“Pet Birds as Emerging Reservoirs of Multidrug-Resistant Virulent Enterobacteriaceae in Noakhali, Bangladesh”

M.Sc THESIS

“A dissertation submitted to the Department of Microbiology, Noakhali Science and Technology University, for the partial fulfilment of the Degree of Master of Science in Microbiology”

Submitted By

Roll No: BFH2305MS108F

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Department of Microbiology

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, NOAKHALI SCIENCE AND TECHNOLOGY UNIVERSITY, FOR PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN MICROBIOLOGY

SUBMITTED TO

DEPARTMENT OF
MICROBIOLOGY NOAKHALI
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ROLL: BFH2305MS108F
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DEPARTMENT OF
MICROBIOLOGY, NSTU

Dedicated to…

My beloved Father Md. Feroj Miah

&

My beloved Mother Mst. Amena Begum

Quotation…

“If you're hard-working and determined, you will make it, and that's the bottom line. I don't believe in an easy way through.”

-Isabel dos Santos

CERTIFICATION OF APPROVAL

It is hereby certified that, student bearing Roll no: BFH2305MS108F, Session: 2022-2023 has carried out the research work described in this thesis entitled “Pet Birds as Emerging Reservoirs of Multidrug-Resistant Virulent Enterobacteriaceae in Noakhali, Bangladesh” by Sabia Afroj Moury, for the partial fulfillment of her Masters of Science Degree in Microbiology from the Noakhali Science and Technology University, Bangladesh under my academic supervision in the Microbiology Laboratory, Noakhali Science and Technology University.

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-By the Author

December, 2025

Abstract

Pet birds are widely kept companions, particularly in South Asia, where their popularity has surged, leading to close human-avian interaction. This intimate association provides an increasingly significant route for the zoonotic transmission of bacterial pathogens and antimicrobial resistance (AMR), presenting a critical One Health challenge. This study was conducted to investigate the prevalence, antimicrobial resistance profiles, and molecular characteristics of key pathogenic bacteria isolated from pet birds in the Noakhali region of Bangladesh. A total of 156 bacterial isolates were successfully recovered from 92 clinical and environmental samples, identifying dominant species as *Escherichia coli* (n=75), *Klebsiella* spp. (n=66), *Pseudomonas* spp. (n=9), and *Citrobacter* spp. (n=6).

Phenotypic susceptibility testing revealed high levels of multi-drug resistance (MDR) across the isolates, with the majority showing resistance to medically important broad-spectrum agents, including amoxicillin, cephalosporins, tetracycline, and sulfonamides. Molecular analysis confirmed the underlying genetic mechanisms of resistance, with prevalent detection of genes such as *blaTEM* (78%), associated with resistance to β-lactams, *tetA* (63.46%), conferring tetracycline resistance, and *sul1* (41.02%), mediating sulfonamide resistance. Crucially, the extended-spectrum β-lactamase (ESBL) gene *blaCTX-M* was detected in nearly 30% of isolates. Furthermore, the presence of virulence genes, including *fimH* and *eaeA* in *E. coli*, and *lasB* in *Pseudomonas*, highlights the pathogenic potential. A substantial 76.28% of isolates exhibited a Multiple Antibiotic Resistance (MAR) index greater than 0.2, confirming a high-risk scenario driven by intense antibiotic selection pressure. These findings unequivocally establish pet birds as significant reservoirs for high-risk, virulent, and MDR bacteria, necessitating immediate implementation of evidence-based biosecurity measures and robust antimicrobial stewardship programs under the comprehensive One Health framework.

Keywords: Pet birds, Zoonosis, Antimicrobial Resistance (AMR), Multi-Drug Resistance (MDR), Enterobacteriaceae, β-lactamase, Virulence genes, One Health, Public health, Transmission.

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List of Abbreviation

Symbol	**Abbreviation**
β	Beta
%	Percentage
≥	Greater than equal
≤	Less than equal
µl	Microliter
µg	Microgram
mm	Milimeter

General term	**Abbreviation**
XLD	Xylose Lysine Deoxycholate
TSB	Trypticase Soy Broath
TSI	Triple Sugar Iron
PCR	Polymerase Chain Reaction
Kbp	Kilo base pair
bp	Base pair
rpm	Rotation per minute
CLST	Clinical Laboratory Standard Institute

Chapter 1

1. Introduction and Review of Literature

1.1. General Introduction

Pet birds such as parrots, budgerigars, cockatiels, lovebirds, doves, finches, and mynas are widely kept as domestic companions because of their aesthetic value, intelligence, and strong emotional attachment with owners. Over recent years, the global craze for pet birds is skyrocketing in South Asia and small household aviaries, indoor bird keeping, and commercial bird markets are increasingly common (Uddin et al., 2024). This close interaction between birds and humans creates significant opportunities for zoonotic transmission of bacterial pathogens, through contact with feces, aerosols, feathers, contaminated cages, feeders, and water bowls (H. A. Ahmed et al., 2021).Birds naturally carries diverse intestinal microbiota. They contain beneficial commensals, opportunistic pathogens, and primary pathogenic species. Of particular concern are *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas spp*., and *Salmonella spp*., *Citrobacter spp.* organisms well known for their zoonotic potential and their increasing levels of antimicrobial resistance (Boseret et al., 2013). Asymptomatic pathogen shedding in bird feces poses unrecognized transmission risks to vulnerable people such as children, elderly individuals, immunocompromised persons, and people with frequent bird contact (Evans, 2011).

Globally, antimicrobial resistance (AMR) has become one of the major threats to public health. This problem has arisen by excessive, improper and unregulated use of antibiotics in human medicine, veterinary, agricultural, aquaculture and in the care of companion animals (World Health Organization, 2025). Moreover, commercial poultry and pet birds are often treated with antibiotics for either therapeutic, growth-promoting or prophylactic reasons. In many low- and middle-income countries, including Bangladesh, distant access to antibiotics is promoting misuse and resistance to drugs (Rahman et al., 2020). Furthermore, exposure to antibiotics in birds favors the gastrointestinal tract with resistant bacteria. Importantly, resistant bacteria from birds have commonly carried resistant genes such as extended spectrum β-lactamase (ESBL) including *blaCTX-M* and *blaTEM*, carbapenase such as *blaNDM* and *blaKPC*, fluoroquinolone such as *qnrA, qnrB and qnrS*, in addition to a range of aminoglycoside, tetracycline and sulfonamide resistance determinants such as *tetA* and *sul1*. These resistance genes play a role in the development of multidrug resistant and extensively resistant (XDR) strains, which cause great problems in terms of veterinary and medical treatment (Hasib et al., 2025; Rahman et al., 2020).

A crucial reason for the high speed of spread of AMR is the role of mobile genetic elements (MGEs) such as plasmids, integrons, insertion sequences and transposons. These elements allow the capture and spread of resistance and virulence determining via horizontal gene transfer (HGT), even between non homologous bacterial species (Partridge et al., 2018). The avian gut harbour high density of microbes and they are frequently exposure to antibiotic, which is the optimal environment for horizontal gene transfer (HGT) and high-rate bacterial evolution (Roth et al., 2019). Among the bacteria of concern in pet birds, *E. coli* is notable because of the broad range of pathogenic variants i.e. enteropathogenic (EPEC), enterohemorrhagic (EHEC), avian pathogenic (APEC), and uropathogenic strains (UPEC), which also have virulence genes including *stx1*, *stx2*, eae, *fimH* and *hlyA* (Kaper et al., 2004). *Klebsiella pneumoniae*, another important pathogenic bacteria, is known worldwide for its capability to produce ESBLs and carbapenases and frequently carries the gene *blaCTX-M*, *blaSHV*, *blaTEM*, *blaNDM* and genes related to hypervirulence such as *rmpA* (Navon-Venezia et al., 2017). *Pseudomonas aeruginosa* is known to be intrinsically resistant to multiple antibiotics because of the efflux pump activity and decreased outer membrane permeability and can be linked to respiratory disease, septicemia, and long-term contamination in the environment of birds (Pang et al., 2019). Meanwhile, *Salmonella spp*. are still considered important foodborne and zoonotic agents capable of colonizing bird intestines and disseminating through feces, with virulence being associated often with *invA*, *stn* and *Salmonella* pathogenicity island (SPI) genes (Foley et al., 2013). *Citrobacter spp.* are opportunistic imprinting, enteric bacteria bridge along with gastrointestinal infections and are carrying multiple AMR genes generating increasing concern the zoonotic and environmental transmission (Pang et al., 2019).

For the clinical significance of these pathogens, accurate identification and characterization of this and their pathogens require more than culture-based techniques. While culture and biochemical tests are still important for and form the basis of preliminary detection, they cannot reliably distinguish virulent pathotypes or identify particular resistance determinants. Therefore, molecular diagnostic tools such as polymerase chain reaction (PCR) are widely used for confirmation of species identity, virulence determinant detection and identification of AMR genes. Combined with antibiotic susceptibility testing (AST) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines, PCR-based analysis provides a complete method of assessing bacterial pathogenicity and difficulties in treating.

Despite the rising popularity of pet birds in Bangladesh, relatively less scientific attention has been paid on avian as reservoirs of pathogenic and MDR bacteria. Existing research in Bangladesh is mostly focused on the commercial poultry sector and there are significant gaps concerning the role

of household pet birds and their potential role in spreading AMR on the community level (Hoque et al., 2020). In many home environments, pet bird cages, feeders and water bowls are not cleaned regularly, owners are often giving antibiotics without professional advice and birds will often be living in close quarters with children and family members. Moreover, many pet shops and aviaries do not have proper sanitation, quarantine or controlled antibiotic use. Considering these factors, pet birds may be previously underrecognized sources of harmful pathogens and resistance genes. Therefore, the early detection and molecular characterization of these bacteria is imperative in establishing evidence-based guidelines in the One Health model that stresses the interconnectedness of human, animal and environmental health.

This study addresses these gaps by isolating key zoonotic bacteria (*E. coli*, *Klebsiella spp.*, *Pseudomonas spp*. and *Citrobacter spp.*) from pet bird fecal samples, detaching virulence and AMR genes using polymerase chain reaction (PCR), finding antibiotic resistance patterns by standardised advanced standard assay (AST) and finding the prevalence of MDR strains. Furthermore, the study discusses correlations between molecular-determinants and phenotypic resistance which provides important background data that will feed into future biosecurity guidelines, public health awareness programs, and antimicrobial stewardship programs. Although the research relies on culture-based isolation, molecular identification, PCR detection and AST for selected genes and antibiotics, and lacks further advanced technologies such as whole genome sequencing, etc., it offers invaluable basic data to support future research and surveillance

1.2 LITERATURE REVIEW

1.2.1 Pet bird as an economic industry

The pet-bird industry can be seen as an important sector with multifaceted ramifications and calculations in terms of legal and unlawful business transactions and profitability. According to Grand View Research (2025), the pet-bird health industry market size was valued at US$958.8 million in 2024 and anticipated to grow beyond 2030 with a compound annual growth rate of 8.03%, reaching US$1.50 billion. This pattern rightly indicates the commercial significance of health, healthcare services, and products in the care of caged birds. At the same time, the market of bird food products in different capacities and varieties from pet birds and caged birds also indicates increase; in fact, the pet and caged bird category of the global bird food market indicates strong momentum in the online retail segment (Mordor Intelligence, 2024).

With respect to the composition of animal species, psittaciforms (parrots) are one of the most traded animal groups and are of high commercial value. Analyzing the international trade of parrots in China from 1981-2022 shows that psittaciforms constitute about 90% of the live birds traded under the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), affirming their eminence in the legal international animal trade (Sánchez-Mercado et al., 2021). The high number can be attributed to the pressure exerted by their colorful appearance, intelligence, and human speech mimicry abilities in the pet animal market (Zhang et al., 2024). On the other hand, songbirds (passeriforms) consist of an exceedingly large number in terms of animal trade; although not many in the appendices of CITES agreements, they constitute a considerable percentage of animal species in international animal trade (BirdLife International, 2024; CITES Secretariat, 2023).

The illicit songbird trade remains another prominent aspect of the black market in birds. A case study from Brazil's northeastern regions recorded 34 species being channeled through the illicit songbird trade and showed how desirability—that is, the possession of singing prowess—is a significant factor in setting the prices and profitability of individual birds. The prices recorded are quite variable: untrained birds caught in the wild may be purchased at remarkably low prices, while those raised in captivity may be worth appreciably more (de Oliveira et al., 2020). At the same time, market surveys in urban areas (such as Mexico City) point towards the continued commercial requirements for both indigenous passerine birds and exotic birds like budgerigars or monk parrots kept in urban areas (Gómez-Álvarez et al., 2023), reflecting the social and commercial viability of what may otherwise be seen as the pet-bird commercial sector.

For many small business owners, breeders, and traders, the pet-bird industry is an important earning point. Even while fixed or institutional breeders and pet shops get access to this income stream, others like market vendors, roving vendors, or poachers get access to this income stream as a proximate means of earning money (Maksuda Aziz, 2023). The association of this industry with feed makers, accessory makers, and pet care centers further strengthens this point. In terms of policy objectives, the overlap between formal and informal trade in birds, together with the following welfare, conservation, and health-related dangers, poses challenges. The illicit songbird trade impacts biodiversity, and the commercial parrot trade sparks fears about the sustainability of harvest and captive breeding (de Oliveira et al., 2020; Sánchez-Mercado et al., 2021). Moreover, husbandry conditions in unauthorized markets may pose threats of zoonotic diseases, emphasizing the significance of effective regulation and enforcement (BirdLife International, 2024). Specialists recommend the combination of approaches that are effective in lessening negative impacts: improving captive-bred bird certification, traceability scheme deployment, cooperation with internet platforms in combating illicit ads, and capacity enhancement in avian vets (Sánchez-Mercado et al., 2021). Despite this information being known, there are still significant gaps in information. Market report information regarding health, food, and products can be commercially sensitive in terms of methodology. The other gap in information lies in academic papers that seem to focus more on conservation rather than the economic significance of the matter. The publication of information in academic journals regarding the employment and sustainability of pet-bird industries in terms of being regulated but legal still remains rare (BirdLife International, 2024; de Oliveira et al., 2020).

1.2.2 Contribution of pet birds to society

Pet birds are essential in ensuring that human life is amplified through companionship/association, cognitive functions/engagements, therapy/emotional support, and social interactions/social engagement. Many pet bird owners display significant levels of emotional association with birds through felt comfort in their companionship; they display action-oriented behaviors in the forms of voices-to-be-heard songs and pleasant interactions that can be impactful in stress reduction and combating anxiety and loneliness (Cheeky Beaks, 2025; Mika Birds Farm, 2024). The cognitive functions and trainability exhibited by many different varieties of pet birds make engagement with birds significantly cognitive-functioning and significant cognitive stimuli achievable through tricks and action-oriented problem-solving endeavors in both birds and pet owners (Jennifer clem, 2023; PetsCareWorld, 2023). For children in particular, pet birds are invaluable in equipping kids through the most effective process of nurturing and responsible behavior in pet-keeping functions like caring, cleaning, social interactions—that dramatically empowers kids in exhibition of compassion and life

functions (PetsGrail, 2023). Again and again, pet birds are clean birds requiring less grooming attention in comparison with other domesticated pets like dogs and cats and have longer life spans—they are accessible companions especially in situations requiring minimal human attention or those requiring little human mobility in grooming or in social engagement (Al Bawaba, 2023; Larry Gilliam, 2023). At social levels, pet birds are social catalysts foundational in triggering social interactivity in various social forms through providing social gatherings among pet owners through birds in starting new social interactions and in combating social isolation in society (Mika Birds Farm, 2024). More importantly, pet birds are essential voices and agents in providing therapy in mental health-related requirements through display-oriented behaviors in forms of voices, actions, and needs in caring through cognitive functions in impacting moods in therapy in both birds and those in need in stabilized human society (Cheeky Beaks, 2025; Mika Birds Farm, 2024).

Figure 1.1: Interaction of pet bird with children and elderly person.

1.2.3 Scenario of pet birds in Bangladesh

The pet bird business is flourishing in Bangladesh, both in the offline markets and a dynamic online marketplace. Even though it is prohibited by law (e.g., Wildlife Conservation and Security Act of 2012 and the Pet Birds Management Rules, 2020), most vendors openly sell on social media sites (Facebook and YouTube) and few attempts are actually made to enforce the regulations (Maksuda Aziz, 2023; Rashad Ahamad, 2025). These markets are commonly inhabited by such species as scaly-breasted munia (Lonchura punctulata), blossom-headed parakeet (Psittacula roseata), Alexandrine parakeet (Psittacula eupatria), common myna (Acridotheres tristis), hill myna (Gracula religiosa), and doves/pigeons (Bd-pratidin English, 2025; Maksuda Aziz, 2023). In a raid in Dhaka,

41 parakeets, 10 munia, 5 common mynas, and 3 pigeons or doves were rescued, meaning that these species were the most common in the local trade (Bd-pratidin English, 2025). Prices of these birds are wide ranging depending on species, age, rarity and whether found in the wild or as captive species. The price of budgerigars (parakeets) sold in pairs at Dhaka at the weekly pigeon and bird market in Banasree is about Tk 800-1,000, a baby common myna (also known as Shalik) costs about Tk 1,000, and a pair of feather-footed fancy pigeons can cost about Tk 2,000 (bdnews24, 2022). At the top-end, more costly talking birds like mynas may be sold contactlessly Tk 30,000 a pair and exotic chicks (mother of parrot species) can be sold contactlessly Tk 150,000 (The Business Standard, TBS, 2024; TBS reportage of markets). Even the wild-caught parrots are sometimes sold by street vendors at extremely low prices: one report stated that common parrots were sold at Tk 1,200-1,500 (bdnews24, 2022). One of the issues is the illegal trade. Although most of the species listed are expressly covered through legislation, there is a lack of enforcement. The raids are implemented by the Wildlife Crime Control Unit of the Forest Department, yet, according to the reports; there is a lack of online enforcement; the online bird trading groups are rather unchecked and some of the species traded illegally are endangered macaws and parakeets, which are also imported with rather dubious certifications (Maksuda Aziz, 2023). The law system is also not coping: even though it is mandatory that pet bird stores and farms should have a license under Pet Birds Management Rules, 2020, there are numerous stores that do not have a license, and in recent years, the government has begun to crack down on the issue (The Daily Star, 2024). It was found to be a pet-bird economy that is, in a way both thriving and threatened, featuring a very diverse range of species being traded, extensive informal and illegal components, and a system of regulation that is struggling to both match up with demand and also deal with enforcement issues.

1.2.4 Microbiome and Pathogenic Bacteria in Birds

1.2.4.1 Normal Gut Microbiota of Avian Species

The avian gastrointestinal tract is a rich and diverse microbial community that differs with anatomical location that includes crop, proventriculus, gizzard, small intestine, ceca and colon. Firmicutes, Proteobacteria, and Bacteroidetes are typical dominant phyla, but their proportions depend on species, diet, age, and exposure to the environment. Fermentation and the generation of short-chain fatty acids (SCFAs) in the ceca are one of the primary locations of nutrient use and energy metabolism in many species of birds. These microbial communities help in important physiological processes in terms of digestion, vitamin production, immune control and competitive elimination of pathogens. The establishment of microbiota starts soon after the hatch and depends

mainly on parental contact, environmental microbial sources, and diet, and varies throughout the remainder of life and with seasonal variations and host physiological condition (Pan & Yu, 2014; Sun et al., 2022).

1.2.4.2 Common Gram-Negative Bacteria Found in Pet Birds

Most pet and ornamental birds commonly become hosts to numerous gram-negative bacteria, most of which can be commensals, opportunistic or may be pathogens. Frequently isolated taxa comprise *Escherichia coli*, *Klebsiella spp.*, *Enterobacter spp*., *Salmonella spp*., *Pseudomonas spp*., Proteus spp., and *Citrobacter spp.* Gram-negative isolates that are frequently identified in surveys of captive and clinical avian samples are *E. coli*, *Salmonella spp*. *E. coli* is the most common gram-negative isolate commonly seen, and *Salmonella spp*. are frequently identified during sporadic outbreaks, or as an asymptomatic carrier. A number of these organisms are also known to be carriers of clinically significant antimicrobial-resistance determinants including extended-spectrum b -lactamases (ESBLs), which pose considerable risks to One-Health because of their zoonotic transmission capability (Kabantiyok et al., 2023; ’Qureshi & ’Azam, 2024).

1.2.4.3 Mechanisms of Bacterial Colonization in the Avian Gut

The avian gut ecology is very adaptive to the complexity of the host physiology, microbial nature and environmental environment, and necessitates the intensive interactions to lead to successful colonization. The adhesion and biofilm formation are the most effective mechanisms. The two mechanisms help in attaching and retaining the bacteria onto the mucosal surfaces; niches of bacterial metabolic activity on exploiting dietary substrates or bacterial mucosal carbohydrates; and immune modifications in which bacteria may circumvent or suppress the host defenses. Competitive exclusion, bacteria production of bacteriocins and metabolic cross-feeding are also microbial interactions affecting the success of colonization. Environmental seeding of feed, litter, water, handslers, and wild birds also makes a contribution to the composition and regulation of bacterial populations in pet birds (Bodawatta et al., 2022; Sun et al., 2022).

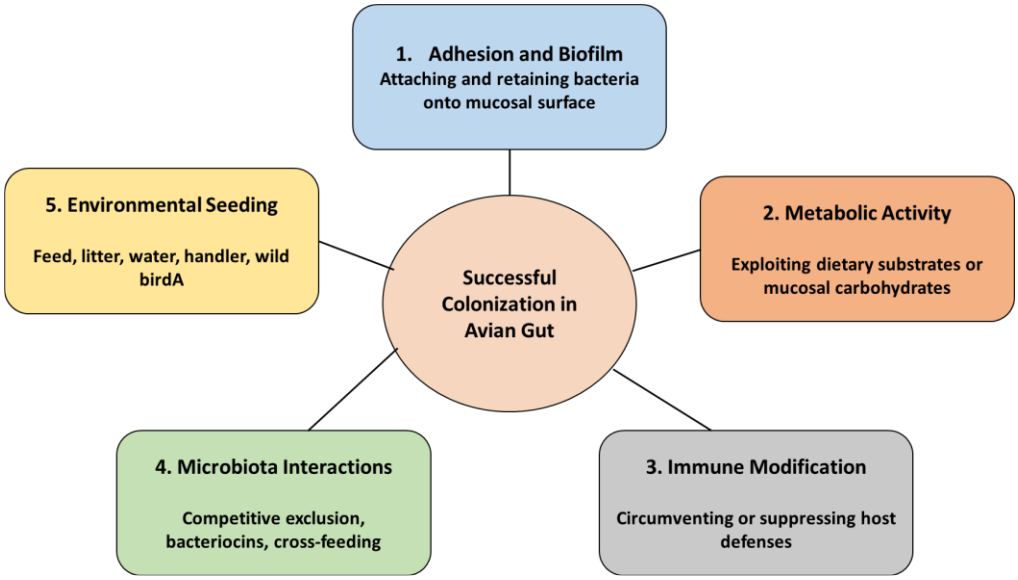


Figure 1.2: Mechanisms of Bacterial Colonization in the Avian Gut

1.2.4.4 Birds as Asymptomatic Carriers of Pathogenic Bacteria

It is also possible that many infected pet birds may be asymptomatic in their carriages of pathogenic bacteria thus serve as asymptomatic carriers of the bacteria. These birds can shed the pathogens periodically or even consistently according to the stress, nutritional condition, environmental hygiene, and even presence of a disease. *Salmonella spp*., *Chlamydia psittaci* and antimicrobial resistant *E. coli* have been widely studied as regards to asymptomatic carriage. This silent carrier makes it harder to keep track of the spread and creates the risk of zoonotic infection of bird owners, breeders, and veterinary personnel (Masud et al., 2024). The health impact on the population is also dramatic, especially in cases of pathogenic or multidrug-resistant strains.

1.2.4.5 Effect of Diet, Environment and Antibiotic Use on Gut Flora

Avian gut microbiome is dependent on diet. Pellet-based, seed-based, insectivorous or fruit-based diets contain different nutrient profiles which select certain media taxa. As an example, diets rich in fiber favour anaerobic fermentation and SCFA generation in the ceca. The type of contact with the wild birds, age, and the quality of water and milk increasingly subject the cages to contamination by new microorganisms that could alter the gut composition. The administration of antibiotics is a potent alterer, which results in most cases in the loss of microbial diversity, colonization resistance dislocation, and the selection of antibiotic-resistant microorganisms. Microbial communities can be restored with the help of such practices as better husbandry, probiotics, and prebiotics, but evidence in pet birds remains scarce in comparison to poultry (Garcia-Mazcorro et al., 2021; Pan & Yu, 2014; Sun et al., 2022).

1.2.4.6 Pathways of Fecal shedding and environmental contention

Fecal shedding is the main pathway of enteric bacteria release by birds which discharges the bacterium into the environment. Contamination is by direct droppings, aerosol feces dried droppings, contaminated feed or water and mechanical transmission of contamination by human hands, cage equipment or transport containers. A large variety of gram-negative bacteria are capable of surviving long tendencies in damp organic matter, and an improper sanitation has become one of the most crucial factors running risk of persistence and dissemination of the pathogen. The role of ensuring a high level of hygiene, regular cleaning of the cages, quarantine of new birds, and responsible use of antibiotics is justified by the fact that asymptomatic carriers can constantly pollute the environment, which is stable (Boseret et al., 2013; Jarma et al., 2021; ’Qureshi & ’Azam, 2024).

1.2.5 Zoonotic Significance of Pet Birds

1.2.5.1. Pet Birds as the Zoonotic Hosts of Disease in humans

Zoonotic bacteria have been identified in many different types of pets and captive birds that have been attributed to recorded outbreaks as well as occasional infection of humans. As well as resistant *E. coli* and *Salmonella*, which have been associated with cases in humans in some cases, Pet birds and their environments have been found to contain enteric bacteria (Boseret et al., 2013; Bueno et al., 2024). Molecular and culture-based surveillance studies indicate that birds could have the same genotypes and resistance determinants as humans and other livestock, indicating the presence of likely transmission pathways between birds and people (Mourkas et al., 2024; Prandi et al., 2023). In the case of human infection, it can be caused by contact and infection of the birds or by contamination of domestic space (cages, feeders) or contact with infected products and droppings by birds, as reported in case studies and outbreaks studies (Boseret et al., 2013; ’Qureshi & ’Azam, 2024).

1.2.5.2 Differential Risks: Children, Older People, and Immunocompromised People

Some human groups have higher risk of severe disease after being exposed to the zoonotic, young children (less than 5 years), older adults, pregnant people, and immunocompromised persons. Both susceptibility and severity of infection are more common and increased by immature or fading immunity, increased frequency of hand-to-mouth behaviour in young children and comorbid conditions in older adults. The former populations can thus always be found in the preventive counselling discussed as a priority population by public-health advice and clinical reviews, as well as more stringent hygiene around a pet (Stull et al., 2015; Varela et al., 2022). In

immunocompromised hosts, even those microbes that are considered to be normal in birds (high-virulence in otherwise healthy individuals) would lead toinvasivedisease and would need specific risk-reduction measures (Stull et al., 2015).

1.2.5.3 Transmission Routes

Aerosols

Aerosol is the transmission of bacteria in respiratory secretions or dried fecal material that gets airborne and is inhaled. This route is common for avian pathogen and can occur in activities that disturb contaminated bedding, dry droppings or during close contact with birds (Boseret et al., 2013). Aerosolized particles, especially can cause exposure to respiratory organisms like *Chlamydia psittaci* and enteric bacteria when fecal dust is airborne.

Fecal-Oral

The fecal-oral route is a critical route of transmission of enteric pathogens such as *Salmonella spp*. and pathogenic *E. coli*. pathogen transmission to humans can occur through contaminated hands (after handling birds, cages or substrates), contaminated food or water, or by direct contact with droppings (Boseret et al., 2013). Poor hand hygiene and household practices (e.g. cleaning cages over kitchen sinks) increase this pathway.

Cage / Feeder / Waterer Contamination

Bacterial contamination of feeders, waterers, toys and cage surfaces form long lasting environmental reservoirs. Studies isolating *Salmonella f*rom cage papers, bedding and feeders show that routine husbandry equipment is capable of harbouring viable organisms for prolonged periods of time as well as being an indirect vector for human exposure (Bueno et al., 2024; Jarma et al., 2021).

Human Handling (Direct Contact/Fomites)

Direct handling of birds and contact with fomites (clothing, hands, grooming supplies) contaminated with bacteria readily transmits bacteria from birds to people, and vice versa. Close and reoccurring interactions (such as kissing birds, allowing birds on household surfaces, or poor hand hygiene) have been shown to be highly linked with increased zoonotic risk (Boseret et al., 2013; Stull et al., 2015).

1.2.5.4 Documented Outbreaks and Case Reports Associated with Pet Birds (*E. coli*, *Salmonella* and Other Enterobacteriaceae)

Many observations link pets or birds to human infections:

- Spread of *Salmonella* relating to pet or feeder birds involve often *S. Typhimurium* (Bueno et al., 2024; Giovannini et al., 2013).
- Studies reveal that multidrug-resistant *E. coli* and other Enterobacteriaceae bacteria are isolated from pet birds and their surroundings (Mohamed et al., 2022; Okunlade et al., 2021).
- Genetics (genotypes) and markers of resistance are shared between bacteria sampled from birds, environment, and humans, supporting One Health perspective of these diseases in large studies (Mohamed et al., 2022; Mourkas et al., 2024; 'Qureshi & 'Azam, 2024).

1.2.6 *Escherichia coli* in Pet Birds

1.2.6.1 Prevalence in Pet Birds Worldwide and South Asia

Escherichia coli is frequently isolated from the gastrointestinal tract of healthy and diseased birds and its prevalence is highly variable depending upon the sampled species, the sampling method (cloacal swab, feces or tissue), husbandry system and geographic region. Studies show that there is a high prevalence of carriage of *E. coli* in both captive and wild birds with prevalence estimates in published studies ranging from low digits to over 70% in certain surveys of poultry (Mbuthia & Hoza, 2025; Pereira et al., 2024). In South Asia including Bangladesh, India and Pakistan, several studies report high levels of carriage of *E. coli* in both poultry and companion/captive avian populations; recent field studies from Bangladesh and neighboring countries report high rates of isolation from cloacal and fecal samples from both poultry and companion c. and high rates of concomitant high-level phenotypic antimicrobial resistance among those isolates (Roy et al., 2025). Differences in reported prevalence exist due to variations in the species of birds used as subjects, sample frames and diagnostic methods employed. Overall, based on the literature, the *E. coli* is a common part of avian gut microbiota and an opportunistic pathogen easily recovered in both pet-bird and production environments (Mbuthia & Hoza, 2025; Pereira et al., 2024).

1.2.6.2 Pathotypes associated with birds (APEC, EPEC, EHEC)

Bird-associated *E. coli* isolates are a diversity of pathotypes. Avian pathogenic *E. coli* (APEC) is extra-intestinal strains that cause colibacillosis in birds, and is the most well-characterized avian pathotype. APEC belongs to the larger group of ExPEC strains, and ones that have some similarities in virulence repertoires with the human ExPEC lineages, indicating a potential for zoonotic transmission (Kathayat et al., 2021; Watts & Wigley, 2024). Intestinal pathotypes such as enteropathogenic *E. coli* and enterohemorrhagic *E. coli* have also been detected sporadically in wild and captive birds as well. Survey data also indicates that eae-positive and stx-positive strains are present among different birds (Mbuthia & Hoza, 2025). The fact that APEC-like ExPEC and

intestinal pathotypes are found in birds means they are not only a reservoir for bird diseases, but a possible reservoir for human ones too.

1.2.6.3 Important virulence genes (*fimH*, *stx1*, *stx2, hlyA, eae, iutA, iss*)

Molecular investigations are commonly used to screen avian *E. coli* for an array of virulence associated genes. The virulence factors are utilized by bacteria for attachment of pathogen to host cell by adhesins such as *fimH*, iron acquisition factors such as iutA, and serum survival genes like iss to allow its survival outside the gut (Kathayat et al., 2021; Ovi et al., 2023). Intestinal virulence markers such as eae and Shiga toxin genes such as *stx1*, *stx2* are found in bird isolates but they are less frequent than in outbreaks involving ruminants or humans (Mbuthia & Hoza, 2025). Hemolysin causing genes such as *hlyA*, have been described in some pathogenic strains and cytotoxicity. The combination and prevalence of these genes vary with the source of the isolate and the pathotype, but *fimH*, *iutA* and *iss* are some of the most consistently correlated to APEC and ExPEC virulence (Kathayat et al., 2021; Ovi et al., 2023).

1.2.6.4 Patterns and originators of resistance to AMR

Antimicrobial resistance (AMR) in avian *E. coli* is being widely reported and is of major concern to veterinary and human health. Systematic reviews and regional studies report the resistance to several drug classes such as β-lactams (including extended-spectrum β-lactamases (ESBLs), tetracyclines, sulfonamides, aminoglycosides and fluoroquinolones (Pereira et al., 2024; F. J. Wibisono et al., 2022). Drivers of this resistance in pet and production birds are the unsparing or prophylactic use of antibiotics, contamination of the environment (e.g. poultry litter, wastewater), human and livestock contact with resistant strains, and horizontal gene transfer via mobile genetic elements (plasmids containing *blaCTX-M*, *qnr, sul, tet* genes). Recent regional-level surveillance technique of South Asia reports the emergence of ESBL-producing *E. coli* in avian isolates as well as the role of local antimicrobial practices and poor stewardship selection for multidrug resistant lineages (Ilyas et al., 2021; Mbuthia & Hoza, 2025; Roy et al., 2025).

1.2.6.5 Role of *E. coli* as One Health threat

E. coli is a typical representative of the One-Health approach: it is being circulated among animal, human and environmental reservoirs and can acquire and spread virulence and resistance determinants with clinical consequence. Because of the ecological connectivity and shared virulence and resistance genes in APEC and human ExPEC and because pet birds have the potential of contributing to local reservoirs of pathogenic as well as resistant *E. coli*, surveillance of avian,

human and environmental resources combined is essential to manage this cross-sectoral risk and improve husbandry (Mourkas et al., 2024; 'Qureshi & 'Azam, 2024; Watts & Wigley, 2024).

Table 1.1: *E. coli* prevalence studies in South Asia

Author (year)	**Country**	**Bird species / Source**	**Sample size (n)**	**Prevalence (%) / positive**	**AMR highlights (key findings)**
(Hasib et al., 2025)	Bangladesh	Pet birds (various psittacines, finches, canaries) — cloacal swabs	150	***E. coli*:** 48.7% (73/150); **ESBL-*E. coli*:** 32/73 (43.8% of isolates).	All ESBL isolates were MDR; susceptible to meropenem; resistant to ampicillin & ceftriaxone. TEM-group ESBLs most frequent.
(Mohsin et al., 2017)	Pakistan	Migratory/wild birds (multiple species; Indus route) — fecal swabs	150	**ESBL-*E. coli*:** 26/150 (17.3%) (fecal carriers of ESBL producers).	WGS: ***blaCTX-M*-15** predominated; 88.4% of ESBL isolates were MDR; high resistance to tetracycline, doxycycline, SXT; one colistin-resistant isolate reported.

(Hasan et al., 2012)	Bangladesh	Wild birds & free-range poultry (Rajshahi region) — feces/cloacal	(various; study-level n reported in paper)	High carriage of antimicrobial-resistant *E. coli*; ESBLs detected in wild ducks/ gulls in some surveys (reported 17–30% ESBL in some bird groups).	Frequent resistance to tetracyclines and older β-lactams; evidence of ESBL genes (CTX-M family) in avian isolates — indicates waterfowl as AMR sentinels.
(Rashid et al., 2015)	Bangladesh	Open-bill storks and nearby water sources (environmental niche study)	(environmental/avian sample set; see paper)	Reported ESBL-*E. coli* in birds and aquatic sources (study demonstrates environmental dissemination).	ESBL producers (CTX-M types) present in both bird and water samples; highlights aquatic environment as reservoir.
(Mandal et al., 2022)	India (multi-region data)	Broilers, layers, backyard poultry (surveillance datasets)	Aggregated across studies (hundreds of farms / samples)	High prevalence in broilers (reported up to ~80–86% colonization in some primary	High MDR prevalence; seasonality noted; widespread resistance to ampicillin, tetracycline;

				studies); MDR *E. coli* frequently reported.	ESBLs and fluoroquinolone resistance commonly recorded.
(Bhattarai et al., 2024)	Nepal	APEC isolates from poultry (clinical and farm sampling)	539 samples (487 APEC isolates reported in one analysis)	*E. coli* (APEC) isolation high in clinical poultry samples (reported proportions depend on farm/region).	AMR common among APEC: resistance to multiple classes; detection of AMR genes; regionally important for poultry disease and potential zoonotic risk.

1.2.7 *Klebsiella spp.* in Pet Birds

1.2.7.1 Occurrence among avian species and environmental reserves

Klebsiella species (mostly *Klebsiella pneumoniae*) are commonly isolated from birds of diverse origins, such as pet/ornamental birds (psittacines, canaries, budgerigars), captive wild birds, poultry and wild waterfowl. Isolation studies and case reports demonstrate *K.pneumoniae* from cloacal swabs, feces, respiratory samples and from cage-environment samples showing that many birds carry, and occasionally become clinically ill from, *Klebsiella* infections (Hoque et al., 2020; Nakhaee et al., 2022). Environmental surveys also reveal the presence of *K.pneumoniae* in water, soil, sewage and feed, thus confirming the existence of environmental reservoirs in the aquatic and terrestrial environments that intersect with avian exposure pathways (Barati et al., 2016). Recent surveillance has revealed the presence of *Klebsiella* in migratory and captive birds and thus indicates the ecological link between wildlife, captive birds, the human environment and potential human exposure (Islam et al., 2025; Raza et al., 2017).

1.2.7.2 Pathogenicity and virulence determinants (*rmpA*, *magA*)

Klebsiella pathogenicity in birds ranges from secondary opportunistic infections that are secondary to stress or co-morbidities of the host to fulminant primary disease in some species of the host. Virulence in *K. pneumoniae* is multifactorial and is usually related to capsule production, siderophore systems, fimbrial adhesins and regulators of mucoid phenotype. Two such genes have been noted previously as being associated with hypermucoidy and hypervirulence: rmpA (regulator of mucoid phenotype A) and *magA* (mucoviscosity-associated gene A, associated with K1 capsule biosynthesis). The occurrence of rmpA and *magA* genes is associated with higher capsule production, resistance to immune cells and severe disease in humans and mammals, but the occurrence of these genes in bird isolates is variable (G. Wang et al., 2020; Yu et al., 2006). Recent studies in avians have been mixed - some of the bird outbreaks have highly virulent and *rmpA*-positive strains, but most of the large surveys have found these markers of hypervirulence to be absent in the majority of bird populations sampled (Hou et al., 2024). However, their identifying and finding any animal reservoir is noteworthy because it indicates potential for severe disease and possible cross-species transmission of hypervirulent genotypes.

1.2.7.3 ESBL producing *Klebsiella* in birds (*blaCTX-M*, *blaTEM*, *blaSHV*)

Extended-spectrum β-lactamase (ESBL) gene, in particular for *blaCTX-M* variants, has been reported to be increasing in birds. Multiple studies from all continents have detected ESBL-producing *K. pneumoniae* and other *Enterobacterales* in faecal specimens from migratory, wild and captive birds. Multidrug-resistant (MDR) phenotypes seem to be a repeatedly reported genotype of the clonal group in avian isolates from Asia and elsewhere; *blaCTX-M*-15 can be associated with MDR phenotypes. Investigations of wet markets, breeder flocks and aquatic habitats reveal that the *blaCTX-M*, *blaTEM* and *blaSHV* resistance genes are found in bird and environmental isolates (Saeed et al., 2023). More recent regionally focused studies (e.g., MDPI Veterinary Sciences, 2025) confirm that ESBL-*Klebsiella* are present in both migratory and captive groups of birds emphasising the role and importance of birds (as sentinels and possible disseminators of clinically important β-lactamase genes).

1.2.7.4 Hypervirulent strains in wildlife and pet species

While hypervirulent strains of *K. pneumoniae* are known to be hypervirulent (i.e. hypermucoidy and high siderophore production) and have been studied for their role in human infection, their markers

have also been found intermittently in animal and environmental isolates. Genomic One-Health studies show the overlap of the same *Klebsiella* populations in humans, animals and environments, even though direct recent transmission events are rarely apparent, but, nevertheless, the identification of loci associated with the hypervirulent scenario (*rmpA, iuc/iut* aerobactin cluster, iro) in nonhuman isolates is a cause for concern arising from potential emergence or spill-over of strains capable of causinginvasivedisease outside humans (Hetland et al., 2025; Ngan et al., 2025). In birds, reports of hypervirulent phenotypes are currently sporadic - but occasionally linked to severe outbreaks in captive collections (e.g. canaries, passerines) - but surveillance is incomplete. The major risk is the recombination of plasmid-borne virulence determinants with resistance plasmids to produce convergent hypervirulent-multidrug-resistant strains with disastrous public health repercussions (Ngan et al., 2025).

1.2.7.5 Genotyping and hypervirulence

While most well-known from human infections, hypervirulent strains of *K. pneumoniae* (further characterized by other features, such as hypermucoidy and hyperproduction of siderophores) have also been detected periodically in animal and environmental isolates. Genomic One-Health studies lead to the discovery of overlapping *Klebsiella* populations between humans, animals and environments although direct recent transmission events may not be always apparent; however, the identification of hypervirulent (*hvKp*) associated loci (e.g. *rmpA, iuc/iut* aerobactin cluster) in non-human isolates is cause for concern with respect to emergence or leakage of strains capable of causinginvasivedisease outside of humans (Hetland et al., 2025; Ngan et al., 2025). In birds, reports of hypervirulent phenotypes are currently sporadic - sometimes linked to serious outbreaks in captive collections (e.g. canaries, passerines) - but surveillance is incomplete. The most important is that virulence determinants carried on plasmids can recombine with resistance plasmids, generating convergent hypervirulent multidrug-resistant strains of major public health concern (Ngan et al., 2025).

Table 1.2: Summary of Major Studies on *Klebsiella spp.* in Pet and Captive Birds

Author & Year	Country	Bird Species / Source	Sample Size	Klebsiella Prevalence (%)	ESBL Genes Detected	Virulence Markers Detected
(Raza et al., 2017)	Pakistan	Migratory wild birds	110	14	*blaCTX-M*-15	Not reported

(Islam et al., 2025)	Bangladesh	Captive wild birds & migratory species	150	18	*blaCTX-M*, *blaTEM*, *blaSHV*	rmpA detected in 5%, *magA* absent
(Nakhaee et al., 2022)	Iran	Canaries (Serinus canaria)	60	12	None detected	rmpA 8%, *magA* 3%
(Hou et al., 2024)	China	Mixed ornamental birds	90	16	*blaCTX-M*, *blaTEM*	rmpA 7%, *magA* 2%
(Ngan et al., 2025)	Singapore	Ornamental birds from retail shops	75	15	*blaCTX-M*	rmpA 5%
(Sundaresan et al., 2022)	Poland	Captive psittacines & passerines	100	11	*blaTEM*, *blaSHV*	rmpA 3%, *magA* 2%
(Saeed et al., 2023)	Egypt	Wild birds & poultry markets	80	17	*blaCTX-M*, *blaTEM*, *blaSHV*	rmpA 6%, *magA* 2%
('Qureshi & 'Azam, 2024)	Pakistan	Pet birds from households	65	12	*blaCTX-M*	rmpA 4%, *magA* 0%

1.2.8 *Pseudomonas spp*. in Pet Birds

1.2.8.1 Ecology and persistence of the environment

Members of the genus *Pseudomonas*, and in particular *Pseudomonas aeruginosa*, are ubiquitous Gram-negative bacteria well adapted to aquatic and soil environments as well as to human-made niches (e.g. sink, humidifiers, bedding, drinking water systems). Their metabolic versatility enables them to survive in habitats containing low nutrient concentration and wide temperature/osmotic conditions; in addition, intrinsic stress response systems (e.g. efflux pumps, membrane impermeability and versatile catabolic pathways) enable persistence of these organisms on fomites

and in moist environmental reservoirs that are common in areas surrounding aviaries and bird-keeping facilities. Environmental surveys and One-Health studies have repeatedly identified *P. aeruginosa* in water, sewage, wetland sediments, hatchery equipment and cage wash water, indicating that birds are in frequent contact with environmental sources and suggested that contact among captive birds with colonized or infected water or substrate is likely to be the mode of colonization or infection. Environmental persistence is facilitated where the organic matter is accumulated (feed spillage, soiled bedding) and where cleaning/disinfection is erratic or where cleaning/disinfection agents are used to which *P. aeruginosa* is tolerant (Abd El-Ghany, 2021; Elfadadny et al., 2024).

1.2.8.2 *P. aeruginosa* as an opportunistic bird pathogen

Although *P. aeruginosa* spp. are commonly isolated from environmental commensal molds, *P. aeruginosa* acts as an opportunistic pathogen in avian hosts. Disease in birds includes localized infections such as pododermatitis, otitis, conjunctivitis to respiratory disease and systemic septicemia especially in neonates, immune compromised animals, or birds which have been stressed by poor husbandry skills, overcrowding, or other concurrent disease. Case reports and minor outbreak investigations in poultry and companion birds report tremendous morbidity and mortality when *P. aeruginosa* has access to vulnerable tissue such as through wounds, following viral/bacterial coinfections, or contaminated water systems. In addition to overt disease, the avian upper respiratory tract and gastrointestinal tract can be colonised by *P. aeruginosa* without evidence of disease and asymptomatic carriers are present which can contaminate the environment (Abd El-Ghany, 2021; Nazem Shirazi et al., 2023).

1.2.8.3 Virulence Factors (*toxA*, *lasB*, *algD*)

Pathogenic *P. aeruginosa* originates from a virulence arsenal. Key determinants that are frequently studied include:

- *toxA i*s an ADP-ribosylating exotoxin that inhibits host protein synthesis and contributes to tissue necrosis and systemic toxicity. Also, toxA is a feature commonly found among clinical isolates and is correlated with increased pathogenicity.
- *lasB* is a secreted metalloprotease that is involved in host extracellular matrix proteins degradation, immune evasion and biofilm maturation. in addition, *lasB* plays a role in tissue invasion and in immune modulation.

- *algD* is a key element of alginate synthesis and mucoid phenotypes involved in bacterial protection from phagocytosis and from antibiotics and underlies the chronic infections in particular

Molecular surveys frequently detect the virulence genes *lasB* and algD at high prevalence in avian and clinical strains and *toxA* varies but is often present in virulent strains. Other effectors like type III secretion effectors such as ExoS/ExoU, phospholipases plcH/plcN and motility/adhesion determinants further modulate virulence host- and strain specific (Bazghandi et al., 2021; Edward et al., 2023).

1.2.8.4 Antimicrobial resistance- intrinsic and acquired

P. aeruginosa has a layered resistance profile, which is difficult to treat. Intrinsic mechanisms are low outer membrane permeability, efflux pumps (Mex family) that are constitutive or inducible, chromosomally encoded AmpC beta-lactamase, and ability to enter slow growing or persister states. Acquired resistance occurs through horizontal gene transfer such plasmid borne carbapenemases, single point mutations, and adaptive phenotypes. Clinically important resistance seen in avian and environmental isolates include resistance to aminoglycosides, fluoroquinolones, extended spectrum cephalosporins and - scare of all - emergence of carbapenemase producers in some One Health surveys. Antimicrobial use in veterinary settings, contamination of water with human/animal wastes, and exposure to resistant strains in human environments are important factors for selection of resistant strains of *Pseudomonas* in bird environments (Elfadadny et al., 2024; Yang et al., 2025).

1.2.8.5 Biofilm formation/chronic infection potential

Biofilm formation plays a key role in *P. aeruginosa* in birds. These collections of organized bacteria adhere to surfaces such as cages or host tissue with the use of a protective matrix and thus become much more resistant to disinfectants, antibiotics and the host immune system. In captive situations, biofilms present in waterlines and on feeders can provide a source of repeated bird exposures and are very hard to eliminate without mechanical cleaning and proper use of disinfectant procedures. Infected birds with biofilm associated infections may develop slow-healing, recurring problems with persistent resistant and virulent bacterial clones. Because biofilms provide an easy way for bacteria to share genes with each other, it is a hot spot for transmission of resistance and virulence among the bacteria that live together (Ren et al., 2025; Y. Wang et al., 2025).

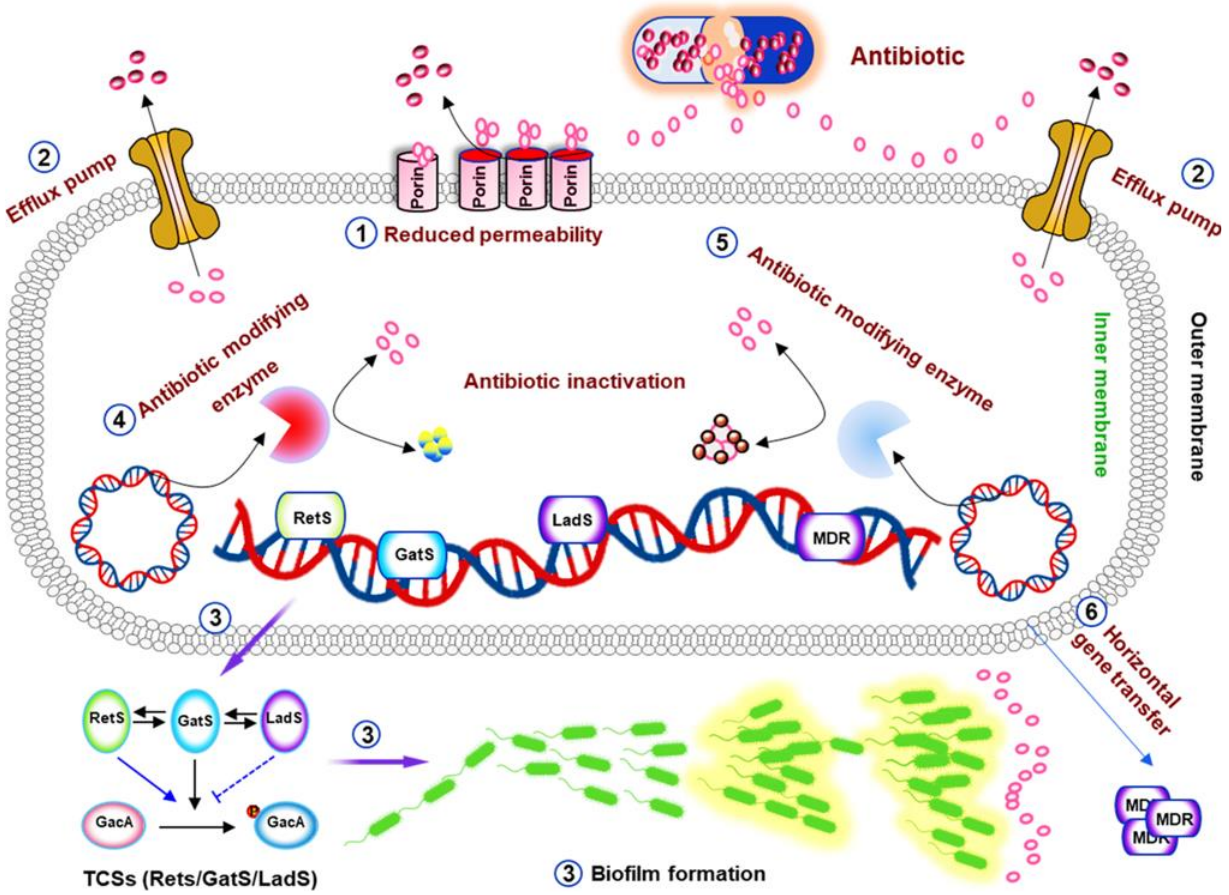


Figure 1.3: Mechanisms of antibiotic resistance in bacteria include decreased uptake, enzyme degradation, resistance mutation efflux pump and biofilm formation (Elshobary et al., 2025).

Table 1.3: Summary of Major Studies on *Pseudomonas spp.* in Pet and Captive Birds

Author & Year	**Country**	**Bird Species / Source**	**Sample Type**	**% Positive for *Pseudomonas spp.* (or *P. aeruginosa*)**	**Key Virulence / Resistance Findings**
(Abd El-Ghany, 2021)	Egypt	Pet birds (parrots, parakeets) & poultry	Cloacal swabs, feces	18–27% *P. aeruginosa*	High prevalence of **toxA**, ***lasB***; multidrug resistance including ciprofloxacin & β-lactams

(Bazghandi et al., 2021)	Iran	Mixed avian isolates (ornamental birds)	Cloacal swabs	15%	*lasB*, algD high frequency; strong association between virulence genes & MDR
(Edward et al., 2023)	Multi-country	Aviary birds & environmental samples	Waterlines, cage surfaces	10–28% environmental contamination	*lasB* 90%, toxA 68%; ESBL-like phenotypes rare but efflux-pump overexpression common
(Y. Wang et al., 2025)	China	Companion birds (budgerigars, cockatiels)	Drinking-water, feces	12–20%	Strong biofilm formation (algD+, psl+); high tolerance to disinfectants
(Ren et al., 2025)	USA	Clinic-submitted samples from exotic birds	Wound & respiratory samples	17% clinical infection rate	Chronic infection strains strongly mucoid; high alginate expression linked to persistent disease
(Elfadadny et al., 2024)	Egypt	Domestic & aviary birds	Water systems	25–30% contamination	Carbapenem resistance emerging; overexpression of MexAB-OprM efflux pump
(Nupur, 2023)	Bangladesh	Pet bird markets (finches, budgerigars, mynas)	Fecal & cage swabs	10–18%	Moderate resistance to tetracycline & ceftazidime; environmental persistence linked to poor hygiene

(Dovč et al., 2016)	Brazil	Parrots undergoing veterinary treatment	Respiratory swabs	13%	Fluoroquinolone resistance due to gyrA/parC mutations
(Pal et al., 2022)	Nepal	Backyard pet birds	Cloacal swabs	9%	Low virulence gene frequency but high intrinsic β-lactam resistance

1.2.9 *Salmonella spp*. in Pet Birds

1.2.9.1 Carriage by asymptomatic birds

Non-typhoidal *Salmonella spp*. are commonly found colonising the intestinal tract of a wide range of birds and are commonly found as asymptomatic colonizers rather than as overtly diseased. The bacteria shed in bird droppings can be quite transient or quite enduring, and the intensity, as well as the duration, will depend on the species of bird and the dose of the infectious agent as well as stressors such as transport, crowding, poor nutrition, presence of other infections and use of antibiotics (Wigley, 2024). Asymptomatic carriage has been repeatedly documented in passerines, psittacines, pigeons, waterfowl and backyard poultry; apparently healthy birds are thus a silent source of environmental contamination, as well as contamination of humans. Environmental studies prove that contaminated cage material, such as paper, feeders, and surfaces, may harbor viable *Salmonella* for weeks (Shen et al., 2023; Wigley, 2024).

1.2.9.2 Important serovars in the avian ecosystem

Salmonella serovars have been identified in a wide range of species of birds; nevertheless, some serovars have a high association with birds and bird-related cases. Among the most commonly reported serovars of *Salmonella typhimurium* and its monophasic variants in passerines, pigeons, and poultry, and the cause of seasonal passerine-human outbreaks in various countries (Söderlund et al., 2019). *S. Enters pertinuitis*, *S. Gallinarum* and *S. Pullorum* are also significant in poultry, and other serovars (e.g. S. Newport, S. Javiana) are sometimes found in wild and captive avifauna, which is again geographically and ecologically dependent (Hudson et al., 2000; Shaji et al., 2023). Distribution of serovars depends on the region, species of birds and ecological environment and this has a surveillance and risk assessment implication.

1.2.9.3 Virulence markers of *Salmonella spp*.

Molecular detectives reveal that canonical *Salmonella* virulence genes are common in avian isolates. *InvA* gene- essential to epithelial cell invasion and a typical target in *Salmonella* PCR detection has been found almost universally in culture confirmed isolates of avian and environmental sources (Shen et al., 2023). Other virulence markers, including *stn* (*Salmonella* enterotoxin), *hilA* (a regulator of genes in *Salmonella* Pathogenicity Island-1), and several SPIs (SPI-1 and SPI-2 loci) encoding the type III secretion systems that form the basis of invasion and intracellular survival are widespread and are linked to invasive phenotypes in experimental systems (Shaji et al., 2023; F. M. Wibisono et al., 2021). Isolation of such loci in isolates of asymptomatic birds confirms that strains involved in carriage always have the pathogenesis genetic toolkit, even when they are not pathogenic to their bird hosts.

1.2.9.4 Global outbreaks associated with birds and bird products

Various studies have linked human infections to contact with wild songbirds at feeders, contact with sick or dead birds, and contact with contaminated surfaces in homes and in the community; cases of outbreaks have occurred in the United States, United Kingdom, Norway and New Zealand among others (CDC, 2021; Söderlund et al., 2019). Besides events in the wild associated with birds, outbreaks of salmonellosis are also associated with commercial or backyard poultry, contaminated eggs and processed bird product are still considered a predominant cause of reported salmonellosis globally. Genetically indistinguishable isolates in birds, the environmental and human-associated sample, and an outbreak case have been frequently recovered with the assistance of molecular typing, which reinforced the concept of attributing sources and proving that the possibility of a bird-human spillover does exist in natural settings (Söderlund et al., 2019).

Table 1.4: Primary South Asia studies of *Salmonella* in birds

Author (year) Country	**Bird species / source**	**Sample size (n)**	**Prevalence (% positive)**	**AMR highlights (key findings)**
(Saiful Islam et al., 2021)	Migratory wild birds (multiple species) sampled on arrival in Bangladesh.	reported in paper (see source).	Reported non-typhoidal *Salmonella* carriage among migratory birds	High proportion of isolates were multidrug resistant (MDR); paper highlights presence

			(study reports notable carriage rates; see source).	of clinically relevant resistance patterns.
(Card et al., 2023)	Wild and migratory birds (multi-species).	aggregated across sites (see article).	Overall prevalence reported ~13.5% in one national dataset of migratory birds; other bird groups showed higher local prevalence in targeted surveys.	High proportion MDR in many isolates; authors emphasize presence of clinically important resistance and One-Health implications.
(Begum et al., 2024)	Captive wild birds and migratory birds (captive collections, zoos).	paper reports aggregated n — see source.	Overall pooled prevalence reported in their sampled population: ~30.9% (study-level prevalence reported in text).	Virulence gene profiling plus AMR: many isolates carried virulence markers and a high fraction were resistant to multiple drug classes (detailed in paper).
(SHARIF et al., 2020)	Migratory birds (various waterfowl/passerines sampled at wetlands).	n reported in the article (see source PDF).	Reported prevalences in specific bird species up to >60% in some targeted captive/wild bird groups in local surveys (see	High MDR rates reported; resistance to commonly used antimicrobials frequently observed.

			source for species breakdown).	
(Faruq et al., 2016)	Captive zoo birds (parakeets, parrots, passerines).	reported in primary/local reports (see cited reviews).	Several captive collections showed very high local prevalences (e.g., 33–46% in some zoo/captive bird subsets reported in regional papers).	Many isolates MDR; captive conditions and poor hygiene identified as risk factors for high carriage and environmental contamination.

1.2.10 *Citrobacter spp.* in Pet Birds

Citrobacter spp. is a Gram-negative, facultatively anaerobic, bacillus, which is a member of the family Enterobacteriaceae. These bacteria are traditionally viewed as opportunistic pathogens in human beings, but they are being detected in more and more animals, including the avians. *Citrobacter freundii* and *Citrobacter koseri* are also the most often identified species in birds and are often acquired in the intestinal tract as a part of the commensal microbiota. *Citrobacter spp.* on the other hand can be opportunistic pathogens that cause enteritis, septicemia, respiratory infection, or localized lesion in response to stress, immunosuppression or bad husbandry conditions, (Funke et al., 2019). The occurrence of *Citrobacter spp.* in pet birds is especially significant since the organisms may survive in the contaminated feed, water, cage surfaces, and shared aviary populations, thus being the possible source of bacteria spread at the household level (Boseret et al., 2013). *Citrobacter spp.* virulence is mediated by a number of factors such as fimbrial adhesins, siderophore systems as well as other toxins that facilitate colonization and invasion of host tissues. An example of such a bacterium is *C. freundii*, which has several virulence-related genes associated with adherence, iron uptake, and serum resistance, which allows it to survive in a variety of hosts (Liu et al., 2018). Notably, Citrobacter species have been known to cause gastrointestinal infections in both animals and humans and a zoonotic spillover may be caused through direct contact with contaminated feces, poor cage hygiene, or cross-contamination within shared living conditions. Due to the close contact of pet birds with humans and their common presence in indoor locations, the identification of the *Citrobacter spp.* in the fecal samples of avian animals should be of interest to the public health (Ahmed et al., 2021). The pathogenicity of *Citrobacter spp.* is of increased interest

as it is capable of containing and spreading antimicrobial resistance (AMR) genes. A number of studies demonstrated that Citrobacter strains acquired in animals frequently possess extended-spectrum β-lactamase (ESBL) genes like *blaCTX-M*, *blaTEM* and *blaSHV* that confer resistance to third-generation cephalosporins (Mehdi et al., 2020). Moreover, it has been reported that C. freundii isolated in the environment and animals have carbapenemase genes such as *blaNDM, blaKPC*, and *blaOXA*, indicating the presence of the organism as a reservoir of clinically significant resistance determinants (Fang et al., 2019). Other common plasmid-mediated quinolone resistance gene (qnrB, qnrS) and tet and sul gene families that provide tetracycline and sulfonamide resistance respectively are also carried by *Citrobacter spp.* (Zhang et al., 2018). These resistance mechanisms are frequently found on mobile genetic elements, including plasmids, integrons, and transposons, which have been implicated in horizontal gene transfer among species, including Citrobacter to other pathogenic species of clinical importance, including *E. coli* and *Klebsiella pneumoniae* (Partridge et al., 2018).

Even though Citrobacter infections are under-reported in birds relative to *E. coli* or *Salmonella*, there is evidence suggesting that the avian species could be the overlooked sources of AMR-bearing Citrobacter strains. From the studies in the companion animals and poultry, there is a mounting isolation rates and high rates of resistance to β-lactams, fluoroquinolones, aminoglycosides, and sulfonamides (Mehdi et al., 2020). The emergence of *Citrobacter spp.* with multi-drug-resistant (MDR) and extended drug resistant (XDR) profile in animal reservoirs stresses the emphasis made on the potential role of these bacteria for the One Health system. Therefore, the detection of the *Citrobacter spp.* in the feces of pet birds has important epidemiological aspects, especially in areas where there is a high misuse of antibiotics, as resistant bacteria can circulate in avian, human, and environmental reservoirs.

1.2.11 Global AMR Crisis

1.2.11.1 Role of Misuse and Overuse of Antibiotics

The modern antimicrobial resistance (AMR) crisis is fundamentally caused by selection pressure due to inappropriate use of antibiotics in both human, veterinary and environmental domains. A significant percentage of antibiotic prescriptions are inappropriate in the human (medical) field, e.g. prescribed when a viral disease occurs, when it is not necessary, and/or taking too long to manage, all of which are aggravated by poor availability to rapid diagnostics (World Health Organization, 2025). Similar pressures are present in low- and middle-income countries where the over-the-counter availability of antibiotics makes it possible to use them without clinical evaluation. In animal

production, the routine prophylactic, and metaphylactic and, in some regions, growth-promotion uses of antimicrobials expose the population of large numbers of microbes to subtherapeutic concentrations of antimicrobials increasing the emergence and perpetuation of resistant bacteria (World Organisation for Animal Health, 2022)). This situation can result in large amounts of antimicrobial residues and resistant bacteria entering the environment with manure and runoff agricultural effluents, which exacerbate environmental selection (Larsson & Flach, 2022). Environmental dissemination connects all the sectors: hospital and municipal wastewater, agricultural discharges and pharmaceutical manufacturing effluent place both antibiotics and organisms resistant to them into the aquatic and soil systems, where resistance genes can build up, combine and propagate in the long term (Larsson & Flach, 2022). The crisis is of a large scale. A seminal global burden analysis estimated in 2019 that AMR had 4.95 million deaths in relation as well as directly caused 1.27 million deaths, thus, it was one of the greatest infectious menaces worldwide (Murray et al., 2024). Resistant *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and other high priority pathogens made a significant contribution to this burden. This evidence proves that misuse and overuse in any sector (human, veterinary or environmental) generates common risks for all systems.

1.2.11.2 AMR between Veterinary and Human Sector

Although the use pattern of antimicrobials for human use and veterinary use differ, both of these fields contribute to significant pressure for global resistance. Quantitatively, a significant portion of mass of antibiotics in the world is applied in animals especially in intensive mode of production of livestock population where antimicrobials are used semi-regularly in healthy population (World Organisation for Animal Health, 2022). The human healthcare systems, in turn, put the selection pressures in the clinical settings. Hospitals being the hotspots of multi-drug treatment and resistant organisms (and their greater infection risk in medical populations), are considered a hotbed area for multi-drug resistance infections and nosocomial transmission particularly multi-drug resistance (WHO, 2023). Complicated by host overlap in antibiotic classes, this makes control actions more difficult. In a lot of areas, livestock are still fed on critical human medicines such as fluoroquinolones, cephalosporins/macrolides which generates cross-sector selection on the same resistance genes (World Organisation for Animal Health, 2022). According to the policy studies provided by the Organisation of Economic Co-operation and Development, (OECD), nothing can be done to turn AMR trends around without more concerted action across sectors; that is, it needs a multisectoral intervention

1.2.11.3 Silent Spread of Resistance in Companion Animals

Another unexplored reservoir in the AMR ecosystem is companion animals - dogs, cats, small mammals, and more recently pet birds. Such animals have high physical interactions with humans and are usually exposed to similar antibiotics as do human medicine. Recent reviews of the literature have shown the presence of clear evidence of bidirectional transmission of resistant bacteria and of mobile genetic elements between pets and their owners (Jin et al., 2023). The transmission is often silent, as colonization with resistant organisms (e.g. ESBL-producing *E. coli*, carbapenemase producers or MRSA) can be infection free in either humans or animals. The environment at home, sharing of sleeping arrangements, close contact, and a weak practice in infection control facilitates such transmission (Jin et al., 2023) The results of these studies hereby indicate that despite the existence of powerful human stewardship societies unseen reservoirs of companion animals may be able to propagate resistance circulation (S. K. Ahmed et al., 2024).

1.2.12 AMR in Birds (Pet Birds vs. Poultry)

1.2.12.1 Comparison of antimicrobial selection pressures

The intensity of antimicrobial selection pressure between pet-bird environment and commercial poultry production varies according to purpose and intensity and ecological impact. Intensive poultry farms routinely administer antimicrobials on a mass basis in therapeutic, metaphylactic and (where legal) prophylactic or growth-promotional modes, exposing large flocks to antibiotics; this results in strong selection pressure since intensive farm animals are being exposed to antibiotic pressure, and this pressure transition leads to high levels of resistance bacteria and amplification of resistance genes in managed microbiomes and excreta (Nhung et al., 2017; World Organisation for Animal Health, 2022). on the other hand, pet birds are treated much less often on population scale. pet birds are exposed to household environments, where they come in contact with human and this increases opportunities for cross species transfer; selection pressure in pet birds is therefore typically episodic but ecologically important because pet birds live in close proximity to humans and household microbiomes (H. A. Ahmed et al., 2021; Hasib et al., 2025). The environmental focal points connect the two niches, as antibiotic residues and resistant strains of bacteria originating in the poultry farm get released into the soil and water through the manure and runoff, creating local reservoirs in which wildlife and companion animals could become exposed (Larsson & Flach, 2022). Therefore, although production of poultry represents a large-volume source of AMR through mass

and continuous selection, pet birds play a role as local reservoirs of the infection and potentially initiate broader dissemination into communities (Hasib et al., 2025; Lappan et al., 2024).

1.2.11.2 Commonly used antibiotics in aviculture

Most commonly used in livestock classes of antibiotics used in poultry include tetracyclines group (oxytetracycline, doxycycline), β-lactam group (amoxicillin, penicillins), aminoglycosides (gentamicin, neomycin), macrolides/lincomycin group (tylosin, erythromycin) and fluoroquinolones (enrofloxacin, ciprofloxacin) patterns financed otherwise documented global reviews and surveys from South Asian countries (Chowdhury et al., 2021). Third-generation cephalosporins and colistins are important in human medicine but they are being used illicitly (World Organisation for Animal Health, 2022). For companion (pet) birds, the licensed antimicrobial choices are narrower and are guided by avian pharmacology: Commonly used antimicrobial agents include doxycycline, enrofloxacin (with caution), trimethoprim-sulfonamides and macrolides for certain infections, choice should reflect avian drug tolerance, tissue distribution and withdrawal periods (H. A. Ahmed et al., 2021). Nevertheless, studies coming from LMICs highlight owner or non-veterinary administration of very broad-spectrum antibiotics to pet birds (including fluoroquinolones, beta-lactams) often without microbiological confirmation and so potentially exposing household microbiota to selection pressure, albeit at smaller scale, similar to the misuse in agriculture. Nevertheless, studies coming from LMICs highlight owner or non-veterinary administration of very broad spectrum antibiotics to pet birds (including fluoroquinolones, beta-lactams), often without microbiological confirmation, and so potentially exposing household microbiota to selection pressure, albeit at smaller scale, similar to misuse of antibiotic in agriculture (Hasib et al., 2025).

1.2.11.3 Self-medication practices among bird owners

Most dangerous element of setting companion animal are self-medication and informal access antibiotics. Surveys of pet owners reveal that many pet shop owners give pets the antibiotics previously prescribed for humans without veterinary oversight, particularly where access to veterinary care is limited or is too expensive (Scarborough et al., 2021). In low- and middle-income countries, these antibiotics are available over-the-counter for both veterinary and human use, and there is an emphasis on sharing medications across the species barrier.In low- and middle-income countries, there is the unregulated dosing of antibiotics, incomplete use of these courses, and use of inappropriate agents due to the over-the-counter availability of veterinary and human antibiotics and the sharing medications across the species barrier (Chowdhury et al., 2021). These behaviours select for resistant strains in the pet microbiome and household environment and complicate stewardship

efforts because household reservoirs can persist despite the improvement in formal veterinary prescribing (Scarborough et al., 2021).

1.2.12 Multidrug-Resistance (MDR) in Avian Bacteria

Multidrug resistance (MDR) in bacterial pathogens of humans is of great public health concern, especially in areas where antibiotic use in animals is not well regulated. MDR is normally defined as resistance to one or more antimicrobial agents in three or more classes of antibiotics, and extensively drug-resistant (XDR) strains as non-susceptibility to all but one or two classes of antibiotics, and pan-drug-resistant (PDR) isolates as resistance to all available antibiotics (Magiorakos et al., 2012). Companion birds, such as parrots, budgerigars, and other birds that are kept in homes, may be reservoirs of MDR Enterobacteriaceae and non-fermenters that can lead to zoonotic transmission. These organisms often obtain AMR determinants from the environment, contaminated feed or water and previous exposure to antibiotics. Among avian bacteria *Escherichia coli* is the most frequently reported MDR organism. Avian pathogenic *E. coli* (APEC) frequently harbors ESBL genes belonging to the class of: *blaCTX-M*, *blaTEM*, *blaSHV*, in conjugation with tet(A/B) and *sul1*/sul2 and plasmid mediated quinolone resistance genes such as qnr and aac(6')-Ib-cr (Ahmed et al, 2020). Studies from both commercial poultry and pet birds have recorded high levels of co-resistance against β-lactams, tetracyclines, sulfonamides and fluoroquinolones (Adzitey & Huda, 2020). Similar trends are seen in *Klebsiella spp.*, particularly *K. pneumoniae* that has a high prevalence of ESBLs and carbapenases such as *blaNDM, blaKPC*, and *blaOXA*-48-like which are posing a significant problem because they also have a high potential for plasmid-mediated gene transfer (Navon-Venezia et al., 2017). *Pseudomonas aeruginosa* in birds is intrinsically resistant because of efflux pumps and because it can form biofilms, and potentially as a result of acquiring the beta-lactamases known as PSE, VIM, and PER, which make them extreme cephalosporin and sometimes carbapenem resistant (Pang et al., 2019). Though less common when isolated from pet birds, MDR Pseudomonas is a cause of persistent respiratory and systemic infections because of its capacity to survive in water, damp cage environments and in disinfectant-resistant biofilms. Increasing attention also has been focused on *Citrobacter spp.* - so-called opportunistic enteric bacteria of the family Enterobacteriaceae which are often present in the avian gut and have the potential to harbor resistance genes that are clinically important. *Citrobacter freundii* and *C. youngae* have been reported to have ESBL genes (*blaCTX-M*, *blaTEM*, *blaSHV*) and in some cases carbapenases (*blaNDM, blaVIM*) like those found in *Klebsiella* and *E. coli* (Fang et al., 2019; Ranjan et al., 2016). Their potential to live in a variety of habitats, form biofilms and share plasmids with one another ensure that they have gained recognition as a contributor of AMR spread in both avian

and human populations (Trevino et al., 2021). Although there is little research on Citrobacter in pet birds, isolation from avian feces indicates that birds may serve as a paedemic reservoir.

Across these bacteria there are major issues of co-resistance and cross-resistance as resistance genes commonly co-occur on mobile genetic elements. For example, the plasmids with the row of genes called *blaCTX-M* also often carry genes for tet, sul, and qnr and therefore are resistant to more than one unrelated class of drugs (Dolejska & Papagiannitsis, 2018). Biofilm-associated resistance only makes treatment even more complicated as bacteria from biofilms are less susceptible to most types of antibiotics (Stewart, 2015), especially true in the case of *Pseudomonas* and *Klebsiella*. Clinically, MDR infections in pet birds are not easy to manage, because there are limited avian-specific antibiotics guidelines, a risk of treatment failure and also potential for transmission to humans (zoonotic disease) in the shared household environment. The emergence of MDR and ESBL-producing *E. coli* and *Klebsiella, Pseudomonas,* and *Citrobacter* highlight the importance of antimicrobial stewardship, better hygiene measures, and surveillance in the One Health concept, as well as the knowledge of the interconnected links between humans, animals, and the environment in the propagation of AMR.

1.2.13 Pet Birds as Vectors of Human Disease

Pet birds (parrots, budgerigars, finches, canaries & other ornamental birds) can serve as a source of asymptomatic carriers and intermittent shedders of a variety of human-pathogenic bacteria (particularly - *Salmonella*, *Escherichia coli*, *Klebsiella spp.* & others). They play role as reservoirs by the help of host infection and shedding dynamics, environmental contamination of cages and household surfaces and multiple transmission scenarios that link birds to people. These are reviewed for evidence on each of these elements, and the public-health implications for households and One-Health surveillance, below.

1.2.13.1 Shedding dynamics

Birds are known to intermittently (as opposed to continuously) shed enteric pathogens. Experimental and field studies demonstrate that the timing, intensity and duration of fecal shedding depend on the strain of pathogen, the host species, immune status, infectious dose and factors that stress the host (environmental or physiological). For example, controlled infection experiments in the laying hen have shown that different serovars of *Salmonella* differ considerably in how often and for how long they are shed in faeces; shedding may be transitory (days) or persistent (weeks to months) and that it is often enhanced by stressors that compromise the resistance to infection (Drauch et al., 2022; Gole et al., 2017). In companion and wild birds, survey result shows that many healthy appearing

birds are positive on one occasion and negative the other that indicates waxing/waning shedding that complicates detection and control (Boseret et al, 2013).

1.2.13.2 Household contamination by the environment

Fecal shedding causes quick environmental contamination in the micro-ecosystem of the household. Cage papers, feeders, waterers, perches, toys and the surrounding surfaces are areas where faeces and droppings are collected. 11 Different Studies Show that Cage Fomites can Contain Viable Bacteria: Several studies show that cage fomites (papers, feeders, waterers, perches, toys and other surfaces surrounding the cage) can contain viable bacteria for days to weeks under favourable conditions and thus act as a persistent reservoir for human exposure (Boseret et al., 2013; F. J. Wibisono et al., 2022). Aimed sampling from pet-bird cages has led to the recovery of *Salmonella* and other Enterobacterales from cage papers and feeder surfaces, even in the absence of apparent clinical illness in birds (Bueno et al., 2024). Environmental contamination is accentuated in areas that are not particularly clean, that do not get cleaned regularly, or use insufficient disinfectants - which are typical not only for private households but also in the context of informal trade/networks of markets. Household practices for cleaning harbored materials that can aerosolize dried fecal dust (e.g. vigorous sweeping, shaking of bedding) can disseminate particulates containing bacteria in the indoor air and onto other surfaces (Raziq et al., 2025a).

1.2.14.3 Transmission scenarios

Multiple, well-documented transmission pathways exist between pet birds and human infection including contact with birds (stroking, kissing, putting birds on clothing or faces) or cage cleaning and exposure to bird saliva or droppings enable the direct transfer of bacteria to hands and mucous membranes (Boseret et al., 2013). Case reports and investigations of outbreaks have repeatedly implicated direct handling of sick or dead birds in human cases of salmonellosis and other infections (Park et al., 2025). Contaminated hands, utensils, kitchen surfaces or foodstuffs are a frequent pathway: hands that are soiled while handling birds or cleaning cages might transfer pathogens to the mouth or food preparation surfaces if hand hygiene is lapsing (Tokuda & Shintani, 2024). Documented outbreaks (including multi-state salmonellosis linked to wild songbirds) are often traced to indirect fecal contamination of environments humans are in contact with (Olaru et al., 2023). Cage parts, feeders, water bowls, clothing and shoes may carry viable organisms from one room or household to another. Studies which isolate *Salmonella* and other enteric bacteria from papers found in cages and from the market environment show that papers of fomites are realistic,

sustained source of exposure (Barancheshme & Munir, 2018; Boseret et al., 2013). Drying of fecal matter generates dust which may contain bacteria, cleaning activities disturbing contaminated bedding or cage papers, generating aerosols and settleable dust which are also inhaled or deposited on food contact surfaces (Park et al., 2025). This is a route of transmission that is extremely relevant in a situation where the birds are being kept indoors and cleaning is being conducted inside the living space. Although discussed more often with regard to poultry and commercial production, the environments of pet birds can contaminate human food or water (e.g. bird droppings on balcony gardens, contaminating outdoor areas where food is prepared) and create additional exposure links (Boseret et al., 2013).

1.2.14 One Health Framework

1.2.14.1 Human–animal–environment interface

A One Health approach acknowledges that antimicrobial resistance (AMR) is not limited to a clinical human context: resistant bacteria and resistant genes are transmitted through the continuum of human-animal-environment, and feedback loops exist between industries, amplifying and perpetuating AMR (One Health Handbook, 2025). Wild and companion birds for instance pick up resistant Enterobacterales and AMR genes from environmental sources like contaminated water, soil, and anthropogenic wast (Mbuthia & Hoza, 2025). Systematic reviews of wild birds have recorded high levels of resistance in even minimally exposed to the environments for direct administration, indicating that environmental pollution and ecological connectivity are leading to circulation of AMRs (Mbuthia & Hoza, 2025). Moreover, we have found from surveillance from avian populated water-bodies that there is an abundance of AMR genes (integrons, mobile elements, resistance markers) in the water, suggesting that the environment acts as a reservoir and channel for resistance (Farkas et al., 2025). In sum, the human-animal-environment interface constitutes not only conceptually central idea in One Health but is empirically demonstrated in the avian AMR systems.

1.2.14.2 Need for AMR surveillance in the pet bird

Pet birds are currently being monitored in most of the national AMR programs under close contact with humans. In certain countries, surveillance data report multidrug-resistant *E. coli* and *Salmonella* from pet birds including the alarming resistance to one of the most important drugs such as colistin. This gap hampers a complete One Health picture, as while surveillance of companion animals usually focuses on dogs and cats, birds are often skipped despite being able to harbor, shed and transmit resistant microbes (Khan et al., 2025). Without routine monitoring, the role of pet birds in

the cycling of AMR especially as a bridge between wildlife, humans and the domestic environment remain poorly understood. Strengthening AMR surveillance systems to explicitly include pet birds (and not merely food animals and wildlife) is therefore critical to closing this One Health surveillance blind spot (One Health Handbook, 2025; World Organisation for Animal Health, 2022).

1.3 Aims and Objectives

I. Overall Aim

The overarching aim of this research is to **assess the role of pet birds as a significant reservoir for multi-drug resistant (MDR) and virulent bacterial pathogens with zoonotic potential** in the Noakhali region of Bangladesh, thereby contributing essential data to the global **One Health** framework for controlling antimicrobial resistance (AMR).

II. Specific Objectives

The specific objectives designed to achieve the overall aim are structured into phases, from isolation to molecular characterization and risk assessment

- To isolate Enterobacteriacea from pet bird feces.
- To confirm virulence and antimicrobial resistance genes using PCR.
- To determine phenotypic antimicrobial susceptibility patterns through disk-diffusion antibiogram testing.
- To assess the prevalence of multidrug-resistant isolates among the recovered bacteria.

Chapter 2

2.0 Method and material

2.1 Study Design and Area

This cross-sectional study was performed between March 2025 and November 2025 in the Noakhali, Bangladesh, area, and the study aimed to identify and classify the pathogenic and multidrug-resistant isolates, specifically the *Escherichia coli*, Klebsiella, Pseudomonas, and *Citrobacter* that are present in the fecal matters of pet birds a. This study uses an investigative design that focuses on laboratory research, which includes microbiologic culture and testing, biochemical testing, and antimicrobial susceptibility testing, as well as the molecular technique of the virulence gene and antimicrobial resistance gene by PCR. This study strictly follows standard protocols for bacterial research conducted on birds (Cheesbrough, 2005).

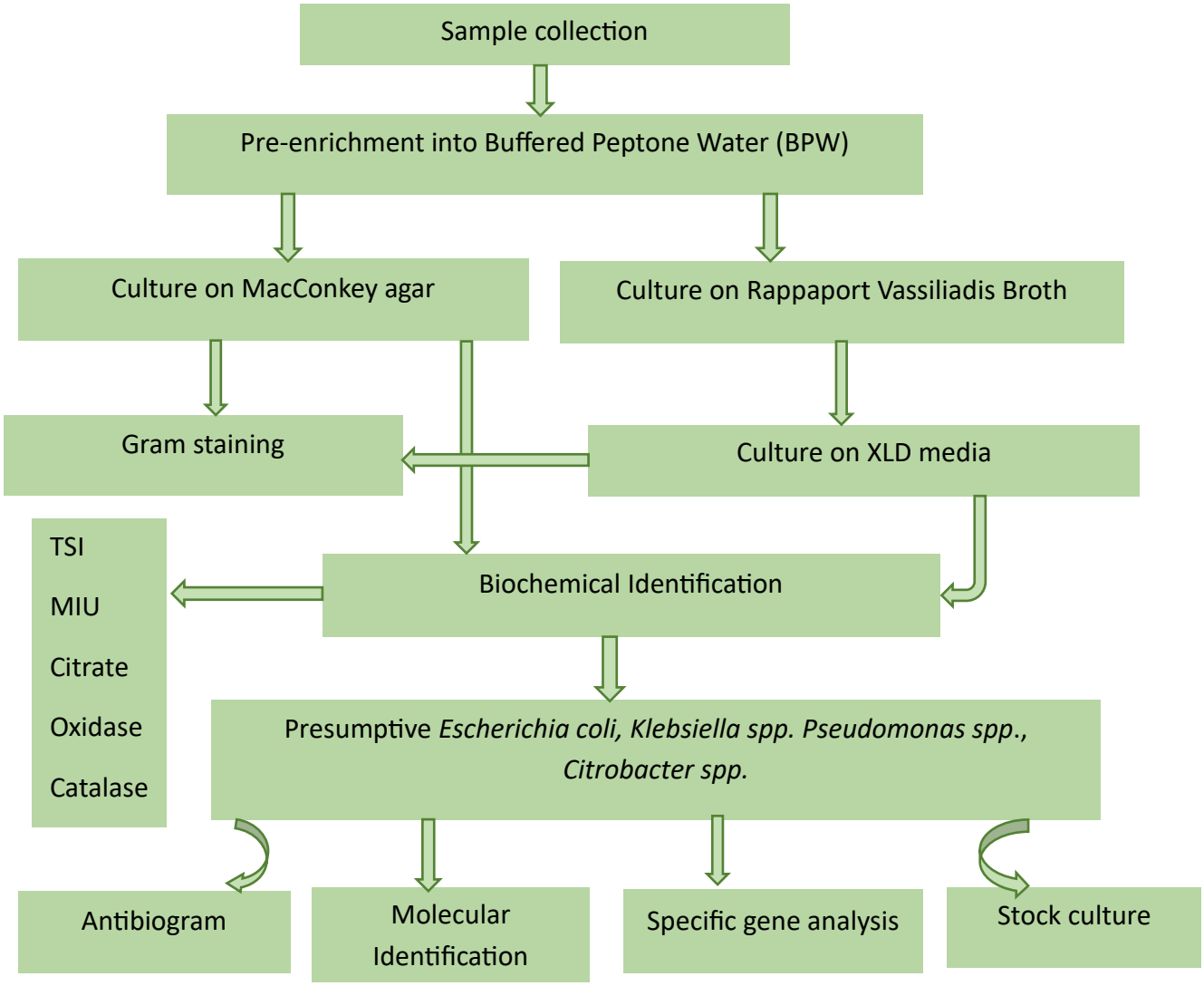


Figure 2.1: Schematic Diagram of the Experimental Procedure

2.1.1 Collection of Pet Bird samples

2.1.1.1 Sample collection site

Samples were collected from ten different pet bird shops located in Noakhali district of Bangladesh. Samples were collected randomly.

Table 2.1: Geospatial mapping of sample collection sites

Serial No.	Location	Latitude	Longitude
1	Dotter haat	22.841432	91.098498
		22.841552	91.097823
2	Maijdee	22.847727	91.096732
		22.874134	91.090276
3	MaijdeeBazar	22.884654	91.096381
		22.883371	91.096203
4	Kalitara bazar	22.814727	91.093222
5	Chowmuhani	22.944882	91.104781
6	Basurhat	22.871036	91.274659
		22.877029	91.281773

2.1.1.2 Sample Type and Source

A total of 92 fecal samples were collected from pet birds including budgerigars, lovebirds, cockatiels, finches, diamond dove, javas, doves. Samples were obtained from pet shops within the selected region.

Figure 2.2: Different types of pet birds in pet shop.

2.1.1.3 Collection Procedure

Collected fresh droppings aseptically from cage linings and used sterile spatulas for this purpose. Approximately 5-10g feed, 1–2 g of fecal matter was transferred into sterile zip lock plastic bag, labeled, and transported in an insulated ice box (4–8°C) to the laboratory within two hours of collection to preserve microbial viability (Cheesbrough, 2005).

Figure 2.3: Pet bird feces sample

2.2 Bacterial Isolation and Culturing

2.2.1 Pre-enrichment and Enrichment

In order to get successful recovery of target enteric pathogens, samples were subjected to a combination of pre-enrichment, enrichment, and selective plating methods. To revive stressed or low-number bacteria which are usually present in environmental or host-derived specimens, one gram of fecal material of each sample was aseptically mixed in suitable liquid media. Pre-enrichment was performed with Buffered Peptone Water (BPW) since it helps to recover injured bacterial cells without suppressing rival flora, thus sensitivity of the isolation and allows proliferation of a wide variety of Gram-negative bacteria. Each enrichment broth was adjusted to 37°C incubation and after 18-24 hours the incubation periods using standard microbiological methods of incubation indicated in (Quinn et al., 2011).

2.2.2 Selective and Differential Media

After pre-enrichment, the fecal samples were cultured on a set of selective and discriminatory media to isolate and differentiate *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas spp*, and *Citrobacter spp*. preliminary plating on MacConkey Agar (MAC) was conducted following enrichment in Buffered Peptone Water (BPW) to recover lactose- and non-lactose-fermenting Gram-negative enteric bacteria. MAC is commonly employed in primary isolation due to the inhibitory effect of the material on Gram-positive flora by bile salts and crystal violet, and differentiation by lactose

fermentation by lactose and neutral red. The presumptive group *E. coli* or *Klebsiella spp.* was indicated by typical pink colonies, and possible Pseudomonas or other non-fermenters by the presence of pale, non-lactose fermenting colonies (Cheesbrough, 2005; Quinn et al., 2011). To isolate *Citrobacter spp*., an aliquot of the BPW-enriched sample was inoculated into Rappaport-Vassiliadis Soya Peptone (RVS) broth which is a selective enrichment medium that favors *Salmonella*, *Shigella spp., Citrobacter spp*. growth under severe or low-level contamination conditions. Following a 24 hours period of selective enrichment, RVS cultures were streaked onto Xylose Lysine Deoxychlate (XLD) Agar, on which *Citrobacter spp* develops the characteristic pattern black centreed colony by lysine decarboxylation and hydrogen sulfide (H_2S) production. One of the features that RVS cultures show as compared to other enteric bacteria (Tegegn et al., 2025). Doubtful *Pseudomonas* fermenting colonies that tested non-lactose fermenting were again streaked on Cetrimide Agar (CA), a selective media with cetrimide or which discourages most bacteria however allowing *Pseudomonas aeruginosa*. Presumptive identification was done by pigment production (pyocyanin or pyoverdine) and typical grape-like odor (Quinn et al., 2011).

Individually, the use of MAC, XLD, and Cetrimide agar offered a methodological process to isolate, differentiate, and presumptively identify the four targeted bacterial genera in fecal samples of pet birds, to be specific and prevent background microbial interference

2.2.3 Morphology and Biochemical Identification of Isolates

Colony Morphology

Initially, presumptive identification of the isolated bacteria was done on the basis of the morphological characteristics of colonies in selective and differential media. On MacConkey agar, *Escherichia coli* was smooth and medium sized colonies with a pink color on the agar which showed the lactose fermentation characteristics. On MacConkey agar, small colonies were observed to be a large mucoid pink color that indicates lactose fermentation and high levels of capsule production. Differentiation of *Pseudomonas aeruginosa* was based on distinctive of the greenish hue (pyocyanin) and clear colonies on Cetrimide agar, which promotes formation pigment and selective-growth of *Pseudomonas spp*. *Citrobacter spp*. were identified in Xylose Lysine Deoxycholate (XLD) agar by the presence of red colonies on the medium with black centers, which represents lysine decarboxylation and production of hydrogen sulfide (H2S), respectively (Forbes et al., 2007).

Gram Staining

To further extend the initial identification of the isolates, gram staining was done to evaluate the microscopic morphology of the isolates. Each culture was stained as a heat-fixed smear with the standard four-step Gram staining technique of crystal violet, followed by iodine, alcohol decolorization and safranin counterstaining. All the four target organisms, which included *E. coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, and *Citrobacter spp*. exhibited the anticipated Gram-negativeism characteristic, which showed as pink colonies using light microscopy. The outcome validated their Gram-negative bacillary characteristics and fitted earlier accounts of such enteric and non-fermentating bacterial species (Beveridge, 2001).

Biochemical identification of isolates

The preliminary identification was confirmed by the series of biochemical tests (TSI agae slant, Motility Indole and Urease (MIU) test, Catalase test, Oxidase test).

2.2.4 Stock preservation of the Isolates

Isolates were aseptically streaked on respective differential media in a stock culture and incubated overnight at 37^0C to allow a single colony. Following incubation period, single colonies of all the isolates were inoculated into sterile Eppendorf tube with 750 ul of Trypticase Soy Broth (TSB) and 250 ul glycerol. Then performed vortex and stock of each isolate were frozen into -20^0C. Eppendorf tubes containing duplicate sets of each of the isolates were stored in -80^0 C

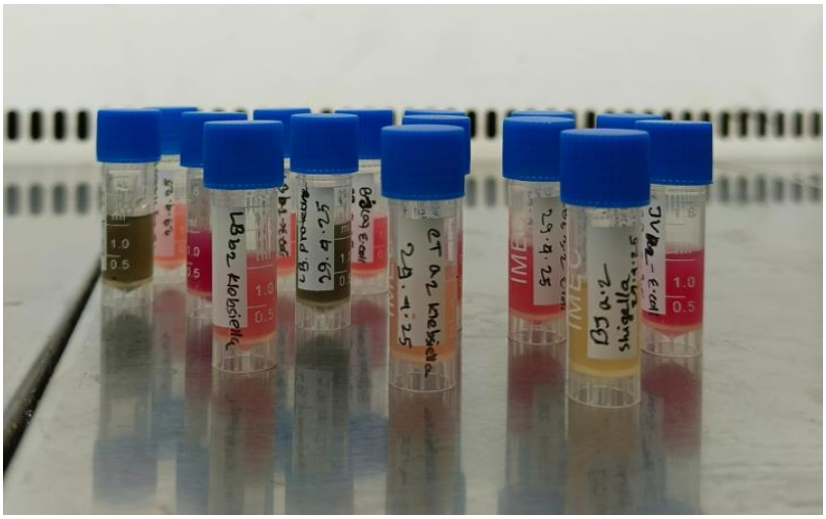


Figure 2.4: Stock culture of the isolates.

2.3 Antimicrobial Susceptibility Test (AST)

The Kirby-Bauer disc diffusion system of antimicrobial susceptibility testing (AST) of the identified bacterial isolates was conducted according to the globally standardized methodology suggested to perform the routine susceptibility testing of bacteria of clinical interest. Mueller-Hinton Agar (MHA), which is supported by the Clinical and Laboratory Standards Institute (CLSI) due to its consistent results across a batch-to-batch operation, optimal cation level, and diffusion of a broad spectrum of antimicrobials, was tested. These were conducted under the strict CLSI M100 (2023) performance standards to take into account all necessary accuracy and comparison with the international datasets. Following the CLSI recommendations, plates were inoculated and antibiotic discs were placed and incubated at 37^0Cof 16-18 hours and the resulting inhibition areas calculated with a digital caliper or calibrated ruler, to the nearest millimeter (CLSI, 2023).

2.3.1 Inoculum Preparation

A fresh bacterial culture was then suspended in sterile normal saline and visually adjusted to the same, using the 0.5 McFarland turbidity standard which represents a one and a half million CFU/mL. When possible, standardization was done on a McFarland densitometer. Use of appropriate inoculum density reduces inconsistency in the size of inhibition zones, as well as adherence to CLSI quality control specifications. Using the aid of a sterile swab, the standardized inoculum was spread onto the surface of the MHA plate to create a uniform bacterial lawn and then discs were placed (CLSI, 2023).

2.3.2 Antibiotic Panel

In order to compare the resistance patterns of *E. coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, and *Citrobacter spp*. against the panel of antibiotics that are largely used in veterinary and human medicine against Gram-negative pathogens was chosen. Discs containing antimicrobial agents were: Amoxicillin, Cephalexin, Cefuroxime, Ceftriaxone, Ciprofloxacin, Norfloxacin, Tetracycline, Co-trimoxazole, Gentamicin, Chloramphenicol, Erythromycin

These antibiotics were chosen on the basis of clinical relevance, frequency of use in avian and human medicine and significance in monitoring multidrug resistance in Enterobacteriaceae and Pseudomonas. The CLSI M100(2017), CLSI M100, CLSI M100(2025) and EUCAST breakpoint tables were used to measure and interpret the inhibition zone diameter of each isolate as Susceptible (S), Intermediate (I) or Resistant (R).

Table 2.2: Sizes of zone of inhibition according to the performance standards for antimicrobial susceptibility testing and organisms were reported as susceptible, intermediate or resistant to various antibiotics. Here in this table different zone interpretation of *Pseudomonas spp*. are not shown. They differ in zone interpretation for Ceftriaxone (≥21, 14-20, ≤13), Ciprofloxacin (≥21, 18-20, ≤17), Tetracycline (≥24, 16-23, ≤15), Gentamicin (≥18, 15-17, ≤12); S, I, R indicate sensitive, intermediate and resistant zone respectively.

Antimicrobial class	**Antimicrobial agents**	**Disc conc. µg**	**Zone interpretation (diameter in mm)**		
			S	I	R
beta lactams (penicillins & cephalosporins)	Amoxicillin	10	≥17	14-16	≤13
	Cephalexin	30	≥18	15–17	≤14
	Cefuroxime	30	≥23	15-22	≤14
	Ceftriaxone	30	≥23	20-22	≤19
Quinolones/Fluoroquinolones	Ciprofloxacin	5	≥26	22-25	≤21
	Norfloxacin	10	≥17	13-16	≤12
Tetracyclines	Tetracycline	30	≥15	12 -14	≤11
Sulfonamides	Co-trimoxazole	25	≥16	11-15	≤10
Aminoglycosides	Gentamicin	10	≥18	15-17	14
Chloramphenicol	Chloramphenicol	30	≥18	13-17	≤12
Macrolides	Erythromycin	15	≥23	14-22	≤13

2.4 Molecular analysis of the isolates

The identification of the isolated bacterial species was carried out through molecular testing and the identification of specific virulence and antimicrobial-resistance genes by polymerase chain reaction (PCR). The process involved extracting the chromosomal DNA, the assessment of purity, the preparation of the PCR reaction mix, amplification at the best conditions, and electrophoresis of the amplified PCR amplicons.

2.4.1 Extraction and Purification of Chromosomal DNA

The conventional boiling technique was applied to extract chromosomal DNA out of freshly grown cultures of Trypticase Soy Broth as described by Bansal et al. (1996). In short, 1.5 mL of overnight bacterial culture in a sterile, labeled Eppendorf tube were centrifuged at 12,000 rpm in 10 minutes to bring together the bacterial cells. The resultant supernatant was disposed cautiously and the cell

pellet was washed once with sterile distilled water and subsequently recentrifugated. The pellet was then washed again into 300 mL sterile PCR-grade water. The suspension was thoroughly vortexed and the tubes were subjected to a boiling water bath at 100 ºC during 15 minutes to lyse the cells and release chromosomal DNA. The tubes were then put on ice immediately after boiling in order to undergo protein precipitation as well as enhance DNA stability. The lysate was then centrifuged at 15,000 rpm over 15 minutes after the cooling period. The apparent supernatant with crude chromosomal DNA was thoroughly pipetted into a clean sterile Eppendorf tube and kept on -20°C until future use in PCR assessments (Queipo-Ortuño et al., 2008). Virulence and resistance genes were amplified as the template using the extracted DNA.

2.4.2 DNA Concentration and Purity Measurement

The concentration and purity of the DNA were measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). A 1-2 uL aliquot of DNA was applied to the pedestal and the value of absorbance at 260 nm and 280 nm were taken. The A260/A280 was used to view the purity of DNA, and the ranges of 1.8 to 2.0 were regarded as fitting to continue further with the PCR amplification (García-Alegría et al., 2020).

Figure 2.5: DNA concentration and purity measurement of the extracted DNA.

2.4.3 Amplification of the 16srRNA sequence

A partial sequence of the 16S rRNA gene, measuring 1.5 kb, was amplified through PCR utilising universal bacterial primers. The universal primers 27F (5′ AGAGTTTGATCCTGGCTCAG-3′)

and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were utilised for PCR amplification of the 16S rRNA gene, in conjunction with nuclease-free water (Promega), GoTaq G2 Green Master Mix (Promega), and 2 µL of templates. The subsequent conditions for PCR were utilised: 4 minutes at 96°C, succeeded by 30 cycles consisting of 30 seconds at 94°C, 30 seconds at 57°C, and 1 minute at 72°C, culminating in a final extension step of 10 minutes at 72°C. Every test incorporated a negative control, where ultra-pure water replaced DNA. A 5 µL sample of each PCR product was put on a 1% (w/v) agarose gel with TBE buffer and analysed.

2.4.4 Amplification of the targeted gene (virulence gene: *eaeA, fimH, stx1, stx2, lasB,invA* and antibiotic resistance gene*: blaCTX-M, blaTEM, tetA, sul*) sequence

2.4.4.1 Preparation of the Reaction Mixture

The preparation of PCR reaction mixture were done in a separate pre-PCR workstation to avoid contamination. PCR reaction mixture was made by mixing the precise amount of the elements in a suitable volume tube in the given sequence as shown in Table 2.4. In a high number of reactions, the master mix without the template DNA was made and aliquoted in PCR tubes. Finally, proper labeling of DNA template was put into a PCR tube. Reaction mixture was capped appropriately and short spin in the PCR tube holding the template DNA was provided. A negative control was used in all PCR involving the lack of template DNA and all other reaction mixture. PCR tubes were put in a thermal cycler (BIO-RAD 96 well, T100tm Thermal Cycler, Hercules, California, USA) and the amplification parameters were adjusted accordingly

Table 2.3: PCR reaction mixture preparation recipe

Solution	Amount (µl)
MASTER MIX	12.5
Forward primer	1
Reverse primer	1
Template DNA	2
Nuclease-free water	8.5
Total Reaction Volume	25

All reagents were kept on ice throughout the preparation process to maintain enzyme stability (Green & Sambrook, 2012).

2.4.4.2 Polymerase Chain Reaction Amplification

Amplification was done by using PCR in a thermal cycle (Bio-Rad, USA). PCR gene-specific virulence and resistance determinants were optimized individually. Each run had negative controls.

2.4.4.3 PCR Conditions

A standard thermocycling profile was used for the majority of target genes, with minor adjustments depending on primer melting temperature (Tm). All the PCR tubes containing the reaction mixtures were heated at 94° C for 3 minutes in the thermal cycler to ensure denaturation of all DNA templates. The PCR reactions was then continued according to the following program:

Step 1: Denatiration at 94°C for 30s.

Step 2: Annealing at 51°C,30s for *eaeA*, 53°C and *magA*,54°C,30s for *lasB*, 56°C,30s for bla CTX-M, 60°C,30s for *fimH*, 55^0C, 30s for *blaTEM*, 53.7°C, 30s for *tetA*, 50.6°C, 30s for *sul1*, 64°C, 30s for *stx1* and *stx2*, 57°C,58°C,30s for *invA*, 30s for 16s rRNA.

Step 3: Extension at 72°C for 30s.

These three steps repeated sequentially 35 cycles with final extension for 5 minutes at 72° C. After completion of the reaction, PCR tubes were hold at 4°C. For further analysis the PCR tubes were stored at -20°C until further analysis.

2.4.4.4 Primer Specificity

A set of highly specific primers was used to identify some virulence factors and antibiotic resistance genes in the bacterial isolates. In *Escherichia coli*, primers to *eaeA*, an intimin adherence factor of enteropathogenic strains, and *fimH*, one of types 1 fimbrial adhesion of an enhanced attachment to epithelium were complemented. Hypervirulence phenotype of *Klebsiella pneumoniae* was determined by amplifying *magA* gene, and *lasB* gene that encode elastase as species-specific virulence factor of *Pseudomonas aeruginosa*. Primers in the *bla-CTXM* and *blaTEM* families of the two most common extended-spectrum β-lactamase (ESBL) families in Enterobacteriaceae were used to detect β-lactam resistance. The resistance of carbapenem was assessed through the vim metallo-β-lactamase gene. Moreover, the *invA,* a conservation-invasion gene that serves as a gold-standard marker in *Salmonella spp*. detection was also added as a species-specific virulence predictor. In the case of antimicrobial resistance determinants unrelated to β-lactams, tet genes, which confer tetracycline resistance (tetracycline-resistance phenotypes are dependent on either an efflux or a ribosomal protection mechanism) and the *sul1* gene, a sulfonamide resistance protein commonly

linked to class 1 integrons, were also added. Random sampling of all primer sequences and annealing temperatures along with anticipated amplicon lengths were chosen in accordance with certified and extensively published protocols to confirm both the accuracy of amplification and diagnostic specificity (CLSI, 2023; Dallenne et al., 2010; Lanotte et al., 2004).

Table 2.4: Peimer sequence used for detection of virulence and multi-drug resistant gene present in avian isolates.

Gene	Primer	Sequence (5'-3')	Tm (^{0}C)	Amplicon size (bp)	References
Universal bacterial primer					
16s rRNA	27F	AGAGTTTGATCMTGGCTCAG	57	1465	https://doi.org/10.1186/s12934-021-01531-4
	1492R	GGT TAC CTT GTT ACG ACT T			
Virulence gene					
invA	F	GTGAAATTATCGCCACGTTCG	58	321	https://doi.org/10.21123/bsj.2023.6806
	R	TCATCGCACCGTCAAAGGAAC			
eaeA	F	ATGCTTAGTGCTGGTTTAGG	51	248	https://doi.org/10.1128/JCM.40.10.3613-3619.2002
	R	GCCTTCATCATTTCGCTT TC			
fimH	F	TGCAGAACGGATAAGCCGTGG	60	508	https://doi.org/10.5812/jjm.17520
	R	GCAGTCACCTGCCCTCCGGTA			
stx1	F	ATA AATCGCCATTCGTTGACT AC	64	180	https://doi.org/10.1128/AEM.69.10.6327-6333.2003
	R	AGAACGCCCACTGAGATCATC			
stx2	F	GGCACTGTCTGAAACTGCTCC	64	255	doi: 10.1128/JCM.36.2.598-602.1998.
	R	TCGCCAGTTATCTGACATTCT			
magA	F	CGAAAGTGAACGAATTGATGCT	53	245	https://doi.org/10.1084/jem.20030857
	R	GTTTCTGCTGCAGATTCGAAGA			
lasB	F	GTTTGGTCGCATATCGCAAC	52	153	https://doi.org/10.5812/jcrps-137394
	R	AATGCGCAGCACCAGGATAG			
Antibiotic resistance gene					
blaCTX-M	F	GACGATGTCACTGGCTGAGC	56	499	https://doi.org/10.1371/journal.pone.0245126
	R	AGCCGCCGACGCTAATACA			
blaTEM	F	CGCCGCATACACTATTCTCAGAATGA	55	445	https://doi.org/10.1371/journal.pone.0245126
	R	ACGCTCACCGGCTCCAGATTTAT			
tetA	F	GTGAAACCCAACATACCCC	53.7	225	https://doi.org/10.5812/jjm.12129
	R	GAAGGCAAGCAGGATGTAG			
sul1	F	CGGCGTGGGCTACCTGAACG	50.6	780	https://doi.org/10.3390/ijerph19074281
	R	GCCGATCGCGTGAAGTTCCG			

2.4.4.5 Analysis of Amplicons by Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to resolve the products of PCR. A 1.5% agarose gel was cast in 1X TBE buffer and 5 uL of the respective amplicon was combined with loading dye and placed in wells. The molecular size marker was a 100 bp DNA ladder. Electrophoresis was conducted at 100 V of duration 30-35 minutes. Ethidium bromide was used to stain gels and visualize them through a UV transilluminator. The presence of bands of the expected sizes was taken as positive in representing the respective genes.

2.4.4.6 Bacterial 16S rRNA Amplicon Sequencing

The bacterial 16S rRNA amplicons were sequenced using the Illumina NextSeq 2000 platform, following standard DNA amplification, sequencing, and data analysis protocols. The amplification of the 16S rRNA gene was conducted using polymerase chain reaction (PCR) on the genomic DNA of bacteria, adhering to the methodology of the next-generation sequence guideline [89–92]. After successful amplification, the PCR products underwent purification using ExoSAP-IT Express reagent for enzymatic clean-up. In this process, 2.5 µL (or 5 µL) of the PCR product was combined with 2 µL of ExoSAP-IT and incubated at 37°C for 15 minutes to activate the exonuclease and the shrimp alkaline phosphatase. The reaction was then inactivated by incubating at 80°C for 15 minutes. The products were stored at −20 °C or directly used in the subsequent cycle sequencing reaction. The BigDye Terminator v3.1 chemistry was utilised for cycle sequencing. The final volume was set to 10 µL with the addition of nuclease-free water. The sequencing reaction mixture included the following components: BigDye Terminator Ready Reaction Mix, 5X sequencing buffer, and a reverse primer at a concentration of 10 µM and 3.5 µL of the purified PCR product. The responses were conducted under thermal cycling conditions, which included an initial denaturation step lasting 1 minute at 96°C. This was followed by 25 cycles comprising 10 seconds at 96°C for denaturation, 5 seconds at 55°C for primer annealing, and 4 minutes at 60°C for extension. The reactions were maintained at 4°C until purification following the cycling process. The BigDye XTerminator Purification Kit was used to purify the sequencing reaction. Purified 16 samples underwent treatment with 55 µL of SAM/BigDye XTerminator bead solution. The tubes were incubated, mixed, shaken, and then centrifuged sequentially. The purified samples were stored at 4°C for the analysis using capillary electrophoresis (CE). For the Illumina NextSeq 2000 sequencing, 20 µL of the purified cycle sequencing product was distributed into a 96-well plate, then properly labelled and sealed. The plate was subsequently placed into the Illumina NextSeq 2000 system. The sequencing module and dye set were chosen according to the system configuration, and the sequencing process was carried out. The data analysis used bioinformatics tools for base calling, quality trimming, and alignment of 16S rDNA amplicons. The sequences were subsequently compared with genomic databases to identify the bacterial species.

2.4.4.7 Bioinformatics and Phylogenetic Analysis

Evolutionary analysis was conducted using the Maximum Likelihood method alongside the Tamura-Nei model. A bootstrap consensus tree was created to illustrate the evolutionary relationships among

the analysed taxa through 1000 replicates. Branches with less than 50% bootstrap support were collapsed, and the percentage of replicate trees in which the associated taxa clustered together (clustered) is shown next to the branches. The first tree was computer generated using the Neighbor-Joining and BioNJ algorithms with a pairwise comparison of differences being performed, and the optimal tree was based on the lowest differences, with the Tamura-Nei model. The analysis included 37 nucleotide sequences, comprising first, second, and third codon positions and noncoding regions, and 1761 positions in the final dataset. Phylogenetic analyses were conducted using MEGA X**.**

Chapter 3

3.0 RESULTS

This chapter presents the findings of bacterial isolation, phenotypic and molecular identification, detection of virulence and antimicrobial resistance (AMR) genes, and antimicrobial susceptibility profiles of the isolates obtained from pet bird fecal and feed samples.

3.1 Isolation and Identification of *E. coli*, *Klebsiella spp.*, *Citrobacter spp.*, *Pseudomonas spp*.

3.1.1 Isolation Rate of Each Organism

A total of 92 fecal samples processed for bacteriological analysis. From these, 156 bacterial isolates belonging to presumptive *E. coli, Citrobacter spp., Klebsiella spp.*, and *Pseudomonas aeruginosa* were recovered. The highest isolation rate was observed for *E. coli* (75), followed by *Klebsiella spp.* (66) and *Pseudomonas aeruginosa* (9), whereas *Citrobacter spp.* showed comparatively lower prevalence (6). Mixed infections were recorded in several samples.

3.1.2. Cultural Characteristics

To get pure colony of the desired organisms, suspected colony were subcultured on respective selective media. Based on cultural characteristics 75 isolates among 156 isolates were selected as presumptive *E. coli*, 66 as *Klebsiella spp*, 9 as *Pseudomonas spp*., and 6 as *Citrobacter spp.*

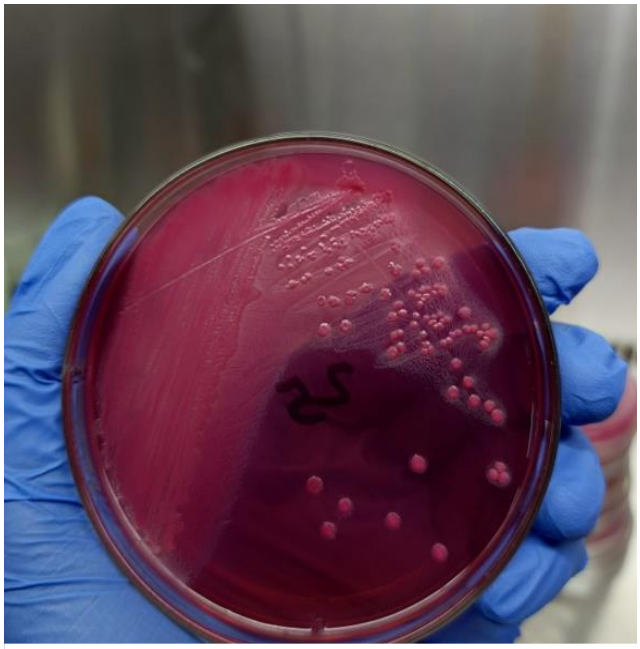


Figure 3.1: Representative *E.coli* colonies on MacConkey agar

Figure 3.2: Representative *Klebsiella spp.* colonies on MacConkey agar

Figure 3.3: Representative *Citrobacter spp.* in mixed colonies on XLD agar

Figure 3.4: Representative *Pseudomonas spp*. colonies on MacConkey agar

Table 3.1: Characteristics of representative *E. coli, Klebsiella spp.*, *Citrobacter spp.* and *Pseudomonas spp*. on selective media.

Isolates ID	Media	Size	Color	Form	Opacity	Consistency
SA2	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SA3	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SA5	MacConkey Agar	Medium	Red	Circular	Opaque	Butyrous
SB8	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SB10	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SC20	MacConkey Agar	Medium	Red	Circular	Opaque	Butyrous
SD25	MacConkey Agar	Medium	Red	Circular	Opaque	Butyrous

SD27	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SE29	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SE30	MacConkey Agar	Medium	Bright Pink	Circular	Opaque	Butyrous
SE31	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SE32	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SA11	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SA12	MacConkey Agar	Large	Pink	Circular	Opaque	Mucoid
SA13	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SA14	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SB16	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SB17	MacConkey Agar	Large	Pink	Circular	Opaque	Mucoid
SC34	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SC35	MacConkey Agar	Medium	Pink	Circular	Opaque	Mucoid
SC51	XLD Agar	Medium	Red with Black Centers	Circular	Opaque	Butyrous
SD52	XLD Agar	Medium	Red with Black Centers	Circular	Opaque	Moist
SA53	XLD Agar	Medium	Red with Black Centers	Circular	Opaque	Butyrous
SB54	XLD Agar	Medium	Red with Black Centers	Circular	Opaque	moist
SE55	XLD Agar	Medium	Red with Black Centers	Circular	Opaque	Moist

SC48	Cetrimide Agar	Large	Greenish-Blue	Spreading	Opaque	Butyrous
SC49	Cetrimide Agar	Medium	Greenish-Blue	Irregular	Iridescent	Butyrous
SC50	Cetrimide Agar	Medium	Greenish-Blue	Spreading	Opaque	Butyrous
SD57	Cetrimide Agar	Large	Greenish-Blue	Irregular	Opaque	Moist
SE58	Cetrimide Agar	Large	Greenish-Blue	Spreading	Iridescent	Butyrous

3.1.3 Morphological Characteristics

All 156 isolates were Gram negative and rod shaped.

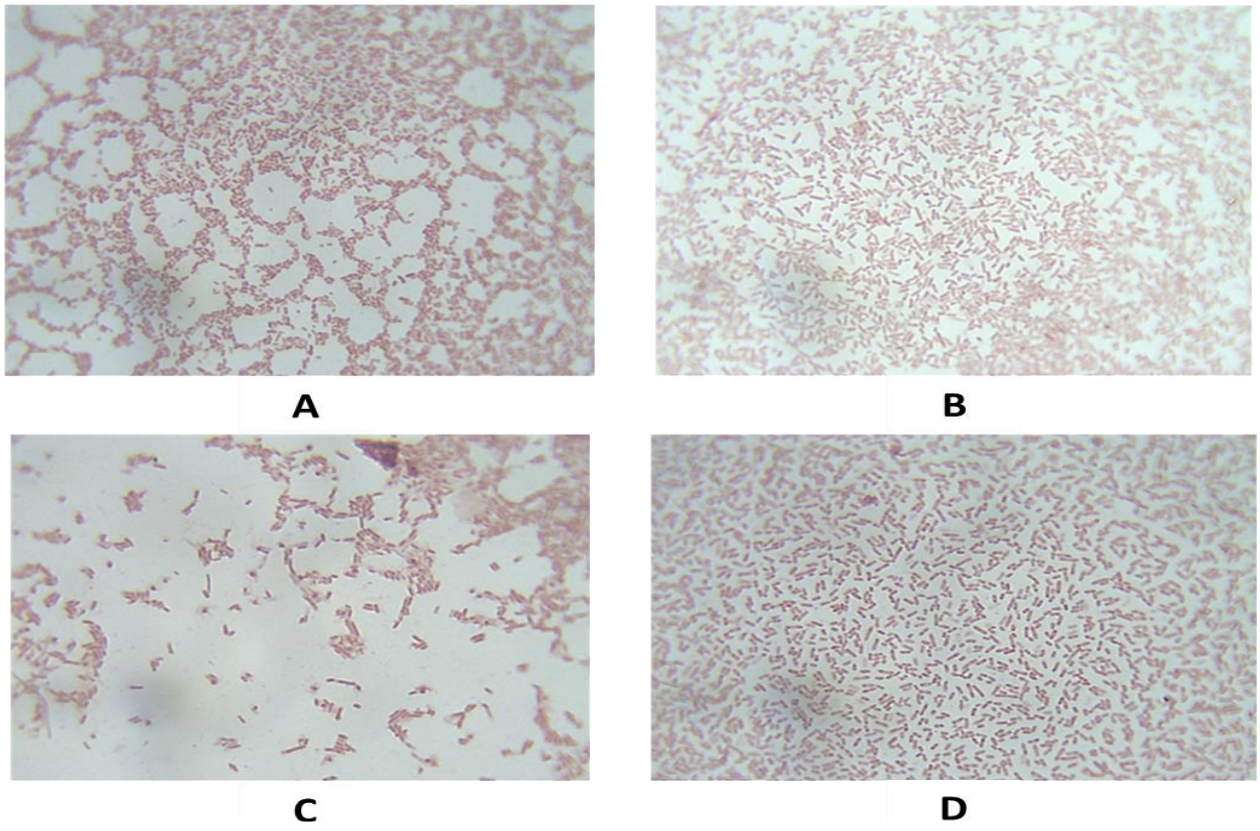


Figure 3.5: Gram staining of representative A) *E. coli,* B) *Klebsiella spp*. C*) Citrobacter spp*. and D) *Pseudomonas spp*

Table 3.2: Cultural characteristics of representative isolates.

Isolates ID	Color	Shape	Arrangement	Staining result
SA2	Pink	Rod	No	-ve
SA3	Pink	Rod	No	-ve
SA5	Pink	Rod	No	-ve
SB8	Pink	Rod	No	-ve
SC18	Pink	Rod	No	-ve
SC19	Pink	Rod	No	-ve
SC20	Pink	Rod	No	-ve
SD25	Pink	Rod	No	-ve
SD27	Pink	Rod	No	-ve
SE29	Pink	Rod	No	-ve
SE30	Pink	Rod	No	-ve
SE31	Pink	Rod	No	-ve
SE32	Pink	Rod	No	-ve
SA11	Pink	Rod	No	-ve
SA12	Pink	Rod	No	-ve
SA13	Pink	Rod	No	-ve
SA14	Pink	Rod	No	-ve
SB16	Pink	Rod	No	-ve
SB17	Pink	Rod	No	-ve
SC34	Pink	Rod	No	-ve
SC35	Pink	Rod	No	-ve
SC37	Pink	Rod	No	-ve
SC39	Pink	Rod	No	-ve
SD43	Pink	Rod	No	-ve
SE46	Pink	Rod	No	-ve
SC48	Pink	Rod	No	-ve
SC49	Pink	Rod	No	-ve
SC50	Pink	Rod	No	-ve
SC51	Pink	Rod	No	-ve
SD52	Pink	Rod	No	-ve

3.1.4 Biochemical Confirmation

Various biochemical tests were performed on selected 156 isolates and they were presumptively identified according to standard guideline (Bergey's manual of determinative bacteriology, 2012) Among all isolates 75 were selected as presumptive *E.coli*, 66 as *Klebsiella spp.*, 9 as *Pseudomonas spp*. and 6 as *Citrobacter spp.*

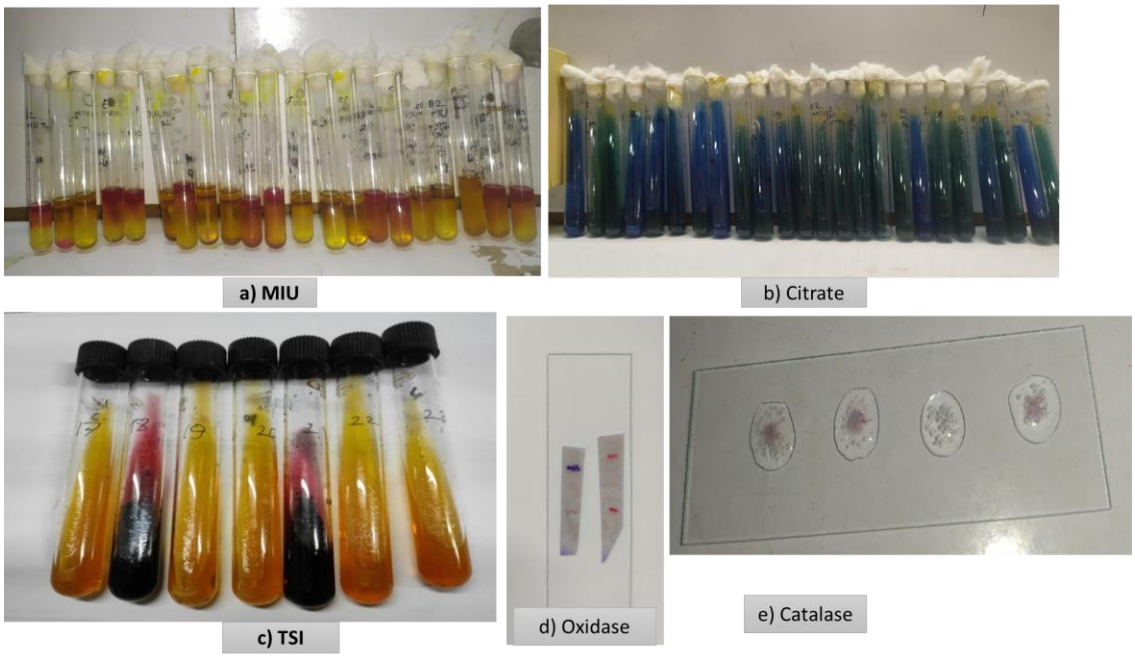


Figure 3.6: Various biochemical test on representative isolates of a) *E. coli,* b) *Klebsiella spp*. c) *Citrobacter spp*. and d) *Pseudomonas spp.*

Table 3.3: Results of Biochemical Test on representative isolates of *E. coli*, *Klebsiella spp.*, *Pseudomonas spp*. and *Citrobacter spp.*

Sample ID	TSI				MIU			Citrate	Oxidase	Catalase
	Slant	Butt	Gas	H_2S	Motility	Indole	Urease			
SA2	A	A	+	-	+	+	-	-	-	+
SA3	A	A	+	-	+	+	-	-	-	+
SA5	A	A	+	-	+	+	-	-	-	+
SB8	A	A	+	-	+	+	-	-	-	+
SC18	A	A	+	-	+	+	-	-	-	+
SC19	A	A	+	-	+	+	-	-	-	+

SC20	A	A	+	-	+	+	-	-	-	+
SD25	A	A	+	-	+	+	-	-	-	+
SD27	A	A	+	-	+	+	-	-	-	+
SE30	A	A	+	-	+	+	-	-	-	+
SA11	A	A	+	-	-	-	+	+	-	+
SA12	A	A	+	-	-	-	+	+	-	+
SA13	A	A	+	-	-	-	+	+	-	+
SA14	A	A	+	-	-	-	+	+	-	+
SB16	A	A	+	-	-	-	+	+	-	+
SB17	A	A	+	-	-	-	+	+	-	+
SC34	A	A	+	-	-	-	+	+	-	+
SC35	A	A	+	-	-	-	+	+	-	+
SD43	A	A	+	-	-	-	+	+	-	+
SE46	A	A	+	-	-	-	+	+	-	+
SC48	K	K	-	-	+	-	-	+	+	+
SC49	K	K	-	-	+	-	-	+	+	+
SC50	K	K	-	-	+	-	-	+	+	+
sSC48	K	K	-	-	+	-	-	+	+	+
sSC49	K	K	-	-	+	-	-	+	+	+
SC51	K	A	+	+	+	-	-	+	-	+
SD52	K	A	+	+	+	-	-	+	-	+
sSC51	K	A	+	+	+	-	-	+	-	+
sSC52	K	A	+	+	+	-	-	+	-	+
SB156	K	A	+	+	+	-	-	+	-	+

A= Acid

K= Alkaline

+ = Positive

- = Negative

3.1.5 Antibiotic Susceptiblity test

One hundred and sixty-six (156) isolates were tested with 11 widely used antibiotics in the avian species (7 different classes of antibiotics). The outcome was determined according to CLSI M100(2017), CLSI M100(2025), EUCAST,2013guideline. Out of 156 isolates, the sensitive, intermediate, and resistant isolates are 51, 21 and 84 respectively. In the case of CL30, there were 15 sensitive, 57 intermediate and 84 resistant isolates. In CXM30, there was low susceptibility with 3 sensitive isolates, and 108 intermediate and 45 resistant isolates. In CTR30, 72, 32 and 52 sensitive, intermediate and resistant isolates respectively. In respect to fluoroquinolones, CIP5 exhibited 30, 36, and 90 sensitive, intermediate, and resistant isolates respectively, and then NX10 had a distinct further sensitivity profile with 111 sensitive, 21 intermediate, and 24 resistant isolates. In the case of tetracycline (TE30), the sensitivity was small (15 sensitive) and there were 42 intermediate and 99 resistant isolates. The use of co-trimoxazole (COT25) gave 21 sensitive, 48 intermediate and 87 resistant isolates. The results of amino-glycoside tests revealed that there were GEN10 sensitive isolates (75), intermediate isolates (48), and insensitive isolates (33) of the bacteria. In the case of chloramphenicol (C30), 120 isolates were sensitive, 6 intermediate and 30 resistant. No sensitivity or intermediate reaction was observed with erythromycin (E15) since all of the 156 isolates were resistant. All in all, NX10 and C30 had the most sensitive isolates, whereas the greatest resistance was made on E15, CIP5, TE30, and COT25. CXM30 recorded the most number of intermediate responses. These results show that there is a significant multidrug resistance burden among the tested isolates.

Of 75 *E. coli* isolates, 36% Cefuroxime, Co-trimoxazole, Ceftriaxone, Ciprofloxacin, Norfloxacin, Tetracycline, Co-trimoxazole, Gentamicin, 52506080120160 were resistant to Amoxicillin, Cefuroxime, Cephalexin and Co-trimoxazole, Cetriaxone. NX10 (80%), C30 (80%), E15 (100%), CIP5 (60%), TE30 (68%), COT25 (68%) were the best with highest sensitivity and resistance rate respectively. These results indicate a high trend of multidrug resistance in avian *E. coli* isolates and resistance is especially susceptible to the β-lactams, fluoroquinolones, tetracycline, and sulfonamides.

Out of 66 isolates of *Klebsiella spp.*, 68.18% 45.45% 31.81% 40.90% 59.09% 27.27% 54.54% 40.90% 36.36% 18.18% 100% were resistant to amoxicillin, cefuloxin, cefuroxime, ceftriaxone, ciprofloxacin, nor the findings confirm that *Klebsiella spp.* have a high multidrug-resistant phenotype. It is observed to have high resistance to b- lactams (AX10, CTR30 CXM30),

fluoroquinolones (CIP5), tetracycline (TE30), and sulfonamides (COT25). The cross-sensitivity of erythromycin is also indicative of universal resistance of macrolides common in Enterobacteriaceae.

The 9 isolates of *Pseudomonas spp* were found to have 9 isolates of resistance to erythromycin (E15) with the 100% of the isolates, which is consistent with the intrinsic resistance to Macrolide. Cefuroxime (CMX30) was also found to have high levels of resistance (88.88) and has the expected resistance of the organism to most β-lactams because of efflux pumps and impermeable outer membranes. There was a significant opposition to chloramphenicol (CL30) (77.77%), ampicillin (AX10) (66.66%), ceftriaxone (CTR30) (66.66%), and tetracycline (TE30) (66.66%). Such observations suggest that cephalosporins, penicillin, and tetracyclines of older generation have little therapeutic effect on *Pseudomonas spp*. and finally 100% resistance rate occurred in case of Amoxicillin (AX10), Tetracycline (TE30), and Erythromycin (E15) among 6 isolates of *Citrobacter spp.* reflecting that the antibiotics are completely unable to work against the investigated Citrobacter isolates.

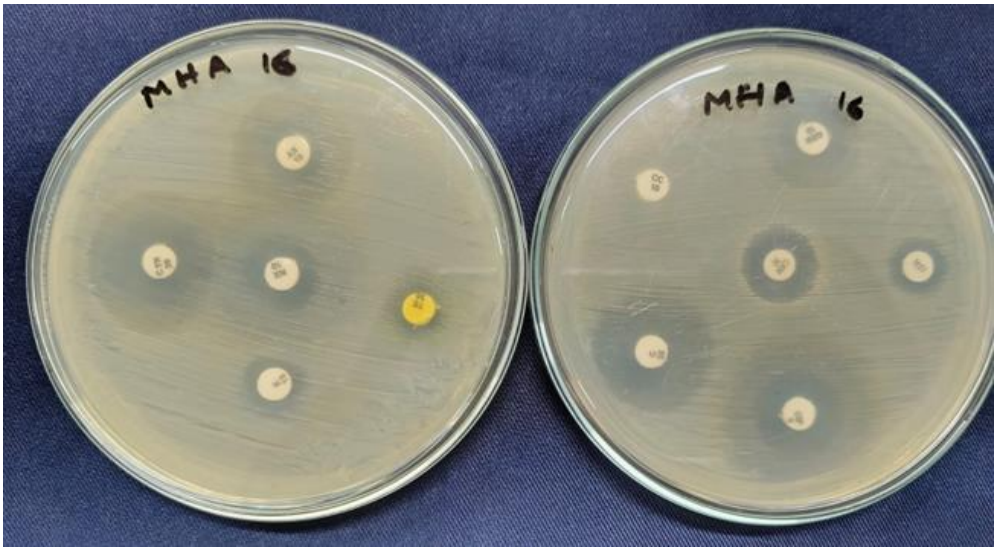


Figure 3.7: Representative figure of Antibiogram of presumptive *Klebsiella spp.* isolate

Table 3.4: Result of antibiotic susceptibility test for representative isolates.

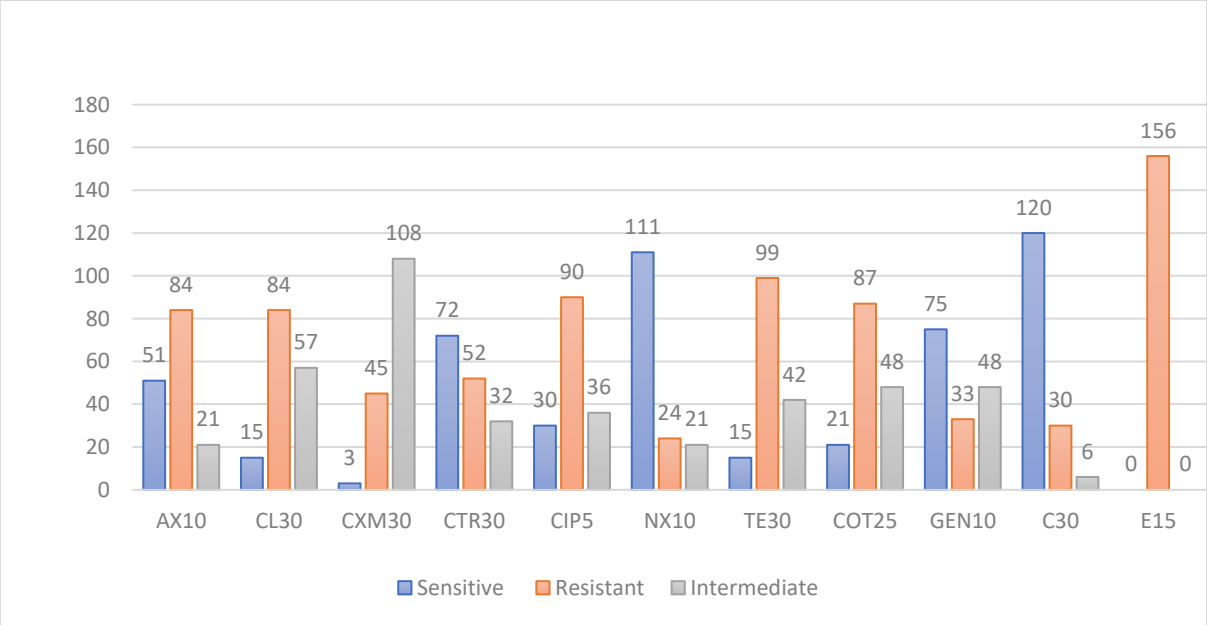
ID	AX10	CL30	CXM30	CTR30	CIP5	NX10	TE30	COT25	GEN10	C30	E15	MAR index
SA2	R	R	R	R	R	R	R	R	R	R	R	1
SA3	I	R	I	S	R	S	I	I	I	S	R	0.27
SA5	S	R	I	S	R	S	S	R	I	S	R	0.36
SB8	R	R	I	R	R	I	R	R	I	R	R	0.72

SB9	R	R	R	R	R	I	R	S	I	R	R	0.72
SB10	R	R	I	I	R	S	R	R	I	S	R	0.54
SC20	R	R	R	R	R	R	R	R	S	S	R	0.81
SD25	S	R	I	I	R	S	R	R	S	R	R	0.54
SD27	R	I	I	S	S	S	R	R	I	I	R	0.36
SE29	R	R	R	R	S	S	R	R	S	S	R	0.63
SE30	S	R	I	I	R	S	R	R	I	S	R	0.45
SE31	R	R	R	R	R	I	I	R	I	S	R	0.63
SE32	R	R	I	I	R	S	S	R	S	S	R	0.45
SA11	R	I	I	I	R	S	R	I	I	S	R	0.36
SA12	R	R	R	R	R	R	I	I	R	S	R	0.72
SA13	R	I	I	R	R	S	R	I	S	R	R	0.54
SA14	R	R	R	R	R	S	R	I	I	S	R	0.63
SB16	S	R	R	S	R	I	I	R	I	S	R	0.45
SB17	R	R	R	R	I	S	R	R	R	R	R	0.81
SC34	R	R	R	R	R	R	R	I	R	I	R	0.81
SC35	R	I	R	R	R	S	I	I	R	S	R	0.54
SC37	R	R	R	R	R	I	R	R	I	S	R	0.72
SC39	R	I	I	I	R	R	R	I	S	S	R	0.45
SD43	R	R	I	I	R	S	I	S	S	S	R	0.36
SE46	S	R	I	S	S	S	R	R	R	S	R	0.45
SC48	R	R	R	R	S	S	R	R	R	R	R	0.81
SC49	S	R	R	S	S	S	S	S	S	S	R	0.27
SC50	R	R	R	R	S	S	R	R	I	R	R	0.72
SC51	R	R	I	I	R	S	R	I	S	S	R	0.45
SD52	R	R	I	I	R	I	R	R	S	S	R	0.54

Here in table 3.4; AX (10) = Amoxicillin (10µg), CL (30) = Cephalexin (30 µg), CXM (30) = Cefuroxime (30µg), CTR(30) = Ceftriaxone (30µg), CIP (5) = Ciprofloxacin (5µg), NX (10) = Norfloxacin (10µg), TE(30) = Tetracycline (30µg), COT (25) = Co-trimoxazole (25 µg), GEN (10) = Gentamicin (10 µg), C (30) = Chloramphenicol (30 µg), E (15) = Erythromycin (15 µg).

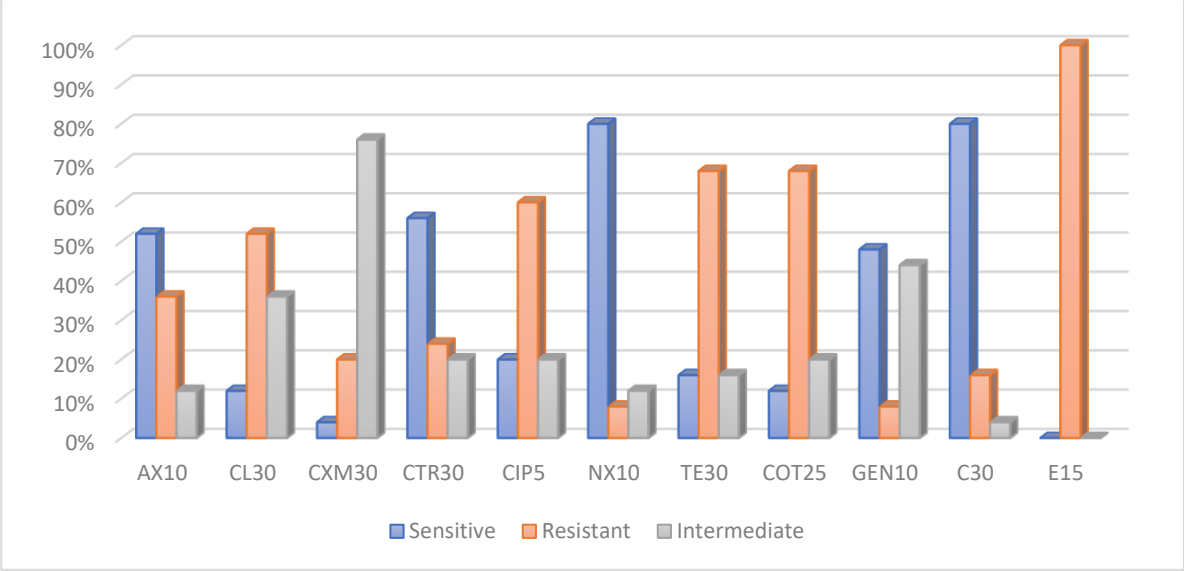
Table 3.5: Multiple Antibiotic Resistance (MAR) index of bacterial isolates

Bacterial species	Number of isolates (n)	MAR Index range	Isolates with MAR>0.2 (n%)
Escherichia coli	75	0.09-1	57(76%)
Klebsiella spp.	66	0.09-0.9	51(77.27%)
Pseudomonas spp.	9	0.18-0.81	7(77.77%)
Citrobacter spp.	6	0.18-0.54	4(66.66%)
Overall	156	0.09-1	119(76.28%)



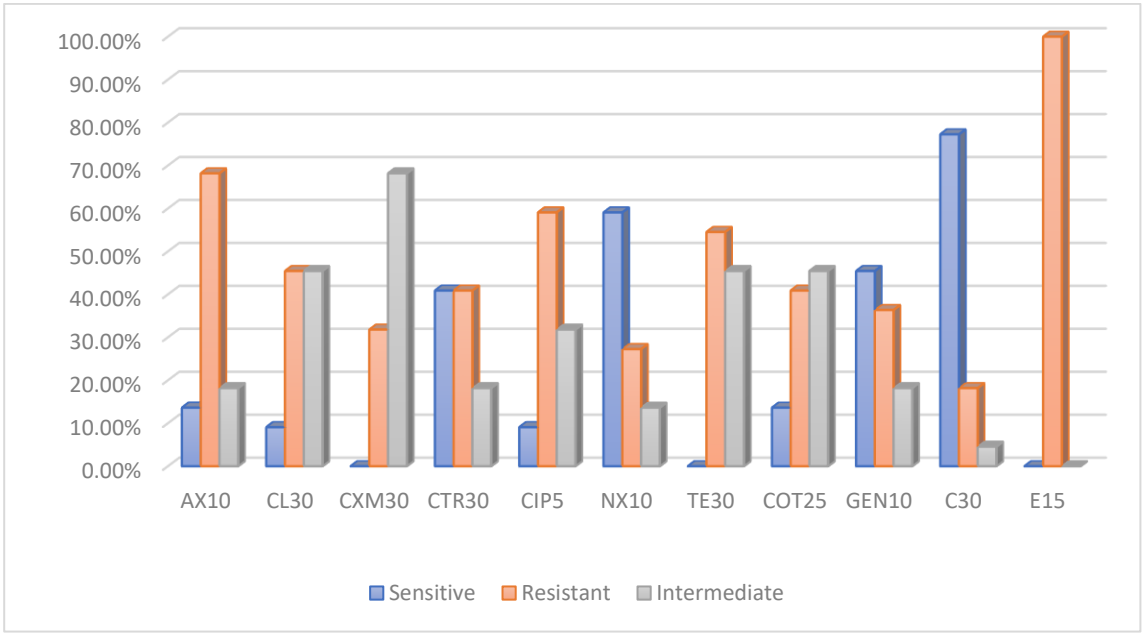
Antibiotics	AX10	CL30	CXM30	CTR30	CIP5	NX10	TE30	COT25	GEN10	C30	E15
Sensitive	51	15	3	72	30	111	15	21	75	120	0
Resistant	84	84	45	52	90	24	99	87	33	30	156
Intermediate	21	57	108	32	36	21	42	48	48	6	0

Figure 3.8: Diagrammatic representation of antibiotic pattern of all 156 isolates.



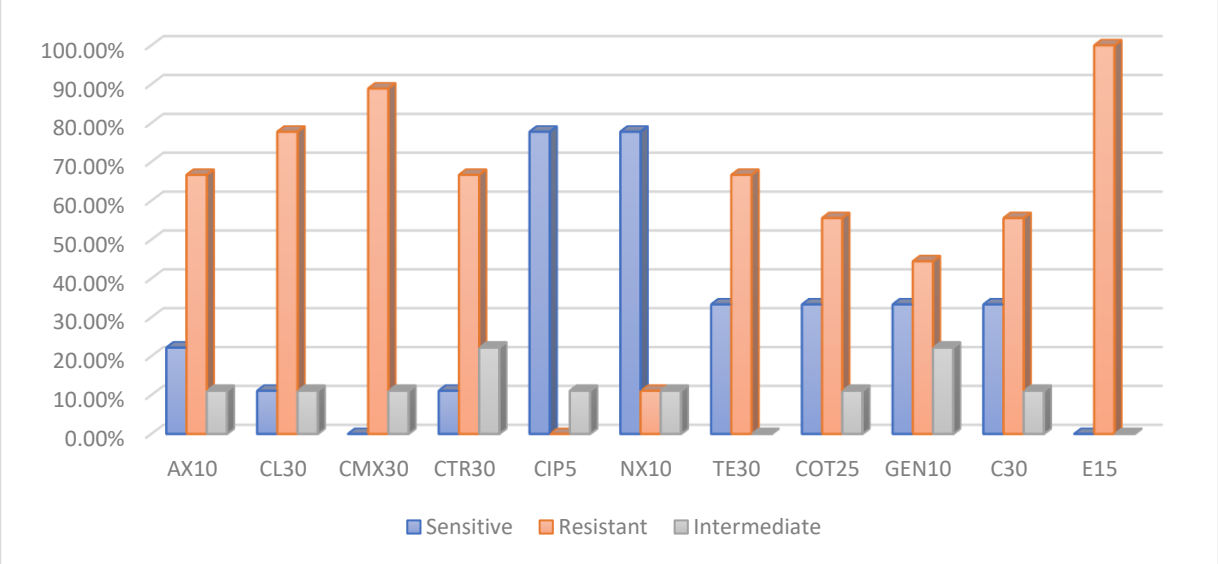
Antibiotics	AX10	CL30	CXM30	CTR30	CIP5	NX10	TE30	COT25	GEN10	C30	E15
Sensitive	52%	12%	4%	56%	20%	80%	16%	12%	48%	80%	0%
Resistant	36%	52%	20%	24%	60%	8%	68%	68%	8%	16%	100%
Intermediate	12%	36%	76%	20%	20%	12%	16%	20%	44%	4%	0%

Figure 3.9: Diagrammatic representation of antibiotic pattern of all presumptive isolates of *E. coli.*



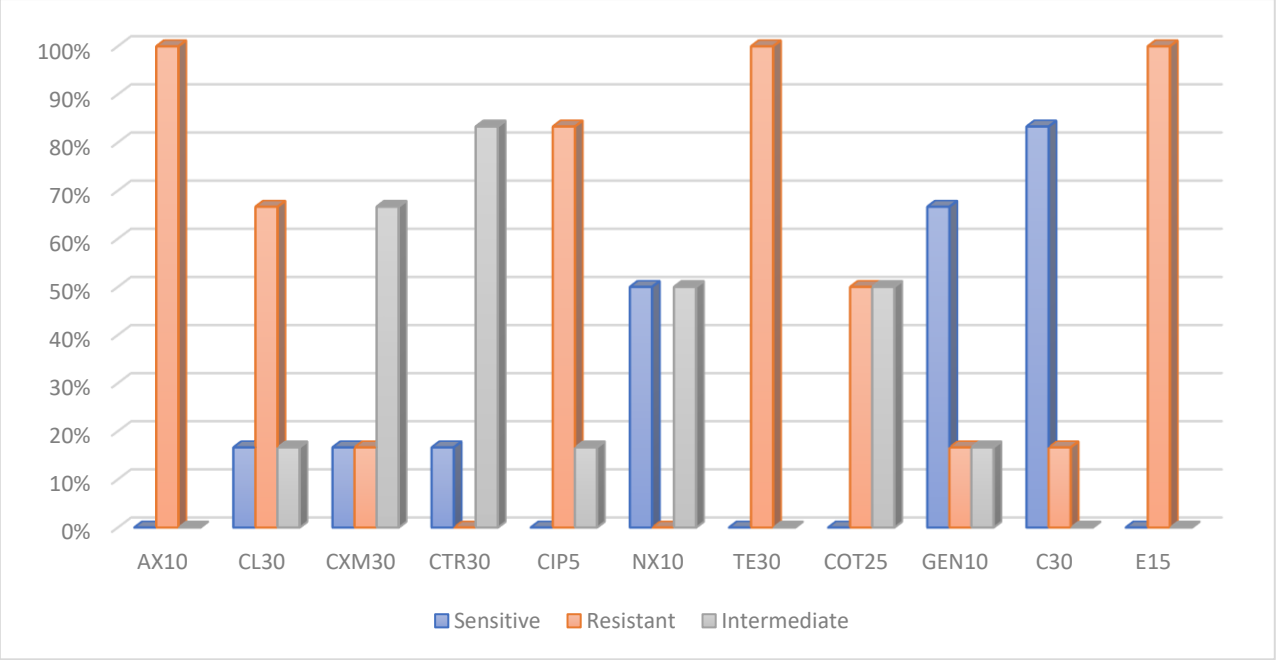
Antibiotics	AX10	CL30	CXM30	CTR30	CIP5	NX10	TE30	COT25	GEN10	C30	E15
Sensitive	13.64 %	9.09 %	0%	40.90 %	9.09 %	59.09 %	0%	13.63 %	45.45 %	77.27 %	0%
Resistant	68.18 %	45.45 %	31.81 %	40.90 %	59.09 %	27.27 %	54.54 %	40.90 %	36.36 %	18.18 %	100 %
Intermediate	18.18 %	45.45 %	68.18 %	18.18 %	31.81 %	13.63 %	45.45 %	45.45 %	18.18 %	4.55 %	0%

Figure 3.10: Diagrammatic representation of antibiotic pattern of all presumptive isolates of *Klebsiella spp.*



Antibiotics	AX10	CL30	CMX30	CTR30	CIP5	NX10	TE30	COT25	GEN10	C30	E15
Sensitive	22.22 %	11.11 %	0%	11.11 %	77.77 %	77.77 %	33.33 %	33.33 %	33.33 %	33%	0%
Resistant	66.66 %	77.77 %	88.88 %	66.66 %	0%	11.11 %	66.66 %	55.55 %	44.44 %	56%	100%
Intermediate	11.11 %	11.11 %	11.11 %	22.22 %	11.11 %	11.11 %	0%	11.11 %	22.22 %	11%	0%

Figure 3.11: Diagrammatic representation of antibiotic pattern of all isolates of presumptive *Pseudomonas spp*.



Antibiotics	AX10	CL30	CXM30	CTR30	CIP5	NX10	TE30	COT25	GEN10	C30	E15
Sensitive	0%	16.66%	16.66%	16.66%	0%	50%	0%	0%	66.66%	83.33%	0%
Resistant	100%	66.66%	16.66%	0%	83.33%	0%	100%	50%	16.66%	16.66%	100%
Intermediate	0%	16.66%	66.66%	83.33%	16.66%	50%	0%	50%	16.66%	0%	0%

Figure 3.12: Diagrammatic representation of antibiotic pattern of all isolates of presumptive *Citrobacter spp.*

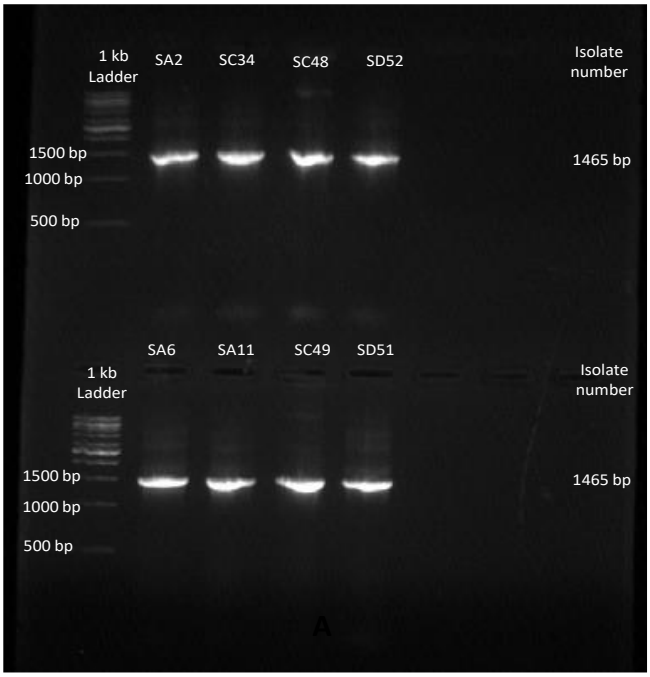
Here in figure 3.12, 3.13,3.14,3.15,3.16; AX (10) = Amoxicillin (10µg), CL (30) = Cephalexin (30 µg), CXM (30) = Cefuroxime (30µg), CTR(30) = Ceftriaxone (30µg), CIP (5) = Ciprofloxacin (5µg), NX (10) = Norfloxacin (10µg), TE(30) = Tetracycline (30µg), COT (25) = Co-trimoxazole (25 µg), GEN (10) = Gentamicin (10 µg), C (30) = Chloramphenicol (30 µg), E (15) = Erythromycin (15 µg).

3.2 Molecular characterization and Genotyping of the isolates

A total of 156 isolates of pet bird fecal and feed samples were subjected to conventional PCR for the identification of pathogenic gene and antibiotic resistance gene. The positive and negative band are visualized by Agarose gel electrophoresis technique.

3.2.1 Molecular identification and phylogenetic analysis

The phylogenetic analysis based on 16S rRNA gene sequences clearly demonstrated the evolutionary placement of the isolates obtained in this study within their respective bacterial lineages. The isolate ***Klebsiella spp*. SC34** clustered tightly with reference sequences of *Klebsiella pneumoniae* and *Klebsiella* sp. strains, supported by strong bootstrap values, indicating a close genetic relatedness and confirming its taxonomic identity. Similarly, ***Citrobacter spp*. SD52** formed a distinct cluster with *Citrobacter freundii* strain BAB-173 and other *Citrobacter* spp., validating the accuracy of its molecular identification. The ***E. coli* isolate SA2** grouped robustly within the *Escherichia coli* clade, showing high similarity to multiple *E. coli* strains reported in GenBank, which further supports its classification as *E. coli*. In the non-fermenter group, ***Pseudomonas spp*. SC48** aligned strongly with *Pseudomonas aeruginosa* reference sequences such as strains K3, HRO3, and AKRC18, indicating its close phylogenetic affinity to *P. aeruginosa*. Overall, the clustering patterns show clear separation between Enterobacteriaceae members (*Klebsiella*, *Citrobacter*, and *E. coli*) and the *Pseudomonas* lineage, reflecting their distinct evolutionary trajectories. High bootstrap values across major nodes provide strong confidence in the phylogenetic relationships inferred. These molecular findings corroborate the biochemical identification results and highlight the genetic diversity of bacterial isolates recovered from pet bird samples, demonstrating that molecular characterization effectively validates species-level identity and evolutionary relatedness among isolates



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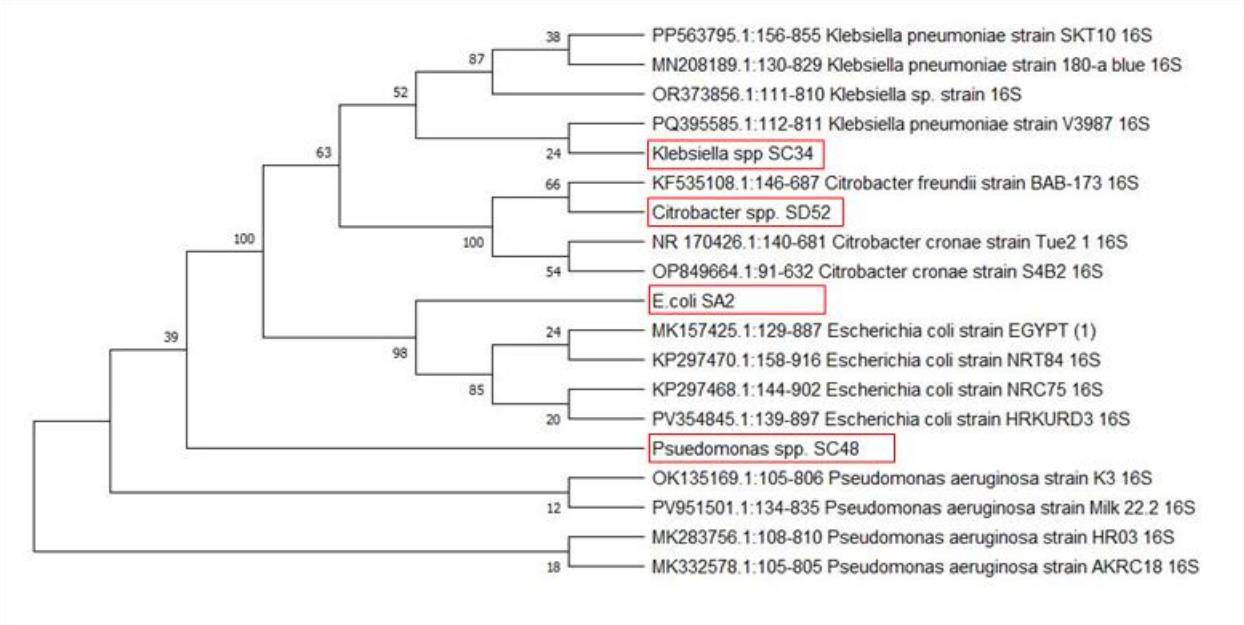


B

Figure 3.13: Gel electrophoresis of 16s rRNA gene amplification (A) and phylogenetic analysis of isolates based on 16s rRNA sequencing (B).

3.2.1 Molecular analysis of Virulence Genes

3.2.2.1 Prevalence of Virulence Genes in isolates of *Escherichia coli*

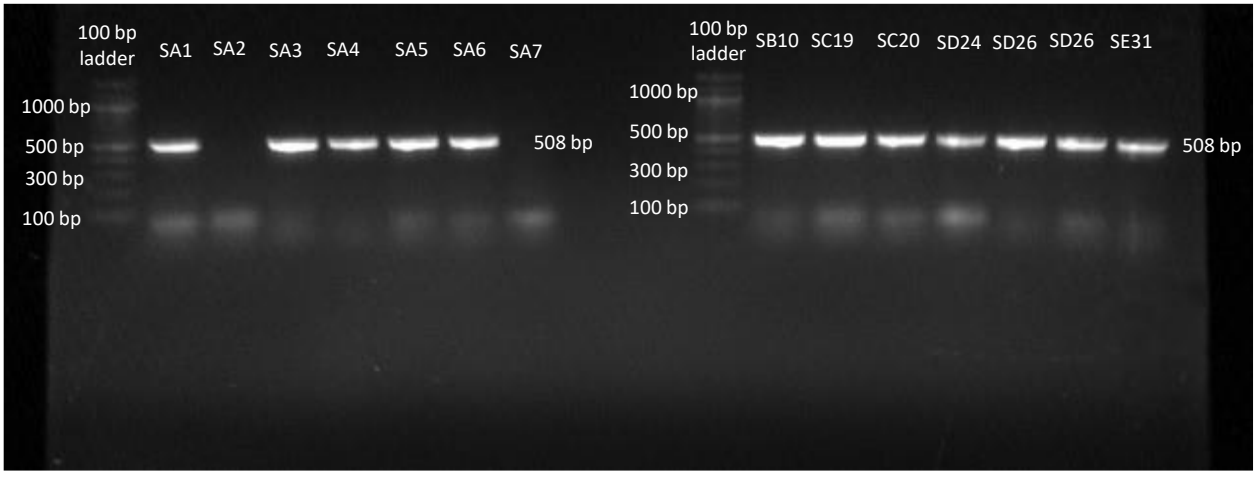
The molecular analysis of the 75 *Escherichia coli* isolates for virulence-associated genes yielded diverse outcomes. The *eaeA* gene which encodes intimin, an outer membrane protein necessary for intimate attachment was detected in 27 isolates (36%). The *fimH* gene which encodes the *fimH* adhesin was identified in 66 isolates (88%), while the *stx1* and *stx2* genes that encodes Shiga toxin was absent in the isolates.

Table 3.6: Outcome of PCR Amplification of *E. coli* Genes (*eaeA, fimH, stx1, stx2*)

Sample ID	*eaeA*	*fimH*	*stx1*	*stx2*
SA1	-	+	-	-
SA2	-	-	-	-
SA3	-	+	-	-
SA4	-	+	-	-
SA5	-	+	-	-
SA6	+	+	-	-
SA7	-	-	-	-
SB8	+	+	-	-
SB9	+	+	-	-
SB10	+	+	-	-
SC18	-	+	-	-
SC19	+	+	-	-
SC20	-	+	-	-
SC21	+	+	-	-
SC22	-	+	-	-
SD23	+	+		-
SD24	+	+	-	-
SD25	-	+	-	-
SD26	+	+	-	-
SD27	-	+	-	-
SD28	-	+	-	-

SE29	-	+	-	-
SE30	-	+	-	-
SE31	-	+	-	-
SE32	-	-	-	-
sSA1	-	+	-	-
sSA2	-	+	-	-
sSA3	+	+	-	-
sSA4	-	-	-	-
sSA5	+	+	-	-
sSA6	+	+	-	-
sSA7	-	+		-
sSB8	-	+	-	-
sSB9	-	+	-	-
sSB10	-	+	-	-
sSC18	+	+	-	-
sSC19	-	+	-	-
sSC20	-	+	-	-
sSC21	+	+	-	-
sSC22	-	-	-	-
sSD23	+	+	-	-
sSD24	-	+	-	-
sSD25	-	+	-	-
sSD26	+	+	-	-
sSD27	-	+	-	-
sSD28	-	-	-	-
sSE29	-	+	-	-
sSE30	+	+	-	-
sSE31	-	+	-	-
sSE32	+	+	-	-
SA105	-	+	-	-
SA106	-	+	-	-
SA107	+	+	-	-

SA108	-	+	-	-
SA109	+	+	-	-
SA110	-	+	-	-
SA111	-	-	-	-
SB112	+	-	-	-
SB113	-	+	-	-
SB114	-	+	-	-
SB115	+	+	-	-
SB116	-	+	-	-
SB117	-	+	-	-
SB118	-	+	-	-
SB119	-	+	-	-
SB120	+	+	-	-
SB121	+	+	-	-
SB122	-	+	-	-
SB123	-	-	-	-
SB124	+	+	-	-
SB125	-	+	-	-
SB126	-	+	-	-
SB127	-	+	-	-
SB128	+	+	-	-
SB129	+	+	-	-



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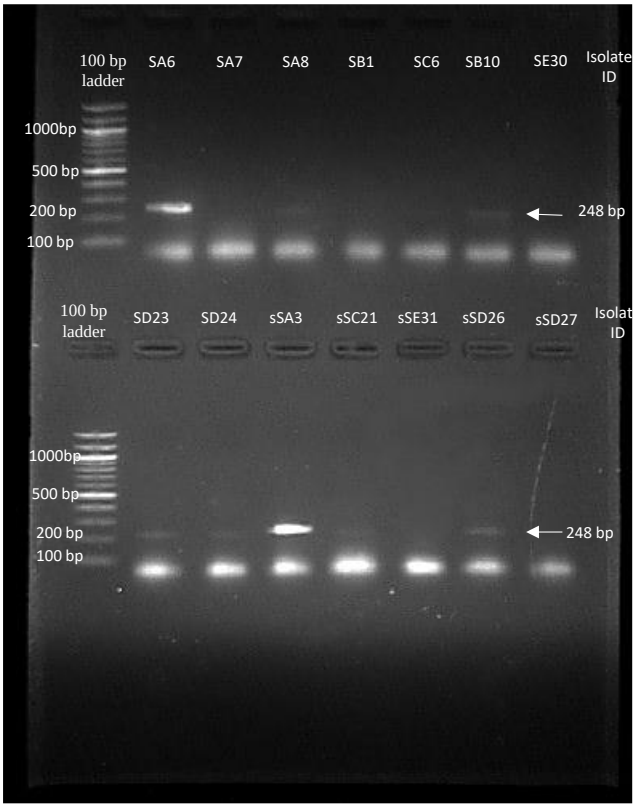


B

Figure 3.14: Gel electrophoresis of A) *fimH*, B) *eaeA* gene amplification; band size at 508 bp and 248 bp

3.2.2.2 Prevalence of Virulence Genes in isolates of *Klebsiella spp.*

The molecular analysis of the 66 isolates of *Klebsiella spp.* against the virulence *magA*, a gene associated with the K1 capsular serotype which encodes Wzy type polymerase that is necessary for the biosynthesis of the capsular polysaccharide leading to a hypermucoviscous phenotype, showed positive result for 12 isolates which indicates 18.18% isolates are *magA* positive. The isolates that showed positive result include ID no. SB17, SD43, SD44, SD45, S2A12, S2C38, S2D43, S2E47, SB138, SB141, SB144, SB149.

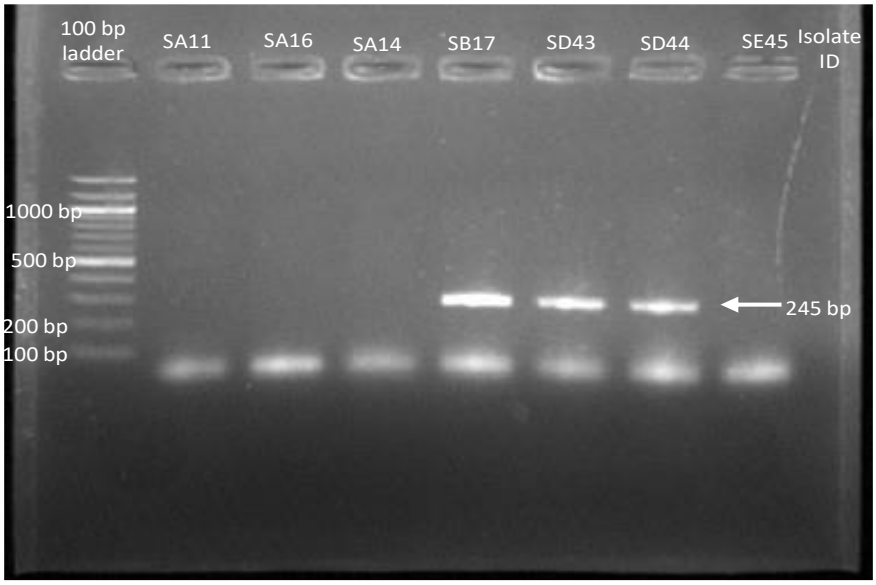


Figure 3.15: Gel electrophoresis of *magA* gene amplification; band size at 245 bp.

3.2.2.3 Prevalence of Virulence Genes in isolates of *Pseudomonas spp.*

The *lasB* gene in *Pseudomonas* species encodes for Elastase B, a key metalloprotease virulence factor that plays a crucial role in infections. This *lasB* gene was successfully amplified and sequenced from 5 *P. aeruginosa* isolates out of 9 *P. aeruginosa* isolates (SC48, SC50, S2C50, SB152, SB153), which is 55.55%.

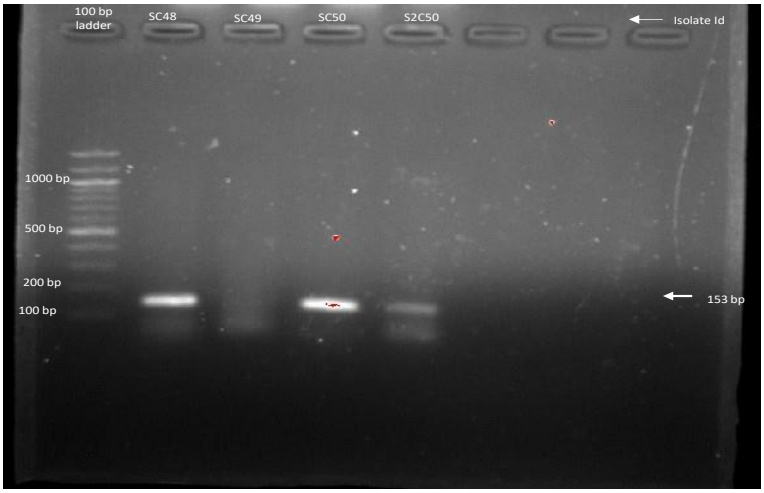


Figure 3.16: Gel electrophoresis of *lasB* gene amplification; band size at 153 bp.

3.2.2.4 Prevalence of Virulence Genes in isolates of *Citrobacter spp.*

The *invA* gene which is considered as a highly specific molecular marker for *Salmonella spp*., 2 *Citrobacter* isolate out of 6 isolates in this study amplified the *invA* target which is 33.33%.

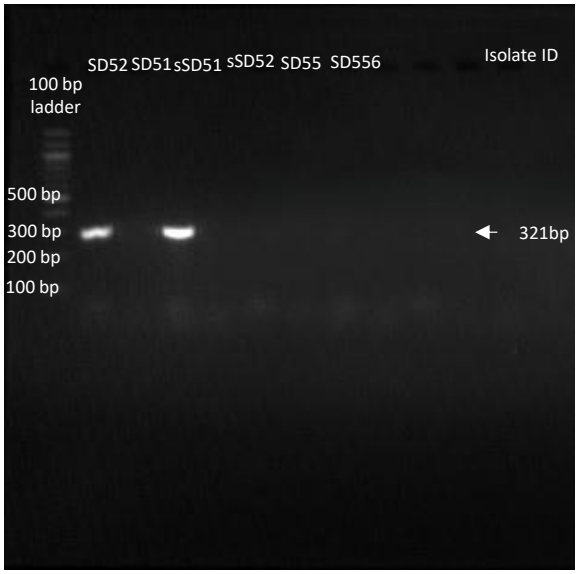


Figure 3.17: Gel electrophoresis of *invA* gene amplification; band size at 321 bp

3.2.3 Molecular analysis of Antibiotic Resistance Gene

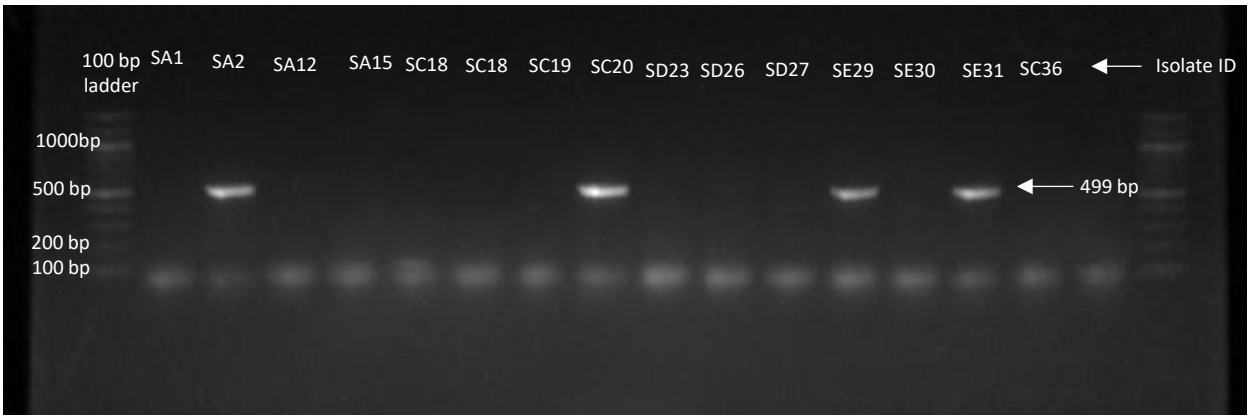
PCR based screening of antimicrobial resistance determinants revealed the presence of several clinically important β-lactamase and non–β-lactam resistance genes among all the 156 isolates of *E. coli*, *Klebsiella spp.*, *Pseudomonas spp.*, and *Citrobacter spp.* The antimicrobial resistant gene that were used are *blaCTX-M*, *blaTEM*, *tetA*, *sul1*. From the molecular analysis we found 46 isolates positive for *blaCTX-M* which is 29.49% of all isolates. Another ESBL gene *blaTEM* showed positive result for 123 isolates (78%). The *tetA* gene was positive for 99 isolates (63.46%) and lastly the *sul1* gene showed positive result for 64 isolates which is 41.02% of all isolates.

Table 3.7: Prevalence of *blaCTX-M*, *blaTEM*, *tetA*, *sul1* genes in one third of total isolates.

Isolate ID	*blaCTX-M*	*blaTEM*	*tetA*	*sul1*
SA1	-	+	-	-
SA2	+	+	+	+
SA3	-	-	+	-
SA4	-	+	-	-
SA5	-	-	+	-
SA6	-	+	-	-
SA7	+	+	-	-
SB8	+	+	+	+
SB9	-	+	+	+

SB10	-	+	+	-
SC18	-	-	+	+
SC19	-	+	-	-
SC20	+	+	+	+
SC21	-	+	+	-
SC22	-	+	+	-
SD23	-	-	+	-
SD24	-	+	-	-
SD25	-	+	-	+
SD26		-	+	-
SD27	-	+	+	+
SD28	-	+	+	-
SE29	+	+	-	-
SE30	-	+	-	-
SE31	+	+	+	+
SE32	-	+	+	+
SA11	-	+	+	-
SA12	+	+	-	+
SA13	-	+	+	-
SA14	+	+	+	-
SA15	-	+	-	-
SB16	+	-	+	-
SB17	+	+	+	+
SC33	+	-	-	-
SC34	+	+	+	+
SC35	-	-	-	-
SC36	-	+	+	-
SC37	-	+	+	+
SC38	-	+	+	+
SC39	-	-	-	-
SC40	-	+	-	-
SC41	-	+	+	-

SC42	-	+	+	+
SD43	-	-	+	-
SD44	-	+	-	+
SE45	-	+	+	-
SE46	-	+	+	-
SE47	+	-	-	-
SC48	+	+	+	+
SC49	-	-	+	+
SC50	+	+	-	+
SC51	-	+	-	-
SD52	+	+	+	+



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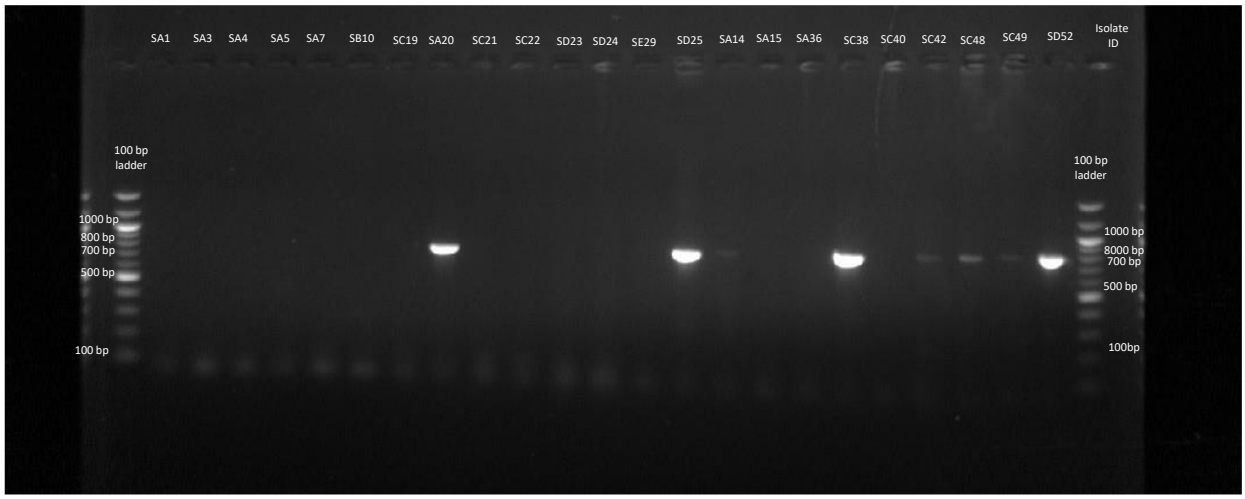

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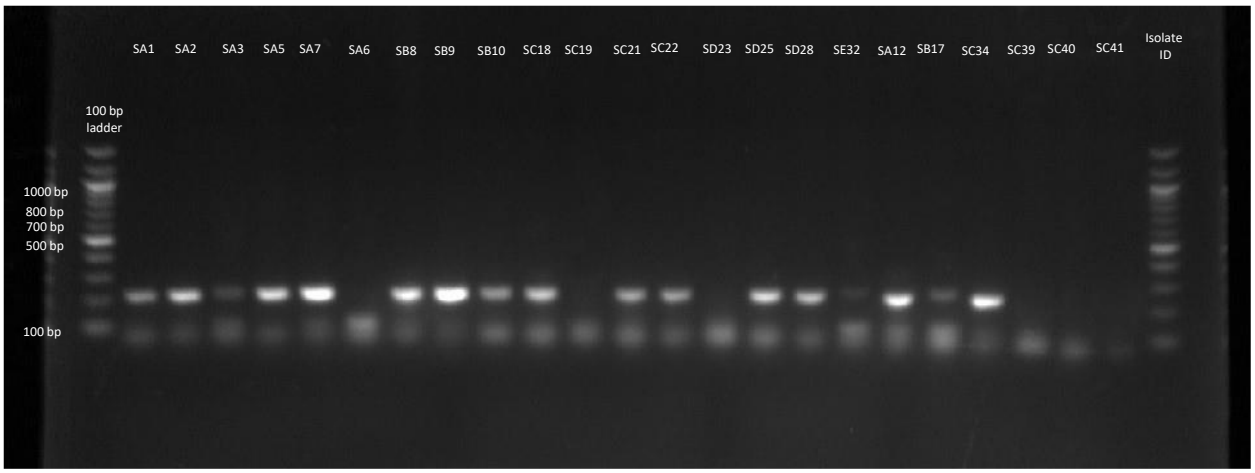

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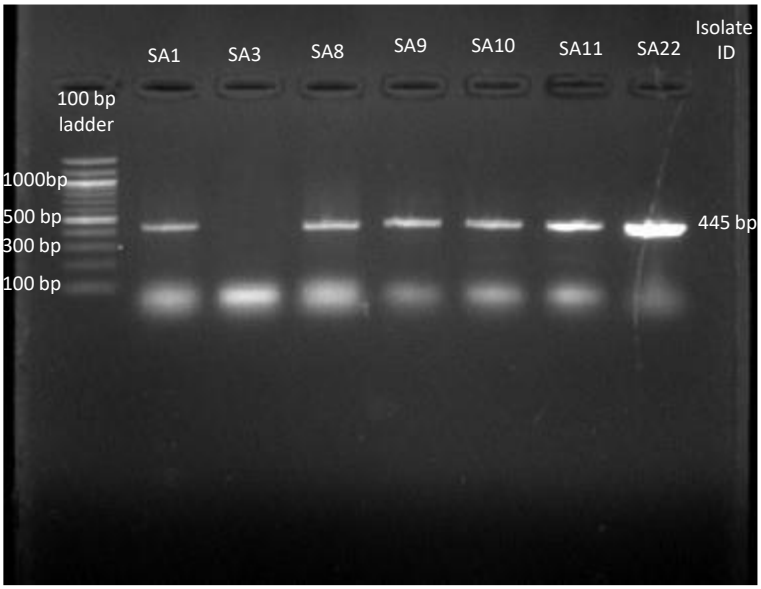
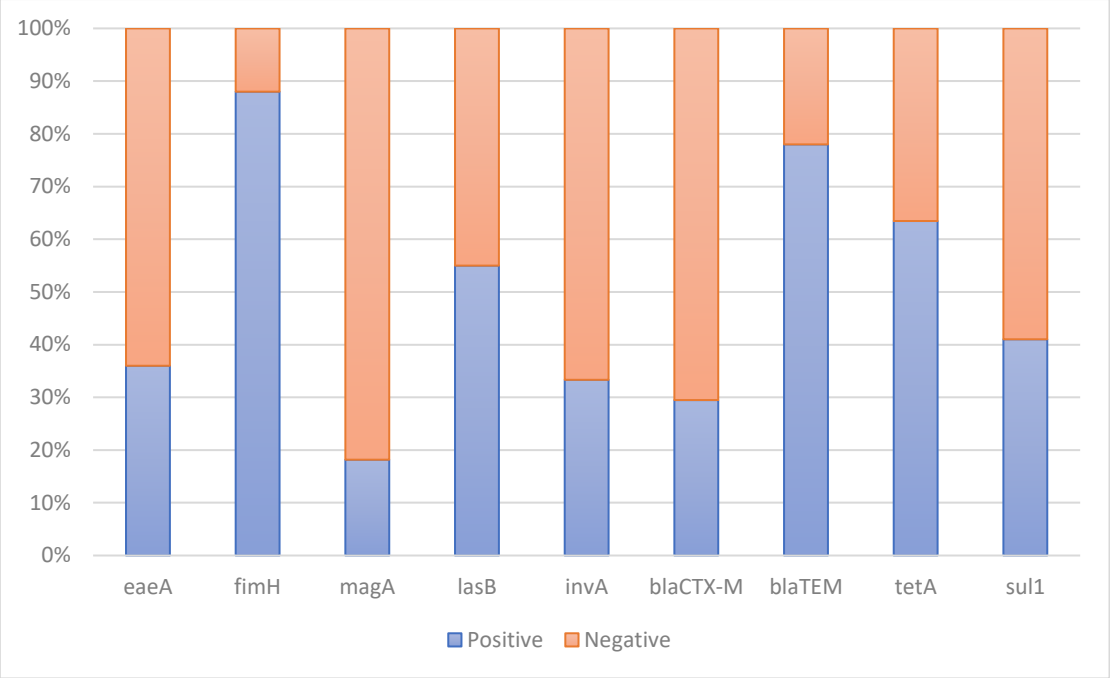


D

Figure 3.18: Gel electrophoresis of A) *blaCTX-M*, B*) sul1,* C) *tetA* and D) *blaTEM* gene amplification; band size at 499bp, 780bp, 225bp, 445bp.

Table 3.8: Summary of Virulence and AMR gene prevalence among isolates.

Gene	Function	*E. coli*	*Klebsiella spp.*	*Pseudomonas spp.*	*Citrobacter spp.*
bla-CTXM	ESBL (third-gen cephalosporins)	18(24%)	21(31.81%)	6(66.67%)	3(50%)
blaTEM	ESBL / broad β-lactam resistance	60(80%)	51(77.27%)	6(66.67%)	6(100%)
tetA	Tetracycline resistance	49(65.33%)	42(63%)	5(55.56%)	3(50%)
sul1	Sulfonamide resistance	29(38.67%)	25(37.88%)	8(88.87%)	2(33.33%)
eaeA	EPEC virulence gene	27(36%)	-	-	-
stx1 & *stx2*	Shiga toxin producing gene	-	-	-	-
fimH	Adhesion gene	66(88%)			
magA	Hypervirulence	-	12(18.18%)	-	-
lasB	Elastase (Pseudomonas)	-	-	5(55.55%)	-
invA	*Salmonella* virulence marker	-	-	-	2(33.33%)
16S rRNA sequencing	Species confirmation	Done	Done	Done	Done



Target gene	*eaeA*	*fimH*	*magA*	*lasB*	*invA*	*blaCTX-M*	*blaTEM*	*tetA*	*sul1*
Positive	36%	88%	18.18 %	55.55 %	33.33 %	29.49%	78%	63.46 %	41.02 %
Negative	64%	12%	81.82 %	45.45 %	66.67 %	70.51%	22%	36.54 %	58.98 %

Figure 3.19: Diagrammatic representation of positive and negative pattern of Pathogenic and antibiotic resistance gene in the isolates.

Chapter 4

Discussion

The current experiment described the isolations of feces derived bacteria in pet birds, in terms of markers of pathogenicity, antimicrobial resistance trends, and MAR (Multiple antibiotic resistance) indexes. The most valuable organisms that were retrieved included *E. coli*, *Klebsiella spp.*, *Pseudomonas spp*., and *Citrobacter spp.* They are generally acknowledged belonging to the Enterobacteriaceae and Pseudomonadales, which is often related to the intestinal colonization and the zoonotic transmission. They also pose a risk to the public health as a result of the intimate contact of humans with the birds and the quality of the environment in which they are found. The observation of multiple Gram-negative bacterial species is consistent with earlier findings that show that micro-animals such as pet birds serve as a reservoir of both opportunistic and pathogenic bacteria (Hoque et al., 2020). Enteric bacteria including, *E. coli*, *Klebsiella spp.*, and *Citrobacter spp.* are often excreted in droppings and may contaminate cages, fooders and common household surfaces. The presence of *Pseudomonas spp*., which has a well-established ability to colonize wet environment, remains in line with the current results reported in the previous study, which finds drinking water pots and wet cages to be optimal environments, particularly due to this organism (Pang et al., 2019). The occurrence of mixed infections in multiple specimens serves as an additional indication of the microbial diversity of the avian gastrointestinal tracts and the possibility of cross-species interaction and gene transfer-a factor to be considered when considering the issue of antimicrobial resistance. The status of PCR screening revealed a series of virulence-related genes, which were dissimilar in species, which validated the assumption that pathogenicity characteristics heterogeneously appear within the enteric bacterial isolates. The *eaeA* (36%), and *fimH* (88) positivity represent the possible existence of potentially pathogenic lineages of *E. coli* and are linked to attaching-effacing strains, which may cause a gastrointestinal disease (Ovi et al., 2023), or ubiquitous virulence factors, commonly associated with extra-intestinal pathogenic *E. coli* (XPEC) (Kathayat et al., 2021). These genes are recovered in pet bird isolates, which agrees with earlier research that has indicated that birds can be carriers of zoonotic-potential strains (Guo et al., 2020). The observation of *magA*(18.18%), in relation to hypermucoid phenotypes and severe infections, indicates that hyperviralent attributes might be held by part of the isolates. Despite being related mostly to human disease, *magA*-positive isolates can be found in environmental and animal reservoirs, which confirms the potential of cross-host transmission (Raza et al., 2017). Pathogenic potential in *Pseudomonas spp*. is confirmed by the presence of *lasB* (55.55%) which encodes elastase. This

enzyme also helps in damage of tissues and immune evasion (Salam et al., 2023). Its identification indicated that the factors which apply to human disease may be deposited even in isolated pet bird environmental strains. Determination of *invA* in *Citrobacter spp.* is not common, but not impossible because horizontal gene transfer to an Enterobacteriaceae may result in the acquisition of *Salmonella* -type genes (Raziq et al., 2025b). The gene presents the ability to cause invasion and can overamplify under the influence of conserved areas amongst Gram-negative enteric pathogens. The findings could thus be explained as: potential horizontal gene transfer, existence of *Salmonella* -like pathogenicity islands or primer set cross-reactivity. This is a significant, yet a prudent finding that should be reported. AMR-associated genes have been detected which supports the phenotypic resistance pattern and there is a genetic reservoir within the genotype of microbiota in pet birds. Enterobacteriaceae with *bla-CTX-M* (29.49%) and *bla-TEM* (78%) are associated with world trends of ESBL-producing organisms in human and animals (Dallenne et al., 2010). Their presence implies an environmental and domestic exposure to β-lactam antibiotics or a response of human handlers. The common occurrence of tet (63.46%) and *sul1*(41.02%) genes by enteric isolates is consistent with the long and extensive use of tetracyclines and sulfonamides in veterinary and agricultural practices. Their ability to remain in commensal gut flora is very well established (Chopra & Roberts, 2001). The antibiogram data showed that it was found to be resistant to a wide range of antibiotic classes. Enterobacteriaceae are likely to be high resistant to ampicillin (AX) and cephalexin (CL), and they usually express β-lactamases. The resistance of *E. coli*, *Klebsiella*, and *Citrobacter* to fluoroquinolone (ciprofloxacin, norfloxacin) is associated with the existence of ESBL genes, where the ESBL producers consistently co-exist with the presence of plasmid-mediated quinolone resistance (PMQR) genes (Robicsek et al., 2006). There was a relatively stronger activity with aminoglycosides (gentamicin), congruent with international information of reduced baseline aminoglycoside resistance among birds. In *Pseudomonas spp*. cephalosporin and aminoglycoside resistance mechanisms are known to utilize intrinsic and acquired resistance mechanisms. The value of MAR index became 76.28% of the total number of isolates having value above 0.2 reflecting high selection pressure on antibiotics. MAR index greater than 0.2 is an indication of exposure to high-risk conditions where antibiotics are widely used (Elshobary et al., 2025). The high MAR value of the isolates obtained in feces and in feed is a pointer of consistent or repeated exposure to antibiotics, probably as a result of the indiscriminate use of antibiotics during the rearing of pet birds or as a result of the entry of the antibiotics through contaminated feed sources. The discussion based on the interpretation of MAR index is a strong argument that pet birds could also contaminate not only households but also serve as reservoirs and transmitters of MDR bacteria. The overall coexistence of virulence factors, AMR genes, and multi-drug resistance, combined with high indices of MAR,

further highlights the zoonotic hazard of the bacteria that are transported by pet birds. The results are congruent with the One Health perspective, and birds have been identified as key contributors to the environmental AMR pool (Monteiro et al., 2025). The results indicate that children, older people, and immunocompromised people are particularly susceptible. AMR bacteria can be transmitted through bird cages, water bowls, and feed, as the same studies carried out in Asia and Europe proved (Raza et al., 2017). ESBL genes suggest that assessing the role at the community level of AMR flow may be carried out by the pet birds because of their high values of MAR. Hygiene issues within pet stores, the misuse of antibiotics and infectious food supplies could all lead to the development and sustenance of resistant bacterial strains

Chapter 5

5.0 Conclusions

The paper examined the presence of antimicrobial-resistant and pathogenic bacteria in the feces of pet birds. Four bacterial genera, including *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas spp*, and *Citrobacter spp.* were successfully isolated and identified by culture, biochemical, and molecular biomarker assays. The results point to the fact that pet birds can be an important source of bacteria that have zoonotic and antimicrobial resistance potential. Significant percentage of isolates had virulence-related genes, such as *fimH* and *eaeA* in *E. coli*, and *lasB* in *Pseudomonas*. These genes denote increased colonization power and pathogenicity. *InvA* present in the Citrobacter isolates is an indication of potential horizontal genetic transfer, which has been identified in earlier studies, and indicates the dynamic nature of the genetic exchange at the mixed microbial setting. The research also found out that there is a prevalence of antimicrobial resistance. Most of the isolates were also resistant to broad spectrum antibiotics like amoxicillin, cephalosporins, tetracycline and sulfonamides. The molecular basis of the observed resistance is proved by the presence of the resistance genes *bla-TEM*, *bla-CTX-M*, *tetA, sul1*, and vim. Some of the isolates were found to be multidrug resistant (MDR), that is, they were resistant to three or more classes of antibiotics. This underscores the increasing worry of the effective use of resistant bacteria within non-clinical settings. These findings were further supported with the Multiple antibiotic resistance (MAR) index. Most of the isolates were found to have MAR values greater than 0.2 which was a sign of exposure to high-risk settings in which antibiotics are being abused or overused. These conditions favor the process of isolating and infecting of resistant strains of bacteria. All in all, the research demonstrates that pet birds have a high probability of carrying a clinically significant bacteria with pathogenic and antimicrobial-resistant properties. These results highlight the importance of hygiene improvement in handling birds, responsible administration of antibiotics, and periodic monitoring through a one health approach. To reduce threats to public health, it is necessary to improve awareness of bird owners and handlers of pet shops. Even with the limited sample size and lack of more sophisticated genomic techniques, the study provided a good basis information that can be used in subsequent surveillance and studies.

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Appendix I

Unless otherwise mentioned, all media were sterilized by autoclaving at 1210C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this study has given below:

Media composition:

XLD

Ingredients	Amount (g/L)
L-lysine	5.0
Yeast Extract	3.0
D-Lactose	7.5
Xylose	3.5
Saccharose	7.5
Sodium Desoxycholate	2.5
Ferric ammonium citrate	0.8
Sodium Thiosulfate	6.8
Sodium chloride	5.0
Agar	13.5
Phenol red	0.08

MacConkey Agar Media

Ingredients	Amount (g/L)
Peptone	20.0
Lactos	10.0
Bile salts	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
pH	7.1

RVS media

Component	Amount (per 1 L)
Sodium Chloride (NaCl)	8.0 g
Sodium Phosphate, Monobasic (NaH_2PO_4)	1.6 g
Potassium Dihydrogen Phosphate (KH_2PO_4)	0.4 g
Magnesium Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$)	13.58 g
Malachite Green Oxalate	0.04 g
Peptone	4.5 g

Cetrimide Agar

Component	Amount (per 1 L)
Pancreatic Digest of Gelatin	20.0 g
Magnesium Chloride ($MgCl_2$)	1.4 g
Potassium Sulfate (K_2SO_4)	10.0 g
Cetrimide (Cetyltrimethylammonium Bromide)	0.3 g
Agar	13.6–15.0 g

Simmons Citrate Agar

Component	**Amount (per 1 L)**
Sodium Citrate	2.0 g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
NaCl	5.0 g

$MgSO_4 \cdot 7H_2O$	0.2 g
Bromothymol Blue	0.08 g
Agar	15.0–20.0 g

Muller Hinton Agar

Component	**Amount (per 1 L)**
Beef Infusion Solids	4.0
Starch	1.5
Casein	17.5
Agar	15.0
pH	7.0

Tryptone Soya Agar

Component	**Amount (per 1 L)**
Pancreatic Digest of casein	15.0
Enzymatic digest of soya bean	5.0
NaCl	5.0
Agar	15.0
pH	7.3

Appendix III

Apparatus Used

Apparatus	**Model / Details**	**Brand / Country**
Autoclave	Model HL-42E	Tokyo, Japan
Centrifuge machine	—	Sigma, USA
Class-1A biological safety cabinet	—	Thermo Forma, USA
Duram bottle	—	Scott, Germany

Electric balance	Model 210S	Sartorius, Germany
Eppendorf tubes (1.5 ml)	—	Eppendorf, Germany
Freezer	–30°C	Thermo Forma, USA
Fridge	4°C	West Host
Fridge	8°C, Model MIR-253	Memmert, Germany
Gel Documentation system	—	Bio-Rad, USA
Incubator	—	Memmert, Germany
Incubator	WTB Binder, Model D-78502	Germany
Glassware	—	Pyrex, USA
Magnetic stirrer	—	Coming, UK
Micro Eppendorf centrifuge	—	Eppendorf, Germany
Micropipettes	—	Lab Systems, Finland
Micropipette tips	—	China
Microscope	Liezacoan 2000	Samsung, Korea
Microwave oven	Model CE2933N	Samsung, Korea
PCR machine	—	MJ Research
Power supply	—	Bio-Rad, USA
pH meter	Model MP220	Toledo, Germany
Shaker incubator	—	Thermo Forma, USA
Sterilizer	—	Memmert, Germany
Vortex mixer	—	Fisher Brand, England