalmated Sandpiper and Red Knot). Although only three species of birds were sampled in the Ryu *et al.* (2014) study, the number of bacteria genera isolated was high. This was because all types of bacteria genera were considered whereas in this study only gram-negative enterobacteria were considered.

Wild birds inhabiting contaminated environments such as areas with high human activities may pick and carry more bacteria, including pathogenic ones than birds that do not live in close proximity to such habitats (Cizek *et al.*, 1994). As such, waterbirds sampled in this study may carry pathogenic strains of bacteria since the study sites experienced human interactions such as fishing, salt mining and open defecation along the shore. Pathogenic bacteria may be ingested inadvertently but may not have any effect on wild birds as observed in this study (all birds were apparently healthy). Thus, wild birds especially migratory species may act as carriers in the spread of pathogenic bacteria to other wild animals, domestic animals, and humans and this may have serious implications for conservation and public health.

From a conservation perspective, continuous surveillance in wild birds will help monitor the occurrence of pathogens and their possible spread to other wild animals, domestic animals, and humans. Although the prevalence may be low, the potential to spread pathogens is considerably high.

## **5.0 CHAPTER FIVE: ANTIMICROBIAL RESISTANCE PROFILES OF BACTERIA ISOLATED FROM FOREST AND WATER- BIRDS**

### **5.1 Introduction**

The emergence of bacterial isolates resistant to antimicrobial drugs is a major public health concern, with serious consequences for both animals and humans. Anthropogenic activities such as the indiscriminate use of antimicrobials in agriculture and hospitals have been associated with the increasing resistance to antimicrobials (Neu, 1992).

In recent years, several studies have reported the occurrence of antimicrobial-resistant bacteria in organisms such as wild animals that were exposed to low levels of antimicrobials or presumably never exposed. Wild animals rarely come into contact with antimicrobial agents yet they have the potential to acquire antimicrobial-resistant bacteria from the contaminated environments or sources shared with humans (Dolejska *et al.*, 2007). The common aspects of transmission of resistant bacteria of human or veterinary origin to wild animals are via contaminated food or water (Cole *et al.*, 2005). While wildlife could be reservoirs of intrinsic genetic determinants for resistance, it is often assumed that antimicrobial resistance in them is acquired from humans and livestock (Vittecoq *et al.*, 2016).

Agricultural lands may also acquire antimicrobial agents from manure or fertilizers applied to them. The frequent application of these manure/fertilizers may lead to some bacteria in the soil becoming resistant to antimicrobial agents they are exposed to. Consequently, run-off from these agricultural lands possibly containing antimicrobial-resistant bacteria could lead to surface water contamination and further spread to animals and humans via direct or indirect contact.

The problem of antimicrobial resistance is even more pronounced in countries where antimicrobials are used as growth promoters. In such countries, antimicrobial agents are incorporated into animal feed at sub-therapeutic levels to promote growth by optimising feed conversion ratios to promote weight gain (Jukes *et al.*, 1950). This practice is known to enhance the selection of resistant bacteria compared to resistance acquired from hospital use (Van den Bogaard *et al.*, 2001). For instance, Avoparcin, a glycopeptide Antimicrobial Growth Promoter (AGP) increased the occurrence of glycopeptide-resistant bacteria (Howarth & Poulter, 1996). Similarly, Colistin use as an AGP in livestock led to the emergence of plasmid-mediated polymyxin resistance (Rhouma *et al.*, 2016). Likewise, Streptomycin resistance was reported in turkeys after experimental feeding with streptomycin (Starr & Reynolds, 1951). Also, an association was found between tetracycline resistance and feeding growth-promoting levels of tetracycline in chickens (Barnes *et al.*, 1979).

Another increasing concern is the discovery of antimicrobial-resistant bacteria in places very far from human activities. For example, Sjölund *et al.* (2008) demonstrated in their study that antimicrobial-resistant bacteria occurred in regions with little human activity such as the arctic regions. Their study isolated antimicrobial-resistant *E. coli* from arctic birds possibly because these resistant bacteria were acquired during migration. Birds that are colonised by antimicrobial-resistant bacteria may become reservoirs of resistant bacteria. In the case of migratory animals particularly migratory birds, they may serve as vectors that spread these bacteria from one locality to the other.

In Ghana, a survey conducted on humans in 2011 reported that the prevalences of resistant bacteria to common and affordable antimicrobial agents such as Tetracycline, Co-trimoxazole, Ampicillin, and Nalidixic acid were largely above 70% while resistances to

Ceftriaxone, Ciprofloxacin, and Amikacin were low (6-11%) (Newman *et al.*, 2011). The high resistance reported for common and affordable antimicrobial agents is probably due to the abuse of antimicrobial agents. This argument is supported by the findings of a study conducted in five African countries which suggests that 30% of people accessed antimicrobial agents without prescription (WHO, 2014). Until 2018, Ghana did not have a policy that guided the use of antimicrobial agents and this could have led to the upsurge in abuse of antimicrobial agents. Hence, In Ghana, the possibility of finding high levels of resistant bacteria in the environment could be high. In the food, agriculture and fisheries sectors in Ghana, the percentage of resistance to antimicrobial agents such as Ampicillin, Tetracycline, Cefadroxil, Erythromycin, Cefotiam, and Penicillin ranged from 61 to 97% (Sackey *et al.*, 2001). Thus, the rate of transferring bacteria along the food chain could be high. Wild animals could be exposed to these antimicrobial agents via shared resources.

The occurrence of antimicrobial resistance in wild animal populations may hamper conservation efforts. Some studies have argued that wild animals, particularly wild birds could be used as sentinels to monitor drug-resistant bacteria. Their ability to migrate from one geographical area to another makes them potential long-range reservoirs for the spread of drug-resistant bacteria (Reed *et al.*, 2003). Ultimately, they may spread resistant bacteria to other wild animals, domestic animals and humans via the food chain. It is thus necessary to pursue the challenge of monitoring for drug resistance by including and increasing surveillance in wild bird populations.

The aim of this chapter was to determine antimicrobial resistance profiles of gram-negative bacteria from forest and waterbirds. Specifically, to determine resistance profiles to Colistin (one of the last resort antimicrobial agents used in the treatment of multidrug-resistant bacteria) and Ciprofloxacin (synthetic quinolone). Subsequently, isolates found

to be Colistin resistant were subjected to multidrug resistance tests including five antimicrobials (Ciprofloxacin, Ampicillin, Oxytetracycline, Streptomycin, and Colistin).

## **5.2 Materials and Methods**

The two hundred and thirty-three cloacal swabs that showed growth on agar plates (see Chapter three, section 4.3.2) were subjected to antimicrobial sensitivity testing. Ninety of the swabs were obtained from forest birds while 143 swabs were from waterbirds. Two methods were employed for antimicrobial sensitivity testing: i) the agar dilution method and ii) the disc diffusion method. Sensitivity to Colistin and Ciprofloxacin was tested using the agar dilution method while the disc diffusion method was employed for multidrug resistance testing of all colistin-resistant isolates obtained by the agar dilution method. Five commercial antimicrobial agents, Ampicillin, Oxytetracycline, Streptomycin, Ciprofloxacin and Colistin representing antimicrobial classes Penicillin, Tetracycline, Aminoglycoside, Quinolone and Polypeptide respectively were used for the multidrug resistance testing.

### **5.2.1 Agar dilution method**

For the agar dilution procedure, MacConkey agar solution was supplemented with Colistin (4 µg/ml). The mixture was swirled to mix thoroughly and poured into 90 mm agar plates to set. Each of the 233 cloacal swabs was streaked on a plate supplemented with Colistin and incubated between 18 – 24 hr at 37 ° C. A maximum of four isolates were picked from each agar plate with growth and identified to the species level by sequencing of 16S rDNA genes.

The agar dilution method was also employed for Ciprofloxacin resistance testing. In this case, MacConkey agar solution was supplemented with 4 µg/ml of Ciprofloxacin and resistant isolates were identified only to the genus level.

### **5.2.2 Co-resistance test by the disc diffusion method (Kirby Bauer method)**

This test was performed for all isolates that were resistant to Colistin by the agar dilution test. Five commercially prepared 6 mm antimicrobial discs were used for this test (Kirby Bauer test) (Shenoy *et al.*, 2002): Ampicillin (AM 10 µg), Colistin (CL 10 µg), Ciprofloxacin (CP 5 µg), Streptomycin (SM 10 µg) and Oxytetracycline (OT 30 µg) (Table 5.1). These antimicrobial agents were selected on the basis of their importance in treating human or animal infections or their use as growth promoters in animal productions. Each isolate was inoculated into 2 ml Mueller Hinton broth and incubated overnight. The turbidity of the inoculum was compared with 0.5 McFarland turbidity standard. A sterile swab was dipped into an inoculum with similar turbidity as 0.5 McFarland standard and was then streaked on freshly prepared 90 mm Mueller Hinton medium agar plate until the whole surface of the agar was covered with inoculum. The five antimicrobial agents mentioned above were placed on the inoculated plates and incubated at 37° C for 18 to 20 h. After incubation, the diameters of the clear zone around the discs were measured using a ruler or precision caliper. Sensitivity profiles were determined by comparing the diameter of the clear zone with disc diffusion breakpoints by the Clinical Laboratory Standards (CLSI) (Table 5.1). *Escherichia coli* ATCC 25922 (American Type Culture Collection) was used as quality control.

**Table 5.1: Breakpoints for antimicrobials used in this study according to CLSI**

Antimicrobial	Code	Disc concentration ( $\mu\text{g}$ )	Breakpoint (mm)
<b>Beta-lactams</b>			
Ampicillin	AM10	10	R $\leq$ 13, I (14-16), S $\geq$ 17
<b>Quinolones</b>			
Ciprofloxacin	CP5	5	R<14, I (15-18), S $\geq$ 19
<b>Tetracyclines</b>			
Oxytetracycline	OT	30	R14, I (15-18), S $\geq$ 19
<b>Aminoglycosides</b>			
Streptomycin	SM10	10	R<11, I (12-14), S $\geq$ 15
<b>Polymyxin</b>			
Colistin	CL10	10	Not yet defined

**R-resistant****I-Intermediate****S-Susceptible****Source: (CLSI, 2018)**

### 5.2.3 Sequencing of Colistin and resistant isolates

To identify resistant species, all Colistin resistant isolates were sent to the Life Science Research Center, Division of Genomics Research, Gifu University, for sequencing of 16S rDNA genes.

### 5.2.4 Data analysis

Based on the CLSI recommendations, the diameter of a clear zone around each antimicrobial agent was reported as susceptible, intermediate or resistant. Bacteria isolates that were resistant to three or all of the antimicrobial agents excluding Colistin were classified as multidrug-resistant. Colistin was excluded because the breakpoints for Colistin have not yet been defined.

Prevalence of antimicrobial resistance to an antimicrobial agent was calculated as follows:

Prevalence of antimicrobial agent resistance to bacteria isolates in the bird

$$= \frac{\text{Number of birds harbouring resistant isolates}}{\text{Total number of birds examined}} \times 100\%$$

Differences in prevalences of resistance between groups were analysed with the chi-square or Fisher's exact test. Difference in mean zone of inhibition for each antimicrobial agent was compared between forest and waterbirds using the non-parametric Mann-Whitney U test.

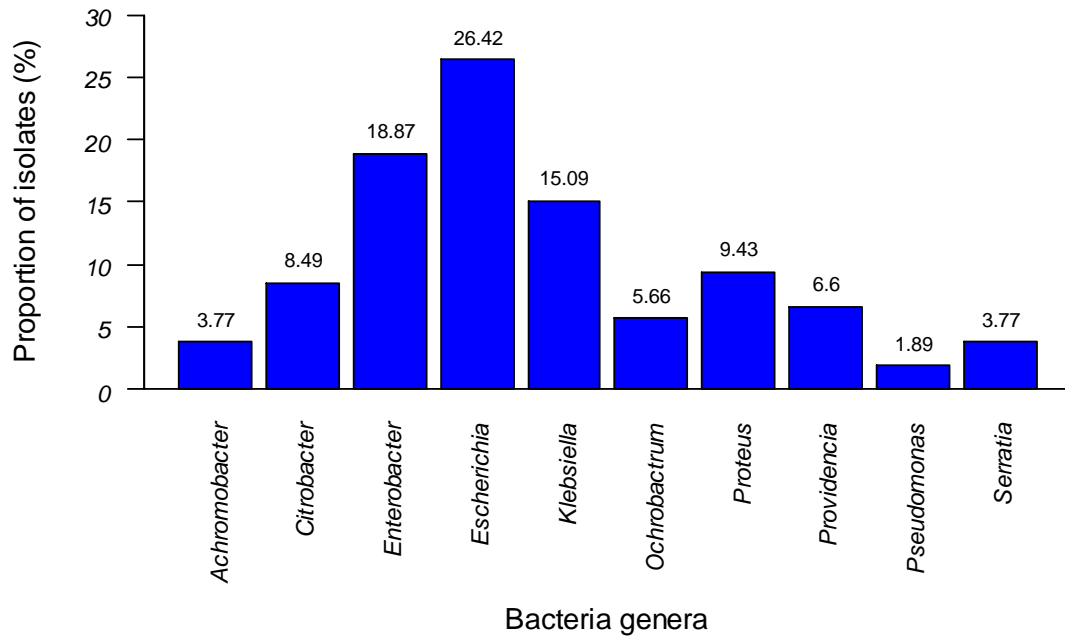
### 5.3 Results

#### 5.3.1 Occurrence of Colistin-resistant isolates from wild birds by the agar dilution method

One-hundred and six Colistin resistant isolates were obtained from all the 233 cloacal swabs. A mean ( $\pm$ SD) of 2.72 ( $\pm$ 1.38) Colistin resistant isolates were obtained per swab/sample. Eighteen different species of Colistin resistant bacteria (Table 5.2) belonging to ten bacteria genera (Figure 5.1) were recorded. The maximum number of bacteria species isolated per genera was four and this was observed in the genera *Enterobacter* (*E. aerogenes*, *E. asburiae*, *E. cloacae* and *E. kobei*). *Escherichia* and *Enterobacter* were the commonly isolated Colistin resistant genera, with prevalences of 6% and 5.6% respectively.

The overall prevalence of Colistin-resistant bacteria in wild birds was 15.5% (36/233). Colistin resistant *Escherichia coli* was the commonest bacteria species isolated and had a prevalence of 6.0% (14/233), followed by *Enterobacter cloacae* with an occurrence of 4.3% (10/233). *Citrobacter freundii* and *Klebsiella variicola* were the next most prevalent (1.7% each). The remaining bacteria species had less than 1% prevalence (Table 5.2).

Colistin-resistant isolates were obtained from 13 host bird species including Forest Robin, Green Hylia, Icterine Greenbul, Olive Sunbird, Red-tailed Bristlebill, White-tailed Anthrush, Yellow-whiskered Greenbul, Black Tern, Common Ringed Plover, Common Tern, Roseate Tern, Sanderling and Whimbrel.



**Figure 5.1: Proportion of isolates for each gram-negative bacteria genus that was resistant to Colistin**

**Table 5.2: Prevalence of bacteria species that showed resistance to Colistin**

<b>Bacteria species</b>	<b>No. of isolates</b>	<b>No. of positive birds (n)</b>	<b>Prevalence (n/233)*100%</b>
<i>Achromobacter xylosoxidans</i>	4	2	0.86%
<i>Citrobacter braakii</i>	4	2	0.86%
<i>Citrobacter freundii</i>	4	4	1.70%
<i>Citrobacter koseri</i>	1	1	0.43%
<i>Enterobacter aerogenes</i>	3	1	0.43%
<i>Enterobacter asburiae</i>	1	2	0.86%
<i>Enterobacter cloacae</i>	15	10	4.29%
<i>Enterobacter kobei</i>	1	1	0.43%
<i>Escherichia coli</i>	28	14	6.0%
<i>Klebsiella oxytoca</i>	5	3	1.30%
<i>Klebsiella pneumoniae</i>	1	1	0.43%
<i>Klebsiella variicola</i>	10	4	1.70%
<i>Ochrobactrum intermedium</i>	6	2	0.86%
<i>Proteus mirabilis</i>	10	3	1.30%
<i>Providencia rettgeri</i>	4	1	0.43%
<i>Providencia stuartii</i>	3	3	1.30%
<i>Pseudomonas aeruginosa</i>	2	2	0.86%
<i>Serratia marcescens</i>	4	1	0.43%
<b>Total</b>	<b>106</b>		

### 5.3.1.1 Occurrence of Colistin-resistant gram-negative bacteria in forest birds

The overall prevalence of Colistin resistance in forest birds was 10% (9/90). Colistin resistant isolates were obtained from seven forest bird species including the Forest Robin, Icterine Greenbul, Olive Sunbird, Red-tailed Bristlebill, White-tailed Ant Thrush, Yellow-whiskered Greenbul (Table 5.3). The prevalence of Colistin-resistant bacteria in bird species with less than 10 sampled individuals was above 15% while the prevalence in bird species with 10 or more sampled individuals was below 15% (Table 5.3) implying that prevalence was high in species with less than 10 sampled individuals.

Twenty-four isolates of Colistin resistant bacteria were obtained from forest birds. These isolates belonged to five genera of gram-negative bacteria and represented nine different species (Table 5.4). The genus with the highest number of species was *Enterobacter* (*E. aerogenes*, *E. cloacae*, and *E. asburiae*) and *Citrobacter* (*C. braakii*, *C. freundii*, and *C.*

*koseri*). The genus *Klebsiella* was represented by two species (*K. oxytoca* and *K. variicola*) while the genus *Escherichia* was represented by only one species (*Escherichia coli*). The maximum number of Colistin resistant species co-existing in an individual bird was three (*E. aerogenes*, *E. coli* and *K. oxytoca*) and this was observed in two bird species (Forest Robin and Yellow-whiskered Greenbul). Statistical differences in prevalence between bacteria species were not determined because of the low numbers of positive birds.

The association between prevalence of colistin-resistant bacteria and proximity of wild birds to human-influenced habitats was performed with data on the Yellow-whiskered Greenbul. Thus, the Yellow-whiskered Greenbuls caught close to the reception/entrance of the protected area and those caught far away from the reception area were considered for the analysis. However, the comparison could not be done because all Colistin-resistant isolates were obtained from birds sampled around the reception area.

**Table 5.3: Prevalences of Colistin-resistant gram-negative bacteria in the forest bird species**

Bird species	No. of birds examined (N)	No. of birds testing positive (n)	Prevalence
Forest Robin	6	1	16.7%
Green Hylia	4	1	25%
Icterine Greenbul	3	1	33.3%
Olive Sunbird	10	1	10%
Red-tailed Bristlebill	3	1	33.3%
White-tailed Antthrush	3	1	33.3%
Yellow-whiskered Greenbul	28	3	10.7%
African Pygmy Kingfisher	1	0	0
Blue-billed Malimbe	1	0	0
Brown-chested Alethe	9	0	0
Dusky-crested Flycatcher	1	0	0
Green-tailed Bristlebill	5	0	0
Grey headed Bristlebill	1	0	0
Little Greenbul	8	0	0
Western Bearded Greenbul		0	0
White-bellied Kingfisher	1	0	0
White-tailed Alethe	5	0	0
Total (90)	90	9	10%

**Table 5.4: Bacteria species obtained from forest birds that showed resistance to Colistin**

Bacteria genera	Bacteria species	No. of isolates	No. of positive birds (n)	Prevalence (n/90)
<i>Citrobacter</i>	<i>Citrobacter braakii</i>	4	2	2.22%
	<i>Citrobacter freundii</i>	3	3	3.33%
	<i>Citrobacter koseri</i>	1	1	1.11%
<i>Enterobacter</i>	<i>Enterobacter aerogenes</i>	1	1	1.11%
	<i>Enterobacter cloacae</i>	2	2	2.22%
	<i>Enterobacter asburiae</i>	2	2	2.22%
	<i>Escherichia coli</i>	5	3	3.33%
<i>Klebsiella</i>	<i>Klebsiella variicola</i>	1	1	1.11%
	<i>Klebsiella oxytoca</i>	5	3	3.33%

### 5.3.1.2 Occurrence of Colistin resistant gram-negative bacteria in waterbirds

Eighty-two isolates of Colistin resistant bacteria were obtained from cloacal swabs from waterbirds. These isolates represented 14 different species belonging to 10 genera of bacteria (Table 5.5). The genus with the highest number of species was *Enterobacter* (*E. kobei*, *E. cloacae*, and *E. asburiae*). The genera *Klebsiella* and *Providencia* were represented by two species each, while the genera *Escherichia*, *Achromobacter*, *Citrobacter*, *Ochrobactrum*, *Proteus*, *Pseudomonas*, and *Serratia* were represented by single species (Table 5.5). The maximum number of Colistin resistant bacteria species co-existing in an individual bird was three and was observed in the Sanderling. *Escherichia coli* was the most frequently isolated Colistin resistant bacteria species (22 isolates).

The overall prevalence of Colistin-resistant bacteria in waterbirds was 18.9% (27/143). They were isolated from six waterbird species; the Black Tern, Common Ringed Plover, Common Tern, Roseate Tern, Ruddy Turnstone, Sanderling and Whimbrel (Table 5.6).

For the analysis of the association between the prevalence of colistin resistance and waterbird species, the Black tern and Sanderling were used for analysis, 20 individuals were randomly selected from each species. The prevalence rates were 30% and 20% for the Black tern and Sanderling respectively. The results showed that the difference in the prevalence of colistin resistance between the species was similar ( $\chi^2$  test value = 0.533, df = 1, p=0.465).

**Table 5.5: Bacteria species obtained from waterbirds that showed resistance to Colistin**

Bacteria genus	Bacteria species	No. of isolates	No. of birds harbouring colistin (n)	Prevalence (n/143)
<i>Achromobacter</i>	<i>Achromobacter xylooxidans</i>	4	2	4.9%
<i>Citrobacter</i>	<i>Citrobacter freundii</i>	1	1	1.2%
<i>Enterobacter</i>	<i>Enterobacter asburiae</i>	1	1	1.2%
	<i>Enterobacter cloacae</i>	13	8	15.9%
	<i>Enterobacter kobei</i>	1	1	1.2%
<i>Escherichia</i>	<i>Escherichia coli</i>	22	11	26.8%
<i>Klebsiella</i>	<i>Klebsiella pneumonia</i>	2	1	2.4%
	<i>Klebsiella variicola</i>	9	3	11.0%
	<i>Ochrobactrum intermedium</i>	6	2	7.3%
<i>Proteus</i>	<i>Proteus mirabilis</i>	10	3	12.2%
<i>Providencia</i>	<i>Providencia rettgeri</i>	5	1	4.9%
	<i>Providencia stuartii</i>	3	1	3.7%
<i>Pseudomonas</i>	<i>Pseudomonas aeruginosa</i>	2	2	2.4%
<i>Serratia</i>	<i>Serratia marcescens</i>	3	1	4.9%
Total	14	82		

**Table 5.6: Prevalence of Colistin resistant gram-negative bacteria in waterbirds**

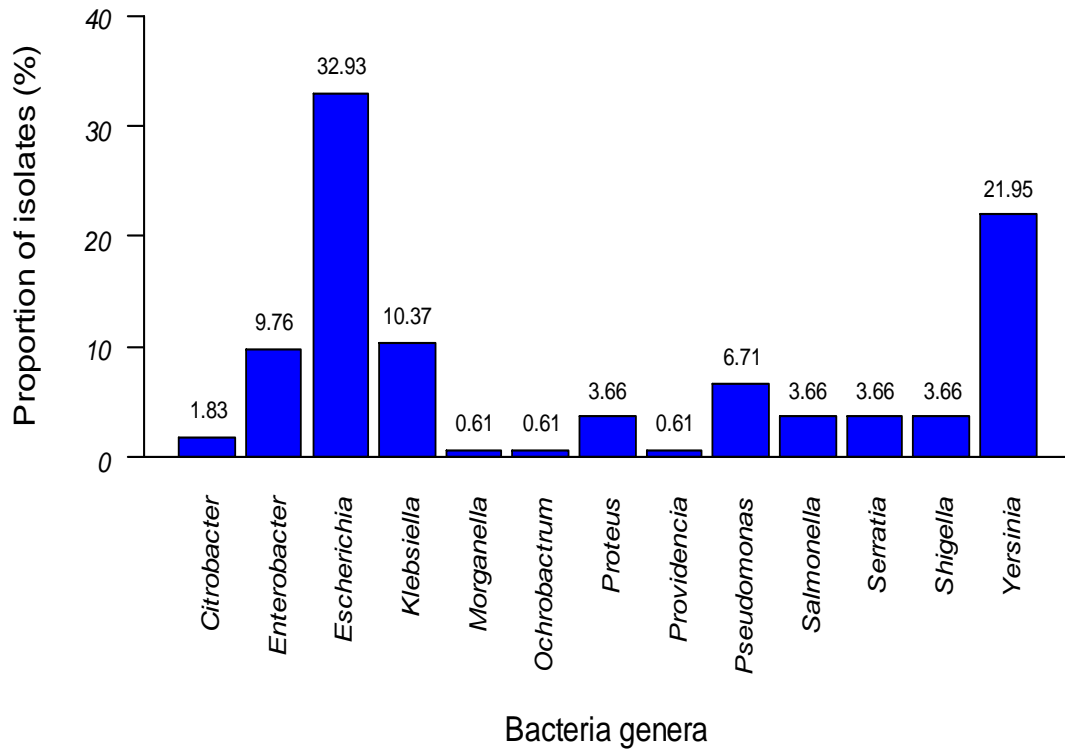
<b>Bird species</b>	<b>No. of birds examined</b>	<b>No. of birds harbouring colistin</b>	<b>Prevalence %</b>
Black Tern	36	10	27.8
Common Ringed Plover	2	2	100
Common Tern	12	1	10
Roseate Tern	4	1	25
Sanderling	73	11	15.1
Whimbrel	4	2	50
Common Sandpiper	2	0	0
Green Shank	1	0	0
Grey Plover	2	0	0
Malachite Kingfisher	1	0	0
African Oystercatcher	1	0	0
Pied Kingfisher	4	0	0
Ruddy Turnstone	1	0	0
<b>Total</b>	<b>143</b>	<b>27</b>	<b>18.9%</b>

### 5.3.2 Occurrence of Ciprofloxacin-resistant bacteria (agar dilution method)

One-hundred and sixty-four Ciprofloxacin-resistant isolates were obtained from 233 cloacal swabs. Ciprofloxacin-resistant isolates were identified to the genus level. In all, Ciprofloxacin resistant bacteria isolates belonged to thirteen genera (Figure 5.2).

The overall occurrence of Ciprofloxacin-resistant bacteria in the birds was 41.7% (97/233). *Escherichia* was the most commonly isolated Ciprofloxacin-resistant genus (Figure 5.2) and constituted 32.93% (54/164) of all isolates.

The prevalence of the genus *Escherichia* had the highest prevalence of 19.3% (45/233) followed by the genus *Yersinia* with a prevalence of 12% (28/233). The remaining genera had prevalences of less than 10%. Ciprofloxacin-resistant isolates were obtained from 20 wild bird species.



**Figure 5.2: Proportions of gram-negative bacteria genera resistant to Ciprofloxacin**

**Table 5.7: Prevalences of bacteria genera that showed resistance to Ciprofloxacin**

Bacteria genus	No. of isolates	No. of positive individuals (n)	Prevalence (n/233)*100
<i>Citrobacter</i>	3	3	1.3%
<i>Enterobacter</i>	16	15	6.4%
<i>Escherichia</i>	54	45	19.3%
<i>Klebsiella</i>	17	15	6.4%
<i>Morganella</i>	1	1	0.4%
<i>Ochrobactrum</i>	1	1	0.4%
<i>Proteus</i>	6	6	2.6%
<i>Providencia</i>	1	1	0.4%
<i>Pseudomonas</i>	11	9	3.7%
<i>Salmonella</i>	6	6	2.6%
<i>Serratia</i>	6	6	2.6%
<i>Shigella</i>	6	6	2.6%
<i>Yersinia</i>	36	28	12%
Total	164		

**5.3.2.1: Occurrence of Ciprofloxacin-resistant bacteria in forest birds**

The prevalence of Ciprofloxacin resistant bacteria in forest birds was 35.6% (32/90).

These resistant bacteria were isolated from eleven different species of forest birds including the Brown-chested Alethe, Green Hylia, Little Greenbul, Olive Sunbird, and Yellow-whiskered Greenbul (Table 5.8).

Fifty-three Ciprofloxacin-resistant isolates were obtained from forest birds. These isolates belonged to 10 genera of bacteria (Table 5.9). The maximum number of Ciprofloxacin resistant genera co-existing in an individual bird was three and these were observed in five bird species: Brown-chested Alethe, Little Greenbul, Olive Sunbird, Red-tailed Bristlebill, White-bellied Kingfisher.

**Table 5.8: Prevalence of Ciprofloxacin-resistant bacteria in each forest bird species**

<b>Bird species</b>	<b>No. of birds examined</b>	<b>No. of birds harbouring resistant isolates</b>	<b>Prevalence (%)</b>
African Pygmy Kingfisher	1	0	0
Blue-billed Malimbe	1	0	0
Brown-chested Alethe	9	4	44.44
Dusky-crested Flycatcher	1	1	100
Forest Robin	6	1	16.67
Green Hylia	4	3	75
Green-tailed Bristlebill	5	3	60
Grey-headed Bristlebill	1	0	0
Icterine Greenbul	3	0	0
Little Greenbul	8	3	37.50
Olive Sunbird	10	8	80
Red-tailed Bristlebill	3	2	66.67
Western Bearded Greenbul	1	0	0
White-tailed Alethe	5	0	0
White-tailed Antthrush	3	1	33.33
White-bellied Kingfisher	1	1	100
Yellow-whiskered Greenbul	28	5	17.85
<b>Total</b>	<b>90</b>	<b>32</b>	<b>35.6%</b>

**Table 5.9: Prevalence of Ciprofloxacin-resistant bacteria genera in forest birds**

<b>Bacteria genera</b>	<b>Number of isolates</b>	<b>Number of positive birds (n)</b>	<b>Occurrence(n/90)*100</b>
<i>Shigella</i>	1	1	1.1%
<i>Yersinia</i>	8	7	7.8%
<i>Salmonella</i>	2	2	2.2%
<i>Citrobacter</i>	2	2	2.2%
<i>Enterobacter</i>	2	2	2.2%
<i>Escherichia</i>	23	18	20.0%
<i>Klebsiella</i>	7	7	7.8%
<i>Proteus</i>	4	4	4.4%
<i>Pseudomonas</i>	3	3	3.3%
<i>Serratia</i>	1	1	1.1%

### 5.3.2.2 Occurrence of Ciprofloxacin resistant bacteria in waterbirds

One Hundred and one Ciprofloxacin resistant isolates were obtained from waterbirds. These isolates belonged to 13 genera of bacteria (Table 5.10). The maximum number of Ciprofloxacin-resistant bacteria genera co-existing in an individual bird was three and these were observed in two bird species (Black Tern and Sanderling).

Overall, the prevalence of Ciprofloxacin-resistant bacteria in waterbirds was 41.3% (59/143). These resistant bacteria were isolated from nine different waterbirds: Black Tern, Common Tern, Grey Plover, Pied Kingfisher, Ringed Plover, Roseate Tern, Sanderling, Whimbrel and Wood Sandpiper (Table 5.11)

**Table 5.10: Prevalence of Ciprofloxacin-resistant bacteria genera in waterbirds**

<b>Bacteria genera</b>	<b>Number of isolates</b>	<b>Number of positive birds (n)</b>	<b>Occurrence (n/143)*100</b>
<i>Shigella</i>	5	5	3.5%
<i>Yersinia</i>	21	21	14.7%
<i>Salmonella</i>	4	4	2.8%
<i>Citrobacter</i>	1	1	0.7%
<i>Enterobacter</i>	14	13	9.1%
<i>Escherichia</i>	31	27	18.9%
<i>Klebsiella</i>	10	8	5.6%
<i>Proteus</i>	1	1	0.7%
<i>Pseudomonas</i>	1	6	4.2%
<i>Serratia</i>	2	5	3.5%
<i>Morganella</i>	1	1	0.7%
<i>Ochrobactrum</i>	8	1	0.7%
<i>Providencia</i>	5	1	0.7%

**Table 5.11: Prevalence of Ciprofloxacin-resistant bacteria in each waterbird species**

<b>Bird species (N)</b>	<b>No. of birds examined</b>	<b>No. of harbouring resistant bacteria</b>	<b>Prevalence (%)</b>
Black Tern	36	19	52.8
Common Tern	12	4	33.3
Common Sandpiper	2	1	50
Common Ringed Plover	2	1	50
Grey Plover	0	0	0
Green Shank	0	0	0
Pied Kingfisher	4	2	50
Malachite Kingfisher	2	1	50
Roseate Tern	4	1	25
Ruddy Turnstone	0	0	0
Sanderling	73	27	37
Whimbrel	4	3	75
Total	143	59	41.3%

### 5.3.3 Occurrence of multidrug-resistant isolates

All Colistin resistant isolates tested for multidrug resistance showed phenotypic results by the disc diffusion method. Some isolates showed clear zones of inhibition around antimicrobial agent discs while others did not show any zones of inhibition (Plate 5.1).

The most frequently encountered form of antimicrobial resistance was resistance to Ampicillin (73.6%, 78/106), followed by resistance to Oxytetracycline (52.8%, 56/106), Streptomycin (50.9%, 54/106), and Ciprofloxacin (8.5%, 9/106) (Figure 5.4). The highest percentage of susceptible isolates was observed for the antimicrobial agents Ciprofloxacin and Streptomycin (23.6% each). About 88% (93/106) of the isolates showed resistance to at least one antimicrobial agent, 22.6% (24/106) showed resistance to two of antimicrobials, 31.1% (33/106) showed resistance to three antimicrobials and 3.8% (4/106) showed resistance to four antimicrobials.

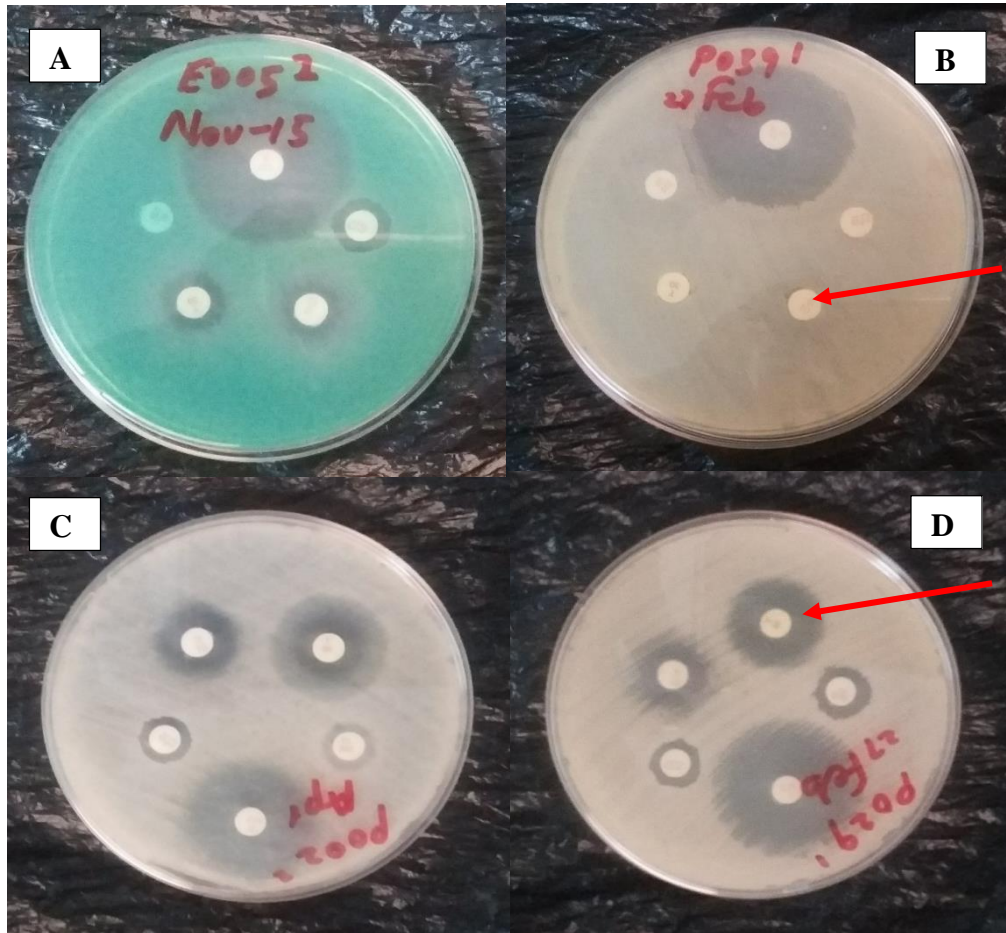
All 18 colistin-resistant bacteria species also showed resistance to Ampicillin and 14 each of Colistin-resistant bacteria species showed resistance to Oxytetracycline and Streptomycin. Only three of the species (*Escherichia coli*, *Citrobacter freundii* and *Citrobacter braakii*) showed resistance to Ciprofloxacin (Table 5.12). Two of the isolates belonging to the species *Enterobacter aerogenes* and *Providencia stuartii* were susceptible to all four antimicrobial agents (Ciprofloxacin, Oxytetracycline, Ampicillin and Streptomycin).

Resistance to Ciprofloxacin occurred in *Citrobacter freundii* (one isolate), *Citrobacter braakii* (one isolate) and *Escherichia coli* (7 isolates). For Ampicillin, resistant isolates were high for the species *Escherichia coli* (16 isolates), *Enterobacter cloacae* (11 isolates) and *Proteus mirabilis* (six isolates). The bacteria species *Escherichia coli* (15 isolates) and *Proteus mirabilis* (9 isolates) recorded a high number of resistant isolates to

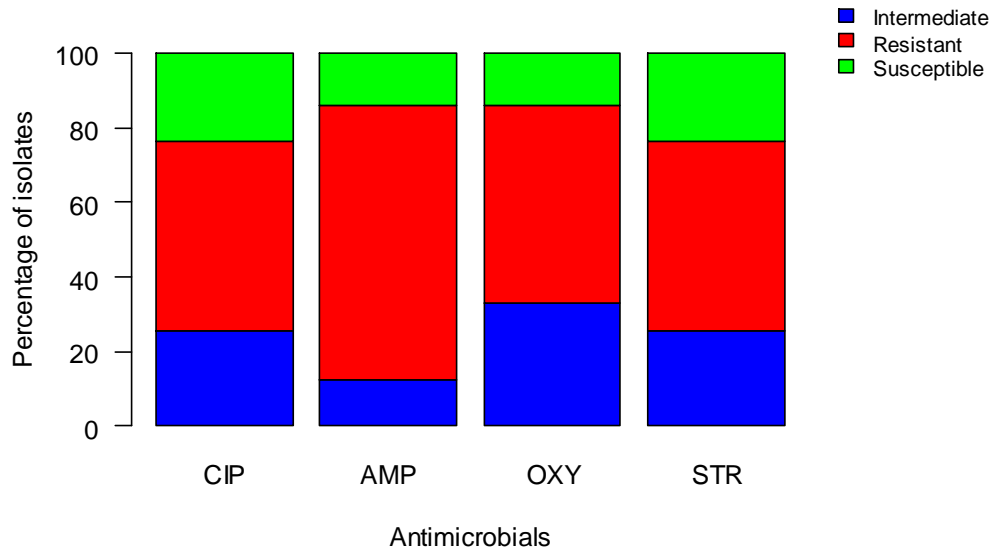
Oxytetracycline. The high resistance to Streptomycin was observed for the bacteria species *Escherichia coli* (13 isolates).

Disc diffusion zone sizes were compared for differences between bacteria species collected from forest and waterbirds collected (Table 5.15). There were no significant differences seen in the diffusion zone sizes for all agents except Ampicillin ( $W = 552$ ,  $p$ -value = 0.000817). The largest diffusion zones (greater susceptibility) were observed in waterbird isolates (Table 5.14). An exception was the diffusion zone for Streptomycin; for this antimicrobial agent, the forest bird isolates had the largest diffusion zone.

Overall, multidrug resistance in the wild birds was 27.8% (10/36) and was observed in the following host bird species: Yellow-whiskered Greenbul, Red-tailed Bristlebill, Olive Sunbird, Icterine Greenbul, Forest Robin, Black Tern, and Sanderling.



**Plate 5.1: Agar plates showing susceptibility profiles of some isolates to the 5 antimicrobial discs used in this study. Arrow in B is pointing to an antimicrobial disc showing no clear zone around the disc while arrow in D is indicating disc surrounded by a clear zone. Clear zones indicate susceptibility.**



**Figure 5.3: Antimicrobial susceptibility profiles. Percentage of antimicrobial susceptibility in the isolates to antimicrobials Ciprofloxacin (CIP), Ampicillin (AMP), Oxytetracycline (OXY) and Streptomycin (STR).**

**Table 5.12: Antimicrobial resistance profiles of bacteria species to four antimicrobial agents**

Bird species	No. of isolates	No. of resistant isolates			
		Amp	Strep	Oxy	Cip
<i>Achromobacter xylosoxidans</i>	4	3	0	4	0
<i>Citrobacter braakii</i>	2	2	1	2	1
<i>Citrobacter freundii</i>	4	3	2	2	1
<i>Citrobacter koseri</i>	1	1	1	0	0
<i>Enterobacter aerogenes</i>	1	1	1	1	0
<i>Enterobacter asburiae</i>	3	2	2	2	0
<i>Enterobacter cloacae</i>	15	11	7	5	0
<i>Enterobacter kobei</i>	1	1	1	1	0
<i>Escherichia coli</i>	30	20	13	15	7
<i>Klebsiella oxytoca</i>	4	2	3	4	0
<i>Klebsiella pneumoniae</i>	2	10	0	0	0
<i>Klebsiella variicola</i>	10	4	5	4	0
<i>Ochrobactrum intermedium</i>	6	2	2	2	0
<i>Proteus mirabilis</i>	10	6	10	9	0
<i>Providencia rettgeri</i>	5	4	4	4	0
<i>Providencia stuartii</i>	3	3	0	0	0
<i>Pseudomonas aeruginosa</i>	2	1	0	1	0
<i>Serratia marcescens</i>	3	2	2	0	0
<b>Total</b>	<b>106</b>	<b>78</b>	<b>54</b>	<b>56</b>	<b>9</b>

Amp = Ampicillin

Strep = Streptomycin

Oxy = Oxytetracycline

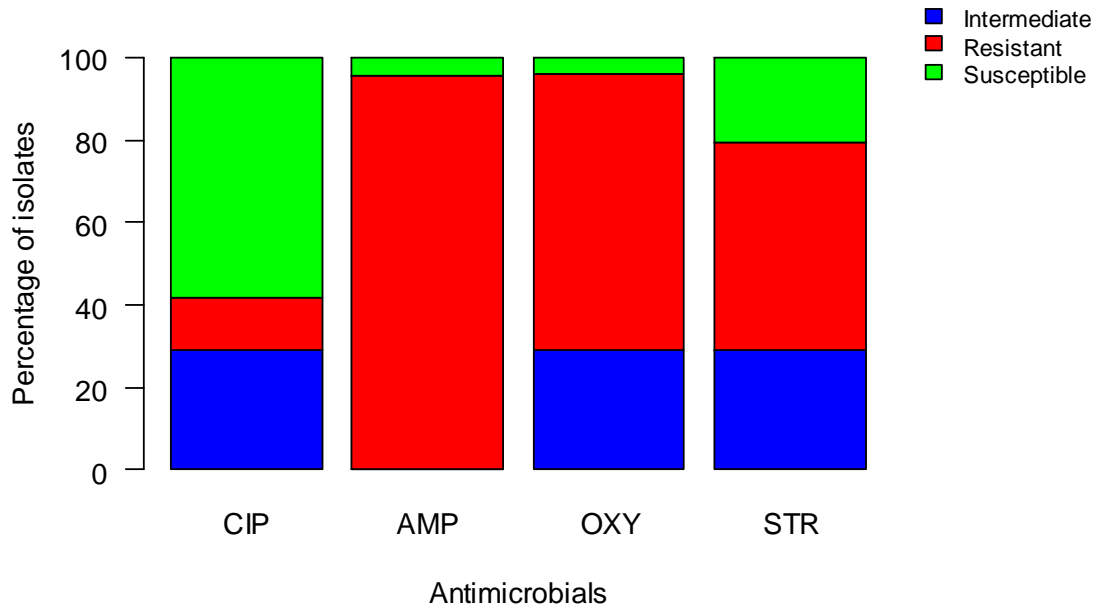
Cip = Ciprofloxacin

### 5.3.3.1 Multidrug resistance in forest birds

Twenty-four of the Colistin-resistant isolates were obtained from forest birds. About Ninety-six percent (23/24) of these isolates showed resistance to at least one other antimicrobial agent tested by the disc diffusion method, 25% (6/24) showed resistance to two other antimicrobial agents and 62.5% (15/24) were multidrug resistant.

All isolates showed resistance to at least one of the antimicrobial agents except one, *Citrobacter freundii*. About 13% (3/24) of isolates showed resistance to Ciprofloxacin, 95.8% (23/24) showed resistance to Ampicillin, 66.6% (16/24) showed resistance to Oxytetracycline and 50% (12/24) showed resistance to Streptomycin (Figure 5.5). There was a significant difference in resistance to the four antimicrobial agents tested ( $\chi^2=21.462$ ,  $df=3$ ,  $p<0.05$ ). Thus, all Colistin-resistant isolates showed high resistance to Ampicillin, Oxytetracycline, and Streptomycin except for Ciprofloxacin.

The overall prevalence of multidrug resistance in forest birds was 66.7% (6/9) (Figure 5.13). Multidrug-resistant isolates were obtained from one individual each of the species: Yellow-whiskered Greenbul, Red-tailed Bristlebill, Olive Sunbird, Icterine Greenbul and Forest Robin. All birds that harboured Colistin resistant isolates also harboured multidrug resistant isolates, except the White-tailed Ant Thrush.



**Figure 5.4: Antimicrobial susceptibility profiles. Percentage of antimicrobial susceptibility in the isolates to antimicrobials Ciprofloxacin (CIP), Ampicillin (AMP), Oxytetracycline (OXY) and Streptomycin (STR).**

**Table 5.13: Prevalence of multidrug resistance in forest birds**

Bird species	No. of individuals examined	No. of birds harbouring multidrug-resistant isolates	Prevalence (%)
Forest Robin	1	1	100
Green Hylia	1	1	100
Icterine Greenbul	1	1	100
Olive Sunbird	1	1	100
Red-tailed Bristlebill	1	1	100
White-tailed Ant Thrush	1	0	0
Yellow-whiskered Greenbul	3	1	33.3
Total	9	6	66.7

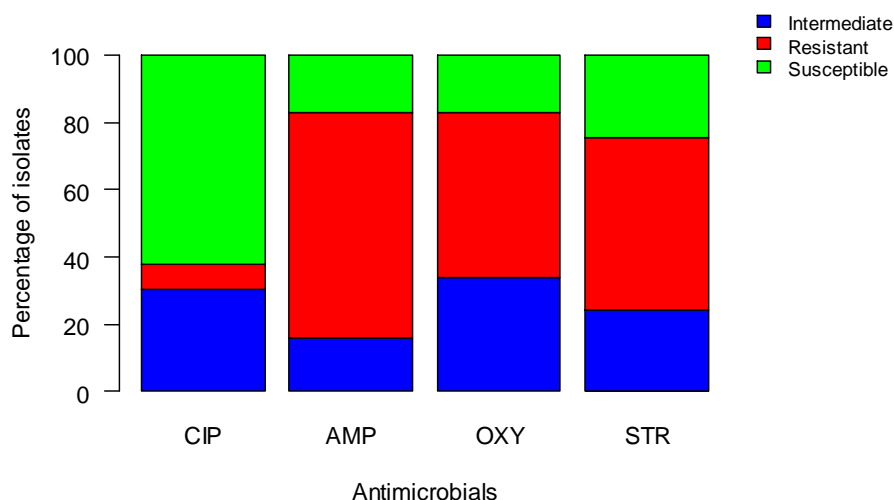
### 5.3.3.2 Multidrug resistance in waterbirds

Eighty-two Colistin-resistant isolates were obtained from waterbirds. About 85% (70/82) of these isolates showed resistance to at least one other antimicrobial agent by the disc diffusion method, 22% (18/82) showed resistance to only two of the antimicrobials and 31.7% (26/82) were multidrug-resistant.

About 7% (6/82) of isolates showed resistance to Ciprofloxacin, 30.5% (25/82) showed resistance to Ampicillin, 24.4% (20/82) showed resistance to Oxytetracycline and 18.3% (15/82) showed resistance to Streptomycin (Figure 5.6).

There was a significant difference in resistance to the four antimicrobials tested ( $\chi^2=21.462$ ,  $df=3$ ,  $p<0.05$ ). All resistance to Ampicillin, Oxytetracycline, and Streptomycin was higher than in Ciprofloxacin.

Multidrug-resistant isolates were obtained from four individual Black Terns and four Sanderlings with prevalences of 40% (4/10) and 36.4% (4/11), respectively.



**Figure 5.5: Antimicrobial susceptibility profiles. Percentage of antimicrobial susceptibility in the isolates to antimicrobials Ciprofloxacin (CIP), Ampicillin (AMP), Oxytetracycline (OXY) and Streptomycin (STR).**

**Table 5.14: Prevalence of multidrug resistance in waterbird species**

<b>Bird species</b>	<b>No. of birds examined</b>	<b>No. of birds harbouring multidrug-resistant isolates</b>	<b>Prevalence (%)</b>
Black Tern	10	4	40
Common Ringed Plover	2	0	0
Common Tern	1	0	0
Roseate Tern	1	0	0
Sanderling	11	4	36.4
Whimbrel	2	0	0
Total	27	8	0

**Table 5.15: Mean disc diffusion zones and resistance breakpoints for gram-negative bacteria isolates, by type of bird**

<b>Antimicrobial agent</b>	<b>Breakpoint (mm)</b>	<b>Mean disc diffusion zone diameter (mm)</b>		
		<b>Forest bird isolates (24)</b>	<b>Waterbird isolates (82)</b>	<b>Mann-Whitney U P-value<sup>a</sup></b>
Ciprofloxacin	≤15	20.42	21.27	0.4305
Ampicillin	≤13	8.69	10.92	0.0008
Oxytetracycline	≤14	13.02	13.98	0.2547
Streptomycin	≤11	10.96	10.91	0.1652

<sup>a</sup> Test for significant differences in disc diffusion zones between types of samples

#### 5.4 Discussion

Wild birds are considered potential reservoirs for the spreading of resistant bacteria. During annual migration, wild birds, particularly migratory birds potentially play a role in the epidemiology of human-associated zoonoses. Interestingly, the first antimicrobial-resistant bacteria from wildlife was isolated from wild birds (pigeons) around 1975 (Sato *et al.*, 1978). Since then many bird species have been found to carry antimicrobial-resistant bacteria. In this chapter, the occurrence of bacterial isolates resistant to antimicrobial agents in forest birds from a protected area and waterbirds from a coastal Ramsar Site and a beach were determined. Resistance to two antimicrobial agents (Colistin and Ciprofloxacin) was investigated. Multidrug resistance to the antimicrobial agents Ampicillin, Streptomycin, Oxytetracycline, Ciprofloxacin, and Colistin was also determined for all bacteria isolates that were Colistin resistant by the agar dilution method.

The first case of antimicrobial resistance in wildlife was from wild birds (pigeons) harbouring *E. coli* showing multiples resistance to some antimicrobial agents (Sato *et al.*, 1978). In this study, Both Colistin and Ciprofloxacin resistant isolates were obtained cloacal swabs from sampled wild birds sampled, implicating these birds as potential reservoirs for the epidemiology of resistant bacteria to other animals (domestic and wild) and humans. The frequency of isolation of gram-negative bacteria resistant to Colistin in the sampled birds was 15.5% (36/233), 18.9% (27/143) in waterbirds and 10% (9/90) in forest birds. A comparison of the prevalence of Colistin resistance in forest and waterbirds shows that the prevalence in waterbirds was almost twice that of forest birds. A probable reason for this could be the difference in numbers of forest and water birds sampled. Also, the water birds sampled share water resource with humans, so may have acquired resistant bacteria from the shared resource contaminated by humans.

Colistin resistance is rare; thus, this antimicrobial is not commonly used; yet, its prevalence in this study was 15.5%. The first case of Colistin-resistant gene in wild birds was detected in a bacteria isolate from a migratory bird (European herring gull *Larus argentatus*) in 2016 (Mohsin *et al.*, 2016) so it is not surprising that resistance to Colistin was observed in this study. Mohsin *et al.* (2016) also found that the Colistin resistant isolate was resistant to Ampicillin, an observation which is similar to what was found in this study.

Also, the prevalence of Colistin resistant bacteria in forest and water- birds were 10% and 18.9% respectively. It is interesting to note that for forest birds sampled birds between the reception area to about 8 km into the interior of the Conservation Area, Colistin resistant bacteria isolates were obtained from birds captured about 300-500 m away from the reception. There were very close to the reception area farms and human settlements, hence, it is possible that these birds may have acquired resistant bacteria from interactions with humans and livestock in the surrounding communities.

The high occurrence of Colistin resistant bacteria in waterbirds could be linked to the fact that they acquired resistance bacteria from the shared water resource. Vittecoq *et al.* (2016) found that natural habitats strongly impacted by human activities are the ones in which the highest diversity of AMR are observed. At Esiama beach, the birds shared the estuaries and beach with livestock, domestic animals and humans. Several anthropogenic activities took place on the beach such as fishing, open defecation, shellfish picking, washing of livestock, among others. The birds could have obtained resistant bacteria via contact with faeces from humans and livestock. At the Densu Delta Ramsar Site, the waterbirds shared the water with humans and domestic animals. Activities such as salt

mining and fishing were prevalent in the area. Therefore, resistant bacteria could have been acquired via contact with water contaminated with faecal matter from surrounding human settlements. Guenther *et al.* (2011) suggested that antimicrobial-resistant bacteria can spread directly via contact or indirectly, through environmental pathways. Birds may consume water polluted with faeces or human waste via food and this seems to be the major source of transmission of resistant bacteria of human and veterinary origin to wild birds. The possibility of acquiring resistant bacteria along the migratory route before arrival in Ghana by migratory birds cannot be ruled out.

Ciprofloxacin resistance, on the otherhand was relatively high (41.7%) by the agar dilution method; overall, 32% in forest birds and 44% in waterbirds. This observation was contrary to expectation as Ciprofloxacin is a completely synthetic antimicrobial agent that has an increased potency and a decreased frequency of spontaneous bacteria resistance (Hooper *et al.*, 1987).

In forest birds, Ciprofloxacin-resistant bacteria isolates were obtained from birds sampled from both near the reception area and also about 8 km away. This suggests that Ciprofloxacin resistance was widespread in the conservation area.

For multidrug resistance, surprisingly, 62.5% of the isolates from forest birds were multidrug resistant, while 31.7% of isolates from waterbirds showed multidrug resistance. In forest birds, resistance to Ampicillin was demonstrated in 95.8% of the isolates. In a similar study conducted on birds from the Brazilian Atlantic Forest, bacteria isolates from the birds were tested for resistance to five antimicrobial agents (Ampicillin, Chloramphenicol, Kanamycin, Streptomycin, and Tetracycline) (Nascimento *et al.*, 2003)

and the results were similar to what was obtained in this study. For example, resistance to Ampicillin was the most frequent in that study. Nascimento *et al.* (2003) also found that 10% of their isolates showed a multidrug-resistant pattern to Ampicillin, Tetracycline, and Chloramphenicol. A similar pattern was observed in this study when Chloramphenicol is replaced with Streptomycin, however this pattern was observed in 29.2% of isolates in this study. Donkor *et al.* (2011) found a high prevalence of resistance (95.7%) to Ampicillin in *E. coli* isolates from farm animals and their keepers in Ghana. The prevalence of *E. coli* isolates resistant to Ampicillin in this study was also relatively high (73.33%).

Resistance to Ciprofloxacin (8.4%, 9/106) was the least observed by the disc diffusion method in this study. The inference that could be drawn from this was that both forest and water- birds had little exposure to this antimicrobial agent compared to the other three antimicrobial agents. Another probable reason is that Ciprofloxacin belongs to the drug class Quinolone which has had restricted use in veterinary medicine since the 1990s after the rapid emergence of resistance to fluoroquinolones (Engberg *et al.*, 2001). Similarly, Newman *et al.* (2011) found that resistance to Ciprofloxacin in human isolates was low (11%). Resistance to Ciprofloxacin by the disc diffusion method was low because isolates used were those that had been found to be resistant to colistin and were obtained from 39 individual birds, however, 233 cloacal swabs were examined for Ciprofloxacin by the agar dilution method, possibly resulting in the high prevalence.

Antimicrobial resistance pattern (Ampicillin-Oxytetracycline-Streptomycin) was observed in both forest and water – birds, suggesting that the level of exposure of these birds to the antimicrobial agents is similar. The similarities in patterns also suggest that

there was a common source of resistant bacteria for both the forest and water- birds. The presence of Ampicillin, Oxytetracycline, and Streptomycin from various species of birds corroborates findings elsewhere on antimicrobial agent resistance to Enterobacteria from a variety of sources worldwide (Erskine *et al.*, 2002; Schlegelova *et al.*, 2002; Schroeder *et al.*, 2002; Klein & Bülte, 2003).

Although some of the birds captured in this study are non-migratory, they could serve to disperse bacteria between widely separated locations and from hotspots to vulnerable populations. Transmission of these bacteria to waterways and other environmental resources via faecal deposition, may therefore constitute a considerable hazard to human and animal health (Bonnedahl *et al.*, 2014). This highlights the linkage between humans, animals and the environment often espoused in the “One Health Approach”.

Evidence from this study suggests that the premise that antimicrobial-resistant bacteria occur generally in hospitals, domestic animals and animal farms should be reconsidered. It is unclear how the birds sampled in this study acquired resistance, but there is reason for concern. Discussions about the expedience of a tight grip policy on antimicrobial release for resistance control is necessary.

## **6.0 CHAPTER SIX: OCCURRENCE OF COLISTIN AND CIPROFLOXACIN RESISTANT DETERMINANTS/GENES IN BACTERIAL ISOLATES FROM FOREST AND WATER-BIRDS**

### **6.1 Introduction**

Antimicrobial resistance is a global health threat that may lead to significant morbidity and mortality. The WHO (2014) estimates that by the year 2050, drug-resistant infections could lead to 10 million deaths a year globally. A major risk factor for this increase is the extensive use of antimicrobial agents and the dissemination of resistant bacteria and their genes in humans and animals (van den Bogaard and Stobberingh, 2000). A growing concern is resistance to newer antimicrobial agents such as third-generation Cephalosporins leading to treatment failures in community and hospital-acquired infections (Batchelor *et al.*, 2008).

The mechanisms of antimicrobial resistance in bacteria include target substitution, target protection, block of intracellular antimicrobial accumulation and antimicrobial detoxification (Middleton & Ambrose, 2005). Thus, the mechanism of acquiring resistance genes may arise by mutation or can be acquired by the horizontal transfer of resistance genes via mobile genetic elements such as plasmids, integrons, and transposon (Middleton and Ambrose, 2005). Plasmid-mediated transmission is the most common mechanism of horizontal gene transfer among bacteria (Davies and Davies, 2010).

Plasmid-mediated resistance is the transfer of antibiotic resistance genes via plasmids (Bennett, 2008). These plasmids can be transferred between the same species of bacteria or different species via a mechanism known as conjugation (Thomas & Nielsen, 2005). In this chapter, the occurrences of plasmid-mediated quinolone and polymyxin resistance

genes were investigated. Unlike intrinsic resistance that is caused by functional expression or mutation of chromosomal genes, plasmid-borne genes have the potential to transfer resistance (Liu *et al.*, 2016).

Plasmid-mediated quinolone resistance (PMQR) was discovered in 1998 in a multi-resistant *Klebsiella pneumonia* isolate (Strahilevitz *et al.*, 2009). Since then, six different plasmid-mediated genes have been identified as responsible for quinolone resistance and they include *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *qnrVC* (Hooper & Jacoby, 2015). These genes code for proteins of the pentapeptide repeat family and inhibit quinolone activity (Schultsz & Geerlings, 2012). They can be found on the chromosome of both gram-positive and gram-negative bacteria (Sánchez *et al.*, 2008). Over the years, studies have reported the occurrence of plasmid-mediated quinolone resistance (PMQR) determinants in both clinical and environmental bacterial samples (Poirel *et al.*, 2012).

Plasmid-mediated Colistin resistance gene was first described by Liu *et al.* (2016). The gene *mcr-1* was the first to be isolated and was identified in *Escherichia coli* from humans and animals in China. Following this finding, four other plasmid-mediated genes (*mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*) have been described (Xavier *et al.*, 2016; Borowiak *et al.*, 2017; Carattoli *et al.*, 2017; Yin *et al.*, 2017).

Resistance to antimicrobial agents is increasing, possibly due to the dissemination of resistance genes. In ecological studies of antimicrobial resistance, there has been perhaps too much focus on resistant organisms and not enough on resistance genes. Due to the capability of bacteria to transfer resistance genes even among distantly related bacteria, analyses of antimicrobial resistance emergence, dissemination and persistence might be better conducted at the gene level (Singer *et al.*, 2007). Hence, identification of resistance

genes is necessary to understand the epidemiology of antimicrobial resistance and also to verify the non-susceptibility of phenotypes. Therefore, the objective of this chapter was to investigate the occurrence of resistance determinants (*qnr* and *mcr*) for two major resistance phenotypes (Ciprofloxacin and Colistin).

## **6.2 Materials and Methods**

All Colistin and Ciprofloxacin resistant isolates obtained from bird samples in this study (Chapter 5, Section 5.3) were examined for plasmid-mediated resistance genes. Deoxyribonucleic acid (DNA) was extracted from the isolates and used in Polymerase Chain Reactions (PCRs) to amplify segments of the target genes.

### **6.2.1 Extraction of DNA templates by the boiling method (adopted and modified from Millar *et al.* (2000))**

To extract template DNA from pure isolates of bacteria, Luria Bertani Broth (LB) was prepared by adding 2 g of Bactotrypton powder, 2 g of NaCl and 1 g of Yeast extract to 200 ml of deionised water. The mixture was stirred using a magnetic stirrer to mix completely and autoclaved at 121 ° C for 15 min. Three to five colonies of each pure Colistin-resistant or Ciprofloxacin isolate were inoculated in 2 ml of LB and incubated at 37 ° C between 18 – 24 h. Thereafter, about 1 ml of inoculum was transferred into a 1.5 ml microtube and centrifuged for 5 min at 10000 rpm (4° C). The cell pellet was resuspended in 500 µl of 70% ethanol and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and the pellet was lysed in 100 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. Then the mixture was centrifuged at 5000 rpm for 5 min and the supernatant was

discarded. The pellet was resuspended in 50 µl of TE buffer and boiled for 5 min and then cooled on ice. After cooling the mixture was centrifuged at 5000 rpm for 5 min and the supernatant was used as template DNA.

### **6.2.2 Polymerase Chain Reaction (PCR) determination of Colistin resistance genes**

Polymerase chain reaction assays were used to amplify target resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*). DNA amplifications were performed in a final volume of 20 µl containing 1 µl of the DNA template from each Colistin-resistant isolate, 2 µl of 10x ExTaq buffer (supplied with *Taq* polymerase), 5 pmol of each corresponding primer (Table 6.1), 2 µM each deoxynucleoside triphosphate (dNTP), and 2.5 U of *Taq* polymerase (Ex Taq, Takara Bio Inc™, Shiga, Japan). Amplifications were performed with TaKaRa thermal cycler Dice system. After an initial denaturation for 1 min at 95 ° C, 30 cycles of 30 s at 95 ° C, 30 s at 55 ° C and 15 s at 72 ° C were run, followed by and a final extension at 72 ° C for 5 min. Amplification of PCR products were analysed by electrophoresis in a 1.5% agarose gel (UltraPure agarose; Invitrogen) in 1x TBE buffer stained with ethidium bromide (0.5 µg/ml) at 100 V for an hour. The electrophoresis results were viewed under UV light.

**Table 6.1: Primers used for the multiplex PCR targeting five families of *mcr* gene**

Accession Number	Primer	Sequence (5`-3`)	PCR product size (bp)
KP347127	<i>mcr-1_f</i>	CGGACAATCTCGGCTTTCTGCTGAC	645
	<i>mcr-1_r</i>	GTCATAGCCATTGAAGCTGACATG	
LT598652	<i>mcr-2_f</i>	GAAAAAGCGATGGGCGGTCTATCCTG	500
	<i>mcr-2_r</i>	CCCACCGAATAAATCGGCGTAATCG	
KY924928	<i>mcr-3_f</i>	TTACCAATATTGCTTGTTCAGCGC	403
	<i>mcr-3_r</i>	TTGAATTGTTGCGCCCCACTGACAC	
MF543359	<i>mcr-4_f</i>	CCCCTTTTCATCGCATCTATGCCGC	306
	<i>mcr-4_r</i>	AAAGGGCTGATAATGAATATCGGCC	
KY807921	<i>mcr-5_f</i>	CTAACTTCTGGAACATGGCTAATGC	201
	<i>mcr-5_r</i>	CATCAGATTCCGCAGCATGGCCTTG	

### 6.2.3 Polymerase Chain Reaction (PCR) determination of quinolone resistance genes

Polymerase chain reaction for the determination of quinolone resistance genes was performed following the protocol described by Kraychete *et al.* (2016). For the preparation of a final reaction mixture of 20 µl, 2 µl of the DNA template from each Ciprofloxacin-resistant isolate was added to 2 µl of 10x ExTaq buffer (supplied with *Taq* polymerase), 5 pmol each corresponding primer *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* and 15 pmol of primers of *qnrVC* (Table 6.2), 2 µM each deoxy nucleoside triphosphate, and 2.5 U of *Taq* polymerase (Ex Taq, Takara Bio Inc™, Shiga, Japan). The quantity of primers targeting *qnrVC* in the reaction was larger because this is degenerated. PCR was performed with TaKaRa thermal cycler Dice system. After an initial denaturation for 10 min at 95 ° C, 25 cycles of 45 s at 95 ° C, 45s at 58 ° C and 15 s at 72 ° C were run, followed by a final extension at 72 ° C for 3 min. PCR products were analysed by gel electrophoresis (2% UltraPure agarose; Invitrogen) at 100 V for an hour.

**Table 6.2: Primers used for the multiplex PCR targeting six families of *qnr* gene**

Primer	Sequence (5`-3`)	PCR product size (bp)	Reference
<i>qnrAm-F</i>	AGAGGATTTCTCACGCCAGG	580	(Kraychete <i>et al.</i> , 2016)
<i>qnrAm-R</i>	TGCCAGGCACAGATCTTGAC		
<i>qnrBm-F</i>	GGMATHGAAATTCGCCACTG	264	(Cattoir <i>et al.</i> , 2007)
<i>qnrBm-R</i>	TTTGCYGYCCGCCAGTCGAA		
<i>qnrCm-F</i>	GCGAATTTCCAAGGGGCAA	136	(Cattoir <i>et al.</i> , 2007)
<i>qnrCm-R</i>	ACCCGTAATGTAAGCAGAGCAA		
<i>qnrDm-F</i>	AGGTGTAGCATGTATGGAAAAGC	691	(Cattoir <i>et al.</i> , 2007)
<i>qnrDm-R</i>	ACATTGGGGCATTAGGCGTT		
<i>qnrSm-F</i>	GCAAGTTCATTGAACAGGGT	428	(Kraychete <i>et al.</i> , 2016)
<i>qnrSm-R</i>	TCTAAACCGTCGAGTTCGGCG		
<i>qnrVCm-F</i>	GAGYKTATGGTTTLAGAYCCTCG	71	(Kraychete <i>et al.</i> , 2016)
<i>qnrVCm-R</i>	TGTCYTG YTGCCACGARCA		

#### 6.2.4 Data analysis

Prevalence of antimicrobial-resistant genes in the wild birds was calculated using the formula below:

$$\text{Prevalence} = \frac{\text{Number of birds harbouring resistance genes}}{\text{Total number of individuals examined}} \times 100\%$$

## 6.3 Results

### 6.3.1 Occurrence of genes responsible for Colistin resistance

Plasmid-mediated Colistin genes were identified from the gel electrophoresis images (Figures 6.1, 6.2, 6.3). Fourteen out of the 106 Colistin-resistant isolates (13.2%) harboured plasmid-mediated Colistin resistant genes. The genes detected included *mcr-3* (observed in 9 isolates), *mcr-4* (observed in 2 isolate) and *mcr-1*, *mcr-2*, *mcr-5* (observed in one isolate each) (Figure 6.4).

Among the bacterial species isolated, *mcr-1* gene was detected in the bacteria species *Enterobacter aerogenes*; *mcr-2* was detected in the bacteria species *Enterobacter cloacae*, *mcr-3* genes was detected in the bacteria species *Enterobacter asburiae*, *Klebsiella oxytoca*, *Enterobacter cloacae* and *Klebsiella variicola*; *mcr-4* was detected in *Escherichia coli* and *mcr-5* gene was detected in *Proteus mirabilis* (Table 6.3). The *mcr-3* gene was observed in both forest and water-birds; *mcr-1* was detected only in a forest bird while *mcr-2*, *mcr-4*, *mcr-5* were detected in waterbird species.

Forty-four percent (4/9) of the forest birds examined harboured *mcr* genes. These were Forest Robin, Icterine Greenbul, Olive Sunbird and Red-tailed Bristlebill (Table 6.4). On the other hand, 25.9% of the waterbird species examined harboured *mcr* resistance genes and they were the Black Tern, Common Tern, Sanderling and Whimbrel (Table 6.5). It should be pointed out that for species with prevalence of 100%, only one individual was examined and it was found to harbour *mcr* resistance genes.

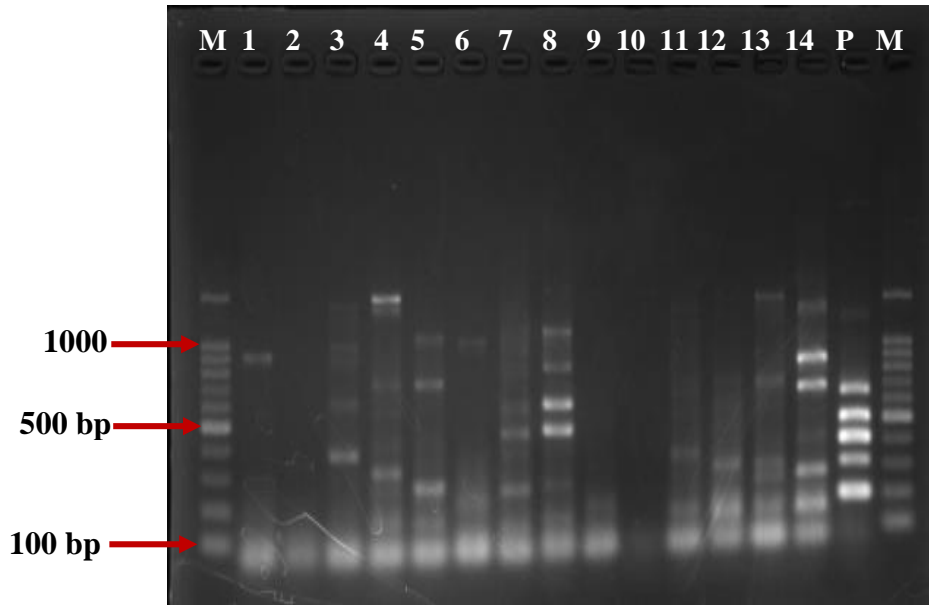


Figure 6.1: Gel electrophoresis results for *mcr* gene detection. M is the molecular size marker, P is the lane for positive controls. Lanes 8 and 14 show band sizes similar to the *mcr-2* gene (500 bp).

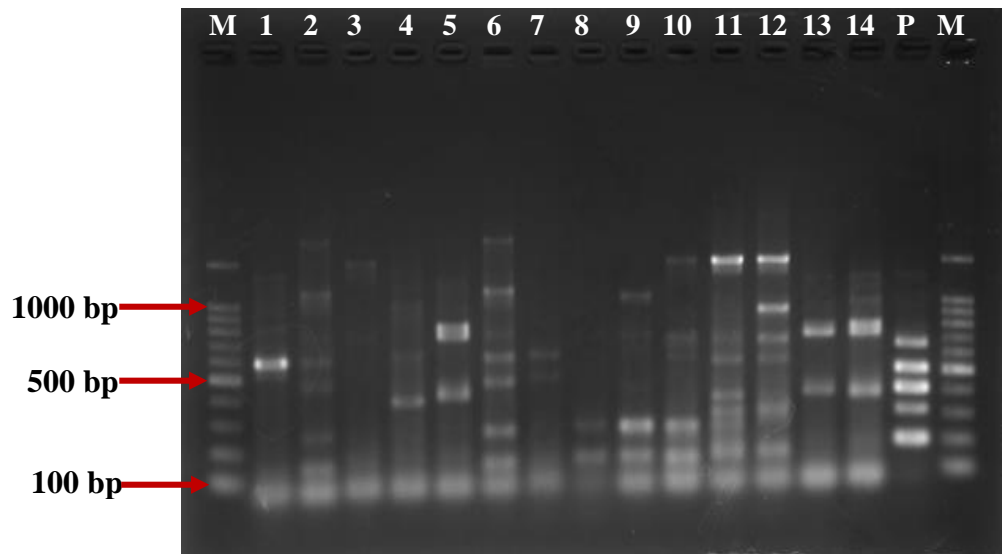
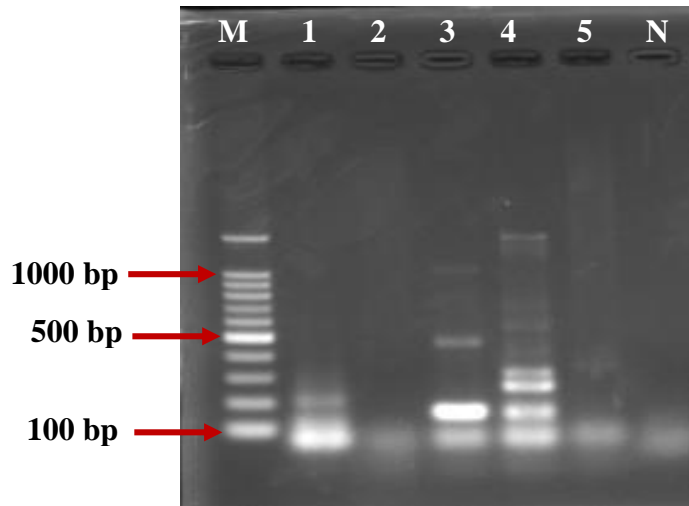
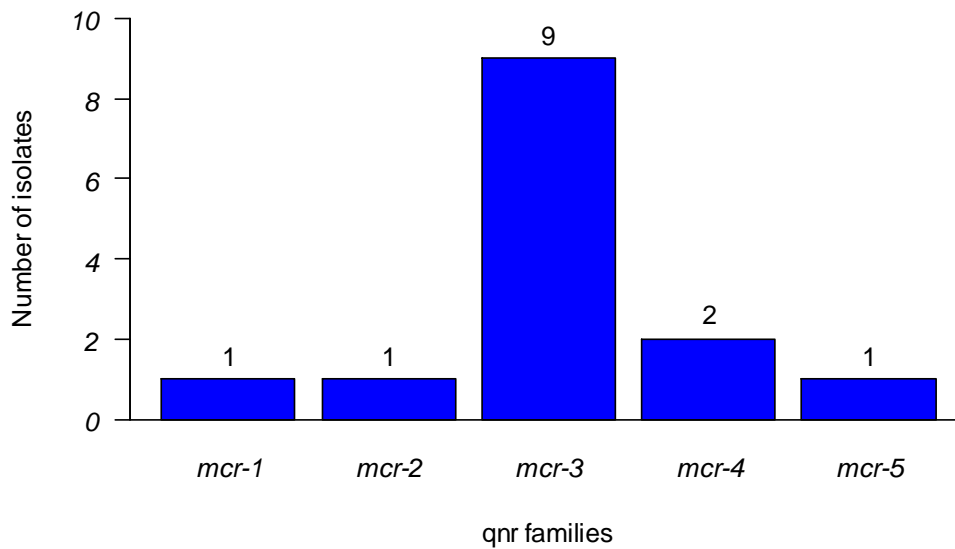


Figure 6.2: Gel electrophoresis results for *mcr* gene detection. M is the molecular size marker, P is the lane for positive controls. Lanes 13 and 14 are showing band sizes similar to the *mcr-3* gene (403 bp).



**Figure 6.3:** Gel electrophoresis results for *mcr* gene detection. M is the molecular size marker, P is the lane for positive controls and N is the negative control lane. Lane 3 is showing band similar to *mcr-5* gene (201 bp).



**Figure 6.4:** Number of colistin-resistant isolates harbouring *mcr* genes

**Table 6.3: Bacteria species found to harbour *mcr* resistance genes**

Bacteria species	<i>Mcr</i> genes					Total
	<i>mcr-1</i>	<i>mcr-2</i>	<i>mcr-3</i>	<i>mcr-4</i>	<i>mcr-5</i>	
<i>Enterobacter aerogenes</i>	1	0	0	0	0	1
<i>Enterobacter cloacae</i>	0	1	3	0	0	4
<i>Enterobacter asburiae</i>	0	0	2	0	0	2
<i>Escherichia coli</i>	0	0	0	2	0	2
<i>Klebsiella variicola</i>	0	0	3	0	0	3
<i>Klebsiella oxytoca</i>	0	0	1	0	0	1
<i>Proteus mirabilis</i>	0	0	0	0	1	1
Total	1	1	9	2	1	14

**Table 6.4: Prevalence of *mcr* genes in bacteria from forest bird species**

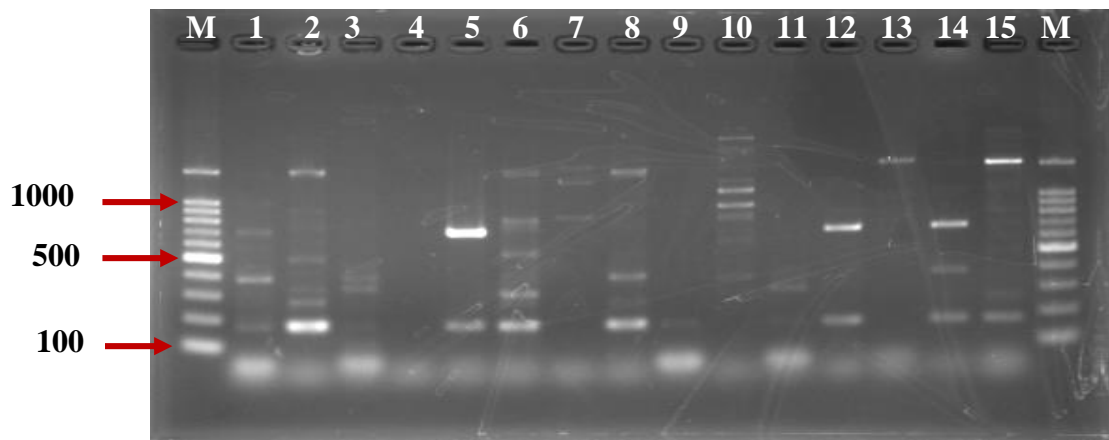
Bird species	No. of birds examined (N)	No. of isolates with resistance genes	No. of positive birds (n)	Prevalence of bacteria with resistance genes in (n/N*100)
Forest Robin	1	2	1	100%
Olive Sunbird	1	2	1	100%
Red-tailed Bristlebill	1	2	1	100%
Green Hylia	1	0	0	0
Icterine Greenbul	1	1	1	100%
White-tailed Antthrush	1	0	0	0
Yellow-whiskered Greenbul	3	0	0	0
Total	9	7	4	44.4%

**Table 6.5: Prevalence of *mcr* genes in bacteria from waterbirds**

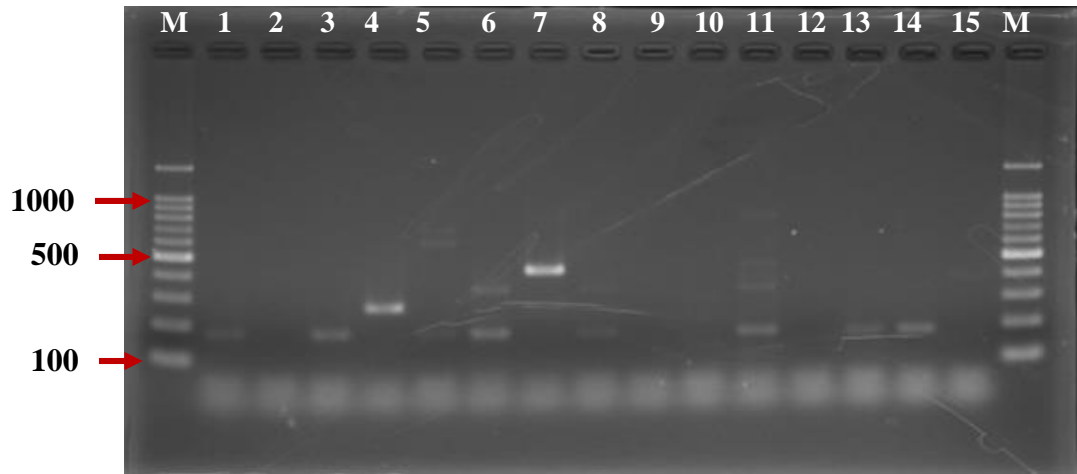
Bird species	No. of birds examined (N)	No. of isolates with resistance genes	No. of positive birds (n)	Prevalence of isolates with resistance genes (n/N*100)
Black Tern	10	2	2	20%
Common Tern	1	1	1	100%
Sanderling	11	3	3	27.3%
Common Ringed Plover	2	0	0	0
Roseate Tern	1	0	0	0
Whimbrel	2	1	1	100%
Total	27	7	7	25.9%

### 6.3.2 Occurrence of genes responsible for Ciprofloxacin resistance in isolates from forest and water- birds

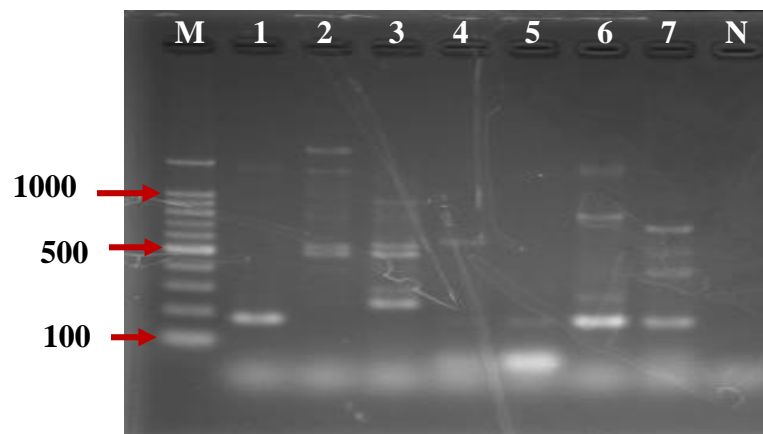
Quinolone resistance genes were identified in the gel electrophoresis images (Figures 6.5, 6.6, 6.7). Out of the 164 isolates that showed resistance to Ciprofloxacin, only 38 (23.2%) harboured plasmid-mediated quinolone gene (*qnr*). Four quinolone resistance gene families were detected namely *qnrB*, *qnrD*, *qnrS* and *qnrVC*. The *qnrB* gene was present in 6.1% (10/164) of isolates, *qnrD* was present in 3.0% (5/164) isolates, *qnrS* was present in 4.3% (7/164) isolates and *qnrVC* was present in 9.8% (16/164) of the isolates (Figure 6.1). About 77% of the Ciprofloxacin resistant isolates did not harbour any of the *qnr* resistance genes.



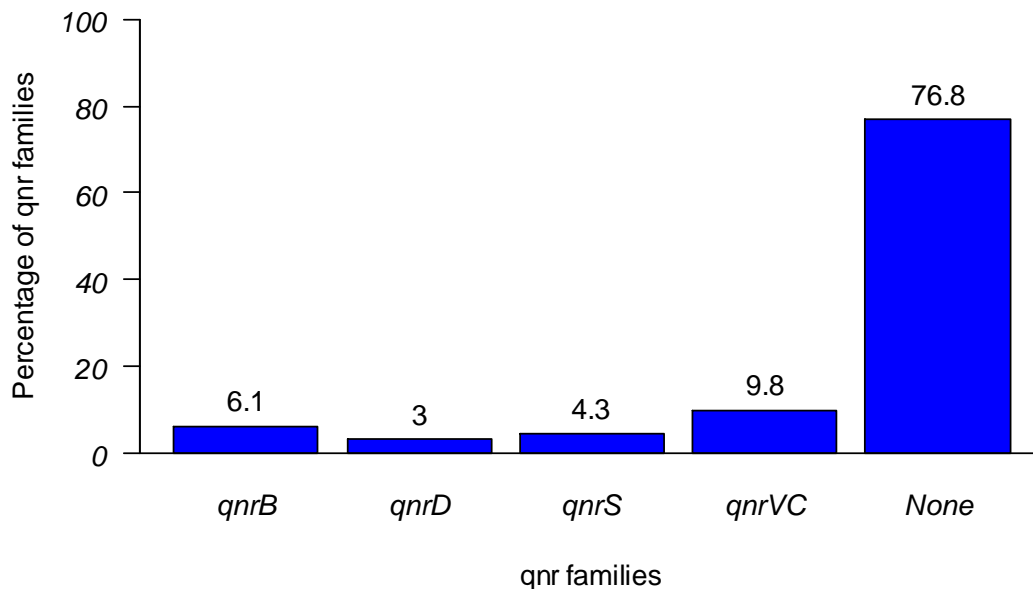
**Figure 6.5:** Gel electrophoresis results for *qnr* detection. M is the molecular size marker. N is the negative control lane. Lanes 5, 12, 14 show similar band sizes as the *qnrD* gene (691 bp)



**Figure 6.6:** Gel electrophoresis results for *qnr* detection. M is the molecular size marker. N is the negative control lane. Lane 4 has similar band size as *qnrB* gene (264 bp).



**Figure 6.7:** Gel electrophoresis results for *qnr* detection. M is the molecular size marker. N is the negative control lane. Lane 5 has similar band size as *qnrVC* gene (71 bp).



**Figure 6.8: Percentage of *qnr* gene families detected in wild birds**

### 6.3.2.1: Occurrence of genes responsible for Ciprofloxacin resistance in isolates from forest birds

Thirteen out of the 53 (24.5%) Ciprofloxacin-resistant isolates from forest birds harboured *qnr* resistance genes. Resistance genes detected represented 3 families; *qnrB*, *qnrS*, and *qnrVC*. The *qnrB* gene was present in 5.7% (3/53) of isolates, *qnrS* was present in 2.4% (1/53) isolates and *qnrVC* was present in 17.0% (9/53) of the isolates (Table 6.7). Isolates that did not harbour any of the *qnr* resistance genes constituted about 75%. About 11% of the resistance genes were detected in *E. coli* isolates.

The prevalence of quinolone resistance genes in forest birds was 18.8% (6/32) (Table 6.6). These resistance genes were detected in isolates from the Brown-chested Alethe, Forest Robin, Olive Sunbird, and Red-tailed Bristlebill. The Brown-chested Alethe and Forest Robin harboured only *qnrVC* genes (three and two respectively). The Olive Sunbird

harboured the genes *qnrS* (1 isolate) and *qnrVC* gene (4 isolates) while the Red-tailed Bristlebill harboured the *qnrB* gene (3 isolates).

**Table 6.6: Occurrence of *qnr* resistance genes in isolates from forest bird species**

Bird species (N)	No. of birds examined	No. of isolates with resistance genes	No. of birds harbouring resistance gene	Prevalence of isolates with resistance genes (n/N*100)
Brown-chested Alethe	4	3	1	25%
Dusky-crested flycatcher	1	0	0	0
Forest Robin	1	2	1	100%
Green Hylia	3	0	0	0
Green-tailed Bristlebill	3	0	0	0
Little Greenbul	3	0	0	0
Olive Sunbird	8	4	3	37.5%
Red-tailed Bristlebill	2	3	1	50%
White-tailed Antthrush	1	0	0	0
White-bellied Kingfisher	1	0	0	0
Yellow-whiskered Greenbul	5	0	0	0
<b>Total</b>	<b>32</b>	<b>12</b>	<b>6</b>	<b>18.8%</b>

**Table 6.7: Occurrence of resistance genes in Ciprofloxacin-resistant gram-negative bacteria genera**

Bacteria genera	Quinolone resistance genes						No resistance gene detected	Total
	<i>qnrA</i>	<i>qnrB</i>	<i>qnrC</i>	<i>qnrD</i>	<i>qnrS</i>	<i>qnrVC</i>		
<i>Shigella</i>	0	0	0	0	0	1	1	1
<i>Yersinia</i>	0	0	0	0	0	5	7	12
<i>Salmonella</i>	0	0	0	0	0	1	1	2
<i>Citrobacter</i>	0	0	0	0	0	0	2	2
<i>Enterobacter</i>	0	0	0	0	0	1	2	3
<i>Escherichia</i>	0	2	0	0	1	4	17	24
<i>Klebsiella</i>	0	1	0	0	2	2	4	9
<i>Proteus</i>	0	0	0	0	0	0	4	4
<i>Pseudomonas</i>	0	0	0	0	0	2	1	3
<i>Serratia</i>	0	0	0	0	0	0	1	1
Total	0	3	0	0	1	9	40	53

### 6.3.2.2: Occurrence of genes responsible for Ciprofloxacin resistance in isolates from waterbirds

Twenty-five out of 111 (22.5%) Ciprofloxacin-resistant isolates from waterbirds harboured *qnr* resistance genes. Resistance genes detected represented the 4 families; *qnrB*, *qnrD*, *qnrS* and *qnrVC*. The *qnrB* gene was present in 6.3% (7/111) of isolates, *qnrD* was present in 4.5% (5/111) isolates, *qnrS* was present in 5.4% (6/111) and *qnrVC* was present in 6.3% (7/111) of the isolates (Table 6.9). About 77% of the isolates did not harbour any of the *qnr* resistance genes.

The prevalence of resistance genes in waterbirds was 18.6% (11/59). These resistance genes were detected in isolates from the Black Tern, Common Tern, Roseate Tern, Sanderling and Whimbrel. The Black Terns harboured *QnrB* (three isolates) and *QnrVC* genes (three isolates), the Roseate Tern harboured *QnrVC* gene only (one isolate), the Whimbrels harboured *QnrS* gene (two isolates) and *QnrVC* gene (two isolates), the Common Terns harboured *QnrD* gene (two isolates) and *QnrVC* gene (two isolates) and

Sanderlings harboured *QnrB* gene (four isolates), *QnrS* (four isolates) gene and *QnrVC* gene (three isolates).

**Table 6.8: Prevalence of quinolone-resistant genes in isolates from waterbirds**

Bird species (N)	No. of birds examined	No. of isolates with resistance genes	No. of positive birds	Prevalence of isolates with resistance genes (n/N*100)
Black Tern	19	6	2	10.5%
Common Tern	4	4	2	50%
Common Sandpiper	1	0	0	0
Grey Plover	1	0	0	0
Pied Kingfisher	2	0	0	0
Ringed plover	2	0	0	0
Roseate Tern	1	1	1	100%
Sanderling	27	11	4	36.4
Whimbrel	3	3	2	66.7%
Total	59	25	11	18.6%

**Table 6.9: Distribution of resistance genes in Ciprofloxacin resistant bacteria genera from waterbirds**

Bacteria genera	Quinolone resistance genes						No resistance gene detected	Total
	<i>qnrA</i>	<i>qnrB</i>	<i>qnrC</i>	<i>qnrD</i>	<i>qnrS</i>	<i>qnrVC</i>		
<i>Shigella</i>	0	1	0	1	0	1	2	5
<i>Yersinia</i>	0	0	0	3	0	4	21	28
<i>Salmonella</i>	0	0	0	0	0	0	4	4
<i>Citrobacter</i>	0	0	0	0	0	0	1	1
<i>Enterobacter</i>	0	2	0	1	1	1	9	14
<i>Escherichia</i>	0	2	0	0	3	1	25	31
<i>Klebsiella</i>	0	1	0	0	2	0	7	10
<i>Morganella</i>	0	0	0	0	0	0	1	1
<i>Ochrobactrum</i>	0	0	0	0	0	0	1	1
<i>Proteus</i>	0	0	0	0	0	0	2	2
<i>Providencia</i>	0	0	0	0	0	0	1	1
<i>Pseudomonas</i>	0	1	0	0	0	0	7	8
<i>Serratia</i>	0	0	0	0	0	0	5	5
Total	0	7	0	5	6	7	86	111

### 6.3.3 Occurrence of *mcr* genes in multidrug-resistant isolates

Out of the 33 multidrug-resistant isolates obtained from chapter 5, section 5.3.3, nine were found to harbour *mcr* genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-5*) (Table 6.10). None of the multidrug-resistant isolates harboured *mcr-4* gene. Five other isolates that were not multidrug-resistant were also found to harbour *mcr* genes. None of the isolates of the species *C. freundii*, *E. kobei*, *E. coli*, *K. variicola*, and *P. rettgeri* harboured any of the *mcr* genes.

**Table 6.10: Distribution of *mcr* genes in multidrug-resistant isolates**

Bacteria species (number of multidrug-resistant isolates)	<i>Mcr</i> genes					Total
	<i>mcr-1</i>	<i>mcr-2</i>	<i>mcr-3</i>	<i>mcr-4</i>	<i>mcr-5</i>	
<i>Citrobacter braakii</i> (1)	0	0	0	0	0	0
<i>Citrobacter freundii</i> (1)	0	0	0	0	0	0
<i>Enterobacter aerogenes</i> (1)	1	0	0	0	0	1
<i>Enterobacter asburiae</i> (2)	0	0	2	0	0	2
<i>Enterobacter cloacae</i> (4)	0	0	2	0	1	3
<i>Enterobacter kobei</i> (1)	0	0	0	0	0	0
<i>Escherichia coli</i> (5)	0	0	0	0	0	0
<i>Klebsiella oxytoca</i> (4)	0	0	2	0	0	2
<i>Klebsiella variicola</i> (4)	0	0	0	0	0	0
<i>Proteus mirabilis</i> (6)	0	1	0	0	0	1
<i>Providencia rettgeri</i> (4)	0	0	0	0	0	0
<b>Total</b>	<b>1</b>	<b>1</b>	<b>6</b>	<b>0</b>	<b>1</b>	<b>9</b>

### 6.4 Discussion

In this chapter the occurrence of resistant determinants (genes) in bacteria isolates from forest and water- birds were investigated. Several antimicrobial-resistant genes exist that can potentially confer resistance to bacteria isolates. The occurrence of Colistin and quinolone resistance genes were investigated in isolates from both forest and water- birds.

Overall, 106 Colistin resistant isolates and 164 Ciprofloxacin resistant isolates were examined.

Four out of the six quinolone resistance gene families (*qnrB*, *qnrD*, *qnrS* and *qnrVC*) were detected in this study. Also, all five Colistin resistant gene families (*mcr1*, *mcr2*, *mcr3*, *mcr-4* and *mcr-5*) were detected.

The overall occurrence of *mcr* genes in the wild birds was 30.5% (11/36) with 44.4% (4/9) and 25.9% (7/27) in forest and water- bird species, respectively. *Mcr* genes were detected in eight bird species: Forest Robin, Olive Sunbird, Red-tailed Bristlebill, Icterine Greenbul, Black Tern, Common Tern, Sanderling and Whimbrel. For some of the species only one individual was examined and hence resulting in 100% prevalence when found positive.

The *mcr-1* gene was the first *mcr* gene to be reported (Liu *et al.*, 2016). The first case was detected in Chinese enterobacterial isolates (Liu *et al.*, 2016). Later, *mcr-1* was detected also in isolates from countries in five continents: European countries such as Belgium (Xavier *et al.*, 2016), Denmark (Hasman *et al.*, 2015), Italy (Cannatelli *et al.*, 2016), Poland (Izdebski *et al.*, 2016), Portugal (Quesada *et al.*, 2016), Germany (Falgenhauer *et al.*, 2016), France (Haenni *et al.*, 2016), United Kingdom, and the Netherlands (Veldman *et al.*, 2016); Asian countries such as Japan (Suzuki *et al.*, 2016), Malaysia (Petrillo *et al.*, 2016), Taiwan (Kuo *et al.*, 2016); North American countries such as Canada (Mulvey *et al.*, 2016) and the USA (McGann *et al.*, 2016); South American countries such as Brazil (Fernandes *et al.*, 2016) and Argentina (Rapoport *et al.*, 2016); African countries such as South Africa (Coetzee *et al.*, 2016), Egypt (Elnahriry *et al.*, 2016), Tunisia (Grami *et al.*,

2016) and Nigeria (Hu *et al.*, 2016). Although several studies have reported on the occurrence of the *mcr-1* gene suggesting wide distribution, in this study, *mcr-1* was identified in only one isolate.

The first case of *mcr* gene in wild birds was detected in bacteria isolated from a migratory species, the European herring gull (*Larus argentatus*); the isolate carrying the *mcr-1* gene was resistant to Colistin and ampicillin (Liu *et al.*, 2016). The finding of this study agrees with the observation from Sims *et al.* (2003); thus, the isolate carrying *mcr-1* gene observed in this study was resistant to both Colistin and Ampicillin.

In previous studies, *mcr-1* genes were observed in the bacteria species *Klebsiella pneumoniae* (Liu *et al.*, 2016), *Shigella sonnei* (Pham Thanh *et al.*, 2016), *Salmonella Enterica* (Hu *et al.*, 2016; Veldman *et al.*, 2016; Webb *et al.*, 2016), *Enterobacter aerogenes* and *Enterobacter cloacae* (Zeng *et al.*, 2016). In the current study *mcr-1* gene was detected in *Enterobacter aerogenes* from the Forest Robin (*Stiphronis erythrothorax*). The *mcr-2* gene has been identified previously in *E. coli* from bovine and swine origin in Belgium. In this study it was identified in an *Enterobacter cloacae* isolate from the Sanderling (*Calidris alba*). *Mcr-3* gene was the most frequently isolated gene in the current study. In previous studies, *mcr-3* was also identified in *Klebsiella pneumoniae*, *E. coli* and *Salmonella* spp. from the United States and Asia, but in this study, it was identified in *Enterobacter cloacae*, *E. asburiae*, *K. variicola* and *K. oxytoca* from the Common Tern, Sanderling, Forest Robin, Icterine Greenbul (*Phyllastrephus icterinus*), Olive Sunbird and Red-tailed Bristlebill (*Bleda syndactylus*). In previous studies, the *mcr-4* gene was identified in *E. coli* and *Salmonella typhimurium* from piglets and pigs respectively (Carattoli *et al.*, 2017). Similarly, in the current study, the *mcr-4* genes were

identified in *E. coli* isolates from the Whimbrel (*Numenius phaeopus*). The *mcr-5* gene was first identified in *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (Borowiak *et al.*, 2017) but in this study it was identified in a *Proteus mirabilis* isolate from a Black Tern (*Chlidonias niger*).

The overall prevalence of *qnr* genes was 40.7% (37/91) with 18.8% (6/32) and 18.6% (11/59) in forest and water – birds respectively. *Qnr* genes were observed in the Forest Robin, Brown-chested Alethe (*Chamaetylas poliocephala*), Olive Sunbird, Red-tailed Bristlebill, Black Tern, Common Tern, Roseate Tern, Sanderling and Whimbrel. Interestingly, *qnr* genes were detected in all the species of terns examined, suggesting wide dissemination among seabirds.

No *qnrA* and *qnrC* genes were detected in this study. The most frequently encountered *qnr* gene was *qnrVC* (identified in 7 isolates). In a previous study, *qnrVC* was identified in isolates belonging to the genera *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Kluyvera*. In the current study *qnrVC* was identified in the genera *Shigella*, *Yersinia*, *Salmonella*, *Enterobacter*, *Escherichia*, *Klebsiella* and *Pseudomonas* from the Forest Robin, Olive Sunbird, Roseate Tern and Whimbrel. *qnrB* and *qnrS* were first identified in *Klebsiella pneumoniae* and *Shigella flexneri* respectively (Hooper & Jacoby, 2015). In another study, *qnrB* and *qnrS* genes were identified in the genera *Enterobacter* and *Klebsiella* (Kraychete *et al.*, 2016).

Literak *et al.* (2010) identified *qnrS* genes in wild birds, specifically from four Cormorants (0.8%, n=499) and 17 mallards (6%, n=305). In a related study on occurrence of five resistance gene determinants (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) in bacterial samples

from rooks in Europe, *qnrS*, *qnrB* and *qnrD* were identified in 156, 13 and 6 samples respectively (Literak *et al.*, 2012). In the current study, *qnrS* was identified in the genera *Enterobacter*, *Escherichia*, and *Klebsiella* from the Olive Sunbird and Whimbrel.

About 27% of multidrug resistant isolates harboured *mcr* resistance genes. Since these genes are plasmid-mediated and can be transferred from one bacteria to the other, there is the potential for an increase in multidrug resistance bacteria in wild bird species when these genes spread between birds. Bird species that harboured bacterial isolates with resistance genes are common forest and water – bird species, thus the detection of quinolone genes in these birds indicate a rapid potential spread of these resistance genes within the environment.

The findings from this study suggest that apparently healthy individual forest birds belonging to species such as the Forest Robin, Brown-chested Alethe, Olive Sunbird, Red-tailed Bristlebill, Icterine Greenbul and waterbirds such as the Common Tern, Roseate Tern, Sanderling, Whimbrel, and Black Tern, may act as reservoirs of resistance determinants (genes). However, it is unclear the origin of bacterial resistance genes, as wild birds are not exposed to antibiotics directly. Therefore, contact with contaminated environments should be considered as an important risk factor for the transmission of resistant bacteria. Finally, considering the fact that wild birds, especially migratory species, can fly from one place to the other, they have the potential to disseminate resistance genes from one place to the other or along their migration route.

## 7.0 CHAPTER SEVEN: OCCURRENCE OF PATHOGENIC ENTEROBACTERIA IN FOREST AND WATER- BIRDS

### 7.1 Introduction

Many species of gram-negative bacteria occur in humans and animals. Some of these species are pathogenic while others are commensal. The pathogenic species are known to cause severe infections (gastric and diarrhoea) in humans and some species of animals. Infections in humans caused by these pathogens are known to lead to malnutrition and stunted growth in children in developing countries (Johansson *et al.*, 2009).

Among the families of enteric gram-negative bacteria are globally important diarrheal agents. These include six groups of *E. coli* Enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli* (STEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), Diffuse adherent *E. coli* (DAEC), Enterohaemorrhagic *E. coli*, Enteropathogenic (EPEC), Adherent invasive *E. coli* (AIEC)) (Croxen *et al.*, 2013) and some other bacteria species such as *Salmonella enterica*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, *Shigella dysenteriae* and *Shigella flexneri* (Bublitz *et al.*, 2014).

The species *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* comprise strains that are pathogenic. Both the pathogenic and non-pathogenic strains of these species have been isolated from birds, mammals, and reptiles, as well as soil and water samples (Thoerner *et al.*, 2003). *Yersinia pseudotuberculosis* is frequently isolated from birds, rodents, rabbits, and hares (Aleksic & Bockemuhl, 1999). On the other hand, *Yersinia enterocolitica* is isolated more from humans and animals such as pigs (Bottone, 1999). Isolation of *Y. pseudotuberculosis* in avian species predominates over *Y. enterocolitica* (Aleksić *et al.*, 1995).

The genus *Salmonella* comprises two species, *S. Bongori* and *S. enterica*. *Salmonella enterica* is the predominant species, with over 2000 serovars (Uzzau *et al.*, 2000). The common serovars are *S. typhi* and *S. typhimurium*; *S. typhi* is responsible for typhoid fever and other systemic infections while *S. typhimurium* causes gastroenteritis (McClelland *et al.*, 2001). *Salmonella typhi* is host specific and infections occur only in humans, whereas *S. typhimurium* has a broad host range (McClelland *et al.*, 2001). *Salmonella typhimurium* has been isolated from both dead (Refsum *et al.*, 2002; Pennycott *et al.*, 2006) and live wild birds (Quessy & Messier, 1992; Morishita *et al.*, 1999).

Shigellosis is a highly contagious food borne disease of humans that is caused by different species of *Shigella*; *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* (Weir, 2002). Symptoms of infection with *Shigella* spp. range from mild gastroenteritis to severe dysentery. Shiga-toxin producing *Shigella dysenteriae* is responsible for epidemics of fatal shigellosis in humans in developing countries (Edwards, 1999). Though animals may act as asymptomatic carriers of *Shigella* spp., they are rarely isolated from wild and domestic animals other than primates (Edwards, 1999).

*Escherichia coli* is the predominant gram-negative bacteria in the gastrointestinal tract of warm-blooded animals (Nataro & Kaper, 1998). Phylogenetic analysis has revealed that *E. coli* is composed of four phylogenetic groups; A, B1, B2, and D, where B2 and D are virulent extra-intestinal groups and A and B1 are commensal groups (Herzer *et al.*, 1990). Virulent strains are known to have diarrheagenic properties (Isidean *et al.*, 2011). Enterotoxigenic *Escherichia coli* (ETEC) is one of the six recognised diarrheagenic *E. coli* groups (Croxen *et al.*, 2013). It produces heat-labile (LT) and/or heat-stable (ST) enterotoxins in the small intestine and is the most common cause of diarrhoea in children in developing countries (Black, 1990, 1993). Illnesses resulting from these organisms

contribute significantly to the overall morbidity and may relate to delayed growth in infected children (Petri *et al.*, 2008). It is estimated that approximately 280 million episodes of diarrhoea are caused by these organisms yearly among children under 5 years of age (Wennerås & Erling, 2004). Only a few outbreaks of avian diarrhoea due to ETEC have been reported (Joya *et al.*, 1990).

Despite the potential for zoonotic transmission, only a few studies have examined the epidemiology of diarrheagenic agents in wild birds. Thus, the role of wild birds to disseminate these pathogens is underestimated.

The occurrence of diarrheal pathogens such as Enterotoxigenic *Escherichia coli*, *Yersinia* spp, (*enterocolitica* and *pseudotuberculosis*), *Salmonella enterica* and *Shigella* spp. (*flexneri* and *dysenteriae*) are investigated in this chapter

## **7.2 Materials and Methods**

All pure isolates of the genera *Escherichia*, *Shigella*, *Salmonella*, and *Yersinia* obtained from bacteria isolation (Chapter 4, Section 4.4.2) were examined for the molecular identification of Enterotoxigenic *E. coli* (ETEC), *S. enterica*, *Shigella* spp. (*flexneri* and *dysenteriae*) and *Yersinia* spp. (*pseudotuberculosis* and *enterocolitica*).

### **7.2.1 Extraction of DNA by the boiling method**

DNA was extracted from all pure isolates of the genera *Escherichia*, *Shigella*, *Salmonella* and *Yersinia* using the procedure described in Chapter 6, Section 6.2.1.

### 7.2.2 PCR amplification of target genes for the identification of Enterotoxigenic *E. coli* (ETEC), *S. enterica*, *Shigella* spp. (*flexneri* and *dysenteriae*) and *Yersinia* spp. (*pseudotuberculosis* and *enterocolitica*)

A multiplex PCR was performed on 2 µl of DNA template, 0.5 µmol of each primer (Table 7.1), 2 ul of 10x *ExTaq* buffer (supplied with *Taq* polymerase), each deoxynucleoside triphosphate at a concentration of 2 µM and 2.5 U of *Taq* polymerase (*ExTaq*, Takara Bio Inc™, Shiga, Japan) in a final PCR mix of 25 µl. The gene *yadA* in *Yersinia* was amplified because it is similar in *pseudotuberculosis* and *enterocolitica* species and could be used to detect both (Thoerner *et al.*, 2003). Similarly, the *ipaH* gene can amplify both *Shigella dysenteriae* and *Shigella flexneri* (Bublitz *et al.*, 2014). The *invA* gene in *Salmonella enterica* also has a conserved nature across serovars (Rahn *et al.*, 1992). The PCR amplification conditions were one cycle of 94 ° C for 15 s, followed by 35 cycles of 94 ° C for 3 s, 50 ° C for 10 s and 74 ° C for 35 s and then a final cycle of 74 ° C for 2 min and 45 ° C for 2s. The PCR products (3 µl each) were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml).

**Table 7.1: Bacterial strain, target genes and primers for bacteria species identification**

Target Gene	PCR primers (5' – 3')*	Product size (bp)
Enterotoxin (LT) gene	f-GAGACCGGGTATTACAGAATC r-GAGGTGCATGATGAATCCAG	117
<i>ipaH</i>	f-CTTGACCGCCTTTCCGATAC r-CAGCCACCCTCTGAGAGTA	610
<i>invA</i>	f-TATCGCCACGTTCTGGGCAA r-TCGCACCGTCAAAGGAACC	275
<i>yadA</i>	f-CTTCAGATACTGGTGTCTGCTGT r-ATGCCTGACTAGAGCGATATCC	681 (849 <sup>a</sup> , 751 <sup>b</sup> )

\*All primer sequences were obtained from Bublitz *et al.* (2014)

<sup>a</sup>Product size with *Y. enterocolitica* serogroup 03 or 09 strains

<sup>b</sup>Product size with *Y. enterocolitica* serogroup 08 strains

### 7.2.3 PCR Amplification of *Escherichia coli* Phylogenetic groups

A triplex PCR was performed by following the protocol by Clermont *et al.* (2000). A reaction mixture of 20 µl contained 2 ul of 10x Ex Taq buffer (supplied with Taq polymerase), 20 pmol of each primer (Table 7.2), each deoxynucleoside triphosphate at a concentration of 2 µM, 2.5 U of Taq polymerase (Ex Taq, Takara Bio Inc™, Shiga, Japan) and 200 ng of DNA.

TaKaRa thermal cycler Dice system was used for PCR process. After denaturation for 5 min at 94 ° C, 30 cycles of 30 s at 94 ° C, 30 s at 55 ° C, 30 s at 72 ° C were run, with a final extension at 72 ° C for 3 min. The primer pairs used are listed in table 7.2. PCR products (3 µl each) were analysed by gel electrophoresis (1% UltraPure agarose; Invitrogen) at 100 V for an hour.

**Table 7.2: Primers for *E. coli* phylogenetic groupings**

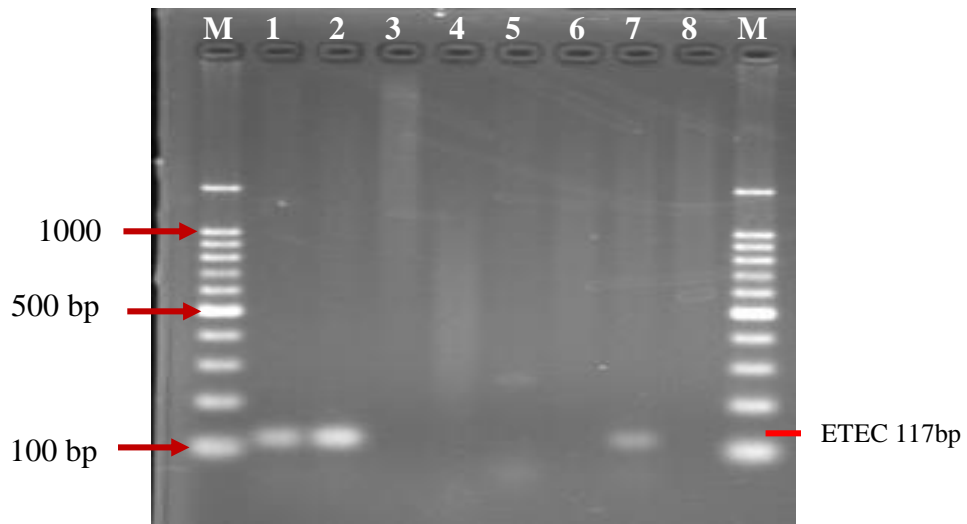
Target genes/DNA fragment	PCR sequence (5` – 3`)	Product size
<i>ChuA</i>	f-GACGAACCAACGGTCAGGAT r-TGAAGTGTCAGGAGACGCT	279 bp
<i>YjaA</i>	f-TGAAGTGTCAGGAGACGCT r-ATGGAGAATGCGTTCCTCAAC	211 bp
TspE4.C2	f-GAGTAATGTCGGGGCATTCA r-CGCGCCAACAAAGTATTACG	152 bp

### 7.3. Results

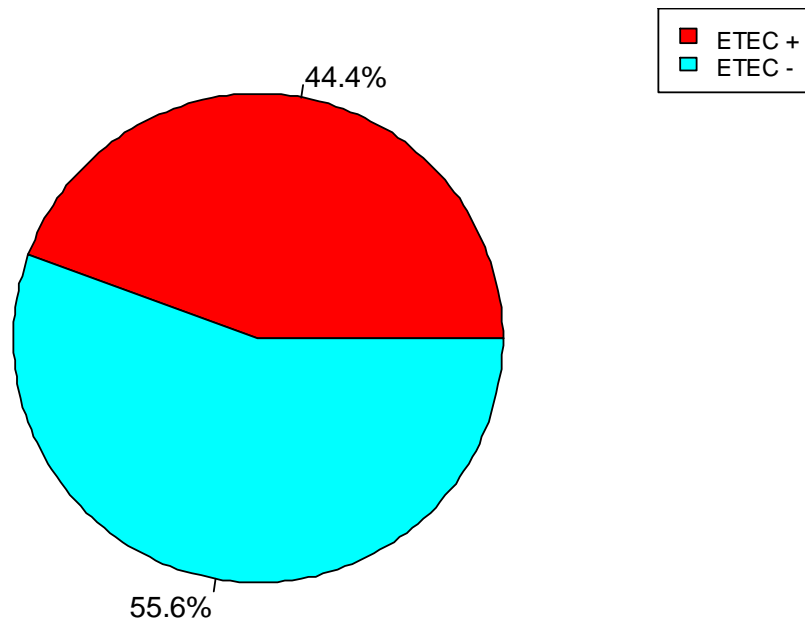
#### 7.3.1 Occurrence of Enterotoxigenic *E. coli* (ETEC), *Shigella* spp. (*flexneri* and *dysenteriae*), *S. enterica* and *Yersinia* spp. (*pseudotuberculosis* and *enterocolitica*)

A total of 279 isolates comprising *Shigella* spp. (n=25), *Yersinia* spp. (n=95), *Salmonella* spp. (n=17) and *Escherichia* spp. (n=142) obtained from both forest and water- birds were investigated. The electrophoresis results showed bands for only Enterotoxigenic *E. coli* (ETEC) (Figure 7.1). None of the isolates was identified as *Salmonella enterica*, *Yersinia* spp. (*pseudotuberculosis* and *enterocolitica*) and *Shigella* spp. (*flexneri* and *dysenteriae*). However, 44.4% (63/142) *Escherichia* spp. were identified as Enterotoxigenic *Escherichia coli* (Figure 7.2). Out of the 88 *E. coli* isolates examined from forest birds, 25 were enterotoxigenic whereas 38 out of the 54 of the *E. coli* isolates from waterbirds were enterotoxigenic.

The overall prevalence of ETEC in wild birds was 50% (42/84) with 38.6% (17/44) prevalence in forest birds (Table 7.3) and 65% (26/40) prevalence in waterbirds (Table 7.4). Enterotoxigenic *E. coli* were isolated from seven forest bird species including the Brown-chested Alethe, Forest Robin, Green Hylia, Green-tailed Bristlebill, Icterine Greenbul, Olive Sunbird and Yellow-whiskered Greenbul. Among the waterbirds, ETEC was observed in five species: Black Tern, Common Sandpiper, Common Tern, Ruddy Turnstone and Sanderling.



**Figure 7.1:** Gel electrophoresis results for the detection of pathogenic gram-negative bacteria. M is the molecular size marker and lanes 1, 2 and 7 are showing bands corresponding to ETEC.



**Figure 7.2:** A pie chart showing the proportions of *E. coli* isolates with the LT gene and those without the LT gene

**Table 7.3: Prevalence of Enterotoxigenic *E. coli* in the forest bird species**

Bird species (N)	No. of birds examined	No. of ETEC isolates	No. of birds harbouring ETEC	Prevalence
African Pygmy Kingfisher	1	0	0	0
Brown-chested Alethe	8	2	2	25%
Dusky-crested Flycatcher	1	0	0	0
Forest Robin	4	0	3	75%
Green Hylia	3	2	2	66.7%
Green-tailed Bristlebill	5	5	2	40%
Icterine Greenbul	1	2	1	100%
Olive Sunbird	3	2	1	33.3%
Red-tailed Bristlebill	3	0	0	0
White-tailed Alethe	2	0	0	0
White-tailed Antthrush	2	0	0	0
Yellow-whiskered Greenbul	11	12	6	54.5%
Total	44	25	17	38.6%

**Table 7.4: Prevalence of Enterotoxigenic *E. coli* in the waterbirds species**

Bird species (N)	No. of birds examined	No. of ETEC isolates	No. birds harbouring ETEC	Prevalence
Black Tern	10	13	10	100%
Common Ringed Plover	1	0	0	0
Common Sandpiper	2	1	1	50%
Common Tern	1	2	1	100%
Pied Kingfisher	1	0	0	0
Ruddy Turnstone	1	2	1	100%
Sanderling	22	20	13	59%
Whimbrel	2	0	0	0
Total (40)	40	38	26	65%

### 7.3.2 Association between bird species and prevalence of Enterotoxigenic *E. coli* (ETEC) in waterbirds

The prevalence of *Escherichia* in waterbirds after random selection of 10 individuals from the Black tern and Sanderling were 100% and 60% respectively. The prevalence rates were significantly different among bird species ( $\chi^2$  test value = 5.000, df = 1, p=0.025).

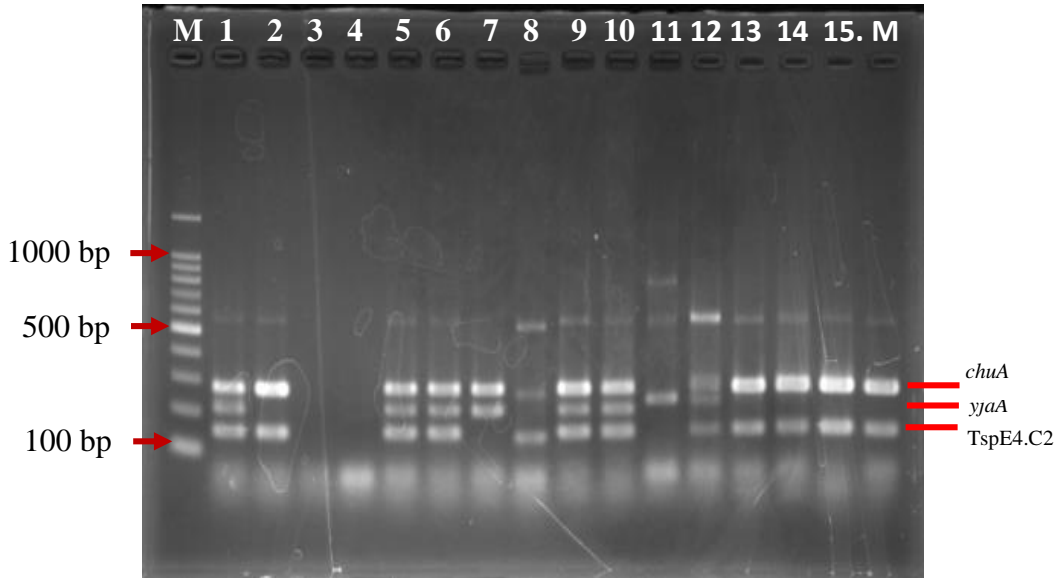
Thus, the chance of isolating Enterotoxigenic *Escherichia* was higher in the Black tern than the Sanderling.

Comparison between prevalence of Enterotoxigenic *E. coli* for forest species was not computed due to low numbers of samples examined. Similarly, comparison of ETEC prevalence between Yellow-whiskered Greenbuls caught close to the reception area of the protected area and the those caught far away from the reception area were not computed because all the Yellow-whiskered Greenbuls that harboured ETEC were caught around the reception area.

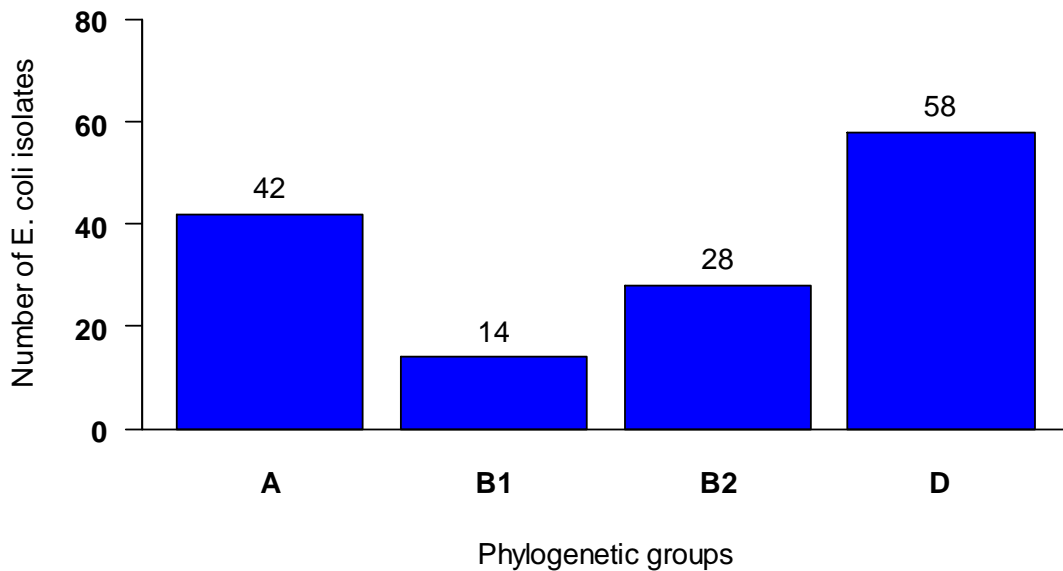
### **7.3.3 Phylogenetic grouping of *E. coli* isolates**

The results of *E. coli* phylogenetic grouping were obtained from electrophoresis gel images (Figure 7.3). Gel results showed that all *Escherichia* spp. isolates were *E. coli*. The *chuA* gene was present in all isolates that belonged to groups B2 and D and was absent in isolates that belonged to groups A and B1. The *yjaA* gene was present in isolates of group B2 and absent in groups D. The clone/DNA fragment TspE4.C2 was present in isolates of group B1 and absent in group D.

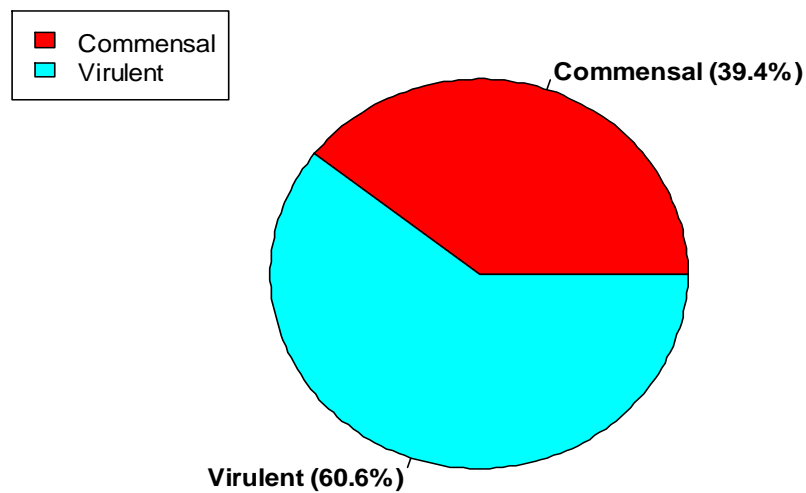
The 142 presumptive *E. coli* isolates were observed to belong to phylogenetic groupings A (n=42), B1 (n=14), B2 (n=28) and D (n=58) (Figure 7.4). The percentages of commensal isolates (Groups A and B1) and virulent isolates (Groups B2 and D) were 39.4% and 60.6%, respectively (Figure 7.5). The overall prevalence of commensal and virulent *E. coli* in the wild birds was 45.5% (20/44) and 60% (24/40) respectively.



**Figure 7.3:** PCR products of *E. coli* phylogenetic groupings visualized by gel electrophoresis. M is the molecular size marker (100 bp, SigmaMarkers™) and lanes 1-15 are the samples. Lanes 1, 5, 6, 9, and 10 are showing bands for phylogenetic group B2; Lanes 2, 12,13,14,15 are showing bands for phylogenetic group D; Lane 8 is showing band for B1; lanes 3 and 4 showing no bands, characteristic of group A).



**Figure 7.4:** Number of *E. coli* isolates identified for each phylogenetic group



**Figure 7.5: Proportion of commensal and virulent isolates of *E. coli***

### 7.3.2.1 Phylogenetic grouping of *E. coli* isolates obtained from forest birds

In forest birds, analysis of *E. coli* isolates revealed the following phylogenetic groups: A (38.6%; n=34), B1 (6.8%, n=6), B2 (20.5%, n=18), D (34.1%, n=30) (Table 7.5). Thus, 45% of the *E. coli* isolates belonged to the commensal groups while 55% belonged to the virulent groups. Phylogenetic group A occurred in all forest bird species (n=12) examined. Group B1, B2 and D occurred in 2, 5 and 8 forest bird species respectively.

**Table 7.5: Distribution of *E. coli* phylogenetic groups among forest birds**

Bird species	Number of birds examined	No of isolates				Total
		A	B1	B2	D	
African Pygmy Kingfisher	1	2	0	0	0	2
Brown-chested Alethe	8	10	1	7	2	20
Dusky-crested Flycatcher	1	2	0	0	0	2
Forest Robin	4	2	0	0	3	5
Green Hylia	3	2	0	1	2	5
Green-tailed Bristlebill	5	1	1	4	5	11
Icterine Greenbul	1	1	0	0	1	2
Olive Sunbird	3	2	0	3	2	7
Red-tailed Bristlebill	3	5	0	0	0	5
White-tailed Alethe	2	1	0	0	0	4
White-tailed Antthrush	2	2	0	0	3	2
Yellow-whiskered Greenbul	11	4	4	3	12	23
<b>Total</b>	<b>44</b>	<b>34</b>	<b>6</b>	<b>18</b>	<b>30</b>	<b>88</b>

### 7.3.2.2 Phylogenetic grouping of *E. coli* isolates obtained from waterbirds

In waterbirds, analysis of *E. coli* isolates revealed the following phylogenetic groups: A (14.8%, n=8), B1 (14.8%, n=8), B2 (18.5%, n=10), D (51.8%, n=28) (Table 7.6). Thus, 29.6% of the *E. coli* isolates belonged to the commensal groups while 70.4% belonged to the virulent groups. Phylogenetic groups A and B2 occurred in three bird species while groups B1 and D occurred in four bird species.

**Table 7.6: Distribution of *E. coli* phylogenetic groups among waterbird species**

Bird species	Number of birds examined	No of isolates				Total
		A	B1	B2	D	
Black Tern	10	3	2	6	7	18
Common Ringed Plover	1	0	0	0	0	0
Common Sandpiper	2	0	0	0	1	1
Common Tern	1	0	0	0	2	2
Roseate Tern	1	1	0	0	0	1
Sanderling	1	4	4	2	18	28
Turnstone	22	0	1	2	0	3
Whimbrel	2	0	1	0	0	1
Total	40	8	8	10	28	54

### 7.3.2.4 Occurrence of Enterotoxigenic *E. coli* among phylogenetic groups

Overall, 76 (53.5%) *E. coli* isolates were not identified as Enterotoxigenic while 66 (46.5%) were identified as enterotoxigenic. None of the isolates from the phylogenetic groups A and B1 were enterotoxigenic (Table 7.7). Sixteen enterotoxigenic *E. coli* isolates belonged to phylogenetic groups B2 while 50 isolates belonged to group D.

**Table 7.7: Distribution of Enterotoxigenic *E. coli* among phylogenetic groups**

Occurrence of ETEC	<i>E. coli</i> phylogenetic grouping				Total
	A	B1	B2	D	
<b>Forest birds</b>					
ETEC positive	0	0	6	22	28
ETEC negative	34	6	12	8	60
<b>Waterbirds</b>					
ETEC positive	0	0	10	28	38
ETEC negative	8	8	0	0	16
Total	42	14	28	58	142

#### 7.4 Discussion

Diarrheal pathogens/agents are important pathogens in humans and animals. They cause severe infections such as gastroenteritis. Wild birds by nature of their movement have the potential to move from one place to the other and are thought to have the potential to disseminate pathogenic agents. However, few studies have examined the gastrointestinal tract of wild birds for pathogenic agents.

In this chapter, the occurrence of six diarrheal pathogens, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Shigella flexneri*, *Shigella dysenteriae*, Enterotoxigenic *E. coli* and *Salmonella enterica* were investigated.

None of the isolates examined were identified as *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Shigella flexneri*, *Shigella dysenteriae*, *Salmonella enterica*. On the other hand, 22.5% (32/142) of the *E. coli* isolates were identified as enterotoxigenic. The proportion of *E. coli* isolates that were identified as enterotoxigenic was 28.4% (25/88) in forest birds and 70.4% (38/54) in waterbirds.

Overall the prevalence of ETEC in wild birds examined was 50% (42/84); thus, 38.6% (17/44) and 62.5% (25/40) in forest and water - birds respectively. Enterotoxigenic *E. coli* was observed in seven forest bird species including the Brown-chested Alethe, Forest Robin, Green Hylia, Green-tailed Bristlebill, Icterine Greenbul, Olive Sunbird and Yellow-whiskered Greenbul. Among waterbird species, ETEC was observed in the Black Tern, Common Sandpiper, Common Tern, Ruddy Turnstone and Sanderling. These birds probably obtained the pathogens from contaminated human sources.

In humans, Enterotoxigenic *E. coli* is a major cause of “traveler’s diarrhoea” (causing about 90% of infections in travelers) and an important cause of diarrhoea in infants in developing countries (Riddle and Tribble, 2008). In a study conducted by Tsuji *et al.* (1988), they purified and characterized LT from chicken isolates of ETEC and found that they were immunologically and physiochemically similar to that of the human strain H10407. In addition, they found that ETEC usually produces heat-labile toxin (LT) and occasionally the heat-stable toxin (Sta) (Tsuji *et al.*, 1988). In another study, ETEC was isolated from chicks from two farms and was demonstrated to be the cause of chick diarrhoea in the two farms (Joya *et al.*, 1990). However, outbreaks of avian diarrhoea due to ETEC have been rarely reported (Joya *et al.*, 1990).

The absence of *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Shigella flexneri*, *Shigella dysenteriae*, *Salmonella enterica* in the wild birds suggests that these species did not occur in the sampled birds or were not widely disseminated. However, some studies have reported the occurrence of these pathogens. For example, in a study conducted on *Corvus* spp. (Large-billed crow), two isolates of *Shigella flexneri* were identified (Yong *et al.*, 2008). In another study, characterising *Salmonella enterica* serotype from wild birds

in England, 90.6% (29/32) of the isolates were identified as *Salmonella typhimurium* and were isolated from nine green finches (*Carduelis chloris*), eight Eurasian siskins (*Carduelis spinus*), six sparrows (*Passer domesticus*), five goldfinches (*Carduelis cardulis*), two Common starlings (*Sturnus vulgaris*), one collared dove (*Streptopelia decaocto*) and one wood pigeon (*Columba palumbus*) (Hughes *et al.*, 2008). It was surprising that none of the isolates was identified as *Yersinia pseudotuberculosis* since wild birds are thought to be significant reservoirs of this species (Schiemann, 1989; Fukushima & Gomyoda, 1991).

There were representations of all four phylogenetic groups; A (42 isolates), B1 (14 isolates), B2 (28 isolates) and D (58 isolates). Phylogenetic group D was the predominant group comprising 41.5% of all the *E. coli* isolates. Contrary to the report that group B1 is predominant in birds (Gordon & Cowling, 2003), only 14 (9.9%) of the *E. coli* isolates in the current study belonged to group B1 and this was the lowest percentage recorded for any of the phylogenetic groups. It is reported that virulent extraintestinal pathogenic strains belong to phylogenetic group B2 and to a lesser extent group D (Picard *et al.*, 1999).

Overall, the virulent phylogenetic and commensal phylogenetic groups constituted 60.6% and 39.4%, respectively, suggesting that most of the *E. coli* strains isolated from the wild birds had virulence determinants.

Twelve and nine isolates belonging to phylogenetic groups B2 and D, respectively, were not enterotoxigenic. Thus, though these isolates belonged to virulent phylogenetic groups, they were not identified as enterotoxigenic. This suggests that these isolates belonged to other diarrheagenic groups including enteropathogenic *E. coli* (EPEC),

enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAaggEC) and diffuse adherent *E. coli* (DAEC).

From this study, it is possible to hypothesize that birds, especially those positive for ETEC (frequently isolated from humans) (Sack, 1975), could be zoonotic reservoirs of this infection and they have possibly acquired these strains through direct or indirect contact with humans and domestic animals.

## 8.0 CHAPTER EIGHT: GENERAL DISCUSSION

### 8.1 Introduction

Wild animals have been implicated in the spread of infectious diseases. A survey by Taylor *et al.* (2001) estimates that 60% of all human infections are zoonotic and about three-quarters of these infections originate from wildlife. Migratory animals have been suggested as important reservoirs owing to their long-distance movements and exposure to diverse habitats (Altizer *et al.*, 2011). Migratory birds, in particular, have far-reaching implications for the emergence and spread of infectious diseases due to their ability to cross geographical borders. The circulation of diseases such as Avian influenza (AIV), *Chlamydia psittaci*, *Campylobacter* spp., *Salmonella* spp. and Avian pneumovirus, Newcastle disease virus (NDV) (Palmgren *et al.*, 1997; Alexander, 2000; Andersen & Vanrompay, 2000; Hubálek, 2004; Wobeser, 2007) have been associated with migratory birds.

Beyond the potential role in pathogen spatial spread, a handful of studies suggest that migratory species themselves encounter a broader range of pathogens from diverse environments throughout their annual cycle compared with species residing in the same area year-round (Altizer *et al.*, 2011).

Although several studies have suggested that wild birds may serve as sources of zoonotic infections and may also encounter pathogens from diverse environments, only a few studies focussing on microbiota of wild birds have been conducted. These studies mainly focused on specific species of birds. These studies are usually conducted during disease outbreaks resulting in high mortalities and do not provide information on the prevalence of pathogens in apparently healthy birds.

In order to monitor disease outbreak events in wild bird populations. There is the need for increased surveillance in apparently healthy birds, as some of these birds may carry pathogens without showing any symptoms of the disease.

Therefore, this study focussed on four main objectives. Firstly, to provide information on the genera of gram-negative bacteria present in wild birds (forest and water- birds). Secondly, the study sought to determine the antimicrobial resistance profiles of gram-negative bacteria isolated from the wild birds. Furthermore, the study aimed to determine whether antimicrobial-resistant bacteria isolates from the birds harboured resistance genes. Lastly, the study sought to investigate the occurrence of six pathogenic species (Enterotoxigenic *E. coli*, *Salmonella enterica*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, *Shigella dysenteriae* and *Shigella flexneri*) in the birds using molecular methods.

Forest birds were trapped from the Ankasa Conservation Area which is a protected area with relatively minimal human interference while waterbirds were obtained from the Esiam beach and the Densu Delta Ramsar site. The Esiam beach and Densu Delta Ramsar site are important areas for waterbirds and these birds share the water resource with humans.

Enterobacteria or enteric bacteria were screened for in this study. These bacteria are found along the gastrointestinal tract because they are usually obtained from food/water. As a result, cloacal swabs were collected from the birds for screening. Also, gram-negative bacteria were considered because they are the most common types of bacteria and quite easy to isolate.

## 8.2 Occurrence of gram-negative enterobacteria

In Chapter Four of this thesis, cloacal swabs from 138 forest bird and 239 waterbirds were screened for gram-negative enterobacteria. The swabs were cultured on MacConkey and Salmonella Shigella agars and pure isolates obtained were identified to the genera level using biochemical tests. The findings of the study revealed that 61.8% (233/377) of the birds harboured some kind of gram-negative bacteria, 65.2% (90/138) and 59.8% (143/239) for forest and water- birds respectively.

Fifteen different genera of gram-negative bacteria were obtained from the birds. Overall, the genus *Escherichia* was the most frequently isolated genus with a prevalence of 22.3% and was isolated from 20 different species of birds. The species *E. coli* under the genus *Escherichia* is considered a normal inhabitant of the gastrointestinal tract (Gordon & Cowling, 2003) and therefore the high occurrence of this genus could possibly be as a result of the high occurrence of *E. coli*.

The occurrence of *Escherichia* in forest and water- birds were 31.9% (44/138) and 16.7% (40/239) respectively, indicating that it was more likely to isolate *Escherichia* from forest birds than waterbirds.

Although the genus *Escherichia* was the most prevalent in forest birds, the case was different for waterbirds. Among the waterbird species, the genus *Yersinia* was the most prevalent, 19.2% (46, 239). This finding suggests that while forest birds from the study area are more likely to harbour *Escherichia* spp., waterbirds on the other hand are more likely to harbour *Yersinia* spp. This could possibly be because forest birds are more susceptible or exposed to the genus *Escherichia* while waterbirds are more susceptible or exposed to the genus *Yersinia* or host physiological conditions that allow for the

colonisation of one bacteria genus than the other. For one of the forest bird species (Olive Sunbird) that had more than 10 individuals examined, the genus *Yersinia* had a higher prevalence than *Escherichia*.

### **8.3 Occurrence of antimicrobial-resistant bacteria**

In chapter five, antimicrobial susceptibility profiles of isolates from forest and water- birds were determined. Cloacal swabs that showed growth of bacteria in chapter four (233 in all) were examined. Each agar plate containing the antimicrobial agents Colistin or Ciprofloxacin, in a procedure referred to as the agar dilution method was streaked with the cloacal swabs/samples. Bacteria that grew on these agar plates were considered as antimicrobial-resistant.

Both Colistin and Ciprofloxacin resistant isolates were recorded in this study. In all, 106 Colistin-resistant isolates were obtained, belonging to 18 different species of bacteria. These resistant isolates were obtained from 15.5% (36/233) of samples examined. The prevalence of Colistin-resistant bacteria in forest birds was 10% (9/90) while the prevalence in waterbirds was 18.8% (27/143). Colistin-resistant *E. coli* was the commonest gram-negative bacteria species isolated, perhaps because it was the most isolated gram-negative genera. Colistin resistant isolates were obtained from seven forest bird species while in waterbirds, it was isolated from six bird species. Though Colistin is rarely used (Kempf *et al.*, 2016), a prevalence of 15.5% was recorded in wild birds in this study suggesting that resistance to this antimicrobial is widely spread. These birds may have acquired these agents from shared contaminated resources via contaminated food and water (Dolejska *et al.*, 2007). This is confirmed by the fact that all colistin-resistant isolates from the forest birds were obtained from birds that were sampled very close to the

reception of the protected area. This part of the protected area usually experiences frequent human interactions. Though wildlife could serve as reservoirs of intrinsic genetic determinants for resistance, it is often assumed that antimicrobial resistance in them is acquired from humans and livestock (Vittecoq *et al.*, 2016). In this study, resistance to Colistin in most of the isolates is suggested to be acquired except for resistance to bacteria genera such *Proteus*, *Serratia* and *Providencia* which have been suggested to show intrinsic resistance to polymyxin (class of antimicrobials colistin belongs) (Hayakawa *et al.*, 2012). Twenty-one out of 106 Colistin-resistant isolates were assumed to show intrinsic resistance to colistin and all of these isolates were obtained from waterbirds. The lack of isolates from forest birds showing intrinsic resistance further confirms that all the Colistin-resistant isolates were acquired possibly from humans or livestock in the surrounding communities and farmlands around the protected area.

Ciprofloxacin-resistant isolates were recorded in 41.7% (97/233) of birds. The prevalence in forest birds was 35.6% (32/90) while the prevalence in waterbirds was 41.3% (59/143). Again, the genus *Escherichia* recorded the highest prevalence. Ciprofloxacin-resistant isolates were recorded in 11 forest bird species and nine waterbird species. Ciprofloxacin is a completely synthetic antimicrobial (Hooper *et al.*, 1987), hence, resistance to this antimicrobial recorded in this study is suggested to be acquired resistance. Ciprofloxacin resistant isolates were obtained from birds that were sampled in all areas of the forest suggesting wide-spread of this antimicrobial agent among forest birds.

Colistin-resistant isolates were further subjected to multidrug resistance tests because this antimicrobial is suggested to be used for the treatment of multidrug-resistant infections. Antimicrobial agents used for the multidrug resistance tests were Ciprofloxacin, Ampicillin, Streptomycin, Oxytetracycline, and Colistin. However, measurements of

diameter of zones of inhibition for Colistin were not considered because no breakpoints have been defined for them (CLSI, 2018). Among all the antimicrobial agents used for the multidrug resistance test, Ciprofloxacin recorded the lowest prevalence 8.5% (9/106) of resistant isolates while Ampicillin recorded the highest prevalence 73.6% (78/106). The other two antimicrobial agents, Streptomycin and Oxytetracycline recorded prevalence of 50.9% and 52.3%, respectively. The difference in the prevalence of resistant isolates to antimicrobial agents was statistically significant ( $p < 0.05$ ), suggesting that resistance by a bacterial isolate to Ampicillin, Streptomycin, and Oxytetracycline were higher than resistance to Ciprofloxacin.

#### **8.4 Occurrence of plasmid-mediated resistance genes**

Colistin and Ciprofloxacin-resistant isolates that were obtained from Chapter five were further subjected to examination for plasmid-mediated genes. In Chapter Six, the 106 Colistin-resistant isolates were examined for the occurrence of five plasmid-mediated polymyxin genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*). The findings showed representations of all five genes in 14 of the isolates, with *mcr-3* recorded as the most prevalent gene. *Mcr-3* was recorded in both forest and water-bird species whereas *mcr-1* was recorded in a forest bird species and *mcr-2*, *mcr-4*, *mcr-5* were recorded in only waterbird species. To the best of my knowledge, this is the first report on the occurrence of *mcr* genes in wild birds in Ghana. The *mcr-1* gene has been reported in human isolates from some African countries such as South Africa, Nigeria, Egypt and Tunisia (Coetzee *et al.*, 2016; Elnahriry *et al.*, 2016; Grami *et al.*, 2016; Hu *et al.*, 2016).

Ciprofloxacin-resistant isolates were also examined for the occurrence of plasmid-mediated quinolone-resistant genes. In all, 164 Ciprofloxacin-resistant isolates were obtained from the birds. There were representations of four (*qnrB*, *qnrD*, *qnrS*, *qnrVC*) out of the six quinolone resistance genes investigated. The *qnrVC* gene was the most prevalent and occurred in 9.8% (16/164) of the isolates suggesting that this gene occurs more in wild birds in the study areas. Nine and 7 isolates from forest and water-birds harboured the *qnrVC* gene. In wild birds, *qnrB*, *qnrC*, *qnrD* and *qnrS* have been isolated from rooks (Literak *et al.*, 2012). In another study, *qnrS* was recorded in isolates from Mallards and Cormorants (Literak *et al.*, 2010). In this study, *qnr* genes were obtained from six forest bird species and 11 waterbird species.

### **8.5 Occurrence of diarrheal pathogens**

In chapter 7 of this thesis, the occurrences of four bacterial pathogens that are known to cause significant illnesses in humans were investigated. Out of the four, only one species, Enterotoxigenic *E. coli* (ETEC) was recorded in the wild birds suggesting that it is unlikely to isolate the other three species from wild birds from the study areas. The prevalence of ETEC in forest and water-birds were 38.6% and 62.5% respectively and were obtained from seven forest birds and five waterbirds respectively. The occurrence of these pathogens in the wild birds suggests that they may serve as significant sources of infection to humans.

Phylogenetic analysis of all *E. coli* isolates obtained also showed a representation of all four phylogenetic groups, A (42 isolates), B1 (14 isolates), B2 (28 isolates) and D (58 isolates). Groups B2 and D which are known to contain virulence characteristics constituted 60.6% of isolates, while groups A and B1 (commensal groups) constituted

39.4% of isolates. This finding suggests the 60.6% of the *E. coli* isolates obtained in this study were likely to be pathogenic.

Overall, 53.5% (76/142) of the *E. coli* isolates were not identified as ETEC. However, 20 of these isolates belonged to the virulent phylogenetic groups. This finding suggests that these isolates could belong to other diarrheagenic *E. coli* groups (Isidean *et al.*, 2011).

### **8.6 Factors that may predispose wild birds to enteric bacteria**

Previous studies have suggested several factors that could expose or make wild birds susceptible to infectious agents such as enteric bacteria. These factors include age, sex, diet, type of bird species and proximity to human-influenced habitats.

The chance of isolating enteric bacteria from birds in relation to the type of species, age and sex were determined. The genus of enteric bacteria considered for analyses was *Escherichia*. This was because it was relatively isolated in most of the bird species. A minimum of 10 individuals were considered for each comparison. Thus, bird species with less than 10 individuals were not included in the analyses.

For forest birds, three species, Yellow-whiskered greenbul (*Eurillas latirostris*), Forest Robin (*Stiphrornis erythrothorax*) and Olive Sunbird (*Cyanomitra olivacea*) were included in the analyses. The Yellow-whiskered Greenbul and Olive Sunbird are primary forest interior generalists while Forest Robin is a primary forest specialist that feeds on insects (Holbech, 2009). The prevalence of the genus *Escherichia* among the bird species was not statistically significant ( $p>0.43$ ). Thus, the prevalence of *Escherichia* among the three bird species was similar. A probable reason for this could be that all three species

feed on insects. Some insects have been suggested to carry high bacteria loads (Pai *et al.*, 2005). Other studies have suggested that generalist species are likely to carry more bacteria (Brittingham *et al.*, 1988; Livermore *et al.*, 2001). However, in this study, the case was different, the prevalence rates of *Escherichia* between the generalist and specialist species were similar.

In determining the association between prevalence of *Escherichia* in relation to age and size of forest birds, only the Yellow-whiskered Greenbul was used for the analysis. This was because the number of individuals of this species was enough to randomly select 10 each of adults and juveniles for comparison. The results of the analyses showed that the chance of isolating *Escherichia* from adult and juvenile birds were the same. Similarly, the size classes of the birds were not statistically significant. Although the results were not significant for this species, it could be significant for other species that were not considered for analyses due to their low numbers.

No attempt was made to compare the associations between ETEC isolated from Yellow-whiskered Greenbuls that were sampled close to the reception of the protected area and those that were sampled far away from the reception area because all the ETEC isolates were obtained from birds sampled near the reception area. Enterotoxigenic *E. coli* is mainly a human pathogen, only a few outbreaks of avian diarrhoea caused by this pathogen were reported in the 1990s (Joya *et al.*, 1990). Therefore, a high occurrence of this pathogen in a habitat relatively influenced by humans suggests that these pathogens were probably acquired from humans.

In addition, all Colistin resistant isolates in the Yellow-whiskered Greenbul were obtained from birds sampled near the reception area. Colistin is an antimicrobial agent mainly used

in animal production. Therefore, an occurrence of resistance to this agent in birds sampled close to the reception area suggests that proximity to human-influenced environments increases the risk of infection in the Yellow-whiskered Greenbul with Colistin-resistant bacteria.

For waterbirds, three species, Black Tern (*Chlidonias niger*), Common Tern *Sterna hirundo*) and Sanderling (*Calidris alba*) were included in the analyses. All three species are migratory. The prevalence of the genus *Escherichia* among the three species was not statistically significant. The Black Tern and Common Tern feed predominantly on marine fish during the non-breeding season; however, their diet may occasionally include insects and crustaceans (Snow & Perrins, 1998). On the other hand, during the non-breeding season, Sanderling's diet consists of small molluscs, crustaceans, polychaete worms and adult, larval and pupal insects (e.g. Diptera, Coleoptera, Lepidoptera, Hemiptera, and Hymenoptera), as well as occasionally fish and carrion (Del Hoyo *et al.*, 1996). Therefore, no association between the prevalence of *Escherichia* and the type of species could be because they have similar diets.

Interestingly, the Black Terns were found to have a high prevalence of Enterotoxigenic *E. coli* in comparison to Sanderlings. All 10 birds that were examined for Enterotoxigenic *E. coli* harboured the pathogen. The population trend of the Black Tern is decreasing according to the BLI (2016) and the high prevalence of Enterotoxigenic *E. coli* could probably be one of the causes of the decline in population.

A study found that the chance of a bird to be colonized by *E. coli* varied with the degree of how close to an environment influenced by human activity the bird was sampled in (Gordon & Cowling, 2003), however, that comparison could not be done in this study

because the number of individuals examined for the different forest bird species were too few for this analyses (Gordon & Cowling, 2003).

It is possible to hypothesize that birds, especially those positive for ETEC, which are frequently isolated from humans (Sack, 1975), could be zoonotic reservoirs of this infection and they have possibly acquired these strains through direct or indirect contact with humans and domestic animals.

## **9.0 CHAPTER NINE: CONCLUSIONS, CONTRIBUTIONS TO KNOWLEDGE, POLICY DIRECTIONS AND RECOMMENDATIONS**

### **9.1 Conclusions**

In conclusion, this study provides useful information on the occurrence of gram-negative enterobacteria in forest and water- bird species. Fifteen different genera of gram-negative bacteria were obtained from the bird species. The most frequently isolated genera were *Escherichia* (22.2%), *Yersinia* (19.4%), *Enterobacter* (12.2%) and *Klebsiella* (9.8%). This information may be useful when handling wild birds as the finding shows that apparently healthy wild birds may harbour enteric bacteria and may also serve as sources of enteric bacteria to other wild animals, domestic animals, and humans.

Secondly, the study provides evidence that wild birds harbour antimicrobial-resistant bacteria. Some of which are intrinsic while most are acquired from contaminated sources. The occurrence of bacteria resistant to Colistin, a last resort antimicrobial suggests that resistance to this antimicrobial is widespread. The occurrence of Ciprofloxacin-resistant isolates further confirms that resistance reported for most of the isolates was acquired. Furthermore, evidence from this study also suggests that wild birds carry multidrug-resistant isolates and these serve as possible sources of transmission to humans, domestic animals, and other wild animals. Moreover, this study has shown that the premise that antimicrobial-resistant bacteria occur generally in hospitals, domestic animals and animal farms should be reconsidered.

Thirdly, this study has shown that bacterial isolates from wild birds harbour resistance genes such as plasmid-mediated polymyxin and quinolone genes. The occurrence of plasmid-mediated genes suggests that resistance could be conferred to other bacterial isolates (possibly commensal bacteria).

Lastly, the study provides useful information on some diarrheagenic bacteria species that occur in wild birds. Enterotoxigenic *E. coli* was the only species of diarrheal pathogens isolated from this study indicating that wild birds could serve as sources of infection of this pathogen.

From a public health standpoint, continuous surveillance in wild birds will help monitor the occurrence of pathogens and their possible spread to other wild animals, domestic animals, and humans. Although occurrence may be low, the potential to spread pathogens is considerable. Similarly, from a conservation perspective, the occurrence of antimicrobial-resistant bacteria could hamper conservation efforts as birds carrying resistant bacteria could serve to disperse bacteria between widely separated locations and from hotspots to vulnerable populations.

## **9.2 Contributions to knowledge**

Although previous studies have demonstrated the occurrence of AMR in wild birds, this study examined a broader range of bird species and a broader range of bacteria species. Most of the studies have focused on one or one or two bird species but this study investigated 36 species of birds from two different ecosystem. Many of the studies also focused on a single bacteria species or two but this study isolated gram-negative bacteria.

There is no published literature on the occurrence of enterobacteria and antimicrobial resistance in about 25 of the bird species that were examined in this study.

Antimicrobial resistance is often linked with human activity (Cizek *et al.*, 1994). Therefore, the occurrence of colistin-resistant bacteria in birds sampled around the reception area only of the Ankasa Conservation Area suggests that the resistant isolates were possibly acquired from sources linked with human activity.

This study has also shown that Black Terns could serve as sentinels of ETEC.

Lastly, to the best of my knowledge this is the first case of colistin-resistant bacteria in forest bird species and ETEC in wild birds in Ghana.

## **9.3 Policy directions**

The occurrence of antimicrobial resistance in the wild birds is an insight that could help practitioners to understand the extent of spread of resistant bacteria.

Practitioners such as managers of protected areas should continuously educate communities surrounding protected areas on the impacts of their activities on wildlife.

People living along the coast especially school children should be sensitized on the risk associated with handling these birds.

There should be continuous education on proper waste management as some of these birds were observed feeding on human excreta and rubbish disposed along the coast.

Antimicrobial resistance is a great threat to the realisation of the Sustainable Development Goal 3 which stresses on ensuring healthy lives and promoting wellbeing for all at all ages. Therefore, there should be proper implementation of the National AMR policy to minimise possible transmissions.

#### **9.4 Recommendations for future studies**

This study recommends the following for future research:

Large sample sizes of birds' species should be considered in order to prevent the abnormally high or very low prevalence due to sampling biases. Furthermore, samples should be collected from other protected areas and important sites for waterbirds. This will possibly increase the number of species investigated.

Secondly, the occurrence of other bacteria types such as gram-positive bacteria should be considered, in order not to miss out on other equally important infectious agents.

Moreover, the number of antimicrobial classes should be expanded to include other antimicrobial classes, as only five antimicrobial classes were used in the current study. Similarly, to provide more information on resistance profiles of isolates to antimicrobial agents under the same class, three or more antimicrobial agents of the same class should be examined under a class of antimicrobial. Obviously, when the numbers of antimicrobial classes are expanded then there is also the need to increase the number of antimicrobial resistance genes investigated.

Lastly, since there are six diarrheagenic *E. coli* groups, the occurrence of all the groups should be investigated to provide information on which of the groups occurs more in wild birds.

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