ISOLATION, IDENTIFICATION & MOLECULAR CHARACTERIZATION OF *Escherichia coli* AND *Salmonella* spp. FROM FECAL MATERIALS OF POPULAR PET BIRDS

A Thesis

By

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MASTER OF SCIENCE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

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CERTIFICATE

This is to certify that the thesis entitled "ISOLATION, IDENTIFICATION & MOLECULAR CHARACTERIZATION OF E. coli AND Salmonella spp. FROM FECAL MATERIALS OF POPULAR PET BIRDS' 'submitted to the Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of Master of Science in Microbiology ,embodies the result of a piece of bona fide research work carried out by Nipu Sen , Registration No. 13-05709 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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Place: Dhaka, Bangladesh

DEDICATED TO MY BELOVED PARENTS

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The Author

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LIST OF ABBREVIATION AND SYMBOLS

ABBREVIATION	FULL WORD
AMP	Ampicillin
AMX	Amoxicillin
Approx	Approximately
AZM	Azithromycin
CFU	Colony Forming Unit
CIP	Ciprofloxacin
DNA	Deoxyribonucleic acid
EMB	Eosin Methylene Blue
ERY	Erythromycin
et al.	and others
etc.	Etcetra
Fig	Figure
GM	Gentamicin
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen Sulphide
hrs	Hours
µg	Microgram
µl	Microlitre
w/v	Weight by volume

LIST OF ABBREVIATION AND SYMBOLS (CONT'D)

ABBREVIATION	FULL WORD
IN	Intermediate
MC	MacConkey
Mg	Milligram
MR	Methyl Red
NCCLS	National Committee for Clinical Standard
NIB	National Institute of Biotechnology
PCR	Polymerase chain Reaction
PBS	Phosphate buffered solution
R	Resistant
Rpm	Revolution Per Minute
S	Sensitive
SAU	Sher-e-Bangla Agricultural University
Sp	Species
SS	Salmonella Shigela
SLT	Shiga-like toxin
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TET	Tetracycline
TSI	Tripple Sugar Iron
UV	Ultraviolet
V	Voltage
VP	Voges-Proskawer
yrs.	Years
°C	Degree Celsius
%	Percentage
~	Tilde
+	Positive

ISOLATION, IDENTIFICATION & MOLECULAR CHARACTERIZATION OF *Escherichia coli* AND *Salmonella* spp. FROM FECAL MATERIALS OF POPULAR PET BIRDS

ABSTRACT

Recently, rearing of pet birds have increased with the increased socio-economic condition of the citizen. These pets population may contribute many zoonotic bacteria to public health. *Salmonella* spp. and *E. coli* are considered as the most common zoonoses in the world, causing important losses to public health. Considering these significances, this study assessed the occurrence of *Salmonella* spp. and *Escherichia coli* in caged Pigeon, Budgerigar & Lovebird. A total of 45 fecal samples were collected as environmental sample from these 3 different species of birds from pet shops of Katabon, Dhaka. Twenty samples from Pigeon (P-1 to P-20), Fifteen samples from Budgerigar (B-1 to B-15) & Ten samples from Lovebird (L-1 to L-10) were collected. Birds with positive cases of *E. coli* were found 22 (48.8%) & positive cases of *Salmonella* spp. were found 8 (17.7%). Rest 15 (33.33%) samples were unidentified. The occurrence of *E. coli* were 7 (35%), 11 (73.33%) and 4 (40%) in case of pigeon, budgerigar and lovebird respectively. The occurrence of *Salmonella* spp. in pigeon was 40%. No isolates of *Salmonella* spp. were found in budgerigar & lovebird. The antibiotic study revealed that all the isolates of *E. coli* were 100% sensitive to Ciprofloxacin, Norfloxacin, Amikacin, Azithromycin & Gentamicin. All the isolates of *E. coli* were 100% resistant to Tetracyclin, Ampicillin & Colistin sulphate. All the isolates of *Salmonella* spp. were 100% susceptible to Amoxicillin, Amikacin & Gentamicin and showed 100% resistance to Tetracycline, Trimethoprim & Colistin sulphate. These results analyze that birds that were analyzed may carry and spread these enterobacteria, and preventive measures for human exposure should be determined, as these microorganisms are public health concerns.

Key words: Pet birds, *Escherichia coli, Salmonella* spp., Zoonosis

CHAPTER-1

INTRODUCTION

Birds are increasingly close to people and many times they are raised as pets. Moreover, they can be beneficial to communities as sources of income from the pet trade economically. The domesticated Budgerigar, Lovebird, Pigeon are the most popular of all pet bird species and widely reared in cage. Pet birds are the source of recreation for human especially for children. Pet birds are excellent companion animals and can form close, affectionate bonds with their owners. These birds as household pets are a hobby and give much pleasure (Forshaw, 1973).

However, pet birds are often suffered from many bacterial diseases with often involvement of normal flora or environmental pathogens in response to stress and immunosuppression. It is known that Psittaciformes are capable of harboring numerous emerging zoonotic pathogens, as well as dispersing infected arthropod vectors (Godoy, 2007). Thus, the presence of Gram-negative bacteria, including those belonging to the Enterobacteriaceae family, in their intestinal microbiota has been considered an indication of potential diseases (Bangert *et al*., 1988; Mattes *et al*., 2005). Currently, microbiological studies with psittacine have increasingly isolated enterobacteria in healthy birds (Serafini *et al*., 2015; Lopes *et al*., 2015; Machado *et al*., 2016) which may indicate a more opportunistic role of these agents (Hidasi *et al*., 2013).

Birds have long played a significant role in human disease, specifically in spreading microbial pathogens (Belshe *et al*., 1998; Reed *et al*., 2003; Johnson *et al*., 2007; Moulin-Schouleur *et al*., 2007). Many zoonotic diseases are transferred from cage or pet birds to human through direct or indirect associations of the carrier or diseased birds. A significant public health threat can be caused by zoonotic pathogens, particularly with regard to bacterial diseases (Taylor *et al*., 2001; Jones *et al*., 2008). The close contact between household pets and people offers favourable conditions for bacterial transmission. Birds, with their broad geographic ranges and close contact with humans, have historically played an important role as reservoirs for drug-resistant bacteria and as carriers of human disease.

First of all, they are able to migrate long distances, colonize new areas, and withstand a range of environments allows for a global distribution (Fournier *et al*., 2000; Rappole *et al*., 2000; Humair *et al*., 2002; Winker *et al*., 2007; Benskin *et al*., 2009; Altizer *et al*., 2011). Secondly, the close association of birds and humans in urban and agricultural area facilitates zoonotic disease transfer (Waters *et al*., 1991; Marzluff *et al*., 2001; Capua and Alexander, 2002; McKinney *et al*., 2002; Atterby *et al*., 2016). Thirdly, birds and humans are host to some of the common bacteria species, many of which are pathogenic. Finally, both domestic agricultural and wild birds can contaminate shared areas and cause human infections (Sacks *et al*., 1986; Graczyk *et al*., 2008; Bonnedahl *et al*., 2009; Ewers *et al*., 2009; Vincent *et al*., 2010; Bonnedahl and Järhult, 2014).

There is a much progress in their diseases studies; the alimentary system was the most concerned system in these studies because of large number of its bacterial isolates (Baker *et al*., 1996). Few surveys were established to detect the normal gastrointestinal tract flora of psittacine birds (all birds commonly known as parrots, cockatoos, cockatiels, macaws, parakeets, lovebirds, lories or lorikeets, and other birds of the order psittaciforme, may also be called hookbills because the upper beak is turned downward) (Flammer and Drewes, 1988). Bacterial enteritis is an Important disease in psittacine birds either a primary intestinal problem or a systemic disease manifestation (Minsky and Petrak, 1982). Bacterial enteritis is often a spontaneous stress associated disease caused mainly by *E. coli, Klebsiella, Salmonella, Pasteurella, Pseudomonas, Aeromonas* and *Citrobacter* (Altman and Robert, 1997). Most of the enteric Salmonellae (*Salmonella Typhimurium, Salmonella enteritidis*) are motile and classified as paratyphoid organisms and the diseases they produce are termed paratyphoid infections (Kirk *et al*., 2002). The CDC recommendations, which were developed in conjunction with the National Association of State Public Health Veterinarians, include sections on the educational responsibilities of venue operators, managing public and animal contact, and animal care and management (CDC, 2005).

With possible increased bacterial migration rates between individuals, antibiotic resistance is also forecasted to evolve and spread rapidly (Perron *et al*., 2007).While zoonotic transmission of pathogens from birds to humans has been more difficult to quantify than conspecific transmission (Tsiodras *et al*., 2008), emerging infectious diseases are predicted

to occur primarily through zoonotic transmission (Jones *et al*., 2008). This, coupled with the large percentage of bacterial pathogens (38%, Taylor *et al*., 2001), makes understanding associations of different bacteria and bird species valuable to public health efforts to combat infectious disease (Kruse *et al*., 2004; Vouga and Greub, 2016). The potentially urgent public health threat of bird-borne infectious diseases suggests that now is the right time to assess bird-bacteria associations.

Bacteria are one of the most common causes of zoonotic diseases. For this, proper isolation, identification and characterization of the bacteria are essential to control zoonotic diseases. Cloacal swabs and faecal samples is a common practice for bacteriological culture used in the routine avian examination (Flammer and Drewes, 1988). Very few works have been studied on the isolation and identification of bacteria from caged birds in Bangladesh and the present study, therefore, was undertaken. Diagnosis of these enterobacteria is based on the isolation of the pathogen in cultures of feces, blood, and urine. Another method used in the detection of enterobacteria is the polymerase chain reaction, PCR (Herrera and Jabib, 2015).

With a great consideration given to the above facts the objectives of the present study were:

- Isolation and identification of bacteria from pet birds fecal materials.
- Determination of antibiotic sensitivity pattern of the isolated bacteria.
- Molecular characterization of the isolated pathogens by PCR and sequencing.

CHAPTER-2

REVIEW OF LITERATURE

Review of related literature is necessity in the sense that it provides scope for reviewing the stock of knowledge and information relevant to the proposed research. Despite the fact that a few numbers of works have been done in Bangladesh related to this research , there are some published reports and related vactivities. However, the limited number of work so far published are mentioned here along with other related works. A short description on the available literature relevant to the present investigation has been presented below:

2.1 Pet birds

The term "Pet bird" designates birds housed and bred for an exclusively ornamental use. This includes to mainly passeriformes (e.g. canaries, finches, sparrows etc.also called songbirds, and psittaciformes (parrots, parakeets, budgerigars, love birds etc.) (Nigel *et al*., 2000; Dorrestein *et al*., 2009; Tully *et al*., 2009).

Many families keep their "kitchen pet bird", which represents a lucrative business for pet shops or local breeders, as a single male canary is sold around 30 euros in Belgium and a female around 20 euros. Prices are about the same for zebra finches or budgerigars, and 50% to 100% higher for "special" finches like Gould diamonds. Bird fairs and live bird markets also gather a lot of people. In addition, some species are bred for their very high value; such as in the case of canaries, male and female breeding stock reproducers with recognized genetic potential are presented in national and international contests for their posture, their color or for their song. As a consequence, their offspring could be sold at high for rising prices. Finally, exotic birds like greater psittaciforms (parrots, e.g. cockatoo), legally or illegally traded from for example Asia or South America, remain high in the ranking of popular pets and are also profusely represented in zoos and parks.

Greater contact with men is due to the fact that these birds may be kept as pets (Passeriformes and Psittaciformes) or their abundant presence in public places, such as parks (Columbiformes and Passeriformes). Although, primary bacterial infections are not only a common cause of disease in parrots and a better understanding of normal microbial flora may help in interpreting the significance of bacterial isolates in sick birds (Bangert *et*

al., 1988; Lamberski *et al*., 2003). A knowledge of normal bacterial flora is also important for identifying potential pathogens that may cause disease during times of stress or immune suppression (Petrak *et al*., 1982).

2.2 Popular pet birds (pigeon, budgerigar and lovebird)

Parakeets are the number one pet bird around the world. They've been a part of many households from the moment they were first brought over to Europe from Australia in the 1840s. What helped launch them to the top spot was sheer personality. Although small, the parakeet has all the charisma of any other parrot in a package only slightly larger than a canary.

Budgerigars are among the most popular pet birds for good reason. These charismatic little parakeets are loveable and affectionate. They are easy to tame if they are acquired at a young age, and are able to mimic speech like larger parrots.

The budgerigar (*Melopsittacus undulatus*) is a long-tailed, seed-eating parrot usually nicknamed the budgie, or in American English, the parakeet. Budgies are the only species in the genus Melopsittacus. The origin of the budgie's name is unclear. First recorded in 1805, budgerigars are popular pets around the world due to their small size, low cost, and ability to mimic human speech. They are the third most popular pet in the world, after the domesticated dog and cat.(Perrins *et al*., 2003).Budgies are nomadic flock parakeets that have been bred in captivity since the 19th century. In both captivity and the wild, budgerigars breed opportunistically and in pairs.

Lovebirds are a favorite among pet birds, often called "pocket parrots,". While there are numerous lovebird species in the world, not all of them are kept as pets. The three most popular species can make charming and loving companions for a bird lover and don't necessarily need a pair of lovebirds to keep them happy. In total, there are nine species of lovebirds. The most common to be kept as pets are the Fischer's lovebird, black-masked lovebird, and peach-faced lovebird.

Lovebirds are known for their short and rather blunt tail feathers. Beyond size, this is one of the primary features that distinguish them from budgerigars. Lovebirds also have a stockier build.

Lovebird is the common name of Agapornis (Greek: agape 'love'; ornis 'bird'), a small genus of parrot belong to the order Psittaciformes, Eight species are native to the African continent, with the grey-headed lovebird being native to Madagascar. Social and affectionate, the name comes from the parrots' strong, monogamous pair bonding and the long periods which paired birds spend sitting together. Lovebirds live in small flocks. Some species are kept as pets, and several coloured mutations have been selectively bred in aviculture. The average lifespan is 20 to 30 years. (Alderton, 2003).

Pigeons and doves have for centuries been kept for use in competitive breeding, homing and in recent history, racing. It is a little known fact that they are also raised as pets. With good reason, they can be very affectionate and loyal, and millions of enthusiasts have learned to love and appreciate them as companion birds.

The domestic pigeon (*Columba livia domestica*) is a pigeon subspecies that was derived from the rock dove (also called the rock pigeon). The rock pigeon is the world's oldest domesticated bird. Mesopotamian cuneiform tablets mention the domestication of pigeons more than 5,000 years ago, as do Egyptian hieroglyphics. Research states that domestication of pigeons occurred as early as 10,000 years ago (Blechman and Andrew, 2007).

2.3 Pet birds are potential carriers or transmitters

There are lot of evidence on the involvement of domestic and companion animals in direct transmission of pathogens to humans, whereas the reservoir for most zoonoses is wildlife (Kruse *et al*., 2004). The high nutrient content of bird excrement gives an excellent sanctuary for potentially harmful organisms. Bird droppings pose a public health risk and cause illness. Humans become infected by inhaling dust containing dried feces, urine, or respiratory secretions of infected birds.

Many of these pathologies could have an important impact on human health, like chlamydophilosis, salmonellosis or even highly pathogenic avian influenza A H5N1, but also have an economic impact if some of these pathogens are spread via carriers or vectors like wild birds, human beings, insects or mites to poultry breeding units or cattle facilities (Carlson *et al*., 2011), then entering the food chain.

According to the opinion of Sick (2001), pigeons are synanthropic birds that are found in large urban areas in Brazil. These birds make their nests in cliffs, and this is the probable reason for their adaptation to urban life, as there are high buildings. Besides, other factors, such as feeding, abundance of shelter and absence of predators enable their disorderly development and reproduction. Therefore, given the close contact between these animals and men, zoonoses may occur in high rates. The same interpretation is valid, in Brazil, for Passeriformes.

The exposure of wild birds to a contaminated environment may create infection accidentally. This occurs commonly in domestic pigeons and colonial water birds. *Salmonella* spp. can be easily transmitted to other animals by contaminated infected birds' feces, since they often gather in very large numbers at feeders. Sources of stress, including food shortage, breeding, poor husbandry with overcrowding and lack of aviary maintenance, poor weather conditions, and the introduction of new birds, may cause the development of salmonellosis and death (Fudge, 2001; Tizzard, 2004). These infected birds may transmit infections to humans, either directly by handling, or indirectly. Small passerines, canaries, and finches are social birds often bred and housed in flock aviaries. Some species like finches, siskins, and sparrows often search food on the ground that may be contaminated by droppings from infected birds. These birds also probably encounter a higher risk, as they often spend a relatively long duration at the feeding site. Due to the zoonotic nature of , it is very important that pet bird owners are trained to practice good hygiene.

Passeriforms and psittacines are housed under different conditions, because of their respective behavior. Actually, psittacines, especially parrots, are more aggressive than passerines and would then rather be kept in pairs than groups (Nigel *et al*., 2000; Dorrestein *et al*., 2009). However, relatively high numbers of budgerigars can be gathered temporarily in the same cage for example in pet shop facilities or markets.

Several times a year, performing birds are brought to shows and competitions, where exchange or selling could occur, and transmission of pathogens could occur (Vanrompay *et al*., 2007; Belchior *et al*., 2011).In the case of the “kitchen-canary”, it could be interesting that in the summer, the cages can be moved outside, in order to allow the bird to sunbathe.

This could be a condition favoring condition of contact between wild and captive passerines (Boseret, personal observations). It is also not rare for canaries to escape from their cage, with a potential risk of disseminating pathogens into a wild avian population, which they could have contracted in their original breeding facility or from humans for example, Chlamydophilosis (Vanrompay *et al*., 2007). Predators, like cats, could also be infected. Finally, we should not forget other potential zoonotic pathogens shedders, like arthropods or rodents, which could also find an easily reachable source of food in cages (Boseret, personal observations) or directly on birds themselves, as this could be the case for haematophagous insects (Lindeborg *et al*., 2012; Loye *et al*., 1995) .

Many times, affected birds do no show clinical signs, but shed the bacteria in eggs or feces. Bird feces contaminate the environment and, in the case of *Salmonella* spp. may remain there for a long period, depending on the environmental conditions. According to (Berchieri *et al*., 2001) the length of fecal shedding and the level of tissue invasion (pathogenicity) depends on the age of the bird at the moment of infection. Therefore, a bird may infect other animals or human for long periods.

Albuquerque *et al.* (2013) studied the experimental infection caused by *Salmonella enteritidis* in chickens and pigeons and observed that birds shed the bacterium in the feces up to 14 days after the experimental infection, demonstrating that contamination of other birds and animals may take place and cause economic losses, besides posing an important public health risk.

2.4 Pet shops, bird fairs and markets act as the source of infection

In direct relationship with local breeders, housing of birds in pet shop facilities increases the risk of transfer of several zoonoses, like for example chlamydophilosis. The overcrowding also induces intense stress to the birds due to fighting for females, territory, food and may cause rapid debilitation of the weakest individuals and higher sensitivity to infections (Boseret *et al*., 2006). This situation is particularly true in live animal markets as represented in several studies performed in Asian countries (Amonsin *et al*., 2008; Wang *et al*., 2006). Unfortunately, no data are available for European countries. But it is frequently observed that pet birds share the same space as poultry, making transmission of pathogens and parasites easier (e.g. *Dermanyssus gallinae*).

Finally, bird fairs are an example of possible contamination. In these regional, national or international gatherings, breeders meet each other and present their production, in a context of championships. Cases of transmission of *Chlamydophila psittaci* from birds-to-humans in France and The Netherlands in such conditions have recently been related by respectively (Belchior *et al*., 2011; Berk *et al*., 2008). In both cases, clinical symptoms were developed by patients and led in several cases to hospitalization.

2.5 Microflora in pet birds

Studies were done by Sousa *et al*. (2010); in Jaboticabal, with 126 free-living pigeons (*Columba livia*) in urban environments, showed the isolation of Salmonella sp. in 10 animals (7.94%).On the other hand, Silva *et al*. (2014); analyzed the presence of enterobacteria in domestic birds (*Cairina moschata*) from households in cities of Ceará and did not find the presence of *Salmonella* spp.

Free living pigeons are potential reservoirs of several pathogenic microorganisms, including *Chlamydophila psittaci* and bacteria belonging to the genus *Salmonella*. In japan, *Salmonella typhimurium* and *Chlamydophila psittaci* were isolated with a high frequency from feral pigeons (Casanovas *et al*., 1995, Pasmans *et al*., 2004). *Chlamydophila psittaci* DNA also has been detected in the feces (16%) of feral pigeons in north-western Italian towns (Magnino *et al*., 2009).

Bangert *et al*. (1988); stated that fecal isolates from healthy parrots included gram-positive bacilli (*Lactobacillus* spp., *Bacillus* spp., *Corynebacterium* spp., *Streptomyces* spp.), gram-positive cocci, (*Staphylococcus epidermidis*, *Streptococcus* spp., *Aerococcus* spp., *and Micrococcus* spp.) and, in a low number of birds, gram-negative bacteria (*Escherichia coli*). They also reported that the number of birds yielding *Corynebacterium* and gram-negative bacteria increased with age; whereas the number of birds yielding lactobacilli decreased with age.

Flammer and Drewes stated that 91% of 506 clinically normal parrots had gram-positive bacilli recovered from cloacal cultures. However, *E. coli* was recovered from 31% of these birds, *Enterobacter* spp. from 4%, *Klebsiella f*rom 0.6%, and *Pseudomonas* spp. from 0.8%. Species differences were noted: *E. coli* was recovered from 60% of the cockatoos

(*Cacatua* spp.) cultured (168 birds), but only 18% from non-cockatoo species (338 birds). All birds were housed in the same facility with similar diets and husbandry, suggesting that these were species-related differences, rather than differences in diet and management.

Khafagyet observed a total of 258 fecal samples from Cockatiles and budgerigars (230 from apparently healthy birds and 28 from clinically diseased) and observed gastrointestinal bacterial infection in love birds, are very limited. The study was concerned with some bacterial pathogens affecting budgerigars and cockatiels, their incidence, distribution, the important pathogens and its susceptibility to different antibiotic. The prevalence of bacterial isolates from apparently healthy birds was (34/230; 14.8%), While its prevalence in diseased birds was (21/28; 75%). The bacterial isolates were identified as *E.coli, Salmonella, Klebsiella, Proteus* and *Enterobacter* was (51.28%), (10.26%), (17.95%), (17.95%) and (2.56%) respectively.

2.6 Harmful effects of Zoonotic diseases from wild and pet birds

Similar to all other vertebrates, birds are susceptible to pathogens and may transmit to humans enteropathogenic organisms of zoonotic potential, but there are few comprehensive studies on the issue with wild and domestic birds (Vasconcelos, 2013). Disease studies on the human population worldwide carried out by Jones *et al*., (2008) showed that emerging infectious diseases are, in most cases, zoonoses (60.3%). From this total, 71.3% are transmitted by wild animals, and 54.3% of these diseases are caused by bacteria and rickettsia, causing an increasing number of microorganisms that are resistant to the pharmaceutical drugs available in the market.

According to the European Centre for Disease Prevention and Control (2010), Salmonellosis and Campylobacteriosis are the two most common zoonoses, and were responsible for 99,020 and 212,064 cases of human disease respectively in the European Union in 2010 (ECDC, 2012).

In addition of the risks it provides for public health, infections impose economical losses to both the public healthcare system and the poultry industry (Collard *et al*., 2008). More than 2,600 serovars of *Salmonella* have been identified, some of which are responsible for human illness and diseases in a wide variety of animals (Gast, 2008). Infections in wild

birds occur frequently, which are a cause of mortality in birds, and can be transferred to humans and domestic animals. Immunosuppressed people are much susceptible to the more common *Salmonella Typhimurium* carried by some pet birds (Fudge, 2001). It appears that the prevalence of in wild birds has been increased significantly in recent years and there have been different studies on this issue to determine and evaluate this risk (Tizzard, 2004). However, compared with research performed in poultry, studies on infections in wild birds have been sparse to date.

It is well known that Psittaciformes are capable of harboring numerous emerging zoonotic pathogens, as well as dispersing infected arthropod vectors (Godoy, 2007). Thus, the presence of Gram-negative bacteria, belonging to the Enterobacteriaceae family, in their intestinal microbiota has been considered an indication of potential diseases (Bangert *et al*., 1988; Mattes *et al*., 2005). Currently, microbiological studies with psittacine have increasingly observed and isolated enterobacteria in healthy birds (Serafini *et al*., 2015; Lopes *et al*., 2015; Machado *et al*., 2016), which may indicate a more opportunistic role of these agents (Hidasi *et al*., 2013).The aviary may include different species or focus only on a single species. Keeping multiple birds in close contact with each other in mixed aviaries provides the ideal environment for infectious disease to spread easily.

Most *Salmonella* spp. serotypes are pathogenic to humans, and clinical signs of the disease vary according to the serotype. The serotypes *S. Agona*, *S. Hadar* and *S. Typhimurium* were considered the most important causes of foodborne diseases in humans. However,*S. Enteritidis* is considered the predominant causing agent of this kind of disease, in several countries. There is great concern, today, about the emergence of serotypes in the genus *Salmonella* that are multi drug resistant to available antibiotics (Shinohara *et al*., 2008).

2.7 Occurrence of *Salmonella* spp. in birds

Salmonellosis is one of the most important zoonotic diseases throughout the world(Gast, 2008). The genus Salmonella is considered a major zoonotic agent, responsible for foodborne infections, especially from poultry products (Tortora *et al*., 2012). Bacteria in the genus *Salmonella* spp., Enterobacteriaceae family, are Gram-negative, facultatively anaerobic, non-sporulating rods (Carvalho, 2006). Nowadays, the genus is divided into two species, *Salmonella enterica* and *Salmonella bongori*. The species *S. enterica*, which is the

pathogenic *Salmonella* species, is divided into six subspecies (enterica, salamae,arizonae, diarizonae, houtenae, and indica), each with different serovars or serotypes. More than 2,500 serotypes are known. Most of them (about 1,500) belong to the subspecies enterica and are associated with clinical conditions both in humans and in animals. This classification in serotypes is based on cell surface structures, such as antigens, flagella lipopolysaccharides, and proteins (Herrera and Jabib, 2015).

The most common characteristics of avian salmonellosis are poor body condition, muscular atrophy, granulomas varying from multifocal to coalescent, transmural ulcerative necrosis of the gastrointestinal tract with clear presence of bacterial aggregation inside and outside the lesions, necrotizing hepatitis, interstitial pneumonia, myocarditis, epicarditis and necrotizing encephalitis (Madadgar *et al*., 2009, Giovaninni *et al*., 2012).

Rahmani et al. (2011); observed 668 samples and 19 isolates (2.8%) were identified. Samples that were positive for *Salmonella* originated from canaries (10 out of 62, 16.1%), pigeons (5 out of 138, 3.5%), psittacines (3 out 130, 2.3%), and eagles (1 out of 2, 50%). In that study, five out of 138 (3.5%) sampled pigeons carried *Salmonella,* among which three isolates were *S. Enteritidis*, one isolate belonged to serogroup B and one isolate to serogroup C.

In 2002, Kirk isolated *Salmonella* spp. from 2.5% of 892 birds tested in California. Kobayashi (2007), isolated *Salmonella* spp. from 5.8% (19 of 328) cloacal swabs obtained from wild birds. Hughes *et al.* (2008); obtained 32 *Salmonella* isolates from wild birds in northern England.

There have been different studies on the prevalence of *Salmonella* infection among wild birds kept in parks, gardens, or in cages. Georgiades *et al*. (2002); observed *Salmonella* from 53 out of 618 pigeons (8.6%), 33 out of 182 canaries (18.1%) and 2 out of 71 psittacines (2.8%) from the Greater Thessaloniki area in Greece. *Salmonella Typhimurium* was the most frequently isolated serotype in pigeons (75.5% of isolates), followed by *Salmonella Enteritidis* (11.3%). In canaries, *Salmonella typhimurium* was also the most frequently isolated serotype (90.9%) followed by *Enteritidis* (6.1%). According to the study by Georgiades , the prevalence of infection in the examined birds was rather rather

low, whereas *S. Typhimurium* and *S. enteritidis* appeared to be the most frequent serotypes in sampled birds (Georgiades *et al*., 2002).

In a study, out of 1,047 fecal swabs sampled at one location in southern Sweden from black-headed gulls during a 3-year period (1998–2000), was found in 28 (2.7%) individuals and *Salmonella Typhimurium* (83%) was the predominant serotype (Palmgren *et al*., 2006). In another study in Iran, Mirzaie (2010), found 18 (3.8%) isolates among 470 samples from house sparrows that were cultured. Nine Typhimurium serovars, eight Enteritidis serovars, and one M serovar were identified among the 18 isolates that were serotyped.

Pigeons have close contact with human in parks, temples and public gardens and can be potential reservoirs for several pathogenic microorganisms including *Salmonella* (Tanaka *et al*., 2005). In some studies, a low prevalence (3%–4%) of infections has been reported in pigeons (Pasmans *et al*., 2004; Tanaka *et al*., 2005). Suphoronski and his colleagues observed 13.36% *Salmonella* spp. in Columbiformes in 2015. *Salmonella* spp. was more commonly isolated among Passeriformes, in a total of 46.67% of the birds.

Akhter *et al.* (2010) observed 21 (46.67%) samples were found positive for *Salmonella* spp., of which 5 (33.33%) were isolated from oral swabs, 9 (60%) from cloacal swabs and 7 (46.67%) from feces; All the suspected *Salmonella* were identified by using *Salmonella* polyvalent 'O' antiserum. Of the 21 *Salmonella* spp. isolated in this study, 4 (19.05%) isolates were identified as *Salmonella Pullorum* when tested with specific antisera against *Salmonella Pullorum*. The occurrence of *Salmonella Pullorum* in psittacine birds is not common (Fowler *et al*., 1986; Allgayer *et al*., 2008). (Shimakura *et al*., 1985; Deem *et al*., 2005) also reported the occurrence of *Salmonalla Pullorum* in psittacine birds.

Almeida *et al.* (2015) studied with 52 samples of Passeriformes and Psittaciformes in the city of Umuarama in Paraná, did not find birds positive for *Salmonella* spp. Hidasi *et al.* (2013); stated that *Salmonella* spp. are habitants of the microbiota of captive and wild life psittacines. The absence of *Salmonella* was also observed in other Brazilian studies, with low detection rates in apparently healthy wild birds, whether raised in captivity or in the wild (Allgayer *et al*., 2009; Santos *et al*., 2010; Lopes *et al*., 2015; Machado *et al*., 2016). Because psittacines are very sensitive to avian salmonellosis, particularly at young ages (Marietto-Gonçalves *et al*., 2010).

2.8 Occurrence of *E. coli* in birds

Escherichia coli is an anaerobic Gram negative bacillary bacterium, which is a potential pathogen of birds, named Avian Pathogenic *E. coli* (APEC) which are responsible for avian colibacillosis. This disease may develop in different forms, such as: colisepticemia, coligranuloma, aerossacullitis, sinusitis, pericarditis, peritonitis, salpingitis, hepatitis, panoftalmitis and osteomyelitis; however respiratory disease followed by septicemia and death is the most frequent process (Janben *et al*., 2001).

Among the enterobacteria relevant to both human and animal health, the role of the bacterium *Escherichia coli (E. coli)* as a potential pathogenic agent has been emphasized. *E. coli* is a commensal resident of microbiota of humans and animals (Schremmer *et al.*, 1999); however, pathogenic strains are responsible for different intestinal and extraintestinal diseases in both domestic and wild birds (Marietto-Gonçalves *et al*., 2007).

According to Croxen *et al.* (2013) *E. coli* may be classified into different serotypes, according to the antigens it presents. There are 173 O antigens, 80 K antigens, and 56 H antigens, producing countless O:K:H serotypes. However, the number of pathogenic serotypes is not much, with wider occurrence of non-pathogenic strains. There are two main groups of these serotypes: those that cause diarrhea, and those that may cause extraintestinal disease (Orskov and Orskov, 1992). However, it may also be a pathogenic agent, as it adapts to diverse conditions, which is mainly related to the loss or gain of bacterial genes. Several different *E. coli* strains cause intestinal and extra-intestinal diseases by means of virulence factors that affect a much variety of cell processes (Kaper *et al*., 2004). According to Croxen and Finlay (2010), there was an impressive worldwide increase in the number of cases of these diseases, with hundreds of millions of people affected annually. Similar to humans, *E. coli* may also cause diseases in the animals.

Colibacillosis is one of the main causes of mortality in birds, and responsible for significant economic losses all over the world (Schouler *et al*., 2012). In studies carried out by Trampel *et al*. (2007) in United States, *E. coli* was isolated from 14 of 15 clinically healthy birds, confirming that these animals carry the bacterium and do not show clinical signs, which may cause disease spreading. Mattes *et al*. (2005); evaluated the influence of biosafety measures in the intestinal colonization of Psittacidae by *E. coli*, in the state of São Paulo.

In Australia, a flock of budgerigars with mortality caused by *E. coli* showed congestion and hemorrhage (Seeley *et al*., 2014). The occurrence colibacillosis in birds with other simultaneous diseases or lesions are randomly found, which may serve as an entry port for the infection by *E. coli* or even *Salmonella* spp. (Crespo *et al*., 2001, Seeley *et al*., 2014).

Braconaro *et al.* (2012); evaluated 253 wild Passeriformes in São Paulo and found 10.7% birds positive for *E. coli*. Brittingham *et al.* (1988); in the analysis of the prevalence of bacteria in Passeriformes and Piciformes birds in the USA and found 1% of 387 samples positive for *E. coli*. When Jones and Nisbet (1980), analyzed 271 birds in the London zoo, they found *E. coli* in 180 sample. They analyzed 26 Columbiformes and showed 81.25% animals positive for *E. coli*. Therefore, even in a bird population with high number of animals positive for *E. coli*, there may be no sick animals, as many serotypes are commensals. The possible pathogenicity of these strains to humans cannot be ruled out.

A study carried out by Vasconcelos *et al.* (2013) in Ceará with Atlantic canaries (Serinus canaria), that belong to the Passeriformes order, showed *E. coli* prevalence equal to 3.62%, with 11 samples of cloacal swabs positive in 487 samples.

Suphoronski and his colleagues did a study in 2015, a total of 471 samples of seven bird orders were analyzed. From the total of samples, 69.38% were positive for *E. coli*. From the total of birds analyzed, 143 (34.29%) were positive both for *Salmonella* spp. and for *E. coli*. When the bird order is taken into account, Columbiformes showed the greatest occurrence of *E. coli*, with a frequency of 82.33% of the birds.

Hidasi *et al.* (2013) studied the intestinal microbiota of parrots belonging to different species (Psittaciformes) seized from illegal trade and also found that *E. coli* was the most prevalent species (33.8%). Vaz *et al.* (2015) detected that 72.7% of wild redtailed Amazon parrot (*Amazona brasilensis*) nestlings sampled from artificial wooden nests harbored *E. coli*; however, they considered this result within the normal microbiological profile of such birds.

2.9 Antibiotic resistance and its mechanism

Antibiotics are also called antibacterials, which are used in the treatment and prevention of bacterial infections (Gualerzi *et al*., 2013). According toWHO (2016) antimicrobial

resistance occurs when microorganisms such as bacteria, viruses,fungi and parasites change in ways that provide the medications used to cure the infections they make it ineffective. Actually antibiotics refer to naturally occurring biomolecules, while the term antimicrobials encompass both naturally occurring and synthetically derived moloecules. Antibiotics are widely used for preventing and treatment of various infections in humans and animals. Their irrational and indiscriminate use in different fields like agriculture,fisheries, livesyock industry etc., has given rise to development of resistant bacteria (Aarestrup, 2005) and this causes in the spread of resistance by transfer of its resistant determinants to other bacteria (Stanton *et al*., 2013). In natural or intrinsic resistance to a drug occurs without any additional changes in genetic elements, whereas acquired resistance causes through frequent mutations or acquisition of foreign genetic material carrying resistance determinants (Hollenbeck and Rice, 2012). The resistant bacteria that are shed in the environment may infect animals, and then travel back through the food chain to humans.

Bacterial resistance to antibiotics has been extensively studied because it is a matter of health concern and of its commercial implications. Studies have shown the increasing role of free-living birds as hosts for pathogens that carry antibiotic-resistance mechanisms (Smith *et al*., 2014; Carroll *et al*., 2015). Therefore, these animals are considered important agents for the emergence of multidrug resistance (MDR) in several environments around the world, (Hasan *et al*., 2012) as well as for the spreading of resistant bacteria to multiple hosts and places. Multi Drug Resistant bacterial isolates of animal origin may spread in human population by direct contacts and through animal-origin foods (Soulsby *et al*., 2008). These resistant bacteria may colonize the human intestinal tract and the genes that encode for antibiotic resistance can consequently be transferred to the bacteria of natural microflora or pathogenic bacteria.

2.10 Antibiotic resistance of *E. coli* and *Salmonella* spp. of birds

Examining the antibiotic susceptibility patterns of pathogens is important toward providing treatment to the ever-changing resistance patterns and distribution of pathogenic bacteria (NCCLS, 2001). Elevated rates of resistance to tetracycline or other antibiotics from the

same group may occur via transference between microorganisms in the microbiota of birds, which may be an indirect or direct risk to the human health (Hu *et al*., 2013).

A study of Morshed and Peighambari (2010), showed that the resistance to antimicrobial agents among serovars isolated from garden or cage birds was much lower than those from commercial poultry . However, Rahmani observed in 2011 that 73.7% of isolates of *Salmonella* spp. and demonstrated the MDR pattern and the number of antibacterial agents varied between two to 11 MDR types. Higher rate of resistance were found in *Salmonella* sp. isolates to certain antimicrobial agents such as nalidixic acid, carbenicillin, streptomycin, lincospectin, florfenicol, tetracycline, and trimethoprim +sulfamethoxazole comparing with those from studies in commercial poultry (Morshed and Peighambari, 2010). A recent study in Iran on 18 isolates from captured house sparrows found all isolates to be sensitive to norfloxacin, flumequine, ampicillin, and sultrim, and 35% were resistant to lincospectin (Mirzaie *et al*., 2010).The presence of a MDR pattern has been previously reported among avian isolates from Iran (Madadgar *et al*., 2009; Mirzaie *et al*.,2010; Morshed and Peighambari, 2010).

Resistance to tetracycline in *E. coli* strains isolated from pet birds was reported in Australia (13.9%), from a total of 594 analyzed samples and multidrug resistance was also reported (Blynton *et al*., 2015). In another study, multidrug resistance to other groups, such as aminoglycosides, quinolones, sulfas and others has been identified in *E. coli* strains isolated from psittacine.

Machado and others studied antimicrobial Resistance Profiles of Enterobacteria from Nestling Grey-Breasted Parakeets (*Pyrrhura Griseipectus*) in brazil and observed 21 (14.9%) isolates presented multidrug resistance, with *E. coli* isolates as the most frequent, accounting for 33% of the isolates (7/21) and presenting resistance to azithromycin, chloramphenicol, sulfametoxazole +trimethropim,sulfonamide,and tetracycline, with a variation ranging from 8.2% to 28.6%.

Akhter *et al*. (2010) observed microflora from apparently healthy caged parrots from zoo. A total of 45 samples (oral swabs, cloacal swabs and feces) were collected from five types of caged parrots and the sensitivity patterns of different bacteria to various antibiotics were so variable that it was difficult to interpret. Ampicillin and amoxicillin were found to be

fully resistant to *E. coli* and *Pasteurella* spp.; and furazolidone to *Salmonella* spp. and *Pasteurella* spp. On the other hand, the antibiotics of fluoroquinolone group such as ciprofloxacin, norfloxacin and enrofloxacin showed moderate to high sensitivity against almost all the bacterial isolates. Ciprofloxacin was found to be consistently highly sensitive to all the bacterial isolates which is consistent with the findings of (Brahmbhatt and Anjaria, 1991; Morishita *et al*., 1996 ; Mukhopadhyay *et al*., 1998).

CHAPTER-3

MATERIALS AND METHODS

The present research work was conducted between December 2019 to October 2020 in the laboratory of Animal Biotechnology division of National Institute of Biotechnology for isolation and identification of bacteria by different microbiological methods. The detailed outline of materials and methods are given below:

3.1 Materials and Methods

3.1.1 Selection of study area

The samples were collected from different pet shops of Katabon, Dhaka where different species of pet birds are kept in cage for selling to customers. Samples were brought to the laboratory of Animal Biotechnology Division (ABD) of National Institute of Biotechnology (NIB) for laboratory analysis.

3.1.2 Collection of samples

A total of 45 fecal samples were collected from 3 different species of pet birds (Pigeon, Budgerigar and Lovebird). Twenty samples from Pigeon (P-1 to P-20), fifteen samples from Budgerigar (B-1 to B-15) and ten samples from Lovebird (L-1 to L-10) were collected. Freshly dropped fecal samples were collected from the tray under their cages. It was collected as an environmental sample (Figure 1 and Figure 2).

Figure 1: Collection of fecal sample from from budgerigars

Figure 2: Collection of fecal sample from pigeon

3.1.3 Transportation and Preservation of samples

The samples were carried out to the laboratory in an ice box contained ice and processed for the isolation, identification and characterization of bacteria subsequently, and the remaining samples were stored at 4^0C for future use.

3.1.4 Media for culture

Different bacteriological culture media and reagents were used for isolation and identification and also propagation of bacteria from fecal samples. Those culture media and reagents that were used in this experiment are mentioned below:

3.1.4.1 Liquid Media

1. Nutrient broth (Difco, USA)

Nutrient broth was used to grow the organisms from the samples collected from the study areas before performing biochemical test and antibiotic sensitivity test (Cheesbrough, 1985).

3.1.4.2 Solid Media for culture

1. Nutrient Agar Medium, (HI-MEDIA, India)

2. MacConkey Agar medium, (HI-MEDIA, India)

3. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)

4. Salmonella-Shigella Agar(SS Agar)(HI-MEDIA, India)

5. Mueller-Hinton agar (Difco, USA)

Solid Media

1. Nutrient Agar Medium, (HI-MEDIA, India)

Nutrient agar is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985)

2. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)

EMB contains dyes that are toxic for Gram positive bacteria and bile salt which is toxic for Gram negative bacteria other than coliforms. EMB is the selective and differential medium for coliforms. *Escherichia coli* colonies grow with a metallic sheen with a dark center . *Salmonella* spp. gives grey color colonies. (Cheesbrough, 1985).

3. MacConkey Agar medium, (HI-MEDIA, India)

A differential medium for the isolation of coliforms and intestinal pathogens in water and biological specimens. Mac-Conkey agar is a culture medium designed to selectively grow Gram-negative bacteria and differentiate them for lactose fermentation. Lactose-fermenting organisms grow as pink to brick red colonies with or without a zone of precipitated bile. Non lactose fermenting organisms grow as colorless or clear colonies (Cheesbrough, 1985).

4. Salmonella-Shigella Agar (HI-MEDIA, India)

Salmonella-Shigella Agar (SS Agar) is a selective and differential medium widely used in sanitary bacteriology to isolate Enterobacteriaceae. In case of *Salmonella* spp. produces black centred colony and *E. coli* produces rose pink colony.(Cheesbrough, 1985).

5. Mueller-Hinton agar (Difco)

Mueller Hinton agar is used for the determination of susceptibility of microorganisms to antimicrobial agents. It has become the standard medium for the Bauer Kirby method and its performance is specified by the NCCLS.

3.1.4.3 Different biochemical test

1) Sugar fermentation test

2) Oxidase test

3) Catalase test

4) Urease test

5) Methyl Red test

6) Voges-proskauer test

7) Simmon's citrate Agar test

8) Triple sugar iron agar slant test

9) Sulphur Indole Motility test

10) Nitrate reduction test

3.1.5 Reagent

- Alcohol
- Kovac's reagent
- Methyl-red solution
- 3% H_2 O_2
- P-Amino dimethylanilin oxalate
- Bromothymol blue
- Phosphate buffered saline (PBS) solution

3.1.6 Glassware and appliances

The different types of important equipment used for this work are listed as follow down:

1. Distilled water
2. Sterile bent glass or plastic spreader Rods
3. Micropipette (1-5 µl,5-50 µl, 10-100 µl, 50-500 µl, 100-1000 µl)
4. Forceps
5. Measuring cylinder
6. Spirit lamp
7. Water bath

8. Vortex Mixture
9. Labeling tape
10. Experimental test tube
11. Bacteriological loop
12. Petri dish
13. Conical flask
14. Durham's tube
15. Slide
16. Electric balance
17. Cotton
18. Incubator
19. Beaker
20. Autoclave etc.

3.1.7 Materials used for bacterial genomic DNA isolation

- TE buffer
- 10% (w\v) Sodium dodecyl sulfate (SDS)
- 20 mg\ml Proteinase-K (stored in small single-use aliquots at -20°C)
- 3 M Sodium Acetate, p^H 5.2
- Phenol/Chloroform/Isoamyl alcohol (25:24:1)
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml microcentrifuge tubes.

3.1.8 Materials used for polymerase chain reaction (Table 1 and 2)

Table 1: PCR reaction mixture

Master Mix	12.5µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Nano Pure Water	9.5 µl
DNA	2.0 µl
Final Volume	25.0 µl

3.1.8.1 Primers used in PCR for *E. coli* and *Salmonella* spp. identification (Table 2)

Table 2 Primer sequence and their sources

***E. coli* Primer sequences (5´- 3´)**	**PCR Product size**	**Source**
16SrRNA *E. coli*:16E1)5- GGG AGTT AAT ACC TTT GCT C-3(F) 16SrRNA *E. coli*:16E2)5- TTC CCG AAG GCA CAT TCT-3 (R)	584 bp	Tsen *et al*., (1998)
***Salmonella* spp. Primer sequences(5´- 3´)**		
S139 5-GTG AAA TTA TCG CCA CGT TCG GGC AA-3(F) S141 5-TCATCGCACCGTCAAAGGAACC-3(R)	284 bp	Rahn *et al*., (1992)

3.1.8.2 Equipment and reagent for PCR:

- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 2% agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)
- 100 bp and 50 bp DNA size marker
- Bromphenicol blue of loading bufter.
- Ethidium bromide (0.5 μg/ml)
- UV trans-illuminator

3.1.9 Antimicrobial discs

Commercially available antimicrobial discs (OXOID Limited, Italy) were used to determine the drug sensitivity and resistance pattern.This method allowed for the rapid detection of the efficacy of drugs against the test organisms by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the discs inhibiting the growth of the organisms. The following antimicrobial agents with their disc concentration were used (Table 3).

Table 3 Antimicrobial agents with their discs concentration

S/N.	Name of antimicrobial agents	Symbol	Disc Concentration (µg/disc)
1.	Amoxicillin	AML	30
2.	Ciprofloxacin	CIP	5
3.	Trimethoprim	TM	5
4.	Tetracyclin	TE	30
5.	Ceftazidime	CAZ	10
6.	Ceftriaxone	CRO	30
7.	Chloramphenicol	C	10
8.	Azithromycin	AZM	15
9.	Colistin Sulphate	CS	10
10.	Enrofloxacin	ENR	5
11.	Amikacin	AK	30
12.	Norfloxacin	NOR	10
13.	Ampicillin	AMP	2
14.	Gentamicin	CN	10

Note: µg = Microgram

3.2 Methods

The following methods were used for the isolation and identification of *E. coli* and *Salmonella* spp. from the key Flock.

3.2.1 Brief descriptions of the experimental design

The entire study was divided into three steps: The first step included selection of sources, collection of samples, isolation, identification and characterization of microorganisms on the basis of their colony morphology, staining properties, and biochemical characteristics. The second step was to molecular characterization for selective isolates. In the third step, the current status of drug sensitivity and resistance pattern of isolates of microorganism were determined by using different antibiotic discs available in the market. The layout of the diagrammatic illustration of the present study is shown in (Figure 3).

Experimental layout

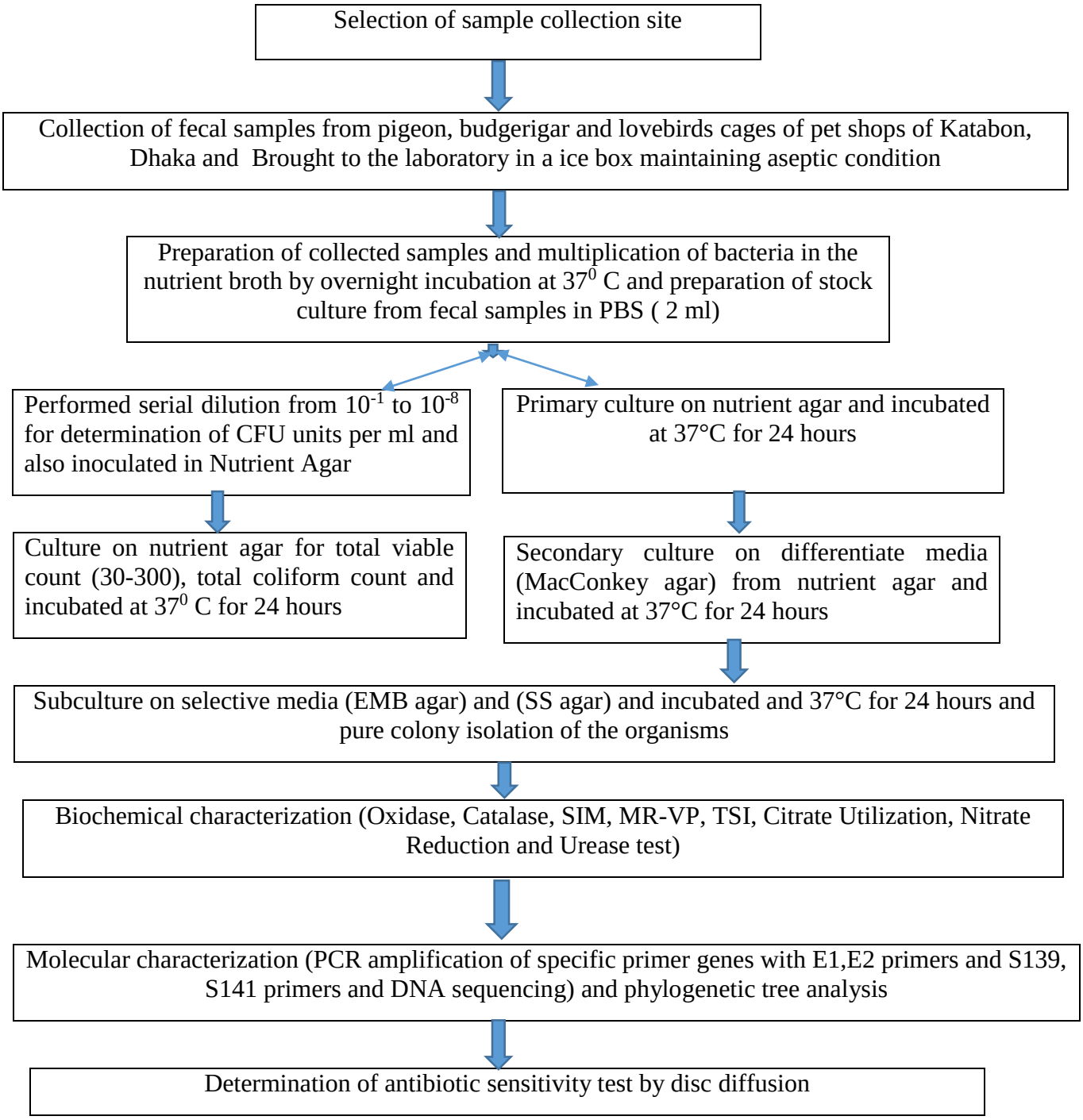


Figure 3: Schematic illustration of experimental layout

3.2.2 Laboratory preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household dishwashing detergent solution ('Trix' Recket and colman Bangladesh Ltd.) overnight. Contaminated glassware was disinfected with 2% sodium hypochlorite solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160°C for 2 hours or by autoclaving for 15 minutes at 121°C under 15 lbs pressure per sq inch. Autoclaved items were dried in a hot air oven over at 50°C. Disposable plastic (items e.g. micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50°C for future use.

3.3 Sampling and Serial dilution for bacterial culture (10 fold dilution method)

Proper care was taken during the sampling procedure to prevent contamination of sample. The samples tubes were completely tied at the time of sampling that prevent contamination. Serial dilution of the stock sample was done to lowering the bacterial for the total viable count (TVC) and total coliform count (TCC). It was done by taking 8 (1-8) Eppendrof tube filled with 900µl of PBS. 100µl of stock sample was transferred from the stock tube (2ml) to the eppendrof tube next to the stock tube. Then 100µl of diluted sample is transferred from the first eppendrof tube to the next. Successive dilution should be made in the same way to the last tube and from the last tube 100µl of diluted sample should be discarded. From the last tube 25µl of liquid sample should be transferred to the nutrient. Agar media and MacConkey agar to elucidate the total viable count and total coliform count. Enumeration of *Salmonella* spp. was done by transferring same amount of liquid sample in the Nutrient agar.

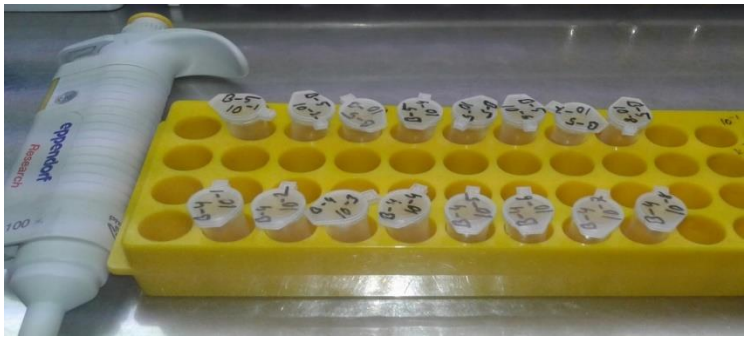


Figure 4: Ten fold serial dilution (10^{-1} to 10^{-8})

3.4 Preparation of various bacteriological culture media

3.4.1 Nutrient Broth

Nutrient Broth was prepared by Suspended 25 grams in 1000 ml purified/distilled water. Heat if necessary to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The broth was filled in the test tubes and incubated at 37°C for overnight to check their sterility and stored at 4°C in the refrigerator until used.

3.4.2 Nutrient Agar

Nutrient agar was prepared by dissolving 28 grams of dehydrated nutrient agar (HiMedia, India) in to 1000 ml of distilled water and was sterilized by autoclaving at 121°C under 15 lb pressure per square inch for 15 minutes. Then the agar was dispensed into pertridish (90 mm and 100 mm) and allowed to solidify. After solidification these were incubated at 37°C for overnight to check their sterility and stored at 4°C in the refrigerator until used (Carter, 1979)

3.4.3 MacConkey's agar

49.53 grams of Bacto MacConkey agar (HiMedia, India) was suspended in to 1000 ml of cold distilled water and was heated for boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121^0 C maintaining a pressure of 15 lb/sq. inch for 15 minutes. After autoclaving, the medium was put into water bath of 45^0 C to decrease its temperature. It was then poured in to sterile petridishes and allowed to solidify. After solidification of the medium in the plates, the plates were then incubated at 37°C for overnight to check their sterility.

3.4.4 Eosine Methylene Blue (EMB) agar

Thirty six grams powder of EMB agar base (HiMedia, India) was suspended in 1000 ml of distilled water. The suspension was heated to boil for few minutes to dissolve the powder completely with water. The medium was autoclaved at 121^0 C maintaining a pressure of 15 lb/sq. inch for 15 minutes.. After autoclaving the medium was put in to water bath at 45°C to cool down its temperature at 40°C. From water bath 10-20 ml of medium was poured in to small and medium sized sterile petridishes to make EMB agar plates. After

solidification of the medium in the plates, the plates were incubated at 37°C for overnight to check their sterility (Carter, 1979).

3.4.5 Salmonella-Shigella agar

Firstly distilled water was autoclaved at 121^0 C maintaining a pressure of 15 lb/sq. According to the direction of manufacturer 60 grams of dehydrated medium was suspended in 1000 ml of sterilized distilled water and heated for boiling to dissolve the medium completely. The medium was put in to water bath of 50°C to decrease its temperature. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37°C for overnight to check their sterility and then stored at 4°C in a refrigerator for future use.

3.4.6 Mueller Hinton Agar

Suspended 38.0 grams in 1000 ml distilled water and heated to boiling to dissolve the medium completely. After the sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooling was done to 45-50°C. Then it was mixed well and poured into sterile petridishes. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37°C for overnight to check their sterility and then stored at 4°C in a refrigerator for future use.

3.5 Preparation of biochemical media and reagents:

3.5.1 Methyl Red and Voges-Prosauer (MR-VP) broth

A quantity of 3.4 gm of MR-VP medium (Himedium, India) was dissolved in 250 ml of distilled water, distributed in 3ml quantities in test tube and then autoclave. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and then stored at 4°C for the future use (Merchant and Packer, 1967)

3.5.2 Sugar solutions

The medium consists of 1% peptone water to which fermentable sugars added. Peptone water was prepared by adding 1 gm of Bacto peptone (Difco, USA) and 0.5 gm of sodium chloride in 100 ml distilled water, boiled for 5 minutes, adjusted to pH 7.6 by Bromothymol blue(0.02%) indicator, cooled and then filtered through filter paper. The solutions were

then dispensed in 5 ml amount into screw caped test tubes containing invertedly placed Durham's fermentation tubes. Then the sugars, dextrose, glucose, lactose, sucrose and mannitol used for fermentation were prepared separately as 10 percent solutions in distilled water (10 gm sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar. These were then sterilized by at 100°C for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. The sugar solutions were incubated at 37°C for 24 hours to check sterility. These solutions were used for biochemical test.

3.5.3 Methyl-Red solution

The indicator MR solution was prepared by dissolving 0.1 gram of Bacto methyl-red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water (Merchant and packer, 1967).

3.5.4 Alpha-Naphthol solution

Alpha-Naphthol solution was prepared by dissolving 5 gram of alpha-naphthol in 100 ml of 95 ethyl alcohol (Merchant and packer, 1967).

3.5.5 Potassium hydroxide solution (H_2O_2)

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in 100 ml of cooled water (Merchant and Packer, 1967).

3.5.6 Kovac's reagent

The solution was prepared by mixing 25 ml of concentrated hydrochloric acid in 5 ml of Amyl alcohol and 5 gram of paradimethyl-aminobenzyldehide crystal were added to this mixture. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1967).

3.5.7 Phosphate Buffered Saline (PBS)

For preparation of phosphate buffered saline, 8 gm of sodium chloride (NaCL), 2.89 gm of disodium hydrogen phosphate (Na2HPO4.12H2O), 0.2 gm of potassium chloride (KCI) and 0.2 gm of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of

distilled water. The solution was heated to dissolve completely and pH was adjusted with the help of pH meter and maintained at 7.0-7.2 p^H. .The solution was then sterilized by autoclaving at 1210 C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at 4°C for future use (Cheesbrough, 1984).

3.6 Isolation of bacteria

3.6.1 Procedure for isolation and identification of bacterial pathogens

3.6.1.1 Primary culture was done on nutrient agar

With the help of sterile inoculating loop the collected samples were directly inoculated into nutrient agar and incubated at 37°C for 24 hours. The incubated media were then examined for growth of bacteria.

Inspection: Morphological characteristics (shape, size, surface texture, edge and elevation, color, opacity etc.) of the suspected colonies on different agar media developed within 18 to 24 hours of incubation were carefully recorded. Growth of microorganisms and their colony characteristics were recorded according to procedures described by Carter, 1979.

3.6.1.2 Secondary culture

The organisms were inoculated into MacConkey agar, EMB agar, SS agar and incubated at 37°C for 24 hours. The incubated media were then examined for growth of bacteria.

1. A inoculum was picked up with a sterile inoculating loop and was streaked on a area of the medium in the petridish.

2. The loop was sterilized by being heated as red hot in a flame.

3. The inoculum was spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inocubated area, then streaking in a single direction in each parallel line. This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.6.1.3 Pure Culture on selective media

EMB agar for *E. coli* and SS agar for *Salmonella* spp.

3.7 Biochemical tests

Isolated organisms with supporting growth characteristics of suspected identified by biochemical test. Several biochemical tests were performed for confirmation of *E. coli* and *Salmonella* spp. isolates. That are as follows:

1) Sugar Fermentation test

2) Oxidase test

3) Catalase test

4) Urease test

5) Methyl Red Broth

6) Voges-proskauer Broth

7) Simmon's citrate Agar (HI-MEDIA, India)

8) Triple sugar iron agar (HI-MEDIA, India)

9) Sulphur Indole Motility agar (HI-MEDIA, India)

10) Nitrate reduction test

3.7.1 Sugar fermentation test

The sugar fermentation test was performed by inoculating a loop full of NB culture of the organisms into each tube containing five basic sugars (e.g. dextrose, sucrose, lactose, maltose and mannitol) separately and incubated for 24 hours at 37°C acid production was indicated by the color change from greenish to yellow in the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube (Cheesbrough, 1985).

3.7.2 Oxidase test

The oxidase test uses Kovac's reagent (1% [wt/vol] solution of N, N, N, N – tetramethyl-ρ-phenylenediamine dihydrochloride) to detect the presence of cytochrome c in a bacterial

organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. The oxidase test was performed on filter paper or on a swab (Cheesbrough, 1985).

3.7.3 Catalase test

This test was used to differentiate bacteria which produce the enzyme catalase. To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide. Then one drop of catalase reagent (3%H_2O2) was added on the smear. The slide was observed for bubble formation. Formation of bubbles within few seconds was the indication of positive test while the absence of bubble formation indicated negative result (Cheesbrough, 1985).

3.7.4 Urease test

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Many organisms especially those that infect the urinary tract, have an urease enzyme which is able to spilt urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink. Sterile urease broth was inoculated with the test organism and following incubation at 37°C for 48 hours.

3.7.5 Methyl Red test (MR)

Sterile MR-VP broth was inoculated with the test organism and incubation was done at 37°C for 48 hours. 5 drops of methyl red solution was added and observed for color formation. If the organism ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the PH, hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.7.6 Voges-Proskauer test (VP)

Voges-Proskauer test – If the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetone which is neutral is converted to diacetyl upon the addition of the VP – reagent – B (40% KOH with 0.3%

creatine) in the presence of VP – reagent – A (5% alpha-napthol in methyl alcohol). Diacetyl is red in color. Yellow color is indicative to negative reaction (Cheesbrough, 1985).

3.7.7 Simmon's Citrate Agar (SCA)

This tube medium is used to identify Gram negative enteric bacilli based on the ability of the organisms to utilize and the sole source of carbon degrades it to ammonia and subsequently converts it to ammonium hydroxide. The p^H of the medium is then increased and this is indicated by a change in color from green to blue (Cheesbrough, 1985).

3.7.8 Triple Sugar Iron Agar (TSI)

This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985).

- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant
- Hydrogen sulfide production – indicated by blackening of the medium. Hydrogen Sulphide (H2S) has been produce due to the sulpher containing compounds. H2S reacts with the ferrous sulphate of the medium producing ferric sulphide, which will appear as a back precipitate.
- Gas production – indicated by presence of a crack, bubble or gas space

PH indicator – bromothymol blue

Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate. (H2S)

TSI agar can also be used to indicate whether Yellow slant, yellow butt, presence of gas bubbles and absence of black precipitate in the butt is positive for *E. coli* and black precipitate in the butt is identical for *Salmonella* spp.

3.7.9 Nitrate reduction test:

All members of the Enterobacteriaceae family metabolize nitrite to other compounds. Anaerobic metabolism requires an electron acceptor other than atmospheric oxygen (O_2).

Many gram-negative bacteria use nitrate as the final electron acceptor. The organisms capable of producing the nitrate reductase enzyme then converts the nitrate (NO_3) to nitrate (NO_2). The reduction of nitrate to nitrate is determined by adding sulfanilic acid and alpha-naphthylamine. Nitrites react with an acid solution of sulfanilic acid and alpha-naphthylamine to from a red color. The sulfanilic acid and nitrite react to form a diazonium salt. The diazonium salt then couples with the alphanapthylamine to produce a red, water-soluble azo dye. In each of the test reactions the appearance of the red dye indicates the presence of NO_3 in the test tube, whether as an of the red dye indicates the presence of NO_2 in the test tube, whether as an unreduced primary substrate, a product of the reduction of NO_3 by the test organism, or a product of the forced reduction of NO_3 with a reducing agent (zinc) for control purposes indicating a negative the nitrate to nitrite, and the nitrite in the medium formed nitrous acid, which reacted with sulfanilic acid. If no color change occurs after the addition of zinc, this indicates that the organism reduced nitrate to one of the other nitrogen compounds. Nitrate reduction test is a test that determines the production, of an enzyme called nitrate reductase, which in the reduction of nitrate (NO_3).

Sterile nitrate broth was inoculated with the test organism and following incubation at 37°C for 48 hours. 5 drops of sulfanilic acid and alpha-naphthylamine solution was added and observed for color formation.

3.7.10 Sulphur motility indole test (SIM test)

The formulation of SIM Medium is designed to allow the detection of sulfide production, indole formation and motility.The medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production is detected when ferrous sulfide, a black participate, is produced as a result of ferrous ammonium sulfate reacting with H_2S gas. Casein peptone, another component of SIM medium, is rich in tryp tophan. Organisms prossessing the enzyme tryptophanase degrade tryptophan to indole. Indole is detected upon the addition of Kovacs Reagent following incubation of the incubated medium. Indole combines with p-dimethylaminobenzaldehyde and produces a red band at the top of the medium. A negative indole test produces no color change upon the addition of Kovacs Reagent.The small amount of agar added to the medium provides a semi-solid structure allowing for the

detection of bacterial motility. Motile organisms extend from the stab line and line and produce turbidity or cloudiness throughout the medium. Non-motile organisms grow only along the stab line and leave the surrounding medium clear.

Sterile SIM media was stabbed with a needle containing organism and incubated at 37°C for 48 hours. *E. coli* isolates were motile, no H_2S production and indole positive was recorded with the red color ring formation by adding Kovac's reagent. *Salmonella* spp. were motile with blackening of media with H_2S production and indole negative with no red color ring formation.

3.8 DNA amplification

3.8.1 Basic protocol of bacterial genomic DNA isolation

Bacteria from saturated liquid culture are lysed and proteins are removed by digestion with Proteinase-K. Cell wall debris, polysaccharides and remaining proteins are removed by Phenol-chloroform extraction and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

3.8.2 Genomic DNA isolation

3.8.2.1 DNA extraction procedure

Materials

TE buffer

10% (w/v) sodium dodecyl sulfate (SDS)

20 mg/ml Proteinase-K (Stored small single – use aliquots at -20°C

3 M Sodium acetate. pH 5.2

Phenol/Chloroform/Isoamyl alcohol (25:24:1)

Isopropanol

70% Ethanol

95% Ethanol

1.5 ml microcentrifuge tubes

Procedure

- Inoculate a 25 ml of liquid culture with the bacterial strain of interest. Grow in conditions appropriate for that stain until the culture is saturated.
- Spin 1.0 ml of the overnight culture in a microcentrifuge tube for 5 minutes at 10000 rpm.
- Discard the supernatant.
- Repeat this step. Drain well into a kimwipe. Resuspend the pellet in 467µl TE buffer by repeated pipetting. Add 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K to give a final concentration of 100 mg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 30 min to 1 hour at 37°C
- Add and approximately equal volume (500 µl) of phenol/Chloroform/ Isoamny alcohol. Mix thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phases are completely mixed.
- Then centrifuge the tube at 12000 rpm for 10 minutes.
- Remove aqueous, viscous supernatant (~ 400 µl) to fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of Phenol/Chloroform/Isoamyl alcohol, extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 min.
- Transfer the supernatant to a fresh tube (~400 µl).
- Add 1/10th volume of 3 M Sodium acetate and mixed.
- Add 0.6 volumes of Isopropanol to precipitate the nucleic acids, keep on ice for 10 min.
- Centrifuge at 13500 rpm for 15 min.
- Decent the supernatant.
- Wash the obtain pellet with 1 ml of 95% ethanol for 5 min. Then centrifuge at 12000 rpm for 10 min.
- Decent the supernatant.
- Dry the pellets well as there is so alcohol.
- Resuspend the pellet in 50 µl of TE and then 7.5 µl of Rnase. Store DNA at 4°C for short term and at -20°C for long term.

3.8.2.2 Concentration of extracted DNA and purity

Quantification of DNA is a very important step to know the amount of DNA that is present when performing the techniques such as PCR. When assessing DNA purity it is important to understand that while A260/A280 ratio is easy to determine and is the most widely used method, it is not particularly robust. DNA absorbs so strongly at 260 nm that it takes significant protein contamination to have a noticeable effect on A260/A280 ratio. On the other hand, the A260/A280 ratio is a particularly robust method for assessing DNA contamination of protein preparations. Because DNA and RNA are so similar, spectrophotometer cannot be used to detect contamination of DNA by RNA and vice versa. A ratio of~1.8 is generally accepted as "pure" for DNA; a ratio of~2.0 is generally accepted as "pure" for RNA and a ratio below 1.70 is generally accepted as ethanol, contamination or other salts are present. DNA concentration and purity is shown in (Table 4).

Table 4 DNA concentration and purity

Sample ID	Ratio	Conc. (ng/µl)
1. P-16	765.6	1.92
2.B-12	402.6	1.83
3.B-8	207.5	1.81
4.P-1	136.6	1.75
5.B-2	296.6	1.90
6.B-1	813.2	1.98
7.B-4	602.9	1.97
8.P-5	277.6	1.77
9.B-6	332.7	1.75
10.P-14	602.7	1.90
11.P-3	251.6	1.83
12.B-3	378.7	1.78
13.P-6	287.1	1.74
14.L-9	356.8	1.86

Table 4 (cont'd)

16.P-8	272.3	1.82
17.L-10	498.7	1.90
18.B-9	459.1	1.82
19.P-17	306.4	1.86
20.L-2	181.4	1.74
21.B-10	271.6	1.85
22.B-13	169.7	1.77
23.B-11	317.4	1.85
24.P-4	92.4	1.90
25.P-19	96.8	1.87
26.P-2	137.5	1.80
27.P-20	120.5	1.69
28.P-10	36.6	1.81
29.P-11	244.3	1.85
30.P-15	220.6	1.92

3.9 Polymerase chain reaction (PCR)

3.9.1 Principals of PCR

PCR is based on the mechanism of DNA replication in vivo: dsDNA is unwound to ssDNA, duplicated and rewounded. This technique consists of repetitive cycle of:

- Denaturation of the DNA through melting at elevated temperature to convert double-stranded DNA to single – stranded DNA
- Annealing (hybridization) of two oligonucleotides used as primers to the target DNA
- Extension of the DNA chain by nucleotide addition from the primers using and DNA polymerase as catalyst in the presence of Mg2+ ions

3.9.2 PCR amplification

3.9.2.1 Thermal conditions (Table 5 and 6)

Table 5 Thermal Condition of PCR for *E. coli*

Step	Temperature	Duration	Cycles
1.Initial denaturation	95^0C	5 min	01
2.Denaturation	95^0C	30 sec	35
3.Annealing	56^0C	30 sec	
4.Extension	72^0C	1.5 min	
5.Final extension	72^0C	10 min	01

Table 6 Thermal Condition of PCR for *Salmonella* spp.

Step	Temperature	Duration	Cycles
1.Initial denaturation	94^0C	1 min	01
2.Denaturation	94^0C	1 min	35
3.Annealing	64^0C	30 sec	
4.Extension	72^0C	30 sec	
5.Final extension	72^0C	7 min	01

3.9.2.2 Precautions of PCR

- PCR tubes were numbered carefully.
- All work was carried out in bio-safety cabinet class II to avoid contamination.
- Melt the vial containing all PCR reaction components.

- All components were taken in correct amounts into tube on PCR color box.
- All tubes were spin down or gently pipetting.

3.9.3 Electrophoresis

Agarose electrophoresis is a routiney used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined by the molecular weight where small weight molecules migrate faster than larger ones. In addition to size separation, nucleic acid fractionation using agars' gel electrophoresis can be an initial step for further purification of a band of interest.

Electrophoresis through agarose is a standard used to identity and purity of DNA fragments. The technique is simple, rapid to perform and capable of resolving fragments of DNA that cannot separated by other procedure.

3.9.3.1 Process of electrophoresis:

- Preparation of gel: Initially 1.05 gm agarose powder was weight out and placed into a 250 ml conical flask. Then 70 ml of electrophoresis buffer (1x TBE buffer) was added into the flask. The flask was then placed into microwave oven for 1 minute. The solution was heated again for 1 minute to dissolve small translucent agarose particles.
- The comb was then placed into the appropriate groove and slot of the casting tray.
- When the agarose solution was cooled to about 50°C (the flask was cooled enough to hold comfortably with bare band), 7µl to 10 mg/ml solution of ethidium bromide was added (the concentration of ethidium bromide in the melted agarose solution may be in the range of 0.5~1.0 µl/ml) and mixed well by gentle shaking to make DNA visible under ultraviolet light and poured into gel tray.
- The gel was allowed to solidify at room temperature for 20-30 minutes.
- The comb was removed carefully from the solidified gel and The casting dams were removed from the edges of the gel tray, so that the gel does not slide off the tray.

- Sample application in the gel and sufficient amount of 1x TBE buffer (about 600 ml) was added to cover the gel. The volume of electrophoresis buffer should not be above maximum buffer mark on electrophoresis system.
- Adjustment of voltage or current (gel- electrophoresis about 70-100 volts). Set up run time about 30-60 minute.
- The separation process was monitored by the migration of the dye in the loading buffer. When the bromophenol blue dye had reached about three-fourths (3/4) of the gel length, the electrophoresis was completed and stopped.

The UV light of the system was switched on; the image was viwed on the monitor, focused, acquired and collected picture of gel.

3.9.3.2 Documentation of the DNA sample

After completion of electrophoresis the gel was taken out carefully from the electrophoresis chamber and placed on UV trans illuminator (WUV-L50, Korea) for primary checking the DNA bands and then placed into the high performance gel documentation chamber (UVD1-254) for further checking and picture storage.

3.10 DNA sequencing and phylogenetic analysis

DNA from a total of sixteen *E. coli* and three *Salmonella* spp. samples were sequenced by Genetic Analyzer 3130 (Applied Biosystems) using dideoxy chain termination method. Sequencing was done using both forward and reverse direction. Obtained sequences were edited and analyzed by Molecular Evolutionary Genetics Analysis (MEGA-X) software (Kumar *et al*., 2018). Phylogenetic tree was made using neighbor joining method with 1000 bootstrap replication (Felsenstein, 1985; Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al*., 2004). Phylogenetic analysis of *E. coli* involved 30 nucleotide sequences and Phylogenetic analysis of *Samonella* spp. involved 27 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

3.11 Antibiotic Sensitivity tests

Kirby-bauer (K-B) antibiotic sensitivity testing:

Materials:

- Test tube rack
- Bunsen burner
- Inoculating loop or needle
- Forceps
- Sterile swabs
- Mueller-Hinton agar plate
- Antibiotic discs

Antimicrobial sensitivity test was performed according to the procedure of Kirby-bauer disk diffusion susceptibility test protocol. First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. The Kirby-Bauer (K-B) disk diffusion test is the most common method for antibiotic resistance/susceptibility testing.

The broth of the test organism was prepared in a test tube containing 5 ml nutrient broth by overnight incubation in shaking incubator. With a micropipette 100 µl of broth culture of the test organism was poured on Muller-Hinton agar plate. Sterile glass spreader was used to spread the culture homogenously on the medium. Inoculated plates were applied aseptically to the surface of the inoculated agar plates at a special arrangement with the help of a sterile forceps. The plates were then incubated at 37°C for 24 hours. After incubation, the diameter of the zones of complete inhibition (incubating the diameter of the disc) was measured and recorded in millimeters. The measurements were done with a ruler on the undersurface of the plate without opening the lid. The value was compared with the zone-size table and The zones of growth inhibition were provided by Clinical and Laboratory Standards Institute (CLSI, 2017). Isolates were classified as susceptible,

intermediate and resistant categories based on the standard interpretation tables updated according to the Clinical and Laboratory Standards Institution (Table 7)

Table 7 Antimicrobial agents with their disc – concentration

Sl. No	Antimicrobial Agents	Symbol	Disc Concentration (µg/disc)	Diameter of zone of inhibition (ZOD)		
				Resistant	Intermediate	Susceptible
1.	Amoxicillin	AML	30	≤13	14-17	≥18
2.	Azithromycin	AZM	15	≤13	14-17	≥18
3.	Gentamicin	GN	10	≤12	13-14	≥15
4.	Norfloxacin	NOR	10	≤12	13-16	≥17
5.	Tetracycline	TE	30	≤14	15-18	≥19
6.	Chloramphenicol	C	10	≤12	13-17	≥18
7.	Colistin Sulphate	CS	10	≤15	16-18	≥19
8.	Trimethoprim	TM	5	≤10	11-15	≥16
9.	Ampicillin	AMP	2	≤13	14-16	≥17
10.	Ceftazidime	CAZ	10	≤14	15-17	≥18
11.	Ceftriaxone	CRO	30	≤24	25-26	≥27
12.	Ciprofloxacin	CIP	5	≤15	16-20	≥21
13.	Amikacin	AK	30	≤14	15-16	≥17
14.	Enrofloxacin	ENR	5	≤16	17-20	≥21

Source: CLSI-2017[Note: S=Sensitive, R=Resistant, I=Intermediate]

3.12 Maintaining stock culture:

Stock culture was maintained with 500 microlitre of organism from nutrient broth with 500 microlitre of 99% glycerin in cryovial and kept in -20^{0}C in refrigerator.

CHAPTER-4

RESULTS

The present research was designed to determine the isolation, molecular characterization and antibiogram study of *E. coli* and *Salmonella* spp. isolated from faecal sample of pigeon, budgerigar and love bird of pet shops of Katabon, Dhaka , Bangladesh. The collected samples were subjected to various bacteriological examinations such as cultural, biochemical techniques and antibiotic sensitivity pattern in the laboratory of the Animal Biotechnology division of National Institute of Biotechnology, for isolation and identification of bacteria. Out of 45 samples, 30 isolates were found to be positive. Results are presented in different tables.

4.1 Total viable count, Total coliform count and Total *Salmonella* count of isolates (Table 8) and (Figure 5, 6 and 7)

Total viable count (TVC) was done on nutrient agar and TVC (CFU/ml) range was 4.76×10^{11} to 1.36×10^{9}. Total Coliform Count (CFU/ml) range was 8.8×10^{8} to 7.2×10^{8} and Total Salmonella Count (CFU/ml) range was 9.4×10^{9} to 2.4×10^{8}.

Table 8 Total Viable Count, Total Coliform Count and Total Salmonella Count from the isolated samples

Serial no. and name of sample	Total Viable Count (TVC) (CFU/ml)	Total Coliform Count (TCC) (CFU/ml)	Total *Salmonella* Count (TSC) (CFU/ml)
1. P-1	1.8×10^{11}	-	2.4×10^{8}
2. P-2	2.3×10^{11}	-	2.6×10^{8}
3. P-3	2.04×10^{11}	7.2×10^{8}	-
4. P-4	1.04×10^{11}	-	9.4×10^{9}
5. P-5	1.56×10^{11}	5.6×10^{9}	-
6. P-6	1.04×10^{11}	8.8×10^{8}	-
7. P-7	4.76×10^{11}	-	-
8. P-8	1.6×10^{11}	2.66×10^{9}	-
9. P-9	2.72×10^{10}	-	-

Table 8 (cont'd)

10. P-10	0.96×10^{10}	-	5.4×10^{9}
11. P-11	1.48×10^{11}	-	3.56×10^{9}
12. P-12	1.04×10^{10}	-	-
13. P-13	1.96×10^{10}	-	-
14. P-14	1.04×10^{10}	1.44×10^{9}	-
15. P-15	1.52×10^{10}	-	4.6×10^{9}
16. P-16	1.48×10^{10}	4.28×10^{9}	-
17. P-17	2.44×10^{10}	2.31×10^{9}	-
18. P-18	1.32×10^{11}	-	-
19. P-19	1.96×10^{11}	-	6.4×10^{9}
20. P-20	8.4×10^{10}	-	3.4×10^{9}
21. B-1	2.8×10^{10}	1.25×10^{9}	-
22. B-2	2.0×10^{11}	4.4×10^{7}	-
23. B-3	9.2×10^{10}	1.9×10^{9}	-
24. B-4	1.04×10^{11}	3.4×10^{9}	-
25. B-5	1.12×10^{10}	-	-
26. B-6	1.48×10^{10}	4.56×10^{9}	-
27. B-7	2.08×10^{10}	-	-
28. B-8	1.32×10^{10}	8.4×10^{8}	-
29. B-9	2.8×10^{9}	3.32×10^{9}	-
30. B-10	1.64×10^{10}	3.76×10^{9}	-
31. B-11	9.2×10^{10}	1.40×10^{9}	-
32. B-12	1.24×10^{10}	8.8×10^{10}	-
33. B-13	5.2×10^{10}	2.6×10^{10}	-
34. B-14	1.88×10^{9}	-	-
35. B-15	1.36×10^{9}	-	-
36. L-1	3.00×10^{10}	6.32×10^{9}	-

Table 8 (cont'd)

37. L-2	1.04×10^{10}	1.84×10^{9}	-
38. L-3	1.2×10^{10}	-	-
39. L-4	5.36×10^{11}	-	-
40. L-5	2.00×10^{10}	-	-
41. L-6	1.52×10^{11}	-	-
42. L-7	3.68×10^{11}	-	-
43. L-8	4.00×10^{10}	-	-
44. L-9	2.5×10^{11}	1.13×10^{9}	-
45. L-10	1.88×10^{11}	3.24×10^{9}	-

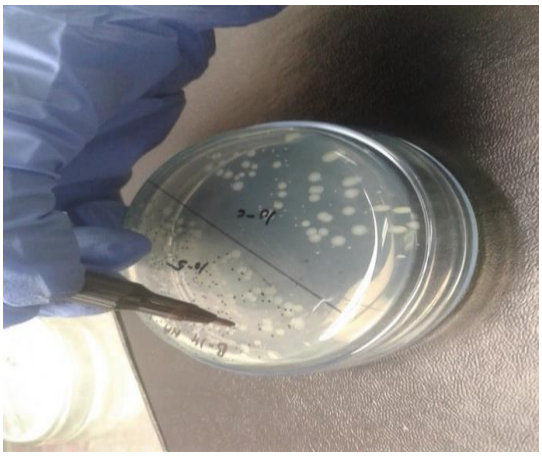


Figure 5: Total Viable Count (TVC)

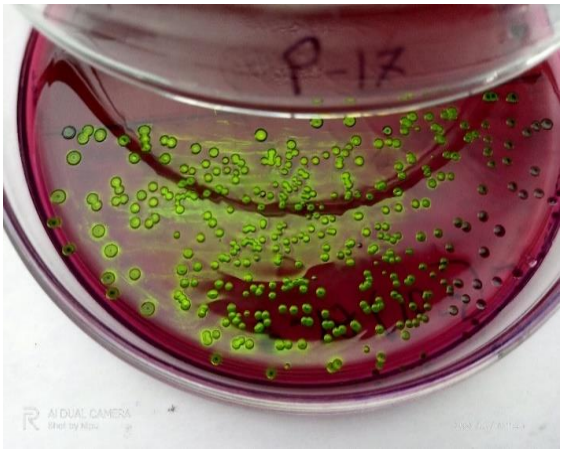


Figure 6: Total Coliform Count (TCC)

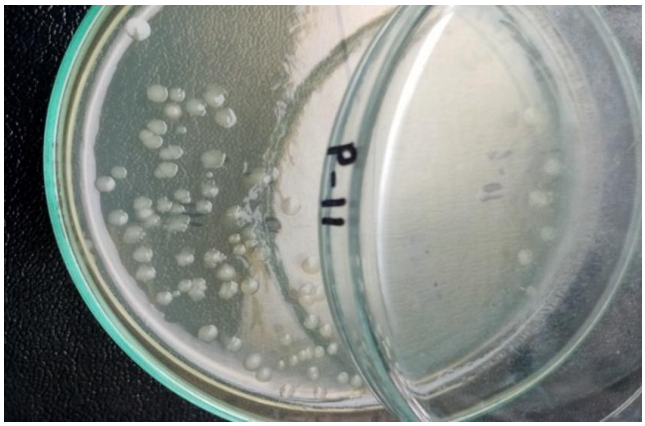


Figure 7: Total Salmonella Count (TSC)

4.2 Cultural characteristics of *E. coli* (Table 9 and Figure 8, 9, 10, 11, 12)

After inoculation of fecal sample on Nutrient agar and Nutrient broth, it was observed that maximum growth was found in Nutrient agar and turbidity in Nutrient broth.

Table 9 *E. coli* colony characteristics

Name of bacteria	Name of Media	Colony Characteristics
E. coli	Nutrient broth	Turbidity
	Nutrient Agar	Large, mucoid, white colony
	Mac-Conkey's Agar	Produce large mucoid rose pink colony.
	EMB agar	Transmitted light blue black centre with a narrow, clear edge.
		Blue-green metallic sheen with reflected light.
	Salmonella-Shigella Agar	*E. coli* on plate with rose pink colony

4.2.1 Nutrient broth

Nutrient broth inoculated with the samples revealed the growth of. *E coli* after 24 hours of incubation at 37°C. The growth of *E. coli* was indicated by the presence of turbidity (Figure 8)

Figure 8: *E. coli* showed turbidity throughout the tube

4.2.2 Nutrient agar

Nutrient agar plates spread with the samples revealed the growth of bacteria after 24 hours of incubation at 37°C and were indicated by the growth of circular, small smooth, convex, and gray white colonies (Figure 9)

Figure 9: *E. coli* showing circular, small smooth, convex, and gray white colonies on NA

4.2.3 Mac-Conkey Agar

Mac-conkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C and were indicated the bright pink color colonies (Figure 10)

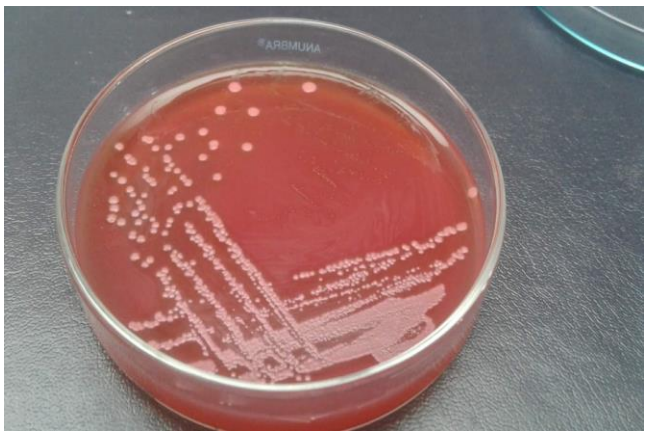


Figure 10: *E. coli* showing bright pink color colonies on Mac-conkey agar

4.2.4 Eosin Methylene Blue agar

Eosin Methylene Blue agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and were indicated by the growth of smooth, circular, black color center with blue-green metallic sheen color colonies (Figure 11).

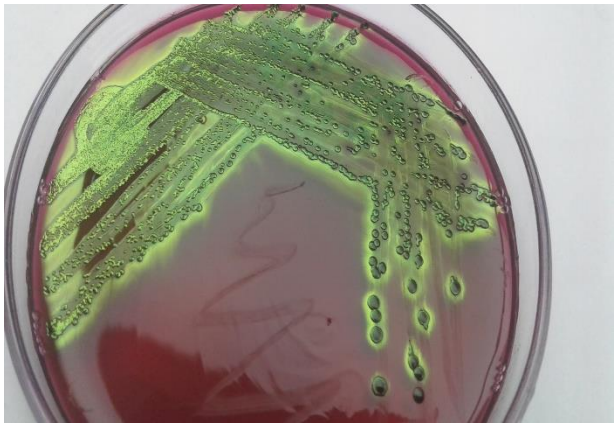


Figure 11 : *E. coli* showing smooth, circular, black color center with blue-green metallic sheen color colonies on Eosin Methylene Blue agar

4.2.5 Salmonella-Shigella agar

Salmonella-shigella agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C and were indicated by transparent colonies, rose pink color colony (Figure 12).

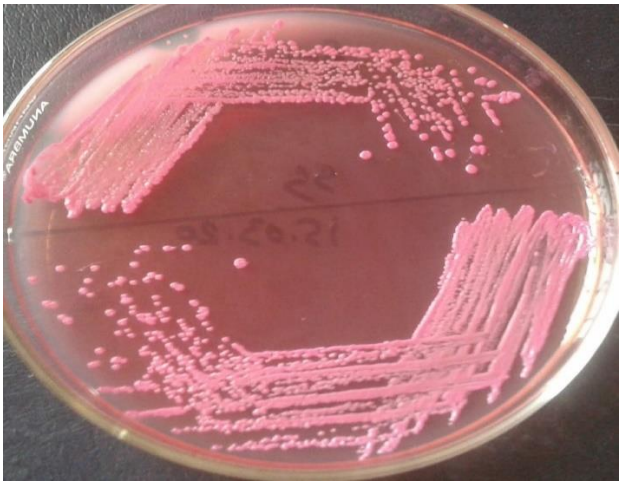


Figure 12: *E. coli* showing rose pink color colonies on Salmonella-Shigella agar

4.3 Different biochemical tests (Table 10)

Table 10 different biochemical tests for *E. coli*

Name of isolate	OX	CT	MR	VP	TSI		SIM			SC	UR	NR
					Butt	Slant	S	I	M			
E. coli	-	+	+	-	A (yellow)	A (yellow)	-	+	+	-	-	+

Legends: + = positive, - = negative, A=acid, OX= oxidase, CT=catalase,MR= methyl-red, VP= voges-proskauer, SC= simmon's citrate, TSI= triple sugar iron,SIM= sulphur indole motility, S=sulphur, I=indole, M=motility, UR=urease, NR=nitrate reduction

4.3.1 Oxidase test

All the isolates of *E. coli* were cofirmed negative for oxidase test with no purple color formation (Figure 13)

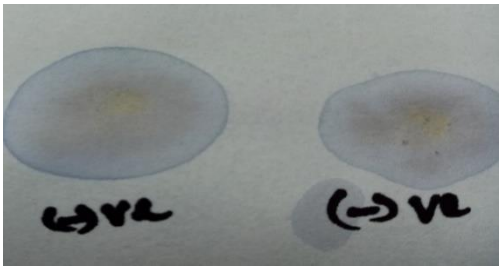


Figure 13: Oxidase test negative for *E. coli*

4.3.2 Catalase test

All the isolates of *E. coli* were confirmed positive as the isolates produced bubbles in catalase test (Figure 14)

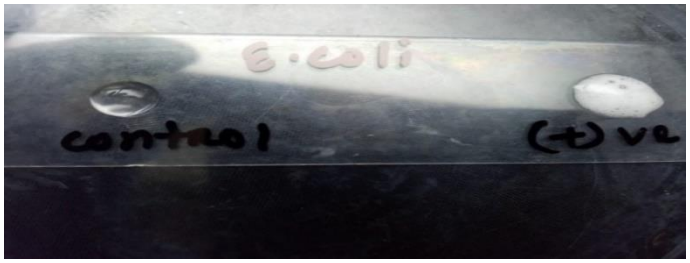
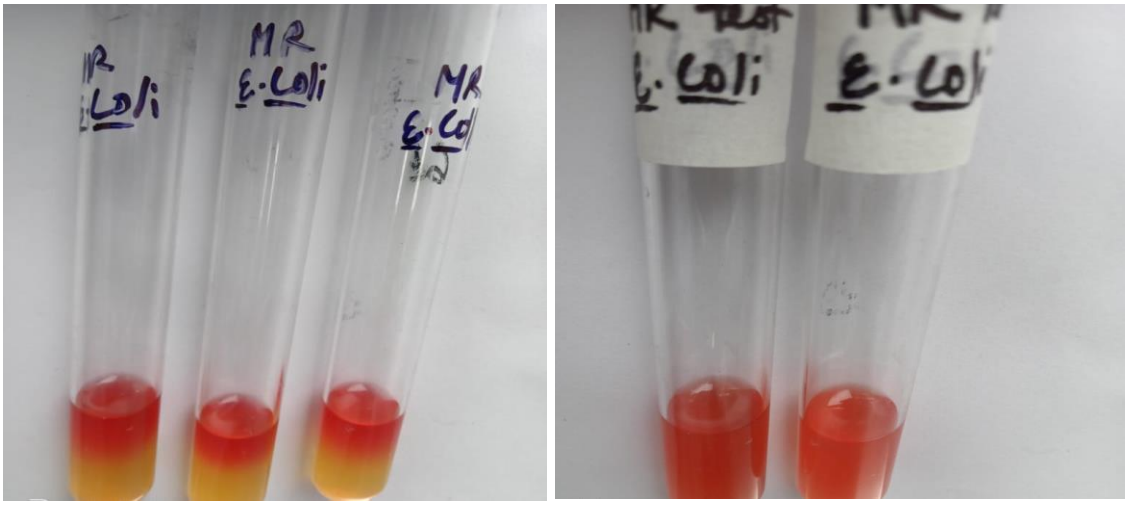


Figure 14: Catalase test positive for *E. coli*

4.3.3 Methyl- Red test

E. coli was positive to MR test. The test was conducted by inoculating colony of the isolated *E. coli* in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. A bright red coloration was produced (Figure 15).



(A) (B)

Figure 15: (A and B) Methyl-Red test positive for *E. coli*

4.3.4 Voges-Proskauer test

E. coli was confirmed negative as they could not give rose coloration for Voges-Proskauer test (Figure 16)

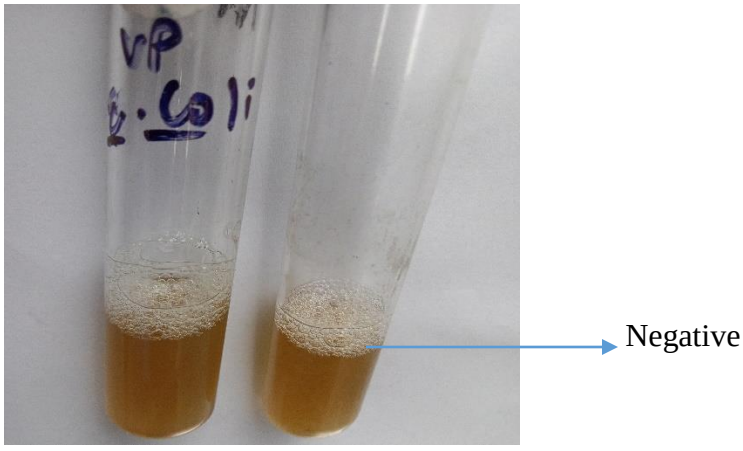


Figure 16: Voges-Proskauer test negative for *E. coli*

4.3.5 Simmons citrate test

E. coli was confirmed negative as they could not change medium green to blue in simmons citrate test (Figure 17).

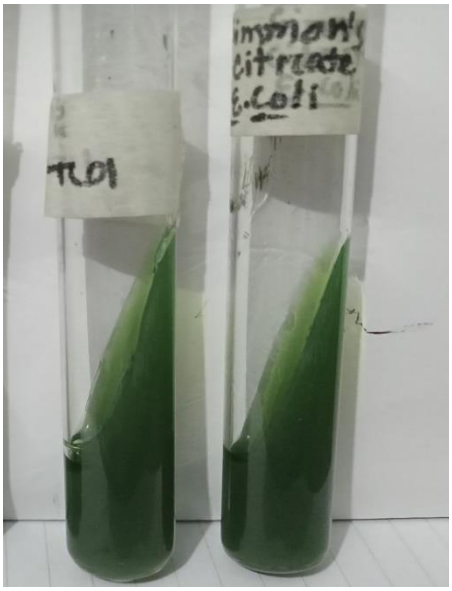


Figure 17: Simmons citrate test negative for *E. coli* (right) and control (left).

4.3.6 TSI (Triple sugar Iron) test:

E. coli showed yellow coloration in both butt and slant with gas production (Figure 18).

Figure 18: *E. coli* showed yellow coloration in both butt and slant with gas production in Triple sugar Iron test

4.3.7 Urease test:

E. coli was confirmed negative with no pink color formation (Figure 19).

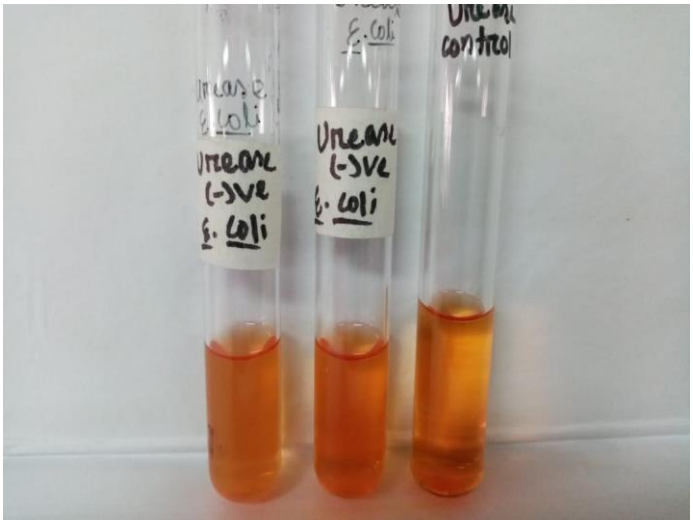
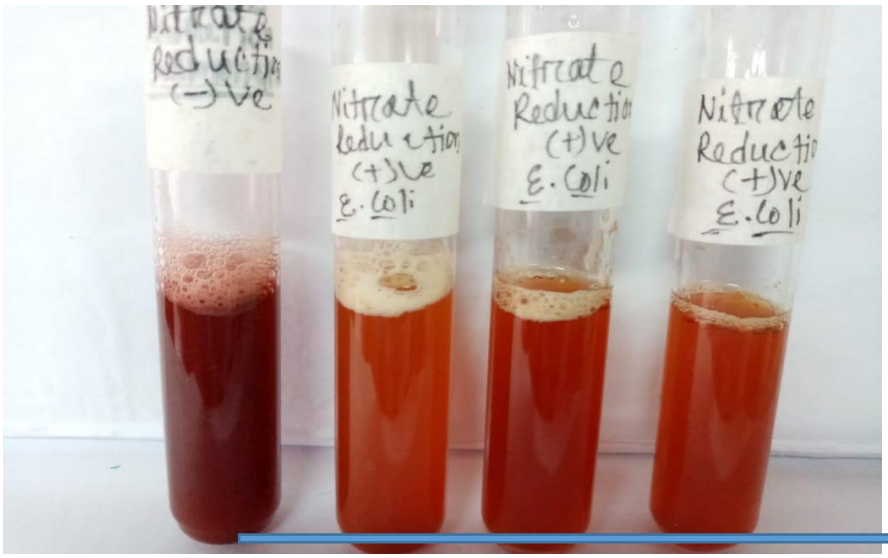


Figure 19: Urease test negative for *E. coli* (left) inoculated and (right) control

4.3.8 Nitrate reduction test:

E. coli was confirmed positive with cherry red color formation after adding sulphanilic acid and alpha naphthol (Figure 20).

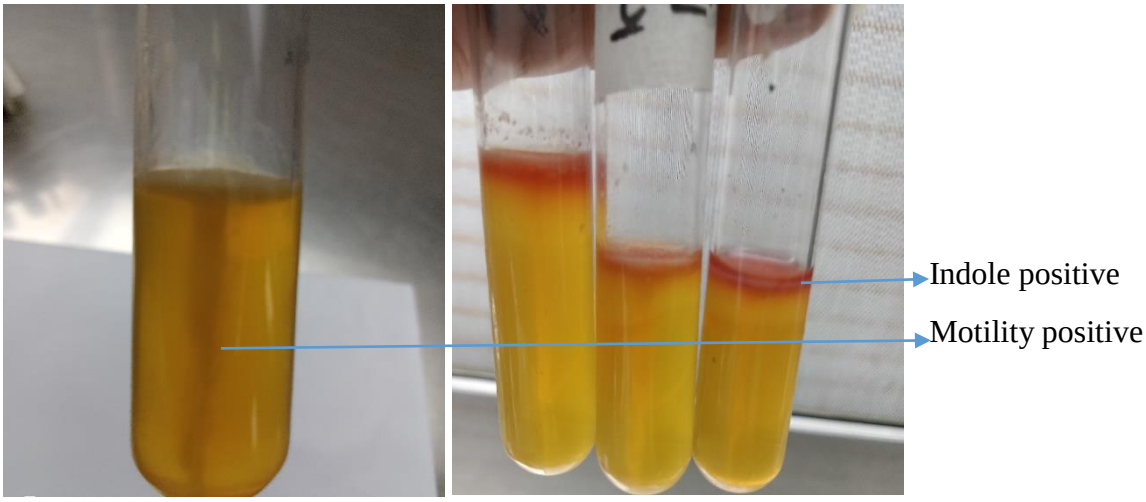


Negative

Figure 20: Nitrate reduction test positive for *E. coli* (left) negative and (right) positive

4.3.9 Sulphur indole motility test (SIM):

E. coli was confirmed positive to motility and cherry red color ring formation after adding Kovacs reagent gives positive result of indole test. No H_2S produced showing no blackening (Figure 21 and 22)



(A) (B)

Figure 21: (A) Motility positive of *E.coli* and no Blackening or Sulphur production
(B) Indole positive of *E.coli* with cherry red ring formation

4.3.10 Sugar fermentation test

All the isolates of *E. coli* were fermented dextrose, glucose, sucrose, lactose and manitol with acid formation showing yellow coloration and gas production showing bubbles in Durham's tube (Table 11 and Figure 22)

Table 11 Biochemical reaction patterns of *E. coli* sugar fermentation test

Name of isolate	**Sugar fermentation properties**				
	Lactose	Dextrose	Glucose	Sucrose	Mannitol
	+	+	+	+	+
E. coli	AG	AG	AG	AG	AG

Legends: +=positive, A= acid production, G= gas production

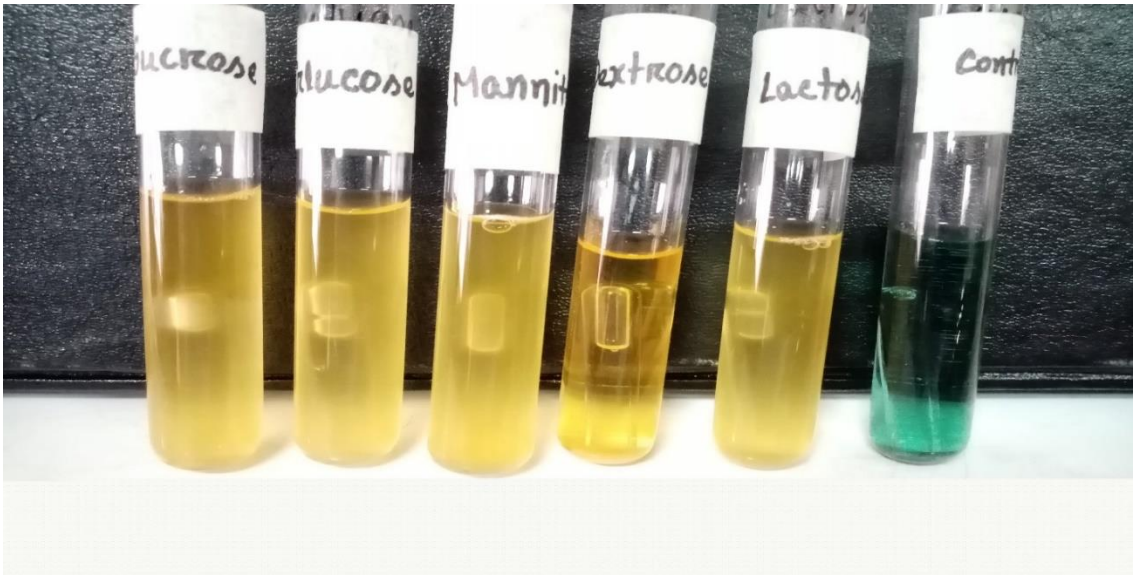


Figure 22: Isolates of *E. coli* fermented dextrose, glucose, sucrose, lactose and mannitol with acid and gas formation

4.4 PCR amplification of *E. coli* DNA with specific primers

Out of 45 samples, 22 isolates were *E. coli*. For molecular identification, PCR was done with E1 and E2 primers (Figure 23)

Gel image:

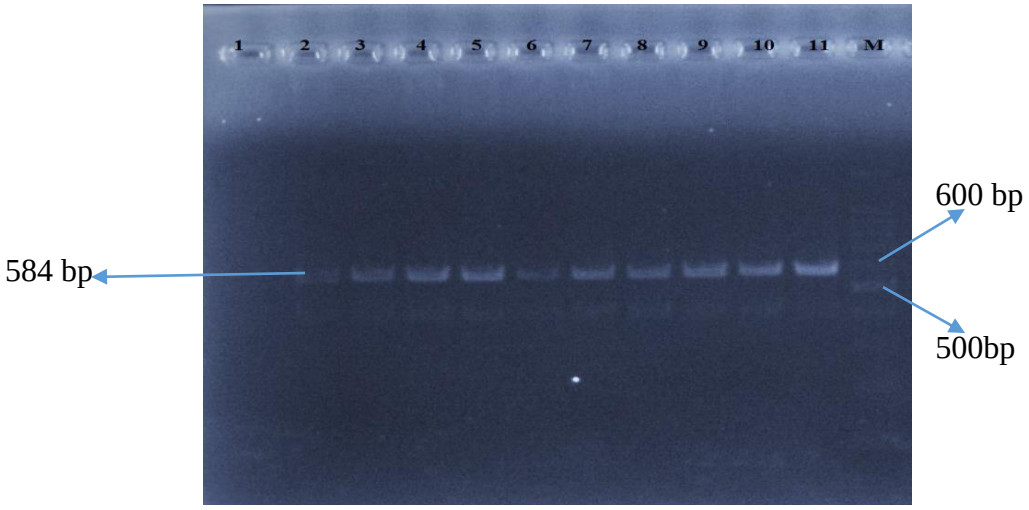


Figure 23: Amplification of 584 bp DNA from 16S rRNA gene of *E. coli*. Lane:1 to 11: test sample and Lane M: Marker(100 bp). Lane 1: negative control.

(Note: PCR=Polymerase Chain Reaction, bp= base pair)

4.5 Phylogenetic tree analysis of isolated *E. coli*

E. coli sequenced (n=16) in this study were found to carry 100% homology with each other irrespective of their host of origin. An *E. coli* isolated namely, CVCaOS-33 BD, accession number MW116771, from oral swab of cat from central veterinary hospital of Bangladesh was fund to carry 100% homology with all of sixteen sequences of this study. Other sequences used in the tree were found to carry 99.590- 99.994% homology. In relation to other sequences our sequences were branched in to two groups. The sequences used in the tree are derived from a variety of hosts of different countries. These include for example isolate accession no.AP022409 and AP022482 from hospital sewage in Japan; CP062211 from river water in New Zealand; CP062967 and CP062970 from human blood culture, Ankara, Turkey; CP062228 from human urine sample in India; AP022549 from human urine sample in Japan; CP055426 from freshwater sample from downstream of wastewater treatment plant, UK; CP018252 from cattle feces, UK; CP062203 from swine feces, South Korea; CP062160 from culture mutant, Pennsylvania USA and LR738975 from fecal samples of weaned piglets, Brazil. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Sequences of this study are marked with red circle in the tree (Figure 24).

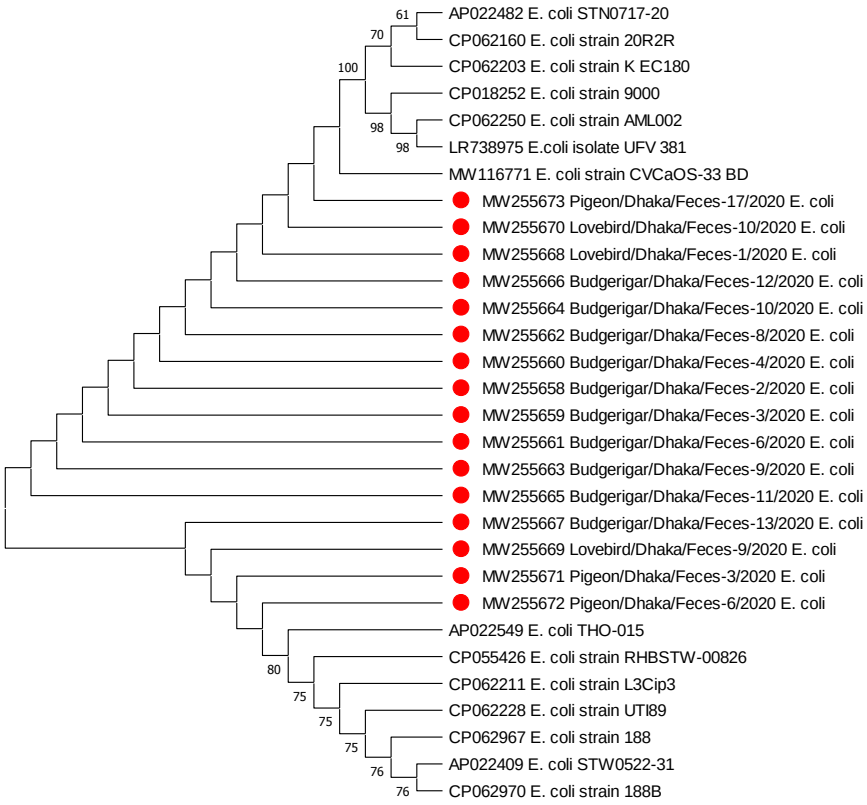


Figure 24: The phylogenetic analysis of *E. coli* isolated from feces of different pet birds

4.6 Cultural characteritics of *Salmonella* spp. (Table 12)

Table 12 Colony characteristics of *Salmonella* spp.

Name of bacteria	Name of media	Colony characteristics
Salmonella spp.	Nutrient broth	Turbidity
	Nutrient agar	Circular, smooth, opaque and translucent
	Mac-conkey agar	Smooth and circular colorless colony
	Eosin Methyline Blue agar	Grey color colony, smooth and circular
	Salmonella-Shigella agar	Black centered,smooth, small,round colony

4.6.1 Nutrient broth

Nutrient broth inoculated with the samples revealed the growth of *Salmonella* spp. 24 hours of incubation at 37°C. The growth was indicated by the presence of turbidity (Figure 25).

Figure 25: *Salmonella* spp. shows turbidity throughout the tube

4.6.2 Nutrient agar

Nutrient agar plates spread with the samples revealed the growth of bacteria after 24 hours of incubation at 37°C and were indicated by the growth of Circular, smooth, opaque and translucent colonies (Figure 26).

Figure 26: *Salmonella* spp. showing Circular, smooth, opaque and translucent colonies on NA

4.6.3 Mac-Conkey Agar

Mac-conkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C and were indicated the Smooth and circular colorless colony (Figure 27).

Figure 27: *Salmonella* spp. showing smooth and circular colorless colony on Mac-Conkey Agar

4.6.4 Eosin Methylene Blue agar

Eosin Methylene Blue agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and were indicated by the growth of smooth, circular, grey color colony (Figure 28).

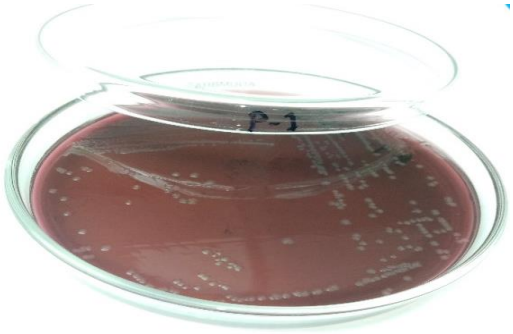


Figure 28: *Salmonella* spp. showing smooth and circular, grey color colonies on Eosin Methylene Blue agar

4.6.5 Salmonella-Shigella agar

Salmonella-shigella agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C and were indicated by transparent black centered, smooth, small, round colony (Figure 29).

Figure 29: *Salmonella* spp. showing black centered, smooth, small, round colony on Salmonella-Shigella agar

4.7 Biochemical properties of different biochemical tests (Table 13)

Table 13 Biochemical properties of different biochemical tests

Name of isolate	OX	CT	MR	VP	TSI		SIM			UR	SC	NR
					Butt	Slant	S	I	M			
Salmonella spp.	-	+	+	-	Yellow With H_2S	Red	+	-	+	-	+	-

Legends: + = positive,- = negative, OX= oxidase, CT=catalase,MR= methyl-red, VP= voges-proskauer, SC= simmon's citrate, TSI= triple sugar iron,SIM= sulphur indole motility, S=sulphur, I=indole, M=motility, UR=urease, NR=nitrate reduction

4.7.1 Oxidase test

All the isolates of *Salmonella* spp. were confirmed negative for oxidase test with no purple color formation (Figure 30).

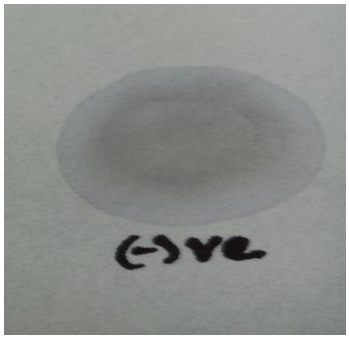


Figure 30: Oxidase test negative *Salmonella* spp.

4.7.2 Catalase test

All the isolates of *Salmonella* spp. were confirmed positive as the isolates produced bubbles in catalase test (Figure 31).

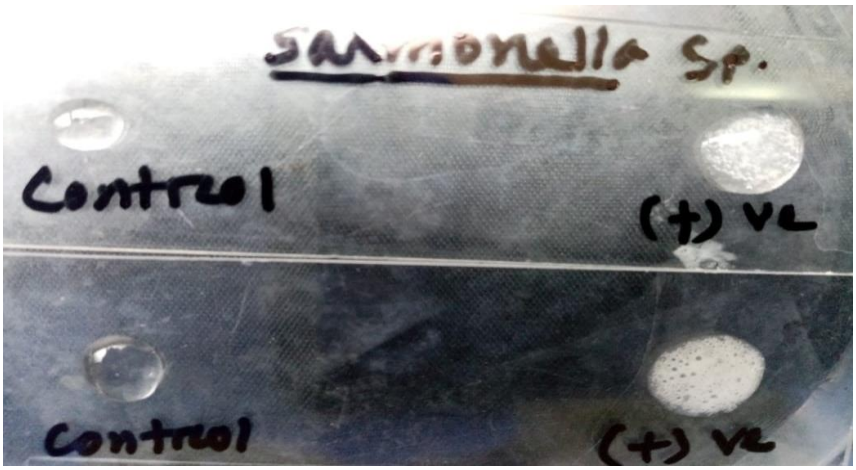
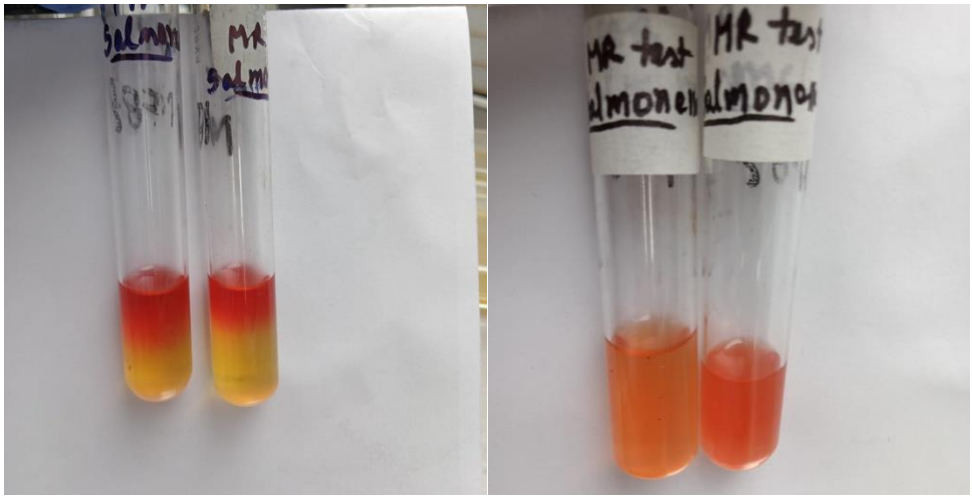


Figure 31: Catalase test positive *Salmonella* spp.

4.7.3 Methyl- Red test

Salmonella spp. was positive to MR test. The test was conducted by inoculating colony of the isolated E. coli in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. A bright red coloration was produced (Figure 32).



(A) (B)

Figure 32: (A and B) Methyl-Red test positive *Salmonella* spp.

4.7.4 Voges-Proskauer test

Salmonella spp. was confirmed negative by no rose coloration for Voges-Proskauer test (Figure 33).

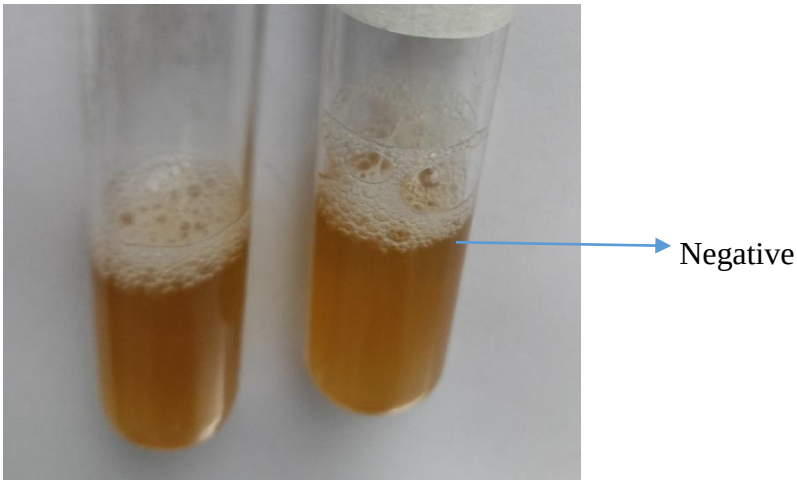


Figure 33: Voges-Proskauer test negative for *Salmonella* spp.

4.7.5 Simmons citrate test

Salmonella spp. was confirmed positive by changing medium green to blue in simmons citrate test (Figure 34).

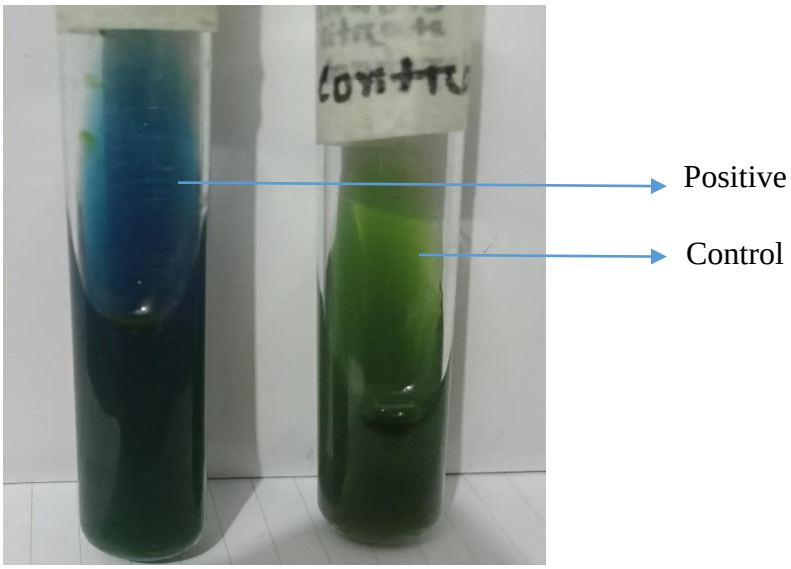


Figure 34: Simmons citrate test positive for *Salmonella* spp. (left) and control (right).

4.7.6 TSI (Triple sugar Iron) test:

Salmonella spp. showed yellow coloration in butt and red in slant with gas production and blackening indicates H_2S production (Figure 35).

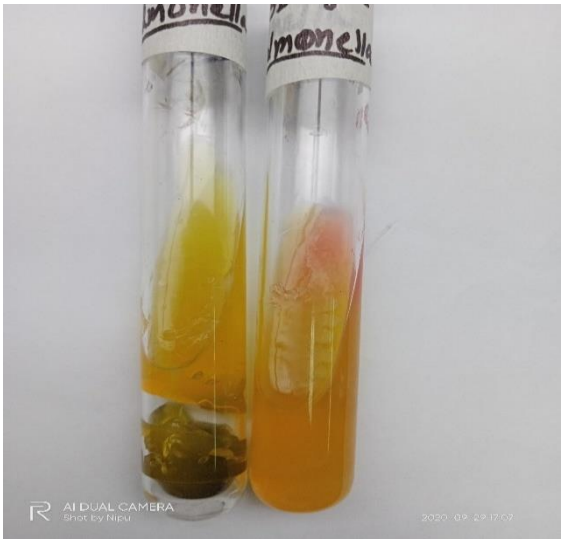


Figure 35: *Salmonella* spp. showed yellow coloration in butt and slant is red with gas and H_2S production in Triple sugar Iron test

4.7.7 Urease test:

Salmonella spp. was confirmed negative with no pink color formation (Figure 36).

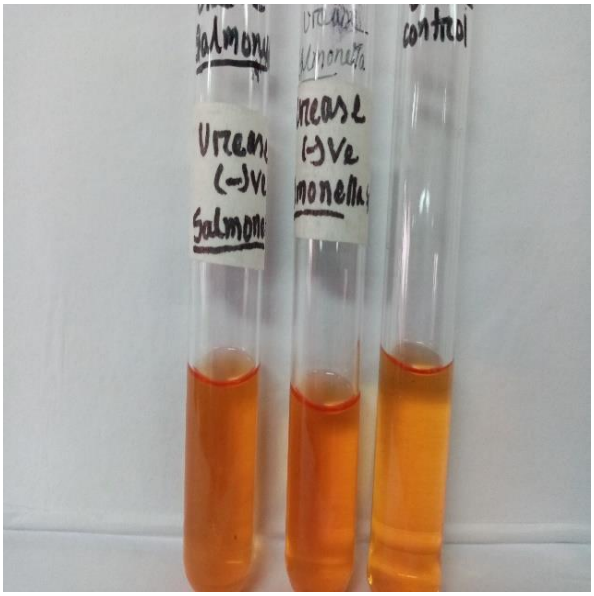


Figure 36 : Urease test negative for *Salmonella* spp. (left) inoculated and (right) control

4.7.8 Nitrate reduction test:

Salmonella spp. showed negative result with no cherry red color formation after adding sulphanilic acid and alpha naphthol. Negative result was indicated by adding zinc dust and showed red color (Figure 37).

Figure 37: Nitrate reduction test negative *Salmonella* spp. (left) and (right) positive

4.7.9 Sulphur indole motility test:

Salmonella spp. showed positive result to motility and no cherry red color ring formation gives negative result of indole test. H2S produced showing blackening (Figure 38).

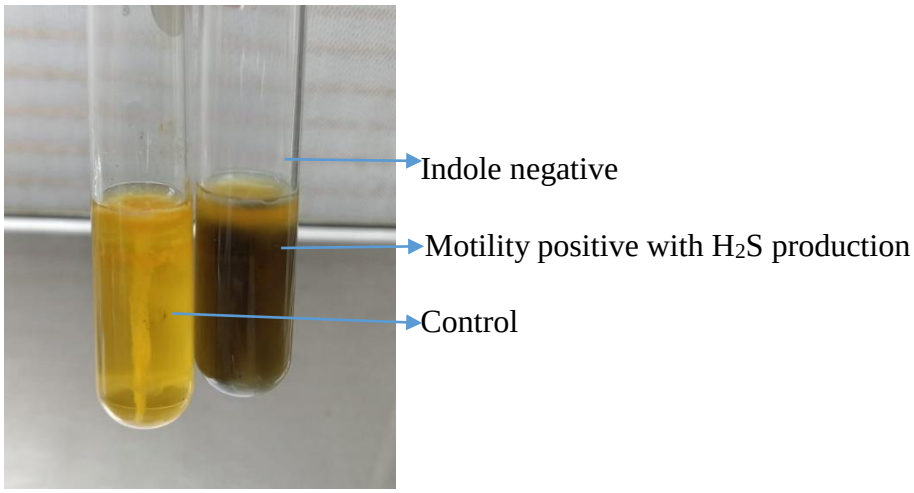


Figure 38: Motility positive *Salmonella* spp. (right) and Indole negative with Blackening or Sulphur production

4.7.10 Sugar fermentation test

All the isolates of *Salmonella* spp. fermented dextrose, glucose, and manitol with acid formation showing yellow coloration and gas production showing bubbles in Durham's tube. But lactose and sucrose were not fermented (Table 14 and Figure 39).

Table 14 Biochemical reaction patterns of *Salmonella* spp. sugar fermentation test

Name of isolate	**Sugar fermentation properties**				
	Dextrose	Sucrose	Lactose	Glucose	Mannitol
Salmonella spp.	+ AG	- NF	- NF	+ AG	+ AG

Legends: + = positive, - = negative, A= acid production, G=gas production, NF= non fermented

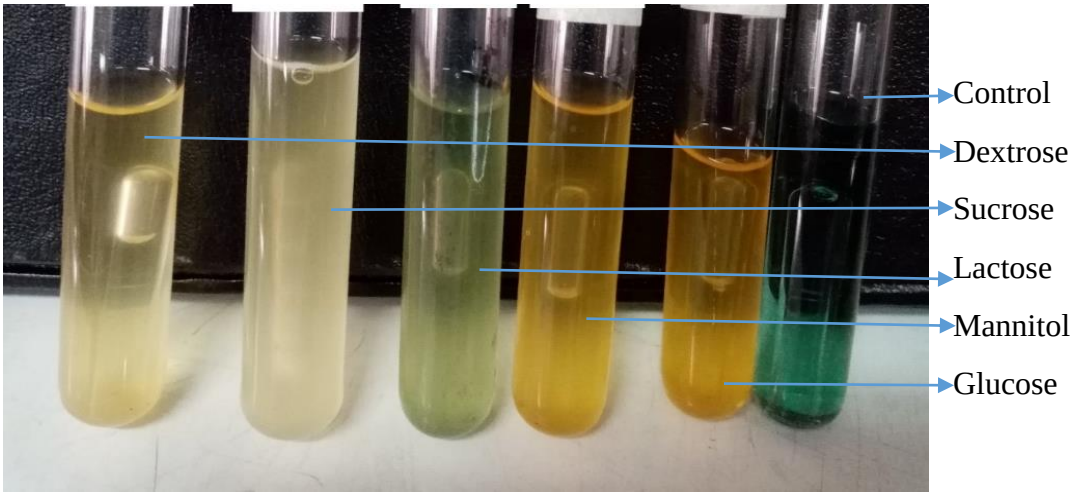


Figure 39: Isolates of *Salmonella* spp. fermented dextrose, glucose, and mannitol with acid and gas formation

4.8 PCR amplification of *Salmonella* spp. DNA with specific primers

Out of 45 samples, 8 isolates were *Salmonella* spp.. For molecular identification, PCR was done with S139 and S141 primers (Figure 40).

Gel image:

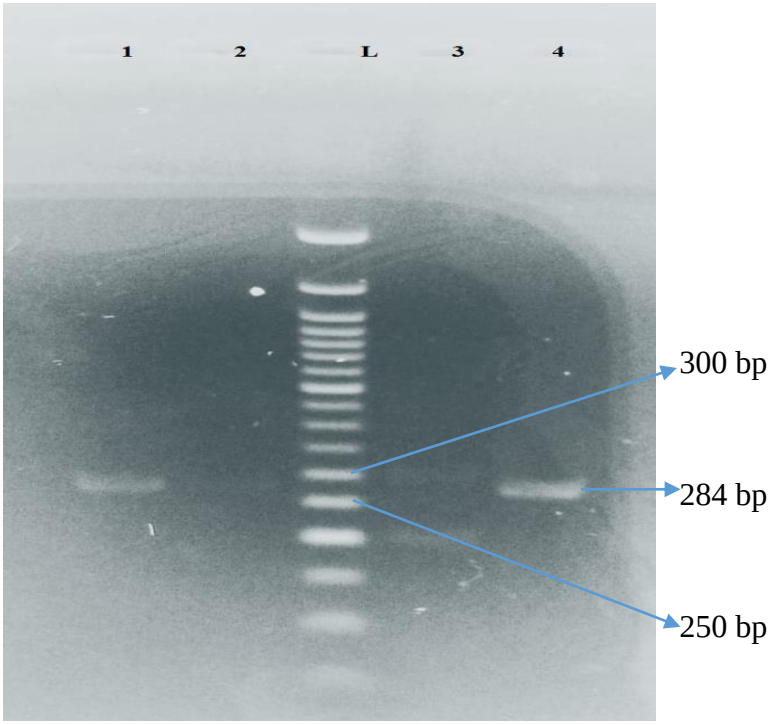


Figure 40: Amplification of 284 bp DNA of *Salmonella* spp. Lane:1 and 4: test samples (p-4 and p-11) of *Salmonella* spp. showed band at 284 bp and Lane M: Marker(50 bp). (Note: PCR=Polymerase Chain Reaction, bp= base pair)

4.9 Phylogenetic tree analysis of isolated *Salmonella* spp.

Salmonella spp. sequenced (n=3) in this study were found to carry 100% homology with each other irrespective of their host of origin. Our sequences were also found to have 100% homology with the sequences of *Salmonella enterica* serovar *Agona*, accession number CP015024 and CP011259 and *Salmonella enterica* strain ST1539 accession number CP035301. These strains are derived from Cereal crop, unsweetened puffed rice cereal, USA, and raw duck meat from South Korea. Other strains are originated from murine gut, Switzerland (LR881463), food material, Russia (CP060515), UK (LR861808, LR862421), horse, Australia (CP058807), minced pork, China (CP053294), human, Australia (CP045831) and so on. High homology was also observed with *Salmonella typhimurium* and ranges from 98.5-99.45%. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Sequences of this study are marked with red circle in the tree (Figure 41).

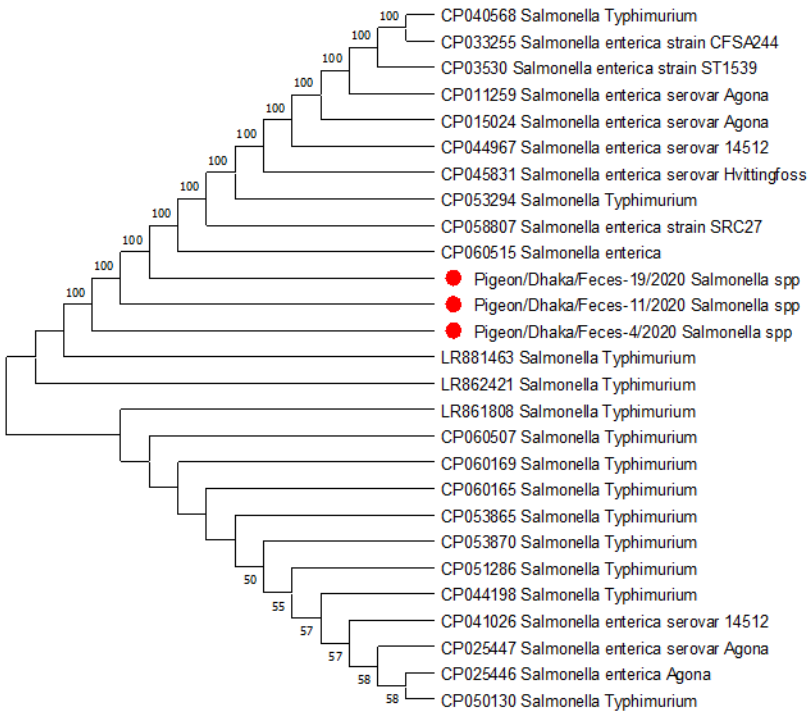


Figure 41: The phylogenetic analysis of *Salmonella* spp. isolated from feces of different pet birds

4.10 Occurrence of microorganism (Table 15)

Table 15 Occurrence of microorganism in different species of birds

Name of the species of bird	No. of sample investigated	No. of sample containing organism	Percentage of occurrence
Pigeon (P-1 to P-20)	20	20	100%
Budgerigar (B-1 to B-15)	15	15	100%
Love bird(L-1 to L-10)	10	10	100%
Total	45	45	100%

4.11 Occurrence of specific organism from sample (Table 16)

Table 16 Occurrence of specific organism from sample

No. of sample investigated	No. of *E. coli* isolates with occurrence (%)	No. of *Salmonella* spp. Isolates with occurrence (%)	Non identified
45	22 (48.8%)	8 (17.7%)	15 (33.3%)

4.12 Occurrence of *E. coli* and *Salmonella* spp. in different species of birds (Table 17 and Figure 42)

Table 17 Occurrence of *E. coli* and *Salmonella* spp. in different species of birds:

Name of the bird species	Isolated organism	No. of isolates with occurrence percentage(%)
Pigeon	*E. coli*	7 (35%)
	Salmonella spp.	8 (40%)
Budgerigar	*E. coli*	11 (73.33%)
	Salmonella spp.	Nil
Love bird	*E. coli*	4 (40%)
	Salmonella spp.	Nil

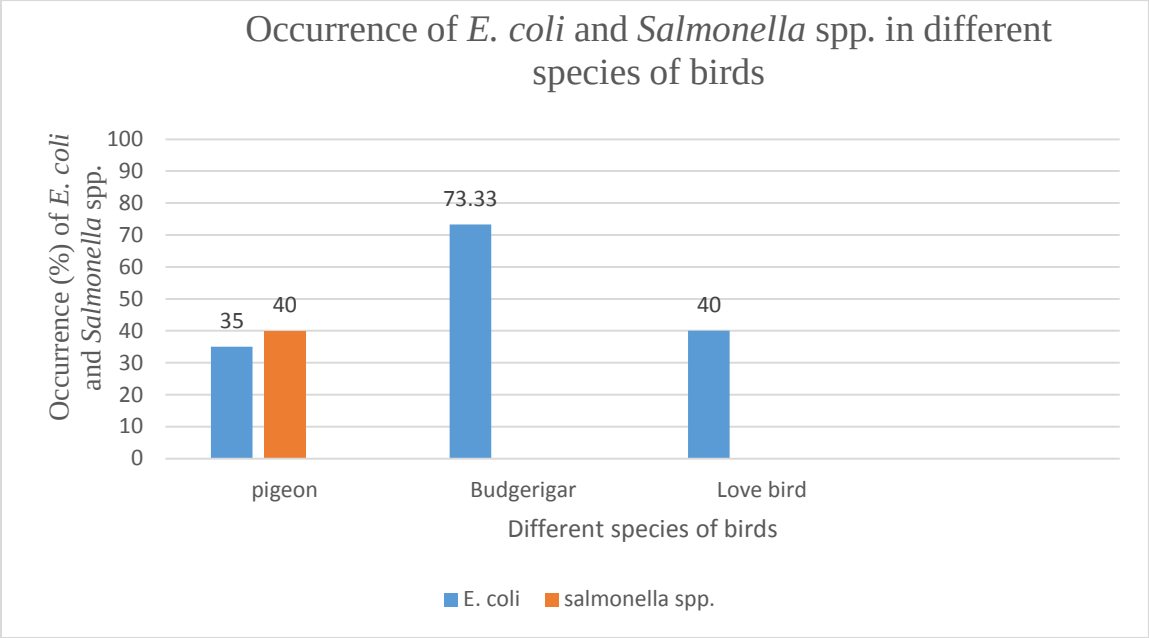


Figure 42: Occurrence of *E. coli* and *Salmonella* spp. from fecal sample of pigeon, budgerigar and lovebird

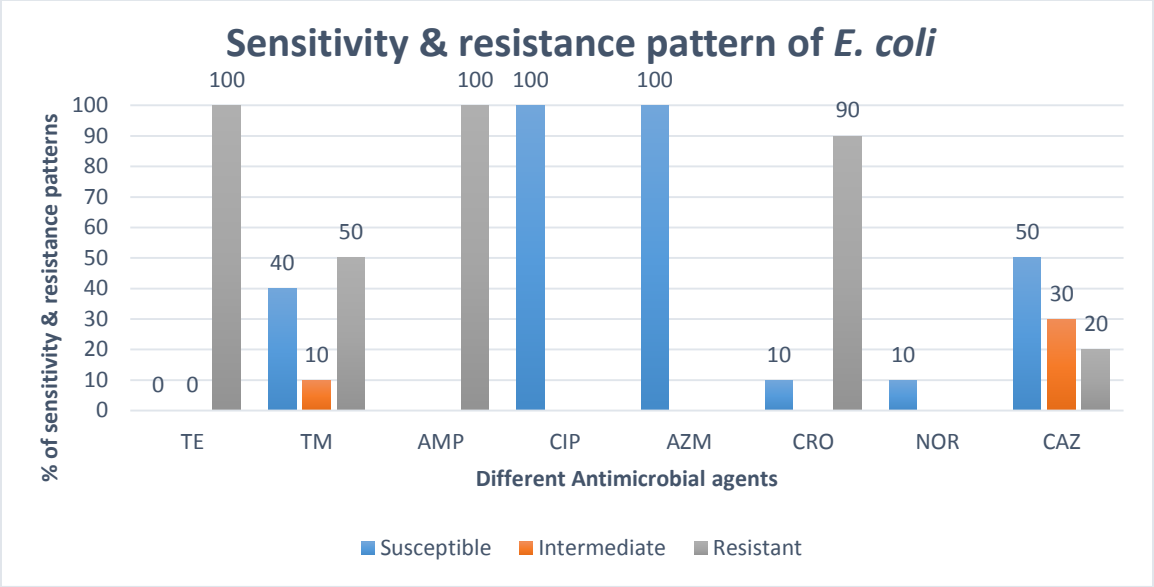
4.13 Results of antimicrobial susceptibility of *E. coli* isolates (n=10) (Table 18 and Figure 43, 44 and 45)

The results of susceptibility analysis showed that all the isolates of *E. coli* (n=10) were 100% susceptible to Ciprofloxacin, Norfloxacin, Amikacin, Azithromycin and Gentamicin. All the isolates of *E. coli* (n=4) of pigeon were also susceptible to Ceftazidime and Chloramphenicol. All the isolates of *E. coli* (n=3) of budgerigar were also sensitive to Chloramphenicol and all the isolates of *E. coli* (n=3) of love bird were sensitive to Enrofloxacin. All the isolates of *E. coli* (n=10) were 100% resistant to Tetracyclin, Ampicillin and Colistin sulphate. All the isolates of *E. coli* (n=3) of budgerigar and *E. coli* (n=3) of love bird were resistant to ceftriaxone.

Table 18 Antimicrobial susceptibility pattern of *E. coli*

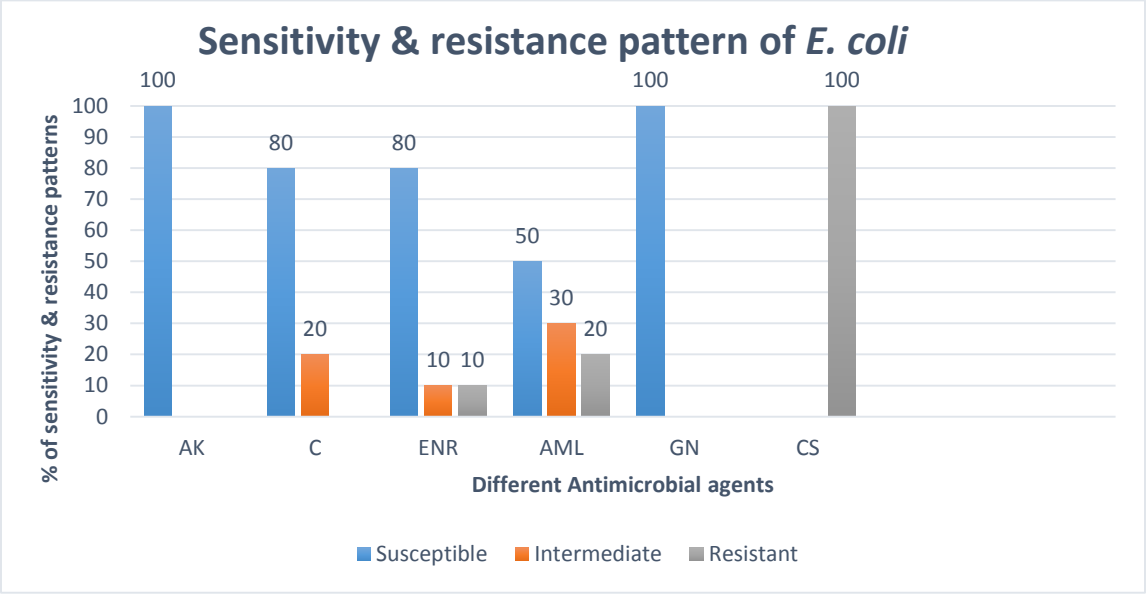
Antimicrobial agents	**No. of *E. coli* isolates (%)**		
	S	**I**	**R**
Tetracycline	0(0%)	0(0%)	10(100%)
Trimethoprim	4(40%)	1 (10%)	5 (50%)
Ampicillin	0(0%)	0(0%)	10(100%)
Amoxicillin	5(50%)	3(30%)	2(20%)
Ciprofloxacin	10(100%)	0(0%)	0(0%)
Azithromycin	10(100%)	0(0%)	0(0%)
Ceftriaxone	1(10%)	0(0%)	9(90%)
Norfloxacin	10(100%)	0(0%)	0(0%)
Ceftazidime	5(50%)	3(30%)	2(20%)
Amikacin	10(100%)	0(0%)	0(0%)
Chloramphenicol	8(80%)	2(20%)	0(0%)
Enrofloxacin	8(80%)	1(10%)	1(10%)
Gentamicin	10(100%)	0(0%)	0(0%)
Colistin sulphate	0(0%)	0(0%)	10(100%)

Legends: S=sensitive, I= intermediate, R= resistant



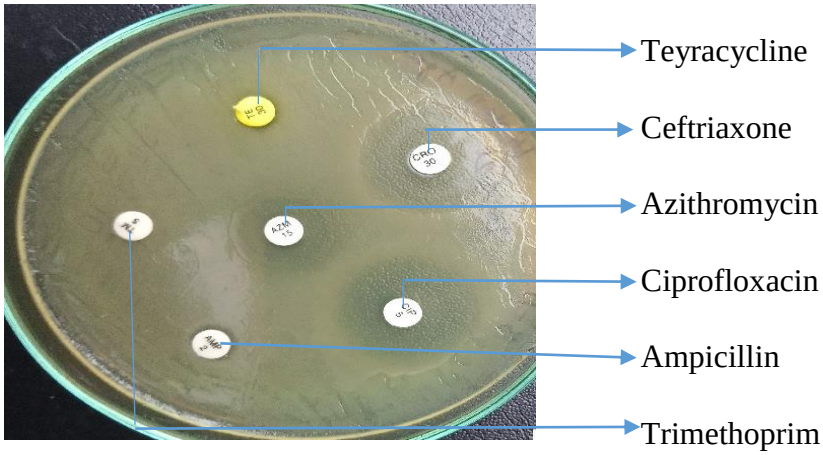
Legends:TE=tetracycline,TM=trimethoprim,AMP=ampicillin,CIP=ciprofloxacin, AZM=azithromycin,CRO=ceftriaxone,NOR=norfloxacin,CAZ=ceftazidime

Figure 43: Antibiotic sensitivity pattern of *E. coli*

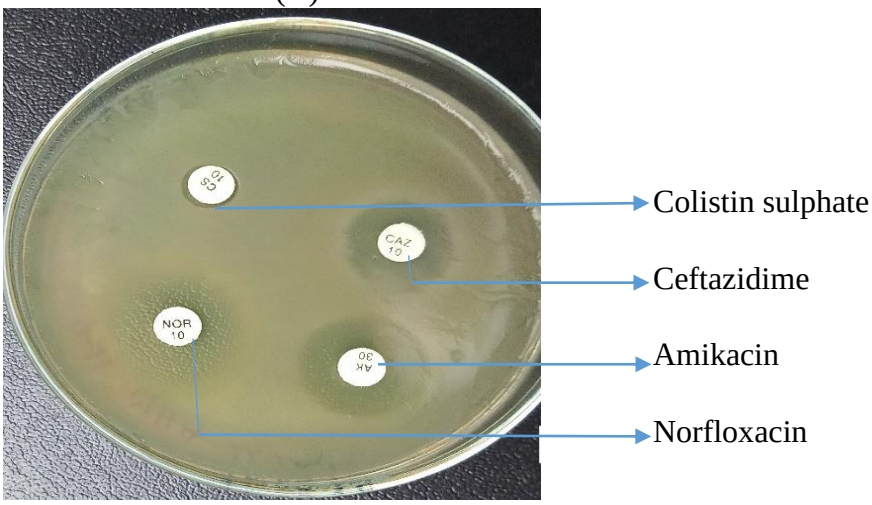


Legends: AK=amikacin, C=chloramphenicol, ENR= enrofloxacin, AML=amoxicillin, GN=gentamicin, CS=colistin sulphate

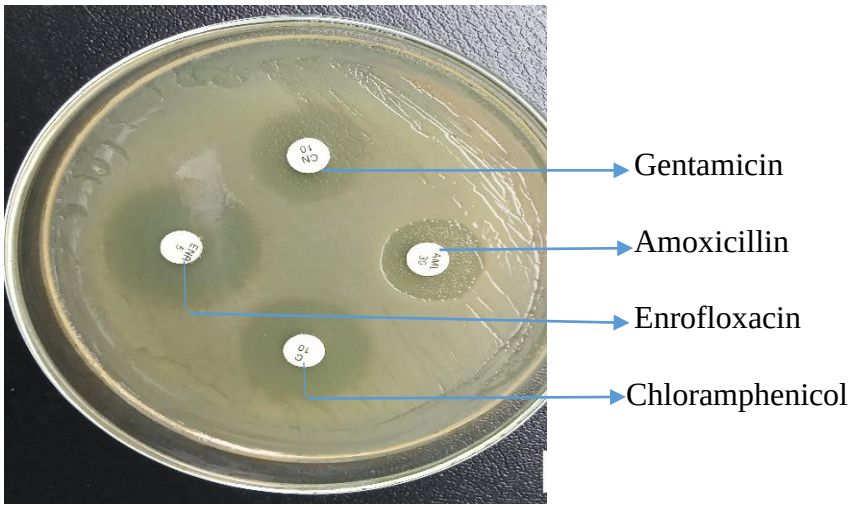
Figure 44: Antibiotic sensitivity pattern of *E. coli*



(A)



(B)



(C)

Figure 45: (A, B, C) Antibiotic sensitivity test of *E. coli*

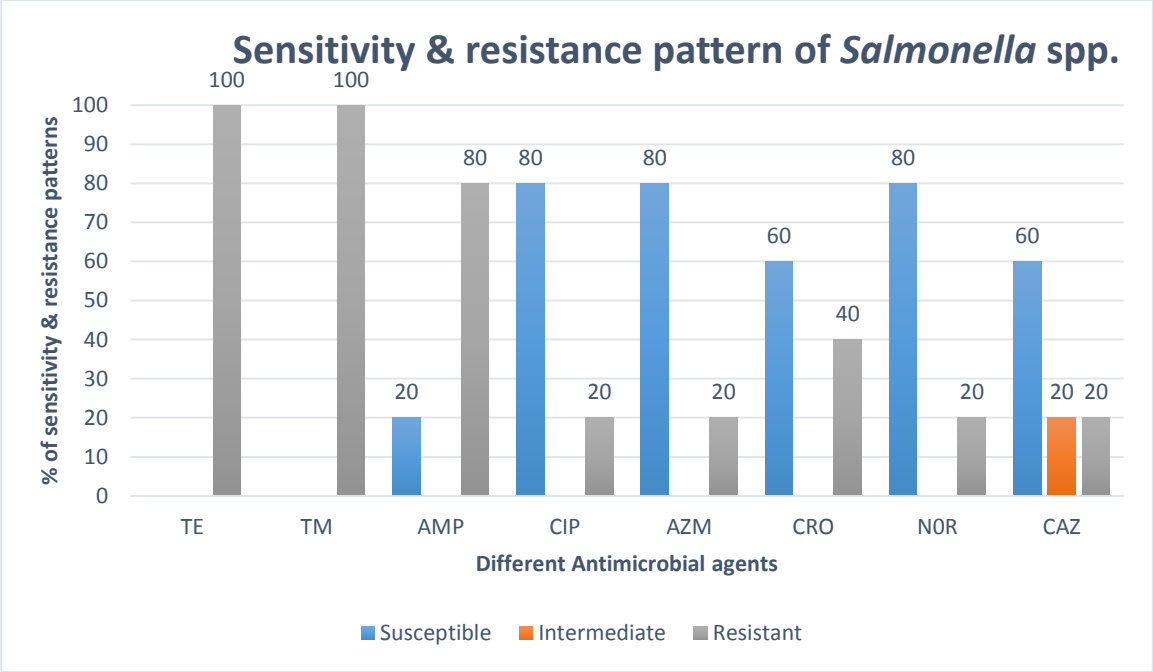
4.14 Results of antimicrobial susceptibility of *Salmonella* spp. isolates (n=5) (Table 19 and Figure 46, 47 and 48)

The results of susceptibility analysis showed that all the isolates of *Salmonella* spp. (n=5) were 100% susceptible to Amoxicillin, Amikacin and Gentamicin. All the isolates of *Salmonella* spp. (n=5) were 100% resistant to Tetracycline, Trimethoprim and Colistin sulphate.

Table 19 Antimicrobial susceptibility pattern of *Salmonella* spp.

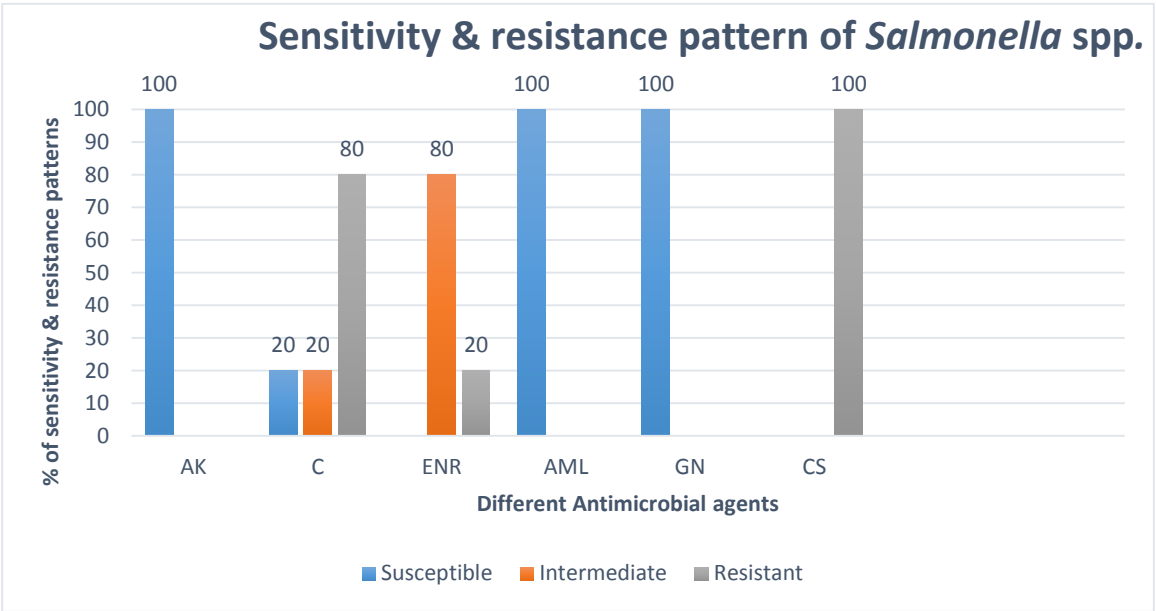
Antimicrobial agents	**No. *Salmonella* spp. of isolates (%)**		
	S	**I**	**R**
Tetracycline	0(0%)	0(0%)	5 (100%)
Trimethoprim	0(0%)	0(0%)	5(100%)
Ampicillin	1(20%)	0(0%)	4(80%)
Amoxicillin	5(100%)	0(0%)	0(0%)
Ciprofloxacin	4(80%)	0(0%)	1(20%)
Azithromycin	4(80%)	0(0%)	1(20%)
Ceftriaxone	3(60%)	0(0%)	2(40%)
Norfloxacin	4(80%)	0(0%)	1(20%)
Ceftazidime	3(60%)	1(20%)	1(20%)
Amikacin	5(100%)	0(0%)	0(0%)
Chloramphenicol	1(20%)	2(20%)	4(80%)
Enrofloxacin	0(0%)	4(80%)	1(20%)
Gentamicin	5(100%)	0(0%)	0(0%)
Colistin sulphate	0(0%)	0(0%)	5(100%)

Legends: S=sensitive, I= intermediate, R= resistant



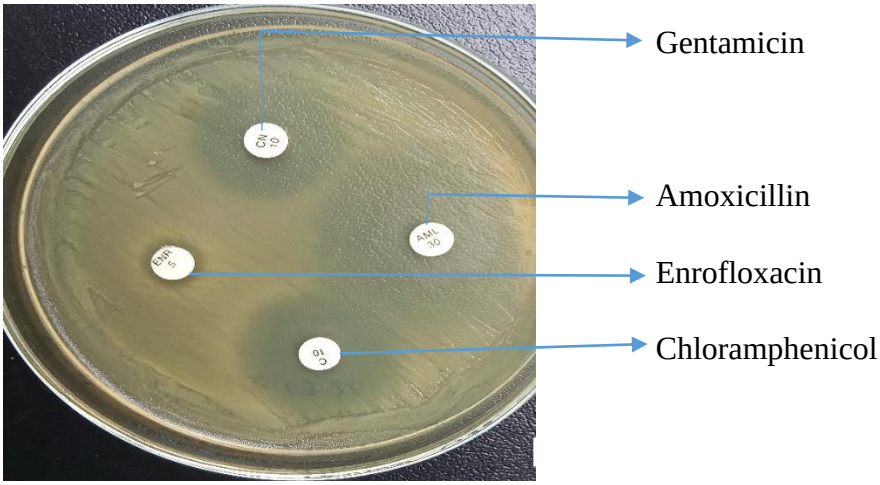
Legends:TE=tetracycline,TM=trimethoprim,AMP=ampicillin,CIP=ciprofloxacin, AZM=azithromycin,CRO=ceftriaxone,NOR=norfloxacin,CAZ=ceftazidime

Figure 46: Antibiotic sensitivity pattern of *Salmonella* spp.

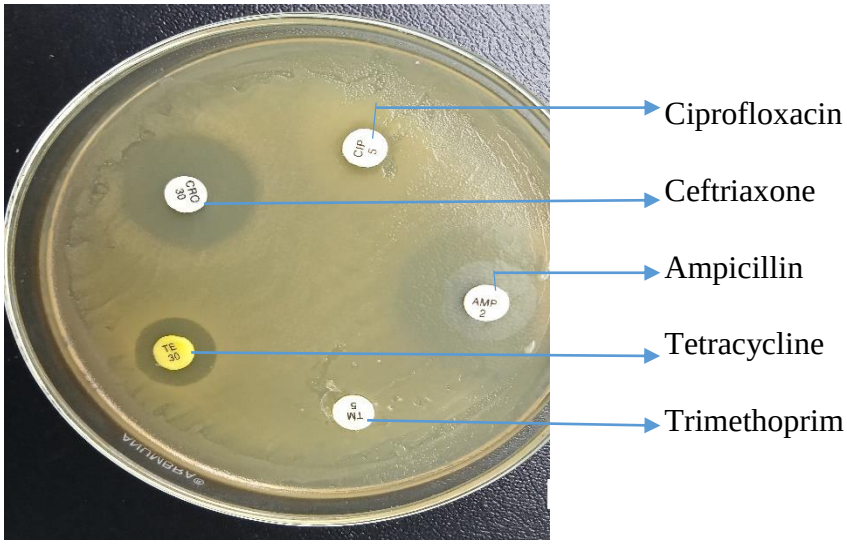


Legends: AK=amikacin, C=chloramphenicol, ENR= enrofloxacin, AML=amoxicillin, GN=gentamicin, CS=colistin sulphate

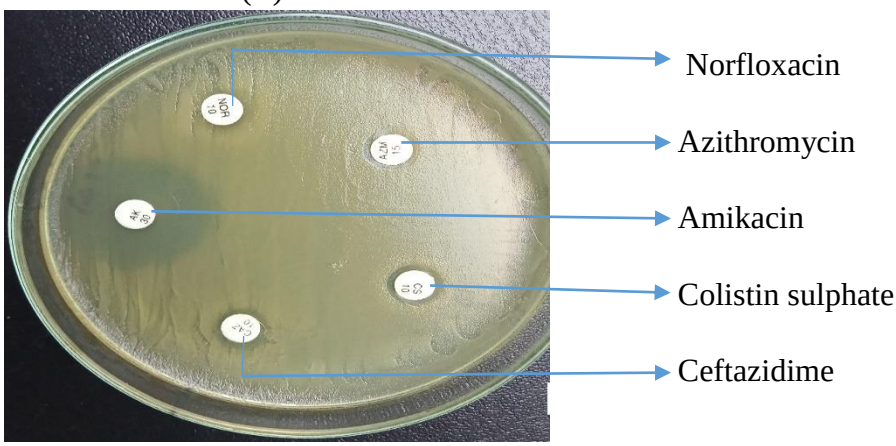
Figure 47: Antibiotic sensitivity pattern of *Salmonella* spp.



(A)



(B)



(C)

Figure 48: (A,B,C) Antibiotic sensitivity test of *Salmonella* spp.

CHAPTER -5

DISCUSSION

The experiment was conducted for the isolation, identification, molecular characterization and antibiotic sensitivity study of *E. coli* and *Salmonella* spp. which were isolated from fecal samples of caged pigeon, budgerigars and lovebird. Similar to all other vertebrates, birds are susceptible to and may also transmit to humans enteropathogenic organisms of zoonotic potential (Vasconcelos, 2013). Most of these bacterial infections are caused by food or water contaminated with feces of infected animals. Similarly, contamination may also be caused by direct contact between infected and suspected animals (Kuroki *et al*., 2013). Birds have a significant role in the dissemination of these diseases, although many of them do not show any clinical signs. Birds may also be potential carriers of many other bacteria, besides virus and parasites (Dovc *et al*., 2004).

In this study , colony characteristics of *E. coli* (Table 9) observed in NA, Mac-conkey, EMB and SS agar were similar to the findings of (Nayak *et al*., 2004; Buxton and Fraser, 1977). Colony characteristics of *Salmonella* spp. (Table 12) observed in NA, Mac-conkey, EMB and SS agar were similar to the previous reports (Buxton and Fraser, 1977; Freeman *et al*., 1985; Jones *et al*., 1987).

Another fundamental basis for the identification of *E. coli* and *Salmonella* spp. Organism was determining the ability or inability of fermentation of five basic sugars with acid and gas production. The *E. coli* isolates revealed complete fermentation of five basic sugars as stated by (Sandhu *et al*., 1996; Beutin *et al*., 1997). Isolates of *Salmonella* spp. of this study fermented dextrose, glucose, and mannitol with acid and gas formation but did not ferment lactose and sucrose which satisfied the statement of (Buxton and Fraser, 1977; Hossain *et al*., 2001; Han *et al*., 2011).

The results of Catalase, MR and indole test of the *E. coli* isolates were positive but VP test was negative as reported by (Buxton and Fraser, 1977). The result of Indole test for *Salmonella* spp. was negative but positive for MR test which is similar to the statement of (Buxton and Fraser, 1977).

Molecular characterization of *E. coli* was done by PCR and amplification of 584 bp DNA of *E. coli* was done with E1, E2 primers (Tsen *et al*., 1998) and amplification of 284 bp DNA of *Salmonella* spp. was done by S139, S141 primers (Rahn *et al*., 1992). In the present study 16S rRNA gene of *E. coli* and invA gene of *Salmonella* spp. were sequenced and analyzed. The isolates were clustered with respective organisms from different countries of the world. The sequences were found closely related with microorganism isolated from various host species. These finding indicate that the isolates are not host specific. For example, sequences of *E. coli* isolated from pet birds have close identity with same from various types of sample. Similar observations were found in case of *Salmonella* spp. Further analysis of genes of these microorganisms are necessary to ascertain their host specificity as well as pathogenicity status.

In this study, *E. coli* and *Salmonella* spp. were isolated from 45 fecal samples of 3 different pet bird species. The overall occurrence of *E. coli* and *Salmonella* spp. were 22 (48.8%) and 8 (17.7%), respectively.

In this study, The occurrence of *Salmonella* spp. in pigeon was 40%. Rahman *et al*. (2016) found 28.57% prevalence of *Salmonella* in pigeon while Hosain *et al*. (2012) reported the prevalence as 35.71%. From this study, it may be concluded that, prevalence of *Salmonella* spp. is increasing day by day. It may be due to cross contamination of pigeon with other wild and/or pet birds.

In this study, no *Salmonella* spp. was found in fecal sample of psittaciformes birds (budgerigar and lovebird) which was similar to the previous report of (Almeida *et al*., 2015). *Salmonella* shedding is usually intermittent (Gerlach *et al*., 1994) and possible explanation for the absence of *Salmonella* spp. may be the young age of the sampled parakeets, since enteric microbiota of birds gradually changes with age as they grow up (Kohl, 2012).

In this study, the occurrence of *E. coli* in budgerigar, lovebird and pigeon was 73.33%, 40% and 35% respectively. Psittaciformes birds (budgerigar and love bird) showed the highest occurrence of *E. coli*. The similar prevalence (73.94%) of *E. coli* in Psittaciformes was observed by (Mattes *et al*., 2005). It may be due to, relatively high numbers of budgerigars gathered temporarily in the same cage for example in pet shop facilities or

markets. Cages are actually often overcrowded, filled with birds of mixed origin (Vanrompay *et al*., 2007). The overcrowding also stimulates intense stress to the birds due to fighting for females, extremely limited territory in this case or food. This will cause fast debilitation of the weakest individuals and higher sensitivity to infections (Boseret *et al*., 2006).

This study showed low occurrence of *E. coli* in pigeon comparing to other studies. In a previous study Dey *et al*. (2013) found an overall prevalence of *E. coli* in pigeon as 69.64% while Zigo *et al*. (2017) found 50.8% *E. coli* from pigeon cloacal swab. On the other hand, Suphoronski *et al*. (2015) observed that Columbiformes showed the greatest occurrence of *E. coli*, with the prevalence of 82.33% of the birds. In this study, fecal samples were collected from caged pigeon where only two or three pigeon were raised in a cage. There was no overcrowding, therefore, it may result in low prevalence or it may be also due to their healthy nutritional diet.

The antibiotic sensitivity study revealed that all the isolates of *E. coli* were 100% resistant to Tetracyclin, Ampicillin and Colistin sulphate. All the isolates of *E. coli* of budgerigar and *E. coli* of love bird were resistant to ceftriaxone. All the isolates of *E. coli* were 100% sensitive to Ciprofloxacin, Norfloxacin, Amikacin, Azithromycin and Gentamicin. Khafagyet *et al*. (2015) studied that Ciprofloxacin and Gentamycin were the most effective drugs against the isolated *E. coli*. A previous study of Zigo *et al*. (2017) showed increased frequency of tetracycline (82.3%), ampicillin (48.1%), amoxicillin (45.1%) and gentamicin (6.3%) resistant strains of *E. coli.* The significance of occurrence of antibiotic resistance in food-borne pathogens has increased sharply and probably linked with the increased use of antimicrobial agents in veterinary medicine and human. It may be concluded that, Ciprofloxacin, Norfloxacin, Amikacin, Azithromycin and Gentamicin can be effectively used in case of treatment.

The results of susceptibility analysis showed that all the isolates of *Salmonella* spp. were 100% susceptible to Amoxicillin, Amikacin and Gentamicin. All the isolates of *Salmonella* spp. were 100% resistant to Tetracycline, Trimethoprim and Colistin sulphate; 80% resistant to Ampicillin and Chloramphenicol. Rahmani *et al.* (2011) revealed in a study that all *Salmonella* isolates were 100% susceptible to danofloxacin, norfloxacin,

levofloxacin, amikacin, gentamicin, and tobramycin and showed (47.4%) resistance to tetracycline. There is great concern, today, about the emergence of serotypes in the genus *Salmonella* that are multiresistant to available antibiotics (Shinohara *et al*., 2008).

This study reveals the alarming zoonotic bacterial infection in pet birds of Katabon pet shops where many customers gather everyday to visit them. It may cause direct zoonotic transmission. These types of bacteria affect not only human but also livestock. Determination of periodical antibiotic sensitivity and resistance pattern should also be analyzed. Otherwise, indiscriminate use of antibiotics may lead to the development of drug resistant mutants causing serious health hazards of different animals, birds and human being also.

CHAPTER- 6

CONCLUSION

From the present study it may be concluded that fecal samples were collected from pigeon, budgerigar and lovebird which are the reservoirs of *E. coli* and *Salmonella* spp. They might make the pet birds vulnerable for easy access of infection.

The prevalence of *E. coli* and *Salmonella* spp. were 22 (48.8%) and 8 (17.7%), respectively. The prevalence of *Salmonella* spp. in pigeon was 40%. No *Salmonella* spp. was found in fecal sample of psittaciformes birds (budgerigar & love bird).

The prevalence of *E. coli* in pigeon, budgerigar and lovebird was 35%, 73.33% and 40%, respectively. Psittaciformes birds (budgerigar & love bird) showed the highest prevalence of *E. coli*. The bacterial *E. coli* and *Salmonella* spp. may pass through the feces to the environment and can easily spread to other animals, human via soil, food, water contamination or by handling pet birds and direct or indirect contact with pet birds.

There is presence of the prevalence of multi drug resistant *E. coli* and *Salmonella* spp. in the fecal samples of the study area ; which suggests an ill use of antimicrobials in pet birds. Antimicrobial drug resistancy is becoming a major threat to global public health. Indiscriminate use of antimicrobial agents should be avoided in order to eliminate health hazards in man and animals caused by *E. coli* and *Salmonella* spp. through preventing the development of multi-drug resistant mutants in nature. This study highlights that, as a result of the close physical contact that is possible between these birds and humans, caged birds pose a risk to public health.

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APPENDICES

APPENDIX I

Composition of different media

1. Nutrient broth

Peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Distilled water	1000 ml
Final P^H (at 25°C)	7.4 ± 0.2

2. Nutrient Agar

Peptone	5.000 gm
Sodium chloride	5.000 gm
HM peptone B#	1.500 gm
Yeast extract	1.500 gm
Agar	15.000 gm
Final P^H (at 25°C)	7.4 ± 0.2

3. MacConkey Agar

Peptones (meat and casein)	3.000 gm
Pancreatic digest of gelatin	17.000 gm
Lactose monohydrate	10.000 gm
Bile salts	1.500 gm
Sodium chloride	5.000 gm

Crystal violet	0.001 gm
Neutral red	0.030 gm
Agar	13.500 gm
P^H after sterilization (at 25°C)	7.1 ± 0.2

4. Eosin Methylene Blue Agar

Peptic digest of animal tissue	10.000 gm
Dipotassium phosphate	2.000 gm
Lactose	5.000 gm
Sucrose	5.000 gm
Eosin - Y	0.400 gm
Methylene blue	0.065 gm
Agar	13.500 gm
Final P^H (at 25°C)	7.2 ± 0.2

5. Salmonella-Shigella agar

Proteose peptone	5.000 gm
Lactose	10.000 gm
Bile salts mixture	8.500 gm
Sodium citrate	8.500 gm
Sodium thiosulphate	8.500 gm
Ferric citrate	1.000 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	13.500 gm

Final P^H (at 25°C)	7.4 ± 0.2

6. Mueller Hinton Agar

HM infusion B from	300.000 gm
Acicase	17.500 gm
Starch	1.500 gm
Agar	17.000 gm
Final P^H (at 25°C)	7.4 ± 0.1

7. (MR-VP) broth

Buffered peptone	7.000 gm
Dextrose	5.000 gm
Dipotassium phosphate	5.000 gm
Final P^H (at 25°C)	6.9 ± 0.2

8. Sugar media

a. Peptone water

Bacto-peptone	10.0 gm
Sodium chloride	5.0 gm
0.5% Phenol red	0.1 ml
Distilled water	1000 ml

b. Sugar solutions

Individual sugar	5 gm
Distilled water	100 ml

c. Sugar media preparation

Peptone water	4.5 gm

Sugar solution	0.5 ml

9. Simmons citrate agar

Ingredients	**g/L**
Magnessium sulphate	0.20
Ammuniumdihydrogen phosphate	1.0
Dipotasium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothvmol blue	0.08
Agar	15.0

10. TSI Agar slant

Ingredients	
Lab Lamco Powder	3.00 gm
Yeast extract	3.00 gm
Peptone	20.00 gm
Sodium chloride	5.00 gm
Lactose	10.00 gm
Sucrose	10.00 gm
Glucose	1.00 gm
Ferric citrate	0.3 gm
Sodium thiosulphate	0.3 gm
Phenol red	0.3 gm
Agar	12.00 gm

Distilled water	1000 ml
Final P^H	7.4 +/-0.2 (at 25°C)

APPENDIX 2

B. Preparation of reagents

1. Pepton water

Pepton	1 gm
Distilled water	1000 ml

2. Kovac's reagent for indole preparation

P-dimethyl aminobenzaldehyde	5 gm
Amyl alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-1

5% alpha-napthanol in absolute ethyl alcohol

4. V-P regent-2

40% potassium hydroxide containing 0.3% creatine.

The ingredient was Dissolved by heating gently over a steam bath.

When in solution, added 0.052 gm of cotton blue dye.

5. Methyl Red Indicator

Methyl red	0.200 gm
Ethyl alcohol	60.000 ml
Distilled water	40.000 ml

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Phosphate buffered saline solution

Sodium chloride	8.0 gm
Disodium hydrogen Phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium dihydrogen phosphate	0.2 gm

8. Oxidase reagent

Tetramethyl-p-phenylenediamine	0.1 ml
Distilled water	10 ml

9. 3% Hydrogen per oxide (H_2O_2) for catalase test

H_2O_2	3 ml
Distilled water	97 ml