Chapter 1

INTRODUCTION

The introduction of antimicrobial agents in human clinical medicine and animal husbandry has been one of the most significant achievements of the 20th century. But after sometime emergence of antibiotic resistance was started and becomes an immense threat to human and animal health. (Aarestrup 2005)

Commonly, it is usual that the principal risk factor for an increase in this situation is the extensive use of antibiotics leading to the dissemination of resistant bacteria and resistance genes in animals and humans. The appearance of multi-drug resistant bacteria of human and veterinary origin is probably accompanied by co-contamination of the environment. (Radhouani *et al.* 2012)

Antimicrobial resistance in *Enterobacteriaceae* poses a critical public health threat, especially in developing countries. Much of the problem is due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species.

Wildlife is normally not exposed to clinically used antimicrobial agents but can acquire antimicrobial resistant bacteria through contact with humans, domesticated animals and the environment, where water polluted with faeces seems to be the most important vector. (Guenther *et al.* 2011)

According to the Centers for Disease Control and Prevention (CDC) most of *E. coli* are harmless and actually are an important part of a healthy human intestinal tract and some strains of *E. coli* cause a variety of intestinal and extra-intestinal infections like diarrhoea, septicemia, urinary tract infections, nosocomial and community acquired infections and neonatal Meningitis in animals. It is also a useful marker of faecal pollution and the persistence of *E. coli* in natural environments as through its ability to grow as a biofilm. (Beloin *et al.* 2008)

Beta-lactams antibiotics mainly extended spectrum cephalosporins, carbapenems and fluoroquinolones constitute the main therapeutic choices to treat infections caused by these microorganisms. Acquired resistance to beta-lactams is mainly mediated by extended spectrum beta-lactamases (ESBLs) that confer bacterial resistance to all beta-lactams except carbapenems and cephamycins, which are inhibited by other beta-lactamase inhibitors such as clavulanic acid (Coque *et al.* 2008). All beta-lactams interfere with the final stage of peptidoglycan synthesis through acting on penicillin-binding proteins, thereby preventing the bacterial cell wall from forming. The peptidoglycan constitutes a layer between the outer membrane and the cytoplasmic membrane which maintains the cell shape and protects the bacterium against osmotic forces. The most common resistance mechanism of *Enterobacteriaceae* spp. against beta-lactams is the inactivation of the drug by hydrolytic cleavage of the beta-lactam ring system. (Greenwood 2000)

Many genera of gram-negative bacteria possess a naturally occurring, chromosomally mediated beta-lactamase. These enzymes are thought to have evolved from penicillin-binding proteins, with which they show some sequence homology. This development was likely due to the selective pressure exerted by beta lactamase producing soil organisms found in the environment. Beta-lactamases are those enzymes which open the beta-lactam ring and inactivate the antibiotic. The first plasmid-mediated beta-lactamase in gram-negative bacteria, TEM-1, was described in the early 1960s. The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM.

Currently, the most widely used classification scheme is Ambler system, which divides beta lactamases into four classes (A, B, C, and D) based on their amino acid sequences. The majority of ESBLs belong to Ambler class A and to the Bush group 2be. Class A enzymes are characterized by an active-site serine, which has the molecular mass of approximately 29,000 Da. ESBLs have been found in a wide range of Gram-negative bacteria, but the vast majority of bacterial hosts belong to the family of *Enterobacteriaceae* including *Klebsiella* spp., *E. coli*, *Salmonella enterica*, *Citrobacter* spp., and *Enterobacter* spp. (Guenther *et al*. 2011)

More than 400 different beta-lactamase enzymes are currently known, sharing the same resistance mechanism but differing in their range of substrates and susceptibility against inhibitory substances. The term extended-spectrum determines the ability of ESBLs to hydrolyze a broader spectrum of beta-lactam antimicrobials than the parent beta-lactamases they were originally derived from. While they are capable of inactivating beta-lactam antimicrobials containing an oxyimino-group such as oxyimino-cephalosporins (e.g. ceftazidime and cefotaxime) as well as oxyimino-monobactam (aztreonam), ESBLs are not active against cephamycins and carbapenems. They are usually inhibited by beta-lactamase-inhibitors like clavulanic acid and tazobactam, which marks a difference between ESBL and AmpC-beta-lactamases producing bacteria. (Bradford 2001)

Antimicrobial resistant *E. coli* isolates originating from wildlife species were reported for the first time at the beginning of the 1980s from Japanese wild birds (Kanai *et al.* 1981 and Tsubokura *et al.* 1995) and after 5 years in South African baboons feeding on human refuse (Rolland *et al.* 1985 and Routman *et al.* 1985). The detection of ESBL *E. coli* of wildlife origin dates back to 2006 only. (Costa *et al.* 2006).

The level of resistant bacteria observed in wild animals seems to correlate well with the degree of association with human activity (Skurnik *et al.* 2006 and Allen *et al.* 2010). Most studies on ESBL *E. coli* in wildlife originate from Central Europe, an area with high livestock and human density and an assumable frequent interaction of wildlife with human influenced habitats of any kind like livestock farms, landfills, sewage systems, or wastewater treatment facilities, resulting in a higher risk for wildlife acquiring antibiotic-resistant bacteria (Allen *et al.* 2010). It has previously been shown that gulls shared strains of *E. coli* with isolates cultured from landfills and wastewater treatment plants (Nelson *et al.* 2008). This underlines the possibility of bacterial exchange between human sewage and birds.

The main objective of this study is to determine the occurrence of and to detect multidrug resistance and ESBL-producing *E. coli* and their susceptibility pattern against various antibiotics in captive mammals and birds.

Objectives of Investigation:

a. To study the occurrence of *E. coli* among captive birds and animals
b. To determine the occurrence of Multidrug resistant *E. coli*
c. To detect the Extended spectrum β-lactamases producing *E. coli* and their antimicrobial susceptibility pattern.

Chapter 2

REVIEW OF LITERATURE

E. coli is a bacterium belongs to *Enterobacteriaceae* family, a most abundant and commensal organism of warm-blooded animals. *Escherichia coli* are a non-spore-forming, Gram-negative bacteria, usually motile by peritrichous flagella. The different pathotypes of enteric *E. coli* which include Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Enteropathogenic (EPEC), and Vero cytotoxigenic (VTEC). These strains of *E. coli* cause a variety of intestinal and extra-intestinal infections, like diarrhea, septicemia, urinary tract infections, and neonatal meningitis and fall into four main phylogenetic groups (A, B1, B2 and D) in which most of the commensal strains belong to group A and major virulent extra-intestinal strains belong to group B2 (Clermont *et al.* 2000). It is a very useful indicator of faecal pollution and thus became an important marker in food and water hygiene.

2.1. Occurrence and antibiotic resistant pattern of *E. coli*

Rolland *et al*. (1985) examined three groups of wild baboons (*Papio cynocephalus*) to determine the prevalence of aerobic antibiotic-resistant faecal bacteria in nonhuman primates with and without contact with human refuse. They found antibiotic-resistant gram-negative enteric bacteria in two groups of baboons and which have limited or no contact with humans. However, resistance was significantly higher among enteric bacteria from the third group of baboons living close to a tourist lodge and having daily contact with unprocessed human refuse.

Routman *et al*. (1985) examined faecal samples from human hosts and free-ranging African yellow baboons for resistance to antibiotics. The frequency of antibiotic resistance in *E. coli* isolated from baboons was similar to that in *E. coli* isolated from humans. Of these 25 (5.04%) of the strains were resistant to only one antibiotic, whereas 31 (6.25%) demonstrated multiple resistance that was to streptomycin (37 strains), followed by tetracycline (28 strains), ampicillin (21 strains), kanamycin (17 strains), and chloramphenicol (7 strains).

Tsubokura *et al*. (1995) evaluated drug resistance of 554 strains of *E. coli* isolated from faeces of migratory waterfowl including whistling swans, pintails and black-tailed gulls. Seven antimicrobial agents were tested: dihydrostreptomycin, kanamycin, spectinomycin, ampicillin, oxytetracycline, chloramphenicol and sulfadimethoxine. All the strains were resistant to sulfadimethoxine. Both multiple drug resistant strains and drug resistance patterns occurred most frequently in strains isolated from whistling swans, followed by black-tailed gulls, and pintails, respectively.

Souza *et al*. (1999) screened samples from wild animals from different continents for *E. coli*. Samples were taken from captive wild animals and birds at the end of which 202 *E. coli* strains were obtained from 81 mammalian species, representing 39 families and 14 orders in Australia and America, a strain was obtained from a reptile and 10 from different families of birds collected in Mexico.

Gopee *et al*. (2000) were conducted a longitudinal study of *Escherichia coli* strains isolated from faecal samples that were randomly collected from the enclosures of animals from captive mammals, birds, and reptiles in Trinidad. The frequency of *E. coli* isolation was significantly higher in mammals compared with birds and reptiles. Overall, the frequencies of isolation of *E. coli* from omnivores, herbivores, and carnivores, 87.2%, 70.0%, and 57.3%, respectively and most (99.6%) of the *E. coli* isolates tested for antibiotic sensitivity exhibited resistance to one or more of the eight antimicrobial agents used in the study.

Middleton and Ambrose (2005) studied that more than 95% of isolates from migrating Canada Geese were resistant to at least one of these antibiotics like penicillin G, ampicillin, cephalothin and sulfathiazole, and many isolates had multiple resistance.

Cole *et al*. (2005) showed an increased proportion of resistant *E. coli* isolates from Canada goose (*Branta Canadensis)* that had utilized swine waste laggons.

Dolejská *et al.* (2007) tested 257 isolates of *E. coli* from young Black-headed Gulls for sensitivity to eight antibacterial substances by disk diffusion method and 75 samples (29.9%) were resistant to one or more antimicrobial agents. The dominant type of resistance was to tetracycline, detected in 49 (19.1%) isolates. Resistance to ampicillin was detected in 30 (11.7%), cephalothin in 11 (4.3%), streptomycin in 24 (9.3%), sulphonamides in 20 (7.8%) and chloramphenicol in 5 (1.9%) isolates.

Ahmed *et al.* (2007) isolated 49 (21.1%) multidrug resistance phenotypes from 232 isolates of gram negative bacteria which were recovered from mammals, reptiles and birds housed at Asa zoological park, Hiroshima prefecture, Japan.

Nelson *et al*. (2008) compared the populations of *Escherichia coli* among herring gulls (*Larus argentatus)*, great black-backed gulls (*L. marinus)*, wastewater, and landfill trash. Strains of *E. coli* from gulls matched (≥90% similarity) more strains from wastewater (39% matching) than from trash (15% matching).

Redimersky *et al.* (2010) detected for antibiotic resistant genes in faecal bacteria in feral pigeons and *E. coli* was found in 203 of 247 samples. Antibiotic resistance was recorded in three (1.5%) from 203 isolates against streptomycin, tetracycline and sulphonamides.

Akhtar *et al*. (2010) isolated and identified the microflora from 45 samples (oral swabs, cloacal swabs and faeces) of five types of caged parrots (Gray cockatiels, Rose ringed parakeet, Alexandrine parakeet, Red breast parakeet and Blossom headed parakeet) which were apparently healthy. They found most frequently *E. coli* that was 64.44%. In antibiotic sensitivity *E. coli* was completely resistant to ampicillin and amoxicillin and ciprofloxacin, norfloxacin and enrofloxacin showed moderate to high sensitivity against the bacteria.

Radhouani *et al*. (2012) were recovered 36 *Escherichia coli* and 31 enterococci isolates from 42 common buzzard faecal samples. The *E. coli* isolates showed high levels of resistance to streptomycin and tetracycline.

Carvalho *et al.* (2012) characterized the extraintestinal pathogenic *Escherichia coli* isolated from captive wild felids with bacteremia.

Clayton *et al*. (2014) characterized *E. coli* in healthy non-human primates. Of the 229 faecal samples collected, *E. coli* was successfully isolated from 162 samples (70.7%). Phylogenetic groups A, B1, B2, and D were all found to be present in the captive population of non-human primates tested in this study.

Bonnedahl *et al*. (2014) reviewed the antibiotic resistance in wild birds that it can carry the resistant bacteria and ESBL-producing *E. coli* have now been isolated from wild birds from all continents of the world except Australia and Antarctica**.**

Mureithi *et al*. (2015) analyzed the phenotype and genetic characteristics of antimicrobial resistance in ninety-seven *E. coli* isolates recovered from 100 faecal samples of two groups of captive baboons. Susceptibility to 16 antibiotics was studied in these isolates, and the most common resistance observed in *E. coli* isolated from both groups of baboons was to ampicillin (34.4-36.1%), sulphamethoxazole (33-36.1%), amoxyclav (26.2-30.6%), piperacillin (22.2-23%), tetracycline (19.7-22.2%), streptomycin (11.1-21.3%), and co-trimoxazole (9.8-25%). The percentage of resistance to chloramphenicol, ceftazidime, ceftriaxone, gentamicin, amikacin and ofloxacin was below 8.2%, and no resistant isolates were detected for meropenem and ciprofloxacin. Multi-drug resistance was found in case of 7-8 antibiotics for all strains tested.

Jobbins *et al*. (2015) evaluated 150 faecal samples from African wild animals, 41.3% contained *E. coli* isolates that were resistant to one or two of 10 tested antibiotics and 13.3% of isolates demonstrated multidrug resistance.

Wildlife can contribute to environmental contamination with bacterial pathogens and their transfer to the human food chain. Global usage and frequent misuse of antimicrobials contribute to the emergence of new antimicrobial resistant (AMR) strains of foodborne pathogens. (Greig *et al*. 2015)

Alcalá *et al*. (2016) analyzed 100 faecal samples belonging to 15 different wild avian species. A total 16 Cefotaxime-resistant *E. coli* isolates were identified, which corresponded to 9 animal species i.e. griffon vulture, yellow-legged gull, black kite, red kite, white stork, spotless starling, golden eagle, common cuckoo and barn owl. Fifteen isolates harboured ESBL encoding genes.

Atterby *et al*. (2016) Screened 160 faecal samples collected from large-bodied gulls glaucous, herring and potentially hybrid gulls. From them, 55% of *E. coli* isolates were resistant to one or more antibiotics and 22% were resistant to three or more antibiotics. In addition, a total of 16% of the gull samples harboured extended-spectrum cephalosporin-resistant *E. coli* isolates.

Oludairo *et al*. (2016) studied 160 faecal samples of wild mammals, birds and reptiles. Out of the 58 confirmed *E. coli*, eighteen were from primates, five from carnivores, five were also from herbivores, and 28 from birds while two were from reptiles.

2.2 Extended spectrum beta-lactamase (ESBL) producing *E. coli*

Arcos *et al.* (1968) detected β-lactamase and penicillinacyclase both coexisted in *E. coli and* said that β-lactamases is not present only in *E. coli.*

Pai *et al.* (1999) investigated two hundred ninety (290) isolates of *Escherichia coli* for the production of extended spectrum β-lactamases (ESBLs) and 4.8% (14) were found to produce ESBLs. The samples were collected from human hospitals of Seoul, Korea.

Costa *et al.* (2006) detected the *Escherichia coli* harbouring extended spectrum β-lactamases of CTX-M, TEM, and SHV classes in the faecal samples of wild animals. They took 72 faecal samples from Natural park and 56 (78%) were found positive for *E.coli* and 9 from these showed an intermediate susceptibility or resistant phenotype to cefotaxime and/or ceftazidime, and a positive screening test for ESBLs was obtained in all of them.

Coque *et al.* (2008) reviewed the increasing prevalence of ESBL producing *Enterobacteriaceae* among human and non-human host and analyzed that *E. coli* mostly cause the community-acquired infections and possesses antibiotic resistant genes.

Bonnedahl *et al*. (2009) reported the zoonotic dissemination of ESBL producing *E. coli* between humans and yellow legged gulls. They detected that 47.1%

isolates were having the resistance to one or more antibiotics and 9.4% isolates from gulls carried ESBL producing *E. coli.*

Guenther *et al*. (2010) isolated 2.3% CTX-15-type ESBL producing *E.coli* from wild birds which were recovered from two Eurasian blackbirds, one rock pigeon and a greater white-fronted goose.

Literak *et al*. (2010) detected 27%, 11% and 1.4% antibiotic resistance *E. coli* in mallard ducks, herring gulls and water bird faeces respectively and characterized the nine ESBL producing *E.coli* isolates in which the most prevalent gene was *bla*$_{\text{CTX-M}}$ (6 isolate).

Pinto *et al*. (2010) detected Extended-spectrum beta-lactamase-containing *Escherichia coli* isolates in 32 of 119 fecal samples (26.9%) from birds of prey and these isolates contained the following beta-lactamases: CTX-M-1 (n = 13), CTX-M-1 and TEM-1 (n = 14), CTX-M-1 and TEM-20 (n = 1), SHV-5 (n = 1), SHV-5 and TEM-1 (n = 2), and TEM-20 (n = 1).

Silva *et al*. (2011) evaluated 220 faecal samples and one sample revealed one ESBL-containing *E. coli* isolate that belongs to the 'A' phylogenetic group, showed a phenotype of resistance to β-lactams and tetracycline, and harboured the bla$_{\text{(CTX-M-14)}}$, *bla*$_{\text{(SHV-12)}}$ and the tet(A) genes.

Simões *et al*. (2010) obtained 139 *E. coli* isolates from seagulls, of which 45 (32%) displayed an ESBL phenotype.

Wild birds have been speculated as sentinels, reservoirs, and potential spreaders of antibiotic resistance. Antibiotic-resistant bacteria have been isolated from a great number of wild bird species and various studies strongly indicate the transmission of resistant bacteria from human rest products to wild birds and vice versa. Some evidences are suggesting that wild birds can spread resistant bacteria through migration also. (Abulreesh *et al*. 2011)

Guenther *et al*. (2011) summarized the current knowledge on ESBL *E.coli* in wildlife and underlined the need for more large scale investigations because it can be another form of environmental pollution.

Garmyn *et al.* (2011) identified the extended-spectrum β-lactamase-producing *E. coli* in wild geese. They isolated two ceftiofur resistant *E. coli* from 396 faecal samples originated from geese and a Canada goose (*B. canadensis*).

Gonçalves *et al*. (2012) tested 128 faecal samples of *Iberian lynx* (wild and captive animals). Eleven samples contained cefotaxime-resistant *E. coli* isolates (all belonging to captive animals) and 10 were ESBL-producing isolates.

Stephan *et al.* (2012) investigated the faecal samples of 84 red deer, 64 roe deer, 64 chamois, and 27 ibex and one sample from a roe deer found positive for ESBL-producing *E. coli*.

Tausova *et al.* (2012) isolated 1.6% ESBL-producing *E. coli* strains in great cormorants and 6% in mallards.

Ma *et al.* (2012) studied the 245 (230 cloacal swab and 15 environment samples) samples taken from duck and duck farm respectively for the detection of extended spectrum β-lactamases producing bacteria and total of 116 and 7 Ceftiofur-resistant *E. coli* strains were isolated from 230 duck samples and 15 environment samples, respectively, from the same duck farm. Using the double-disk synergy test, ESBL production was confirmed in 119 out of the 123 Ceftiofur-resistant *E. coli* isolates.

Guenther *et al*. (2012) analyzed 281 wild birds, mostly raptors from Germany and Magnolia. The proportion of ESBL-producers among *E. coli* (Germany: 13.8%, Mongolia: 10.8%) were similar in both regions. We identified sequence types (STs) that are well known in human and veterinary clinical ESBL-producing *E. coli* (ST12, ST117, ST167, ST648) and observed clonal relatedness between a Mongolian avian ESBL- *E. coli* (ST167) and a clinical isolate of the same ST that originated in a hospitalized patient in Europe.

Hasan *et al*. (2012) studied the occurrence of multidrug resistance *Escherichia coli* that was found in 22.7% of isolates from bird samples and 30% produced extended-spectrum β-lactamases, including clones of CTX-M genes among wild and domestic birds.

Radhouani *et al*. (2013) isolated two cefotaxime-resistant *E. coli* (4 %) from 52 faecal samples and both were ESBL producers and contained β-lactamase genes.

Kumar *et al.* (2013) highlighted the fact behind the contribution of zoo and wildlife in occupational zoonoses to veterinarians.

Dobiasova *et al*. (2013) investigated the dissemination of ESBL and plasmid mediated quinolone resistance. From the 160 faecal samples of various animal species, 69 (43%) cefotaxime and 94 (59%) ciprofloxacin-resistant *E. coli* isolates were found. Forty-nine (71%) cefotaxime resistant and 15 (16%) ciprofloxacin-resistant *E. coli* isolates harboured ESBL or PMQR genes, respectively. All the ESBL-producing *E. coli* isolates were multi resistant. ESBL positive isolates showed resistance to tetracycline (98%) sulphonamides (96%), nalidixic acid (18%), streptomycin (16%), trimethoprim, sulphamethoxazole and gentamicin (both 4%).

Veldman *et al.* (2013) isolated cefotaxime resistant *E. coli* from wild birds. Their study included 414 cloacal swabs originating from 55 different bird species and they found cefotaxime-resistant *E. coli* isolates in 65 birds (15.7%) from 21 different species. In all, 65 cefotaxime-resistant *E. coli,* ESBL genes were detected.

Báez *et al*. (2015) identified 91 *E. coli* isolates from 124 faecal swabs with high rates of antibiotic resistance. 67 of the 91 (54%) isolates exhibited an ESBL phenotype due to the presence of CTX-M-15 (61.3%), CTX-M-2 (19.3%), CTX-M-22 (16.1%), and CTX-M-3 (1.6%) coding genes.

Rashid *et al.* (2015) isolated 76 *E. coli* from 170 Openbill stork and 8 *E. coli* isolates from three river sources. In total, 29% of *E. coli* isolated from Openbill stork. Multidrug resistance was observed in 2.6% of Openbill stork and 37.5% of the water isolates. Also, 1.2% of the ESBL-producing *E. coli* was isolated from open bill stork, whereas 50% of the *E. coli* isolated from water sources were ESBL producers possessing the CTX-M-15 gene.

Parker *et al*. (2016) characterized *Escherichia coli* isolated from 75 birds including great horned owls, crows and American robins. The recovery rate of *E. coli* varied significantly between species from 44·8% of robins to 92% of crows. Among isolates, ampicillin resistance was most commonly identified. Three birds carried multidrug-resistant isolates and extended-spectrum β-lactamase (ESBL)-producing organisms (CTX-M-15 and SHV2a) were isolated from two.

Cristóvão *et al.* (2017) analyzed the clonal diversity of extended spectrum β-lactamase (ESBL) producing *E. coli* isolates from nine different species of wild animals and out of the 53 ESBL-positive *E. coli* isolates that were analyzed, 28 showing ESBL types.

Borges *et al.* (2017) described the role of wild birds and pigeons in the transmission of Shiga toxigenic *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) to humans and other animals, and they found multidrug resistance (MDR) in 25.0% of the pigeon strains and 57.0% of the wild bird strains; the wild birds also yielded one isolate carrying extended-spectrum β-lactamases (ESBLs) gene.

Bachiri *et al*. (2017) recovered 47 ESBL-producing isolates from 216 faecal samples. Out of the 40 (44%) were from 90 wild boars and 7 (6%) were from 126 barbary macaques.

Waititu *et al*. (2018) isolated *E. coli* from stool samples collected from sixty-two captive and sixty-two wild baboons and isolates from both groups of animals were resistant to all 13 antimicrobial agents except ciprofloxacin which were used for testing. Prevalence of ampicillin resistance was high in *E. coli* isolated from both captive (32.3%) and wild (35.5%) baboons. There was a higher prevalence of ESBLs in *E. coli* isolated from wild (17.7%) than captive (14.5%) baboons.

Kariuki *et al*. (2018) isolated *E. coli* from stool samples of sixty-two captive and sixty-two wild baboons that were resistant to all antimicrobial agents except ciprofloxacin. Prevalence of ampicillin resistance was recorded from both captive (32.3%) and wild (35.5%) baboons. There was a higher prevalence of ESBL producing *E. coli* isolated from wild (17.7%) than captive (14.5%) baboons.

Chapter 3

MATERIALS AND METHODS

The structure of research gives a snap-shot view, which has been ideally suited to assess the prevalence and antibiotic susceptibility patterns of ESBLs among *E.coli* isolated from faecal samples based on the investigation made at one specific point of time from September 2018 to June 2019.

3.1 Place and design of the study

The study was performed at the Department of Veterinary Microbiology, Bihar Veterinary College, Bihar Animal Sciences University, (BASU), Patna, Bihar, India. The faecal samples were collected from the Sanjay Gandhi Biological Park, Patna and Kanpur Zoological Park, Uttar Pradesh.

The aim of the study was isolation, biochemical and molecular detection of *E. coli* of captive mammals and birds of different Zoos. The antimicrobial susceptibility pattern was determined to investigate the occurrence of multidrug resistance *E. coli* and ESBL producing *E. coli*.

3.2 Materials

3.2.1. Source of samples

A total of ninety-four (94) fresh faecal samples were collected during the study period from September 2018 to June 2019 from 30 species of captive mammals (53 samples) and 30 species of captive birds (41 samples) of different captive zoo facilities: Sanjay Gandhi Biological Park, Patna, Bihar and Kanpur Zoological Park, Kanpur, U.P.

The captive animals were categorized into Orders: mammals and birds, depending on the biological classification scheme (Table 1 & 2). The animals were also divided into carnivores, herbivores and omnivores based on their feeding habit (Table 3 & 4). The species wise collection of faecal samples from captive mammals and birds and their detailed sample source is presented in Table 5 and Table 6, respectively.

Table 1: Categorization of captive mammals based on their Order

Sl. no.	Order	Species of Mammal
1.	*Carnivora*	Himalayan Black bear Sloth bear White tiger Bengal tiger Asiatic lion Hybrid Lioness Leopard Palm civet cat Jackal Hyena Wolf
2.	*Proboscidea*	Elephant
3.	*Perissodactyla*	One-horned Rhinoceros Zebra
4.	*Artiodactyla*	Hippopotamus Giraffe Indian Gaur Sangai deer Chinkara deer Sika deer Hog deer Swamp deer Sambar deer Black buck Antelope four horned
5.	*Primate*	Rhesus monkey Capuchin Monkey Chimpanzee Langur
6.	*Rodentia*	Porcupine

Table 2: Categorization of captive birds based on their Order

Sl. no.	Order	Species of Mammal
1.	*Accipitriformes*	Eagle, Vulture
2.	*Anseriformes*	Duck, Swan
3.	*Bucerotiformes*	Hornbill
4.	*Casuariiformes*	Emu
5.	*Ciconiiformes*	Painted stork
6.	*Columbiformes*	Pigeon
7.	*Galliformes*	Lady Amherst's pheasant Silver pheasant Kalij pheasant Golden pheasant Grey peacock pheasant Peacock Jungle fowl
8.	*Gruiformes*	Crane
9.	*Passeriformes*	Zebra finch, Hill myna
10.	*Psittaciformes*	Medium sulphur crested cockatoo Alexandrine Parakeet Scarlet macaw Spix’s macaw Parrot Cockatiel Greater sulphur crested cockatoo Budgerigar Love bird
11.	*Strigiformes*	Indian owl Two horned owl
12.	*Struthioniformes*	Ostrich

Table 3: Categorization of mammals based on their feeding habit

Category	**Mammal**
Carnivore	Bengal Tiger
	White Tiger
	Asiatic Lion
	Hybrid Lioness
	Leopard
	Jackal
	Hyena
	Wolf
	Palm Civet Cat
Herbivore	Elephant
	One horned Rhinoceros
	Hippopotamus
	Zebra
	Giraffe
	Indian Gaur
	Sangai Deer (Brow antlered)
	Chinkara Deer
	Sika Deer
	Black Buck (*Krishna mrig*)
	Antelope four horned (*Chowsingha*)
	Hog Deer
	Swamp Deer (*Barasingha*)
	Sambar Deer
	Porcupine
Omnivore	Rhesus Monkey
	Capuchin Monkey
	Chimpanzee
	Langur
	Himalayan Black Bear
	Sloth Bear

Table 4: Categorization of birds based on their feeding habit

Category	**Bird**
Carnivore	Two horned owl
	Indian owl
	Vulture
	Eagle
Herbivore	–
Omnivore	Budgerigar
	Hornbill
	Emu
	Ostrich
	Crane
	Swan
	Duck
	Lady Amherst Pheasant
	Silver pheasant
	Kalij pheasant
	Golden pheasant
	Jungle fowl
	Parrot
	Cockatiel
	Greater sulphur crested cockatoo
	Zebra Finch
	Medium sulphur crested cockatoo
	Alexandrine Parakeet
	Scarlet macaw
	Spix’s macaw
	Peacock
	Pheasant grey peacock
	Painted stork
	Love bird
	Hill Myna
	Pigeon

Table 5. Details of faecal sample collected from captive mammals during the study period

Sl. no.	Mammals	No. of samples from Patna zoo	No. of samples from Kanpur zoo	Total no. of sample
1.	Himalayan Black Bear	02	02	04
2.	Sloth Bear	00	01	01
3.	Bengal Tiger	02	02	04
4.	White Tiger	02	00	02
5.	Asiatic Lion	01	05	06
6.	Hybrid Lioness	01	00	01
7.	Leopard	02	01	03
8.	Jackal	00	01	01
9.	Hyena	02	00	02
10.	Wolf	02	00	02
11.	Palm civet Cat	01	00	01
12.	Elephant	02	00	02
13.	One horned rhinoceros	01	01	02
14.	Hippopotamus	01	00	01
15.	Zebra	01	01	02
16.	Giraffe	01	00	01
17.	Indian Gaur	01	00	01
18.	Sangai Deer	00	01	01
19.	Chinkara Deer	00	01	01
20.	Sika Deer	00	01	01
21.	Black Buck	01	01	02
22.	Antelope four horned	00	01	01
23.	Hog Deer	01	00	01
24.	Swamp Deer	01	00	01
25.	Sambar Deer	01	00	01
26.	Rhesus Monkey	00	01	01
27.	Capuchin Monkey	00	01	01
28.	Chimpanzee	02	00	02
29.	Langur	01	01	02
30.	Porcupine	01	01	02
Total		**30**	**23**	**53**

Table 6. Details of sample collected from captive birds during the study period

Sl. no.	Birds	No. of samples from Patna zoo	No. of samples from Kanpur zoo	Total no. of sample
1.	Budgerigar	01	01	02
2.	Hornbill	01	00	01
3.	Emu	01	01	02
4.	Ostrich	01	00	01
5.	Crane	02	01	03
6.	Swan	00	01	01
7.	Duck	00	01	01
8.	Lady amherst pheasant	00	01	01
9.	Silver pheasant	01	01	02
10.	Kalij pheasant	00	01	01
11.	Golden pheasant	01	01	02
12.	Jungle fowl	01	00	01
13.	Parrot	02	00	02
14.	Cockatiel	01	01	02
15.	Greater sulphur crested cockatoo	01	00	01
16.	Zebra finch	01	00	01
17.	Medium sulphur crested cockatoo	01	00	01
18.	Alexandrine Parakeet	00	01	01
19.	Scarlet macaw	01	00	01
20.	Spix’s macaw	01	01	02
21.	Peacock	01	01	02
22.	Pheasant grey peacock	01	00	01
23.	Painted stork	00	01	01
24.	Two horned owl	00	01	01
25.	Indian owl	00	01	01
26.	Love bird	00	01	01
27.	Hill myna	01	00	01
28.	Vulture	02	00	02
29.	Eagle	01	00	01
30.	Pigeon	01	00	01
Total		**24**	**17**	**41**

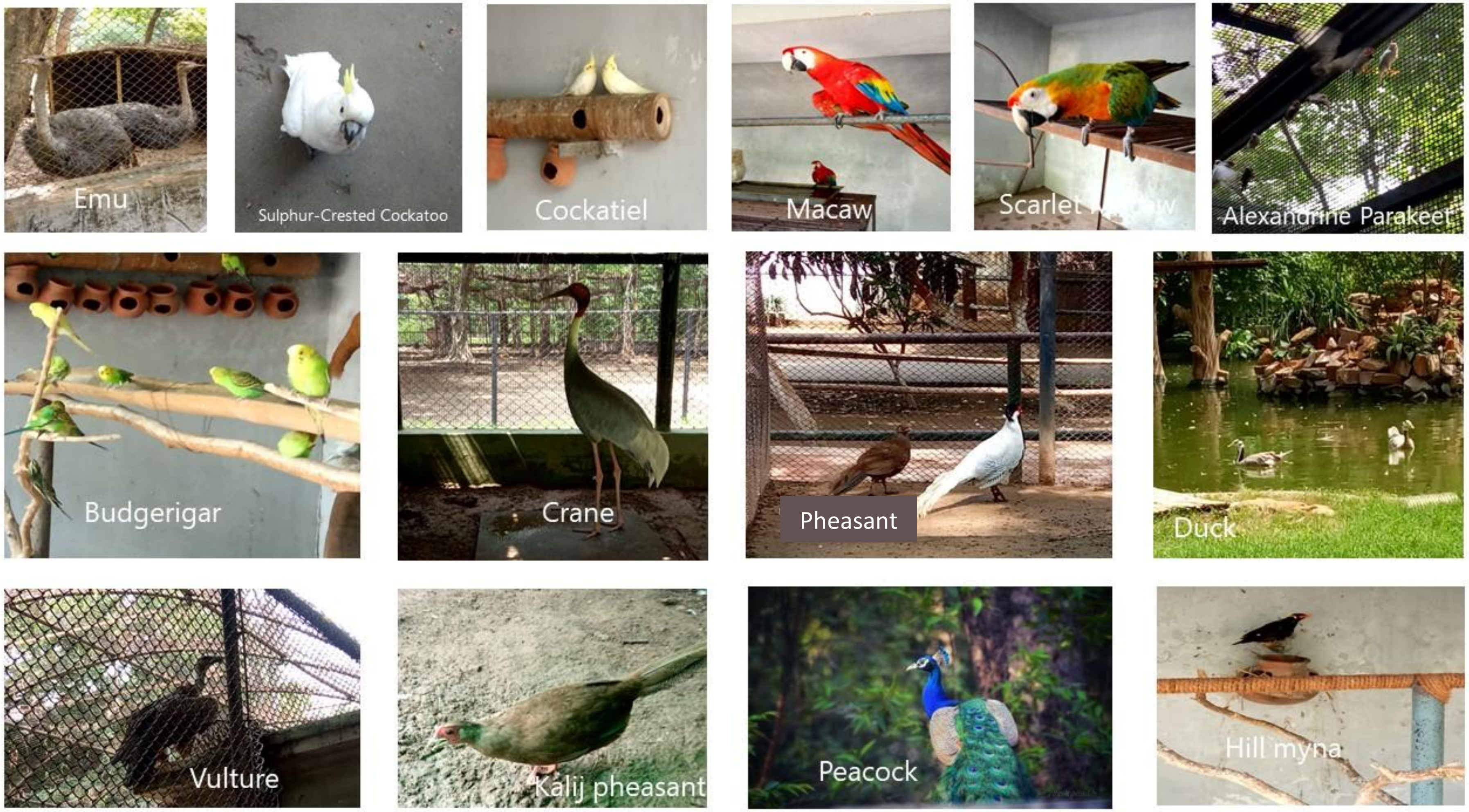


Figure 1: Photograph of representative species of captive birds from which samples collected

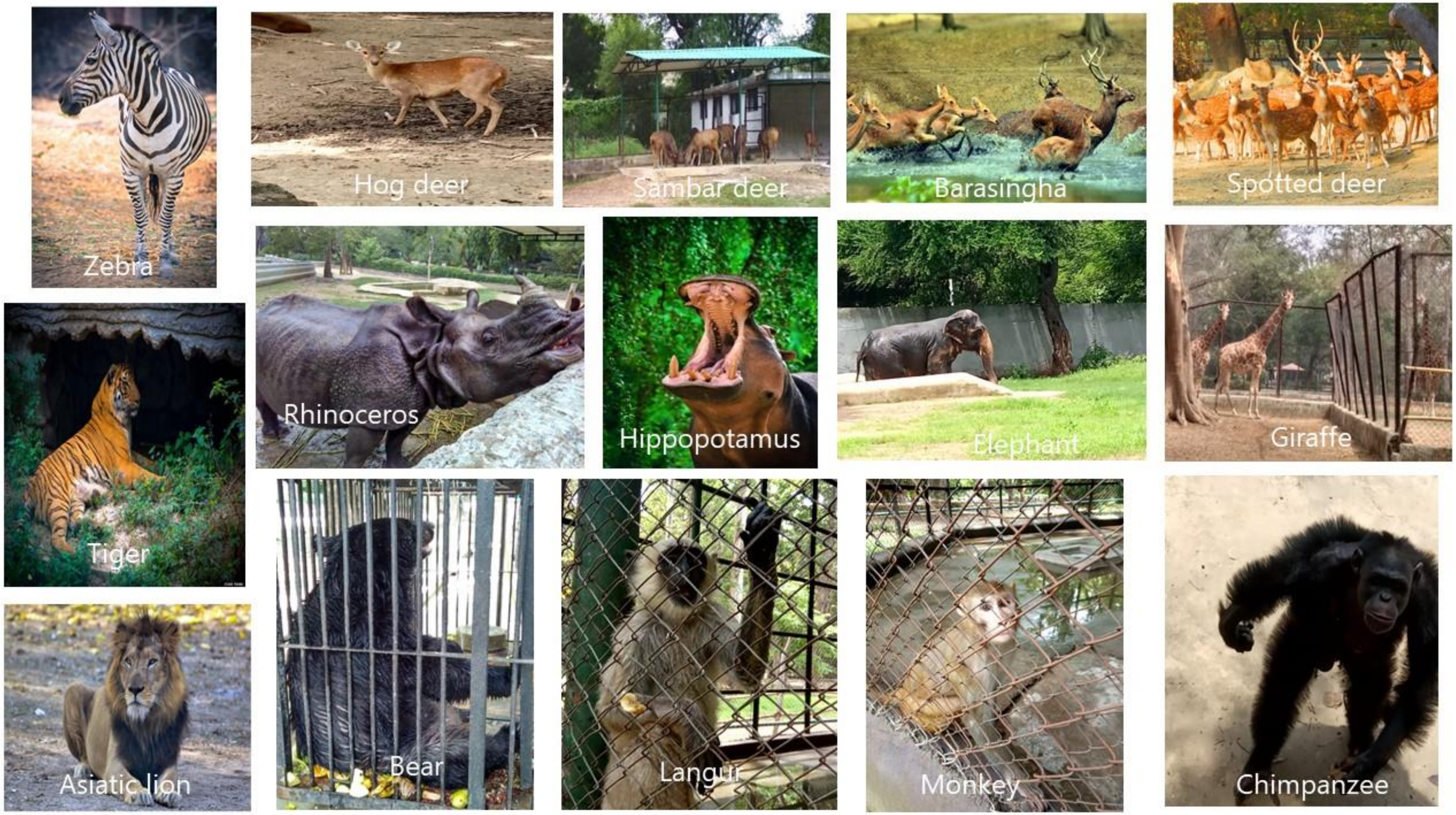


Figure 2: Photograph of representative species of captive mammals from which samples collected

3.2.2. Media, buffers and reagents

The media and reagents used in this study were procured from HiMedia, India. The detail of media and their preparation, buffers and reagents that were used in this study, has listed in appendix no. I, II, III

3.2.3. Chemicals used in molecular studies

- Dream Taq DNA polymerase (Thermo Scientific, USA)
- 10x PCR buffer (Thermo Scientific, USA)
- dNTP mixture (Thermo Scientific, USA)
- Nuclease free water (Thermo scientific, USA)
- 100 bp plus Gene ruler (Thermo scientific, USA)
- 6x loading dye (Thermo Scientific, USA)
- Ethidium bromide (Sigma, USA)
- Agarose (HiMedia)

3.2.4 Plasticware and glasswares

All the plastic wares used in this study were procured from national and international firms i.e. HiMedia laboratories Pvt. Ltd., Mumbai, Axiva (India), Greiner (India) and Axygen (USA) and glasswares were obtained from Tarsons (India), Borosil (India) and Schott Duran (Germany).

3.2.5 Antimicrobial agents

The antimicrobial agents used in the disk diffusion technique are: Gentamicin (10µg), Amikacin (30µg), Ceftriaxone (30µg), Cefotaxime (30µg), Ampicillin (10µg), Amoxicillin-clavulanic acid (30µg), Chloramphenicol (30µg), Oxytetracycline (30µg), Ciprofloxacin (5µg), Enrofloxacin (5µg), Ceftazidime (30µg), Ceftazidime-clavulanic acid (30/10µg), Cefpodoxime (10µg), Cefpodoxime-clavulanic acid(10/5µg) and Cefotaxime-clavulanic acid (30/10µg). They were obtained from Hi-media Labs (India) and listed in Table 8 and 9.

3.2.7 Equipments

- Electronic balance (Denver, USA)
- Centrifuge machine (REMI, India)
- Deep fridge -20°C (Blue Star, India)
- Gel documentation system (Biorad, USA)
- PCR machine
- pH meter (LABMAN)
- Micropipette of various volumes (0.5 µl - 1000µl) (Eppendorf, Germany)
- Horizontal gel electrophoresis apparatus (Thermo scientific, China)
- Water bath (YSI, India)
- Vortex mixture (Tarson, India)
- Autoclave (Instrumentation, India)
- Hot air oven (Sonar, India)
- Incubator (Sonar, India)
- Water distillation apparatus (Millipore India Pvt. Ltd., New Delhi)
- Laminar airflow bench (Ikon instruments, India)
- Microwave oven (LG, India)
- Compound light microscope
- Refrigerator

3.2.8 Oligonucleotide primers

Primers used in this study were custom synthesized from Xcelris (India). The details of the primers are given in Table 7.

Table no. 7: Oligonucleotide primers used in this study

Sl. no.	**Primer Sequences (5’-3’)**	**Target gene/locus**	**Expected product size**	**Reference**
1.	**ECA75F:** GGAAGAAGCTTGCTTCTTTGCTGAC **ECR619R:** AGCCCGGGGATTTCACATCTGACTTA	16S rRNA	544 bp	Sabat *et al.* 2000

F: Forward primer, **R:** Reverse primer

3.3 Methods

3.3.1 Collection and transportation of samples

Before sample collection, permission has been taken from the concerned authority of zoo and noninvasive sampling as per CZA, 2017. Ninety-four faecal samples were collected from captive mammals and birds. Freshly voided faecal samples were randomly collected from all enclosures of mammals and birds early in the morning.

For sampling, up to 2g of fresh faecal material were collected aseptically using sterile swabs (HiMedia, India.) in the pre-labelled sterile sample containers and sealed with parafilm to avoid sample cross-contamination. In most cases, samples were collected within minutes to hours of deposition. Samples were transported to the laboratory in cold box maintaining cold chain (4°C) and processed within an hour of collection.

3.3.2 Enrichment of faecal sample:

Approximately, one gm. of the sample was enriched inoculated in 10 ml sterile buffered peptone water (1:10 ratio) under aseptic condition and incubated at 37°C for 24 hr.

3.3.3 Isolation and cultural identification of *E. coli*

Following enrichment, an aliquot of enriched broth was inoculated on MacConkey agar (differential agar) and incubated at 37°C for 24 hr to differentiate the lactose fermenter. Five typical colonies, showing small, pink, round morphology, were subcultured onto EMB agar plate and incubated at 37˚C for 24 hr. Characteristic black centered purple colony with green metallic sheen were used for further identification.

A loop-full of the culture of BPW was then streaked on MacConkey agar and incubated at 37°C for 24 hr**.** MacConkey agar was used as a differential media to differentiate the lactose fermenter and non-lactose fermenter and EMB agar were used as a selective media. Small, pink, round lactose fermenting colony was selected and further streaked onto EMB agar and incubated at 37˚C for 24 hr. Characteristic black centered purple colony with green metallic sheen were selected for further studies.

3.3.4 Biochemical characterization of presumptive *E. coli* isolates

The colonies showing typical green metallic sheen on EMB agar were confirmed by biochemical tests using KB001 HiIMViC biochemical test kit (HiMedia, India) including, Indole, Methyl Red, Voges Proskauer's, Citrate Utilization and carbohydrates fermentations (Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose and Sucrose). A single presumptive colony from each culture plate were grown in 5 ml. nutrient broth for 4-6 hr at 37°C. The kit was opened aseptically and the sealing tape peeled off. 0.5µl of culture was inoculated on each well of the test kit and incubated for 24 hr at 37°C. The results were interpreted after addition of reagents/indicators in wells as per manufacturer guidelines with the help of result interpretation chart (HiMedia, India).

3.3.5 Preservation of the isolates

All biochemically confirmed isolates were inoculated in nutrient agar stab (0.9% agar) with overnight incubation at 37°C and stored at 4°C.

3.3.6 Subculture of *E. coli* of the preserved isolates

The preserved isolates were revived through inoculation in nutrient broth for 24 hr incubation at 37°C. The DNA templates were prepared from these broth cultures by snap and chill method (Kaushik *et al***.** 2014) for screening with PCR assay and to perform antibiotic sensitivity test for confirmed isolates.

3.3.7 Template DNA preparation by heat lysis method (Snap chill method)

The DNA extraction method by heat lysis was adopted as described earlier by (Kaushik *et al***.** 2014). About 1.5 ml overnight culture grown in the nutrient broth was taken in Eppendorf tube (2 ml), centrifuged at 10,000 rpm for 10 min. to pellet the bacteria. The supernatant was discarded pellets were resuspended in 1 ml distilled water and vortexed. The process was repeated twice and the resulting pellet was suspended in 100 µl nuclease-free water. The suspension was subjected to boiling for 15 min. After boiling, cell lysate was kept immediately at -20°C for 20 min. The tube was removed out of -20°C and kept at room temperature for thawing and centrifuged at 4000 rpm for 4 min. The supernatant was taken as a genomic DNA template.

3.3.8 Polymerase Chain Reaction (PCR) Assay

All the isolates previously confirmed *E. coli*, based on cultural, morphological and biochemical properties, were re-confirmed by genomic DNA based PCR amplification**.** A PCR assay was standardized for direct detection of species-specific 16s rRNA gene of *E. coli* as per the method described by Sabat *et al.* (2000) with slight modification. The amplification reaction was performed by taking 5 µl of DNA template and 20 µl of PCR master mixture. PCR master mixture contained 2.5µl 10x PCR buffer (500mM KCl, 100mM Tris HCl, pH-8.3; 15mM $MgCl_2$), 2.5µl of dNTP (2.0mM), 2µl for each (10 pmol) of forward and reverse primer of 16s rRNA gene, 0.2µl Taq DNA polymerase (5 unit/µl) and nuclease-free water to made the volume 20 µl. was used to amplify the gene of 544bp.

The PCR programme included Hot start at 94°C for 3 min. followed by 40 cycles of denaturation (94°C for 30 sec.), annealing (72°C for 45 sec.) and elongation (72°C for 45 sec) with a final extension at 72°C for 10 min.

3.3.9 Agarose Gel Electrophoresis of PCR products

All the amplicons were subjected to agarose gel electrophoresis in 1.5% agarose gel containing ethidium bromide for visualization and also to measure the size of amplicon. For electrophoresis, a total of 100 ml of 1.5% agarose was prepared in 1X Tris-acetate EDTA buffer and boiled it up. It was allowed to cool about 45°C and ethidium bromide (stock conc.10mg/ml) was added to obtain a final concentration of 0.5µg/ml. The gel was poured into an electrophoresis gel casting tray. The gel Comb was inserted and allowed the medium to solidify for 15 min. The gel electrophoresis tank was filled with 1X TAE buffer. Then prepared electrophoresis gel casting tray was kept in electrophoresis tank after removing the side rubber blocker/cello tapes. Samples were prepared on a parafilm by mixing 10µl of PCR product and 2µl of 6X loading dye. Then samples were loaded in parallel with molecular weight marker (100 bp DNA ladder). The Gel electrophoresis run was allowed for 1 hr 20 min. at 80V. The gel was viewed in Gel-documentation system.

3.3.10 Antibiotic Sensitivity testing by disc diffusion method

The antimicrobial agents and their respective concentrations used for test have been depicted in Table 8 and 9.

The drug sensitivity test was performed by disc diffusion method as per the Kirby-Bauer disk diffusion technique (Bauer *et al*. 1966) and interpretation of antimicrobial resistance phenotype was performed as per Clinical Laboratory Standards Institute guide the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2011). Filter paper disc containing a designated concentration of antimicrobial drugs was used (HiMedia, India). Mueller Hinton agar (HiMedia, India) plates were inoculated with the help of sterile cotton swabs. The plates were allowed to dry at room temperature and thereafter the antibiotic discs were gently and firmly placed on the surface of the inoculated media plate by using sterile foreceps. The plates were then placed in an incubator at 37°C for 24 hr. The antimicrobial activity if present on the plate was indicated by the formation of a zone of inhibition. The diameter of inhibition zone was measured in millimeter (mm.) after 24 hr of incubation by using antibiotic zone scale (HiMedia, India). Results were classified as susceptible, intermediate or resistant based on zone size interpretative chart provided by HiMedia Labs, India according to the approved guidelines of the CLSI, 2011.

Table 8: Class of antibiotics used for Antibiotic Sensitivity Test (ABST)

Sl. no.	**Class of antibiotic**	**Antibiotic drug**	**Disc concentration (µg)**
1.	**Amphenicols**	Chloramphenicol	30
2.	**Aminoglycosides**	Amikacin	30
		Gentamicin	10
3.	**β lactam antibiotic**	Ampicillin	10
		Amoxicillin clavulanic acid	30
		Cefotaxime	30
		Ceftriaxone	30
4.	**Quinolones**	Ciprofloxacin	5
		Enrofloxacin	5
5.	**Tetracycline**	Oxytetracycline	30

3.3.11 Identification of Multi Drug Resistance (MDR) *E. coli* isolates

The Multi Drug Resistance (MDR) character of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics (Magiorakos *et al*. 2012). The *E. coli* isolates were considered as Multi Drug Resistant when they showed resistance to at least three classes of the antibiotics tested.

3.3.12 Phenotypic confirmation of ESBL producing *E. coli*

3.3.12.1 Screening for ESBL-producer: Isolates that exhibited by reduced susceptibility to cefotaxime or ceftriaxone were considered as potential producers of ESBL and spelt for confirmatory tests.

3.3.12.1.1 Confirmatory tests for detection of ESBL *E. coli*

The phenotypic confirmatory test was performed according to CLSI recommendation (CLSI, 2011).

Phenotypic detection for ESBL positive *E. coli* was performed by employing Hexa G-minus 24 kit (HiMedia, India) following the guidelines of the manufacturer's instruction. The kit was composed of six discs. The details of the antibiotics used in the kit are given in Table 9. The isolates were considered as potential ESBL-producer after the initial screening test results of ABST where cefotaxime (30 µg) or ceftazidime disks (30 µg) with or without clavulanate (10 µg) was taken phenotypic confirmation of the presence of ESBLs in *E. coli*. A difference of ≥ 5 mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate disk was considered to be phenotypic confirmation of ESBL production.

In combination disc method, two discs were used i.e. cefotaxime (CTX) and cefotaxime-clavulanic acid (CEC). The interpretation has been made according to the criteria given by Drieux *et al*. (2008) and Taneja and Sharma (2008). A difference of ≥ 5 mm between the zone diameters of cefotaxime and cefotaxime-clavulanic acid discs was considered as phenotypic confirmation of ESBL producing *E. coli*.

Table no. 9: Details of Antibiotic kit (Hexa G-minus 24 kit; Hi-Media, India) used for phenotypic detection of ESBL *E. coli* producers

Sl. no.	**Antibiotic name**	**Concentration (µg)**	**Abbreviation**
1.	Cefpodoxime	10	CPD
2.	Cefpodoxime & clavulanic acid	10/5	CCL
3.	Ceftazidime	30	CAZ
4.	Ceftazidime & clavulanic acid	30/10	CAC
5.	Cefotaxime	30	CTX
6.	Cefotaxime & clavulanic acid	30/10	CEC

Chapter 4

RESULTS AND DISCUSSION

The present investigation was designed to study the occurrence and molecular detection of *Escherichia coli* harbouring extended-spectrum β-lactamases from captive birds and animals. The present study was conducted to explore the occurrence of *Escherichia coli* and their detection by conventional as well as molecular method and exploration regarding their status for possessing ESBL.

4.1. GENERAL OPERATING AND SOURCES PARAMETER

4.1.1. Number and information on the Zoo

There was a total of two Zoos that were selected for the study after due permission and periodically visited. The Sanjay Gandhi Biological Park, Patna, Bihar, and Kanpur Zoological Park, Kanpur, U.P. Both the two Zoos were maintained as per the guidelines of the Central Zoo Authority of India. Permission has been taken from the competent authority for the collection of samples from the premises.

4.1.2. Visit intervals and testing frequencies

The two Zoos were visited following the curriculum. The visit was carried out with the technical preparation and the presentation of the objective at the Zoo.

4.1.3. Description of Captive mammal and bird

For this purpose, sixty species of animals were selected that comprised of 30 species of captive mammals and 30 species of birds maintained at different accredited Zoos namely; Sanjay Gandhi Biological Park, Patna, Bihar, and Kanpur Zoological Park, Kanpur, U.P. The species and distribution into various categories of captive animals (mammals and birds), selected during the period of study, has been presented in Table 1, 2, 3 & 4 and (Figure 1 & 2). The details of the faecal sample collected from each species of mammal and bird have been depicted in Tables 5 & 6, respectively.

The present study encompassed 30 species of mammals across six different Orders (*Carnivora, Proboscidea, Perissodactyla, Artiodactyla, Primate and Rodentia*) and 30 species of birds covering twelve different Orders (*Accipitriformes Anseriformes Bucerotiformes, Casuariiformes, Ciconiiformes, Columbiformes,* *Galliformes, Gruiformes, Passeriformes, Psittaciformes, Strigiformes* and *Struthioniformes*) enabled to examine a range of host factor such as diet type for harbouring ESBL producing *E. coli*.

4.1.4. Sampling from Captive mammal and bird

Altogether 94 faecal samples were collected form captive wild mammals and birds without capturing or manipulating them. In this study the faecal sample collection has been done by non-invasive strategy. The Non-invasive approaches to sampling are a strategy widely used by researchers in field studies and have become a major component of wildlife research (Garshelis 2006; MacKay *et al*. 2008). Advantages of faecal sample-based wildlife research include easy collection, access to large sample sizes and spatio-temporal coverage (Biswas *et al*. 2019)

4.2. Isolation and Identification *E. coli* from faecal samples

In total, 94 different faecal samples examined. Among them, 93 (98.94%) showed a small, pink round lactose fermenting colony on MacConkey agar (Figure 3) whereas 82 (87.23%) samples produced typical green “metallic sheen” on EMB agar (Figure 4).

All the positive isolates showed typical characteristics during cultural, i.e., produced typical “metallic sheen” when grown on EMB agar plates and morphological examinations (pink rods after Gram’s staining).

During the experiment the MacConkey agar medium were used for differential isolation of *E. coli* from faecal sample. MacConkey medium is non-selective to concerning members of the *Enterobacteriaceae* (Ewing 1986), but it inhibits the growth of Gram-positive and many Gram negative species (Gordon & FitzGibbon, 1999). Relative to other members of the *Enterobacteriaceae*, *E. coli* has a distinct colony morphology and colour on MacConkey agar and consequently is unlikely to be overlooked. The EMB agar medium was used as a differential medium. The colonies showing a unique and characteristic green metallic sheen were considered as *E. coli* whereas non green colonies as other coliforms. Several workers have used Eosin methylene blue (EMB) agar for identification of *E. coli* on this agar (Betteiheim 1994; Quinn *et al*. 1994).

Out of 82 isolates, 73 (89.02%) showed biochemical characterization, i.e., positive to indole, methyl red, catalase, and nitrate reduction whereas negative to VP and citrate utilization. The isolates were subjected to sugar fermentation reactions. The result revealed that all the isolates fermented glucose while the fermentation of other sugars showed variable results (Cruickshank *et al*. 1975; Forbes *et al*. 2007).

The molecular identification of 82 samples was done by the amplification of species-specific 16S rRNA gene via polymerase chain reaction revealed that 73 (89.02%) isolates were positive for *E. coli.* (Figure 5). The 73 isolates confirmed through PCR were selected for further study. Seventy-three distinct isolates from captive mammals (n = 46) and birds (n = 27) were included in the assessment.

Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, can be routinely used for identification of bacteria, and can lead to the recognition of novel pathogens and noncultured bacteria (Clarridge 2004).

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Several studies evaluated the usefulness of 16S rRNA for identification of bacteria. Comparative assays were performed by amplification reactions using the same samples as templates and either different sets of previously described PCR primers targeting bacterial genome (Eckburg *et al*. 2005; Wang *et al*. 2005; Palmer *et al*. 2007).

4.2.1. Prevalence of *E coli* among captive animals (mammals and birds)

Out of the 73 confirmed *E. coli* isolates, 46 (63.01%) were from mammals (Table 10) and 27 (36.98%) from birds (Table 11). Further perusal of data (Table 12) showed that among all captive mammals and birds, 21 (77.77%) carnivores, 19 (95.00%) herbivores, and 33 (70.21%) omnivores carried *E. coli* in their faeces. The overall occurrence of *E. coli* among mammals and birds was highest in herbivores followed by carnivores and omnivores.

Previous workers have also screened the *E. coli* from wild animals (Souza *et al*. 1999; Gopee *et al*. 2000; Ahmed *et al.* 2007).

The prevalence of *E. coli* among mammalian hosts species varied from 0 to 100% (Table 10). Overall, *E. coli* was detected in 86.79% of the 30 mammalian hosts examined. Adesiyun and Downes (1999) reported prevalence of *E. coli* isolates from captive mammals as 48.0%. *E. coli* is the predominant indigenous flora in the gastrointestinal tract of the intestines and faeces of warm-blooded animals (Berg 1996; Gordon and Cowling 2003). They reside in the mucus layer that covers the epithelial cells throughout the tract and are shed into the intestinal lumen with the degraded mucus component and excreted in the faeces (Poulsen *et al*. 1994). The frequency of isolation of *E. coli* in present study varied from 0-100% in captive mammalian species. Gordon and Cowling (2003) also documented 0% to 100% *E. coli* from wild mammals, however, Tenaillon *et al*. (2010) reported only 56% in wild mammals. The various *E. coli* hosts have distinct body sizes, gut morphologies, diets, digesta retention times, different niches in the gut and microbiota. These characteristics can have a substantial influence on the prevalence of *E. coli*, which vary from 0% to 100% among host species (Gordon and Cowling 2003; Ley *et al*. 2008).

Table 10. Frequency of isolation of *E. coli* from captive mammals

Sl. No.	Species of mammals	Scientific Name	Total no. of faecal sample	No. of sample positive for *E. coli*	Percentage of sample positive for *E. coli*
1.	Himalayan Black Bear	*Ursus thibetanus*	04	03	75.00
2.	Sloth Bear	*Melursus ursinus*	01	01	100.00
3.	Bengal Tiger	*Panthera tigris tigris*	04	04	100.00
4.	White Tiger	*Panthera tigris*	02	02	100.00
5.	Asiatic Lion	*Panthera leo leo*	06	06	100.00
6.	Hybrid Lioness	*Panthera leo*	01	01	100.00
7.	Leopard	*Panthera pardus*	03	02	66.67
8.	Jackal	*Canis aureus*	01	01	100.00
9.	Hyena	*Hyaena hyaena*	02	01	50.00
10.	Wolf	*Canis lupus*	02	01	50.00
11.	Palm Civet Cat	*Paradoxurus hermaphroditus*	01	0	0.00
12.	Elephant	*Elephas maximus*	02	02	100.00
13.	One horned Rhinoceros	*Rhinoceros unicornis*	02	02	100.00
14.	Hippopotamus	*Hippopotamus amphibius*	01	0	0.00

Sl. No.	**Species of mammals**	**Scientific Name**	**Total no. of faecal sample**	**No. of sample positive for *E. coli***	**Percentage of sample positive for *E. coli***
15.	Zebra	*Equus quagga*	02	02	100.00
16.	Giraffe	*Cervus camelopardalis*	01	01	100.00
17.	Indian Gaur	*Bos gaurus*	01	01	100.00
18.	Sangai Deer (Brow antlered)	*Rucervus eldii eldii*	01	01	100.00
19.	Chinkara Deer	*Gazella bennettii*	01	01	100.00
20.	Sika Deer	*Cervus nippon*	01	01	100.00
21.	Black Buck	*Antilope cervicapra*	02	02	100.00
22.	Four-horned Antelope	*Tetracerus quadricornis*	01	01	100.00
23.	Hog Deer	*Axis porcinus*	01	01	100.00
24.	Swamp Deer	*Rucervus duvaucelii*	01	01	100.00
25.	Sambar Deer	*Rusa unicolor*	01	01	100.00
26.	Rhesus Monkey	*Macaca mulatta*	01	01	100.00
27.	Capuchin Monkey	*Cebinae*	01	0	0.00
28.	Chimpanzee	*Pan troglodytes*	02	02	100.00
29.	Langur	*Semnopithecus entellus*	02	02	100.00
30.	Porcupine	*Atherurus macrourus*	02	02	100.00
	Total		**53**	**46**	**86.79**

Table 11. Frequency of isolation of *E. coli* from captive birds

Sl. No.	**Species of birds**	**Scientific Name**	**Total no. of faecal sample**	**No. of sample positive for *E. coli***	**Percentage of sample positive for *E. coli***
1.	Budgerigar	*Melopsittacus undulatus*	02	02	100.00
2.	Hornbill	*Ocyceros griseus*	01	0	0.00
3.	Emu	*Dromaius novaehollandiae*	02	01	50.00
4.	Ostrich	*Struthio camelus*	01	01	100.00
5.	Crane	*Grus antigone*	03	02	66.67
6.	Swan	*Cygnus cygnus*	01	01	100.00
7.	Duck	*Anas platyrhynchos*	01	01	100.00
8.	Lady Amherst's pheasant	*Phasianus colchicus*	01	01	100.00
9.	Silver pheasant	*Lophura nycthemera*	02	02	100.00
10.	Kalij pheasant	*Lophura leucomelanos*	01	01	100.00
11.	Golden pheasant	*Chrysolophus pictus*	02	01	50.00
12.	Jungle fowl	*Gallus gallus*	01	0	0.00
13.	Parrot	*Psittaciformes*	02	02	100.00
14.	Cockatiel	*Nymphicus hollandicus*	02	0	0.00

Sl. No.	**Species of birds**	**Scientific Name**	**Total no. of faecal sample**	**No. of sample positive for *E. coli***	**Percentage of sample positive for *E. coli***
15.	Greater Sulphur-crested cockatoo	*Cacatua galerita*	01	0	0.00
16.	Finch zebra	*Poephila guttata*	01	0	0.00
17.	Medium sulphur-crested cockatoo	*Cacatua galerita eleonora*	01	0	0.00
18.	Alexandrine Parakeet	*Psittacula eupatria*	01	01	100.00
19.	Scarlet macaw	*Ara macao*	01	0	0.00
20.	Spix’s macaw	*Cyanopsitta spixii*	02	02	100.00
21.	Peacock	*Pavo cristatus*	02	01	50.00
22.	Pheasant grey peacock	*Polyplectron bicalcaratum*	01	01	100.00
23.	Painted stork	*Mycteria leucocephala*	01	01	100.00
24.	Two horned owl	*Bubo virginianus*	01	01	100.00
25.	Indian owl	*Bubo bengalensis*	01	0	0.00
26.	Love bird	*Agapornis spp.*	01	01	100.00
27.	Myna hill	*Gracula religiosa*	01	01	100.00
28.	Vulture	*Gyps himalayensis*	02	01	50.00
29.	Eagle	*Aquila chrysaetos*	01	01	100.00
30.	Pigeon	*Columbia livia*	01	01	100.00
	Total		**41**	**27**	**62.79**

Table 12: Order wise distribution of *E. coli* isolated from captive mammals

Sl. no.	Class of animal	Total no. of samples collected	Positive number of *E. coli*	Occurrence of *E. coli* (%)
1.	*Carnivora*	27	22	84.62
2.	*Proboscidea*	02	02	100.00
3.	*Perissodactyla*	04	04	100.00
4.	*Artiodactyla*	14	13	100.00
5.	*Primate*	06	05	83.33
	Total	**53**	**46**	**86.79**

Table 13: Order wise distribution of *E. coli* isolated from captive birds

Sl. no.	Order of bird	Total no. of samples collected	Positive number of *E. coli*	Occurrence of *E. coli* (%)
1.	*Accipitriformes*	3	2	66.67
2.	*Anseriformes*	2	2	100.00
3.	*Bucerotiformes*	1	0	0.00
4.	*Casuariiformes*	2	1	50.00
5.	*Ciconiiformes*	1	1	100.00
6.	*Columbiformes*	1	1	100.00
7.	*Galliformes*	10	7	70.00
8.	*Gruiformes*	3	2	66.67
9.	*Passeriformes*	2	1	50.00
10.	*Psittaciformes*	13	8	61.54
11.	*Strigiformes*	2	1	50.00
12.	*Struthioniformes*	1	1	100.00
	Total	**41**	**27**	**61.11**

Table 14: Occurrence of *E. coli* for selected captive mammal species based on their feeding habit

Sl. no.	Class of mammal	Total No. of samples collected	Positive Number of *E. coli*	Occurrence of *E. coli*
1.	**Carnivores**	22	18	81.82%
2.	**Herbivores**	20	19	95.00%
3.	**Omnivores**	11	09	81.82%
	Total	**53**	**46**	**86.79%**

Table 15: Occurrence of *E. coli* for selected captive bird species based on their feeding habit

Sl. no.	Class of bird	Total No. of samples collected	Positive Number of *E. coli*	Occurrence of *E. coli*
1.	**Carnivores**	05	03	60.00%
2.	**Herbivores**	0	0	-
3.	**Omnivores**	36	24	66.67%
	Total	**41**	**27**	**65.85%**

Table 16: Occurrence of *E. coli* for all the selected captive mammal and bird species based on their feeding habit

Sl. no.	Class of animal	Total No. of samples collected	Positive Number of *E. coli*	Occurrence of *E. coli*
1.	**Carnivores**	27	21	77.77%
2.	**Herbivores**	20	19	95.00%
3.	**Omnivores**	47	33	70.21%
	Total	**94**	**73**	**77.65%**

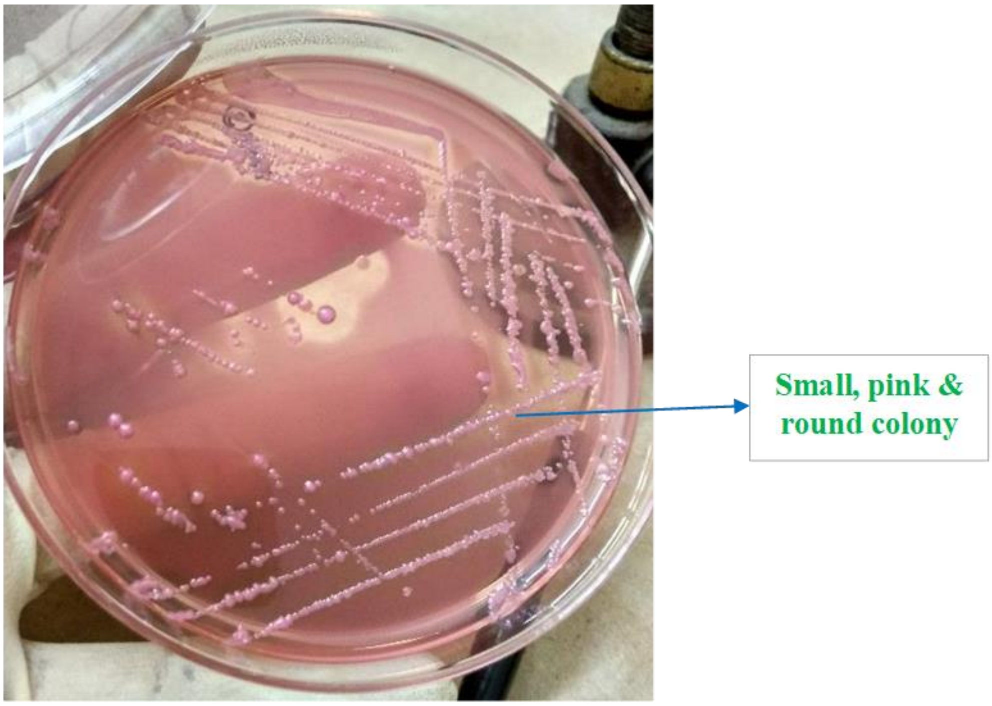


Figure 3: *MacConkey Agar Showing Small, pink and round colony*

Figure 4: *Eosin Methylene Blue (EMB) agar plates representing the selection of E. coli isolates (Green metallic sheen production).*

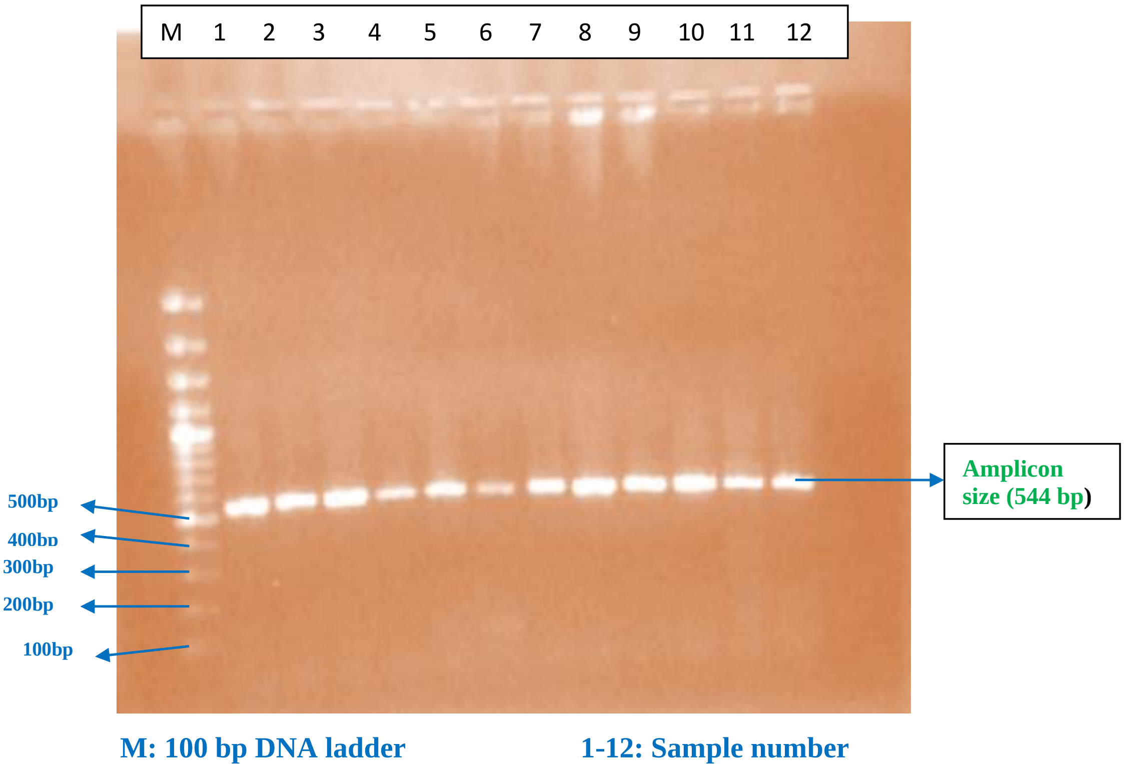


Figure 5 : Amplification product of 16S rRNA gene

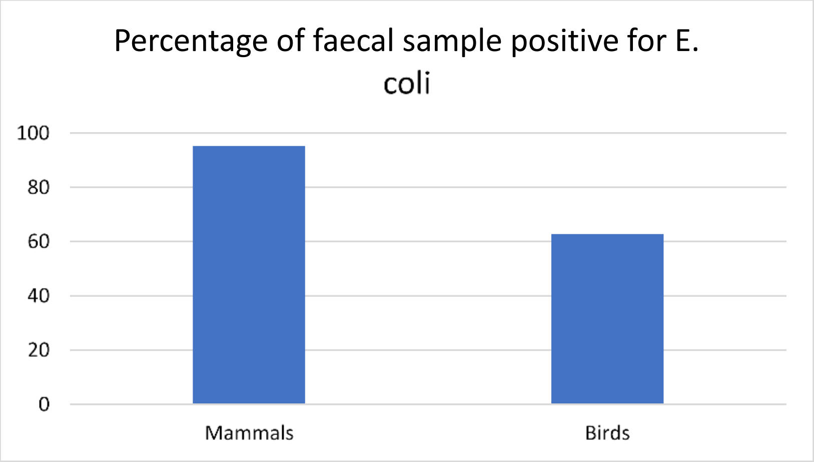


Figure 10: *Bar diagram showing Class wise distribution of faecal sample positive (%) for E. coli*

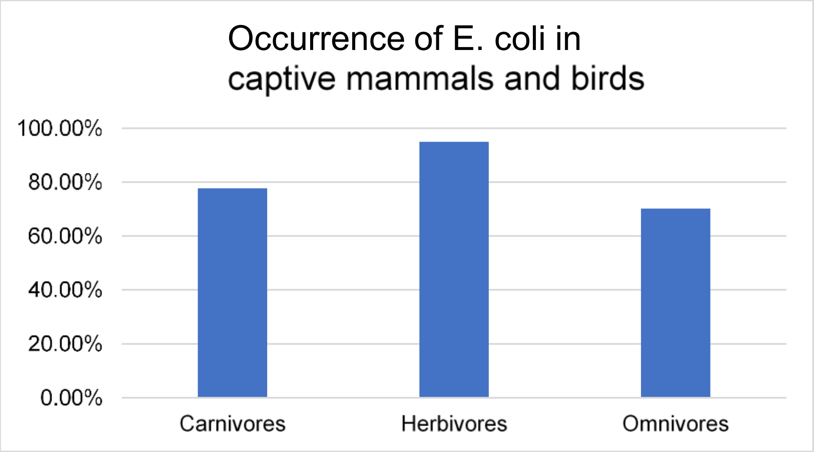


Figure 11: *Occurrence of E. coli in captive mammals and birds*

4.2.1.1. Prevalence of *E. coli* among captive mammals

The Order-wise occurrence of *E. coli* among captive mammals that *Proboscidea*, *Perissodactyla*, and *Artiodactyla* had an isolation frequency of 100% for *E. coli* compared with *Carnivora* and *Primate* with 84.62% and 83.33%, respectively (Table 12). The occurrence of *E. coli* in faeces from various types of mammal was analyzed. The perusal of the data from Table 14, it has been revealed that the occurrence was highest in mammalian herbivores (95.00%) followed by carnivores and omnivores (81.82%). The overall occurrence of *E. coli* in mammals was 86.79%.

The perusal of data showed the highest mammalian frequency of isolation of *E. coli* in *Proboscidea, Perissodactyla* and *Artiodactyla* (100.00%), which include herbivores. The transmission by faecal oral route might have been the reason for colonisation of *E. coli*. Higher values of the three orders, which include herbivorous member and may be interpreted partially by the fact that these animals tend to defecate in their drinking water. The transmission by faecal oral route might have been the reason for high density of colonisation of *E. coli*. Furthermore, the contaminated water acts as potential source of *E. coli* infection in animals and humans (Linton *et al*. 1974; Gordon and Cowling 2003). The carnivores and primates had relatively a smaller number of isolates. Although carnivores and omnivores are usually reported to harbour more *E. coli* than herbivores (Gordon and Cowling 2003). The present finding disagreed with the earlier reports.

The present finding showed herbivores (95.00%), carnivores (81.82%), and omnivores (81.82%) in terms of yield of *E. coli*, which is in agreement with the findings of Gopee *et al.* (2000) who reported isolation of *Escherichia coli* from omnivores, herbivores, and carnivores at the rate of 87.2%, 70.0%, and 57.3%, respectively. Palanivelrajan *et al*. (2018) reported rate of yield *Escherichia coli* as 46.67 % in omnivores captive mammals whereas Oludairo *et al*. (2016) isolated 20.83% *E. coli* from carnivores and 31.25% from herbivores, comparatively lesser than present study. Although carnivores and omnivores are usually reported to harbour more *E. coli* than herbivores (Gordon and Cowling 2003). The present finding disagrees with the earlier reports.

4.2.1.2. The occurrence of *E coli* among captive birds

The perusal of Table 11 showed that in the case of captive birds, the occurrence of *E. coli* in the faecal sample of omnivores was 66.67% followed by carnivores (60.00%). There was no herbivorous bird under this study. The overall occurrence in the bird category was found at 65.85%.

The perusal of Table 21 depicted the order-wise occurrence of *E. coli* in *the* faecal sample of captive birds. The highest percentage of isolate was in *Anseriformes* (100.00%), *Columbiformes* (100.00%), *Ciconiiformes* (100.00%) and *Struthioniformes* (100.00%) followed by *Galliformes* (70.00%), *Accipitriformes* (66.67%), *Gruiformes* (66.67%), *Psittaciformes* (53.85%), *Casuariiformes* (50.00%), *Passeriformes* (50.00%) and *Strigiformes* (50.00%). No *E. coli* was isolated from faecal samples of *Bucerotiformes* (0.00%).

The similar findings were recorded by Suphoronski *et al*. (2015) and Gopee *et al*. (2000) who isolated *E. coli* at 88.33 % and 68.0%, respectively, in zoo birds. However, Roger (2006) isolated *E. coli* at a percentage of 38% and Brittingham *et al*. (1988) and Hedawy and El-Shorbagy (2007) reported lower *E. coli* prevalence rates of 18.7% in free living birds, respectively.

Escherichia coli have been isolated from a range of bird species, including apparently healthy passerines and waterfowl (Brittingham *et al*. 1988; Damare *et al*. 1979; Foster *et al*. 1998). Presence of *Escherichia coli is* 23% in birds (Gordon and Cowling 2003).

The frequency of isolation of *E. coli* from various orders has been presented in Table 13. Prevalence ranged from 50.00% in *Casuariiformes*, *Passeriformes* and *Strigiformes* to 100% in *Anseriformes*, *Ciconiiformes*, *Columbiformes* and *Struthioniformes.*

Table 15 shows the frequency of *E. coli* isolation from birds of different classes. The frequencies for isolation of *E. coli* from avian carnivores and omnivores, 60.00% and 66.67%, respectively. Gopee *et al*. (2000) isolated of *E. coli* from avian carnivores (43.4%) and omnivores (87.2%) which are in agreement with present finding. The variations in *E. coli* prevalence rates may be attributed to many factors, like age of birds, state of health, state of immunity and other factors related to the environmental conditions as temperature and humidity. Also, it may be related to diet, physiology and degree of hygiene of feed, water, air and litter.

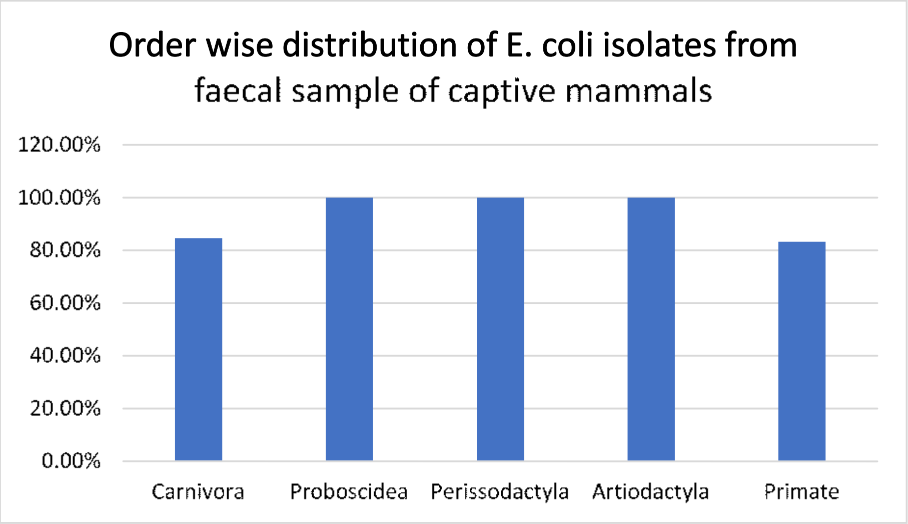


Figure 6: *Bar diagram showing Order wise distribution of E. coli isolates (%) from faecal samples of captive mammals*

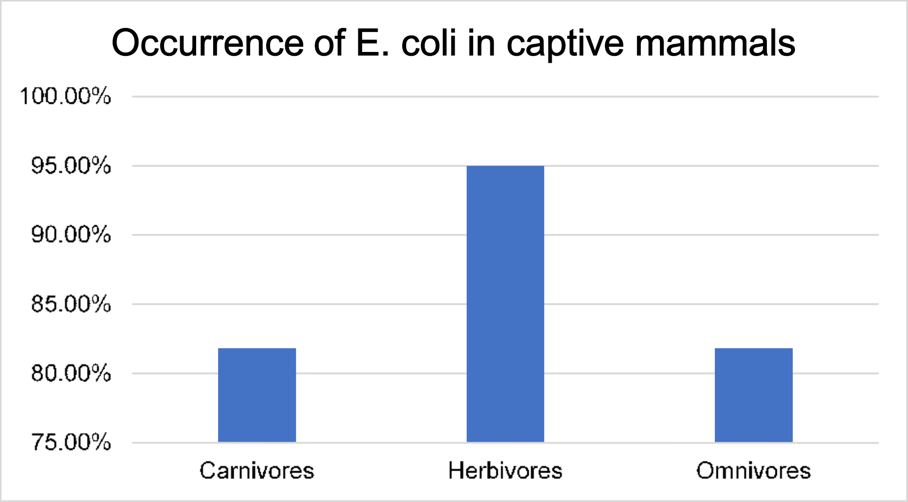


Figure 7: *Occurrence of E. coli in captive mammals*

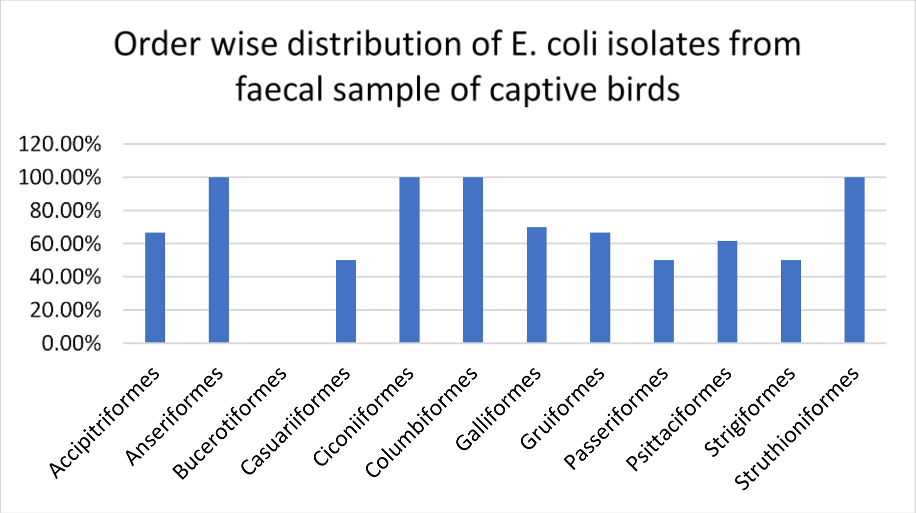


***Figure 8**: Bar diagram showing Order wise distribution of E. coli isolates (%) from faecal sample of captive birds*

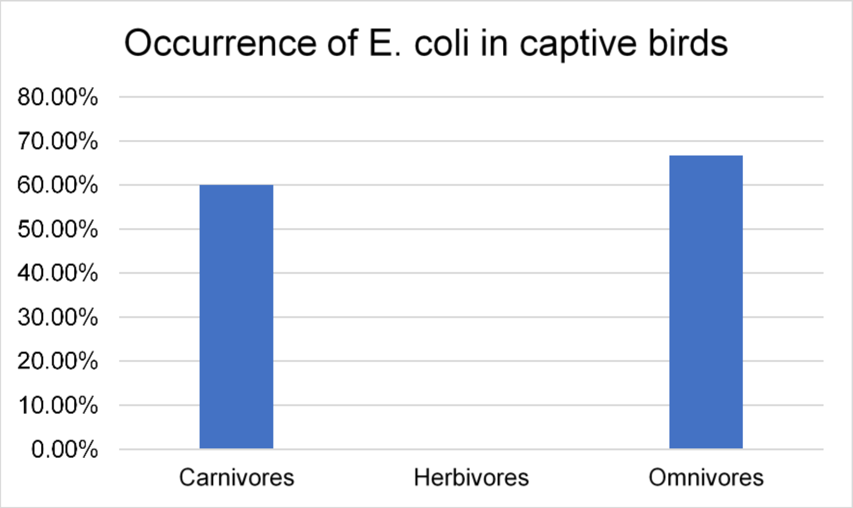


***Figure 9:** Occurrence of E. coli in captive birds*

4.3. *In vitro* Antibiotyping Assay

All the 73 isolates confirmed to be *E. coli* were subjected to antibiotic drug sensitivity as per the guidelines of CLSI (2011) (Figure 12).

4.3.1. Antibiotic sensitivity test and general resistance screening of *E. coli* isolates

The antimicrobial sensitivity pattern of seventy-three *E. coli* isolates from faecal samples of zoo mammals and birds are summarized in Table 17. The degree of susceptibility ranged from 35.62% up to 97.26%. The isolates were highly sensitive to Chloramphenicol and Gentamicin (97.26%) followed by Amikacin (90.41%), Ciprofloxacin (71.23%), Enrofloxacin (72.60%), Amoxicillin-Clavulanic acid (58.90%), Ceftriaxone (50.68%) while the Oxytetracycline showed the least inhibition to the growth (35.62%).

A significant proportion of the ESBL producing strains were found to be resistant to antimicrobial agents. The antibiotic resistance of *E. coli* showed a full range of resistance (19.18% up to 100.00%) for the ten antibiotics (Table 17). The highest rate of resistance was seen against Ampicillin (100%) followed by Cefotaxime (76.71%) and Oxytetracycline (52.05%). Minimum resistance was observed for Chloramphenicol (2.74%) followed by Enrofloxacin (13.70%), Ciprofloxacin (15.07%) and Amoxycillin-Clavulanic acid (19.18%). None of the isolates was found resistant to aminoglycosides (Amikacin and Gentamicin).

It was evident that resistance to antimicrobial agents was generally was high as high as 2.74% to 100.0% for *E. coli* isolates exhibiting resistance to one or more of the antimicrobial agents used. Similar results have also been documented by previous workers (Adesiyun and Downes 1999; Gopee *et al*. 2000; Batalha de Jesus 2018)**.** In both studies, resistance was very high to ampicillin and tetracycline but comparatively low to chloramphenicol and gentamicin, which corroborates the present finding. The relatively high prevalence of resistance to antimicrobial agents amongst captive wildlife could have therapeutic implications due to the fact that transfer of resistance factor is known to be common, particularly amongst enteric bacteria (Anderson 1968; Wierup 1975). Adesiyun and Downes (1999) reported that *E. coli* strains from free-ranging wildlife, especially mammals, exhibited moderate to high resistance to ampicillin, while showing low resistance to chloramphenicol and gentamycin. Adesiyun and Kaminjolo (1992) revealed that chloramphenicol and gentamycin were most effective on *E. coli* isolates from wild animals. Anderson (1968) observed that strains of *E. coli* may have high resistance to tetracycline while moderately susceptible to ampicillin. The findings of present study are in consonance of earlier work.

Antimicrobial resistance is a complex and multifaceted problem involving humans, animals, and the environment. However, the role of wildlife in the emergence of antibacterial resistance might be underestimated. The first report of antibacterial resistance in wildlife revealed chloramphenicol resistance in *E. coli* isolates obtained from Japanese wild birds (Sato et al. 1978). Since then, the occurrence of resistant bacteria in wild animals has been increasingly reported within diverse animal species across different geographical areas (Tsubokura *et al*. 1995; Dobbin *et al*. 2005; Costa *et al*. 2008; Rose *et al*. 2009; Akhter *et al*. 2010; Wasyl *et al*. 2018).

4.3.2. Multi Drug Resistance

The phenotypic resistance profiles of multidrug resistant *E. coli* isolates are summarized in Table 18.

All *E. coli* isolates of this study were screened by disc diffusion test according to the CLSI criteria 1 for phenotypic resistance against important antimicrobial substances from human and veterinary medicine, including third generation cephalosporins, other *beta*-lactams, aminoglycosides, tetracyclines and fluoroquinolones, chloramphenicol and tetracycline. Eleven captive mammals and birds host species were carrying multi drug resistant *E. coli* in their faeces.

Table 17: Antimicrobial susceptibility (%) of *E. coli* isolates obtained from captive mammals and birds

Sl. no.	**Class of antibiotic**	**Antibiotic drug**	**Sensitive**	**%**	**Intermediate**	**%**	**Resistant**	**%**
1.	**Amphenicols**	**Chloramphenicol**	71	97.26	-	-	02	2.74
2.	**Aminoglycosides**	**Amikacin**	66	90.41	07	9.59	-	-
		Gentamicin	71	97.26	02	2.74	-	-
3.	**β-lactam antibiotic**	**Ampicillin**	-	-	-	-	73	100
		Amoxicillin-Clavulanic acid	43	58.90	16	21.92	14	19.18
		Cefotaxime	06	8.21	11	15.07	56	76.71
		Ceftriaxone	37	50.68	08	10.96	28	38.36
4.	**Quinolones**	**Ciprofloxacin**	52	71.23	10	13.70	11	15.07
		Enrofloxacin	53	72.60	10	13.70	10	13.70
5.	**Tetracycline**	**Oxytetracycline**	26	35.62	09	12.33	38	52.05

Number of isolates = 73

Antibiotics included were: gentamycin, amikacin, amoxicillin + clavulanic acid, ampicillin, cefotaxime, chloramphenicol, ceftriaxone, ciprofloxacin, enrofloxacin, oxytetracycline

Table 18. Details of mammals/birds having MDR *E coli*

Sl. no.	Mammal/Bird spp.	No. of *E. coli* isolate	*E. coli* isolate resistant to no. of antibiotics	Resistant antibiotics (code)
1.	Painted Stork	1	5	AMP, AMC, O, CIP, EX
2.	Budgerigar	1	6	CTX , AMP , AMC , O , CIP , EX
3.	Crane	1	6	CTR , CTX , AMP , AMC , O , CIP
4.	Eagle	1	6	CTX , AMP , AMC , O , CIP , EX
5.	Elephant	1	6	CTX , AMP , AMC , O , CIP , EX
6.	Asiatic Lion	1	7	CTR , CTX , AMP , AMC , O , CIP , EX
7.	Chimpanzee	1	7	CTR , CTX , AMP , AMC , O , CIP , EX
8.	Chinkara Deer	1	7	CTR , CTX , AMP , AMC , O , CIP , EX
9.	Jackal	1	7	CTR , CTX , AMP , AMC , O , CIP , EX
10.	Leopard	1	7	CTR , CTX , AMP , AMC , O , CIP , EX
11.	Hybrid lioness	1	8	CTR , CTX , AMP , AMC , C , O , CIP , EX

Abbreviation:

CTR = Ceftriaxone, CTX = Cefotaxime, AMP = Ampicillin, AMC = Amoxicillin + clavulanic acid, O = Oxytetracycline, CIP = Ciprofloxacin, EX = Enrofloxacin, C = Chloramphenicol

Table 19: Order wise occurrence of MDR *E. coli* isolated from captive mammals

Sl. no.	Order of animal	Positive Number of *E. coli*	Number of MDR *E. coli*	Percentage of MDR *E. coli*
1.	***Carnivora***	22	04	18.18
2.	***Proboscidea***	02	1	50.00
3.	***Perissodactyla***	04	0	00.00
4.	***Artiodactyla***	11	1	9.09
5.	***Primate***	05	1	20.00
6.	***Rodentia***	02	0	0.00
	Total	**46**	**7**	**15.23**

Table 20: Order wise occurrence of MDR *E. coli* isolated from captive birds

Sl. no.	Order of bird	Number of *E. coli* isolates	Number of MDR *E. coli*	Percentage of MDR *E. coli*
1.	*Accipitriformes*	2	1	50.00
2.	*Anseriformes*	2	0	00.00
3.	*Bucerotiformes*	0	0	0.00
4.	*Casuariiformes*	1	0	00.00
5.	*Ciconiiformes*	1	1	100.00
6.	*Columbiformes*	1	0	0.00
7.	*Galliformes*	7	0	00.00
8.	*Gruiformes*	2	1	50.00
9.	*Passeriformes*	1	0	00.00
10.	*Psittaciformes*	8	1	12.50
11.	*Strigiformes*	1	0	00.00
12.	*Struthioniformes*	1	0	0.00
	Total	**27**	**4**	**11.11**

Table 21: Occurrence of MDR *E. coli* in Captive mammals

Sl. no.	Class of mammal	Total no. of *E. coli* isolate	Number of MDR positive *E. coli*	Occurrence of MDR *E. coli* (%)
1.	**Carnivores**	18	4	22.22
2.	**Herbivores**	19	2	10.52
3.	**Omnivores**	09	1	11.11
	Total	**46**	**07**	**15.21**

Table 22: Occurrence of MDR *E. coli* in Captive Birds

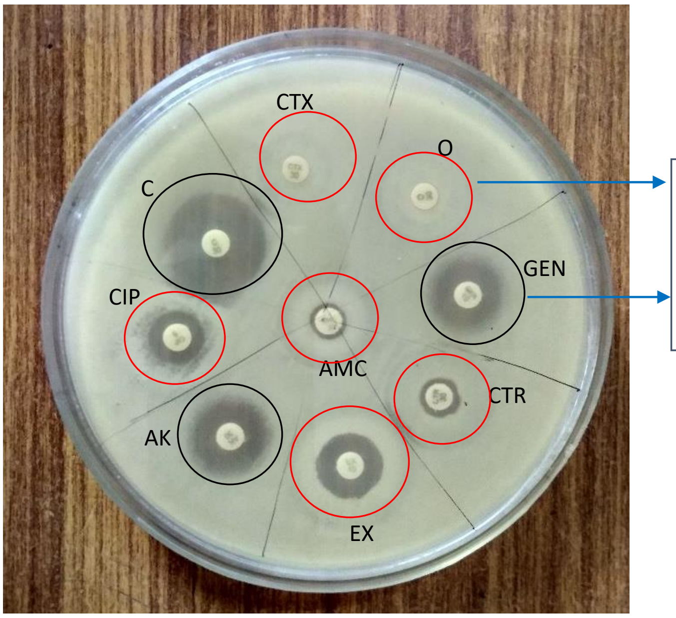
Sl. no.	Class of bird	Total no. of *E. coli* isolate	Number of MDR positive *E. coli*	Prevalence of MDR *E. coli* (%)
1.	**Carnivores**	03	01	33.33
2.	**Herbivores**	-	-	-
3.	**Omnivores**	24	03	12.50
	Total	**27**	**04**	**14.81**

Table 23: Occurrence of MDR *E. coli* in all Captive mammals and birds

Sl. no.	Category	Total no. of *E. coli* isolate	Number of MDR positive *E. coli*	Prevalence of MDR *E. coli* (%)
1.	**Carnivores**	21	05	23.80
2.	**Herbivores**	19	02	10.52
3.	**Omnivores**	33	04	9.90
	Total	**73**	**11**	**15.06**

Zone of Inhibition

Figure 12: *Representative Mueller Hinton agar plates showing antibiotic susceptibility test (Kirby Bauer disc diffusion method) depicting different Zone of Inhibition*



Red circle: Resistant zone

Black circle: Sensitive zone

Figure 13: *Antibiotic resistance pattern of E. coli.*

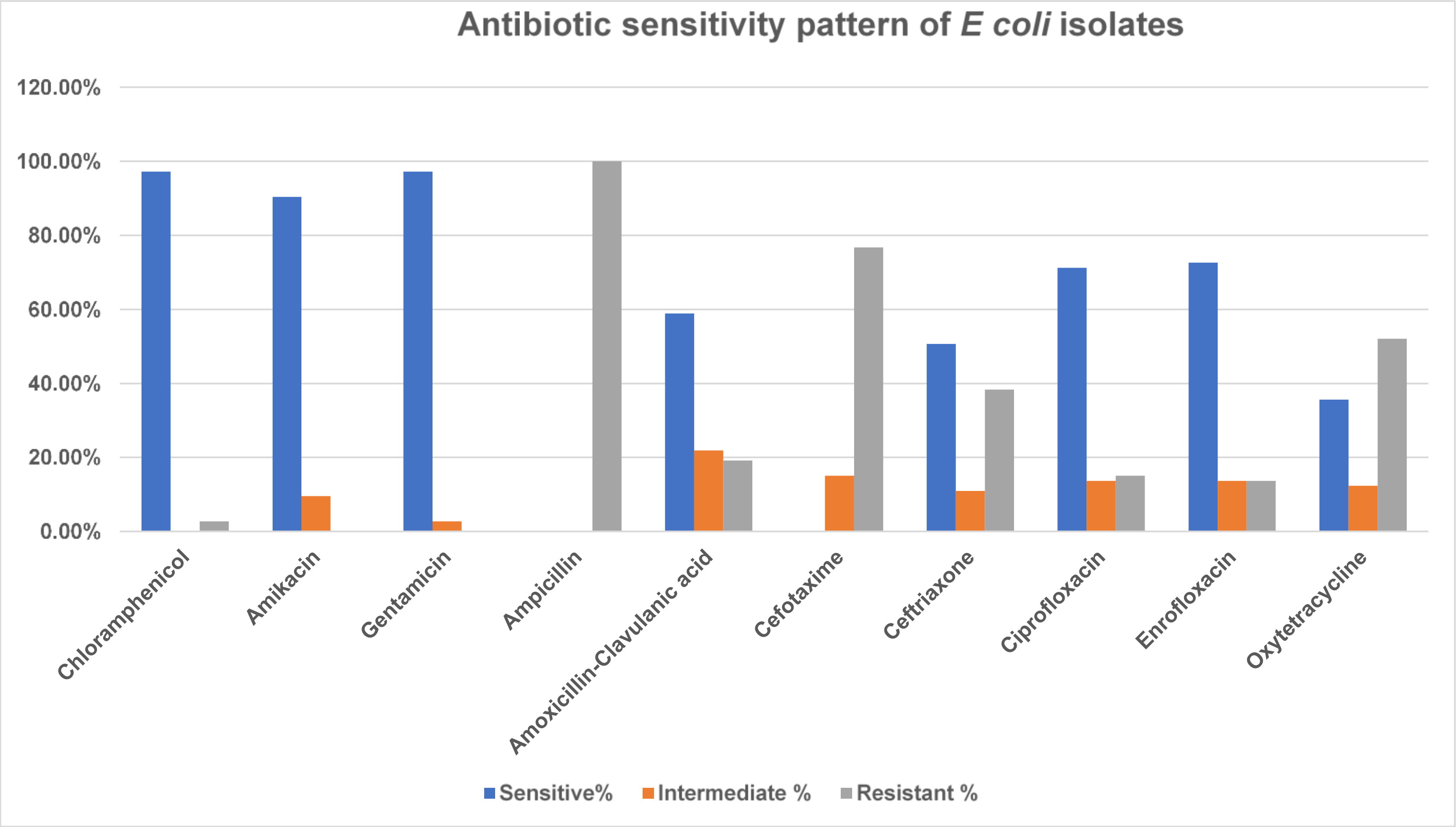


Figure 14: *Bar diagram showing susceptibility pattern (%) of E. coli isolates towards different class of antibiotics.*

The *E. coli* isolates from the captive mammals and birds were having resistant *E. coli* to at least three antibiotics. Resistance to three or more antimicrobial agents was found in approximately 15.07% of the isolates. The most widespread resistance phenotypes were those resistant to Ampicillin (100%), Cefotaxime (76.71%) and Oxytetracycline 52.05% as depicted in Table 17.

Furthermore, among the 10 antimicrobial drugs tested, against *E. coli* isolates and multiple drug resistance ranged from five (01 isolates) up to even eight (one isolate) antibiotic compounds. Four of them displayed resistance to 6 antibiotics, 5 showed resistance to 7 antibiotics, one revealed resistance to 5 antibiotics. One isolate showed resistance to 8 antibiotics (Table 18).

The perusal of Table 17 revealed that most of the isolates were highly resistant to amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, ciprofloxacin, enrofloxacin and oxytetracycline. The entire isolated organisms in this study were 100% sensitive to amikacin and gentamycin.

4.3.2.1. Multi Drug Resistance *E coli* isolates from mammals

The order wise MDR *E. coli* among captive mammal has been summarized in Table 19. The highest prevalence was recorded in *Proboscidea* (50.00%) followed by *Primate* (20.00%), *Carnivora* (18.18%), *Artiodactyla* (9.09%) (25.00%). No MDR *E. coli* was found in the faecal sample of *Perissodactyla* and *Rodentia* (0.00%). The overall occurrence of *E. coli* was 15.23%.

The perusal of the data from Table 21 revealed that the occurrence of MDR *E coli* in the mammal category was highest in carnivores and omnivores faeces (22.22%) followed by omnivore (11.11%) and herbivores (10.52%).

4.3.2.2. Multi Drug Resistance *E coli* isolates from birds

Further, the perusal of Table 20 revealed order wise trend of prevalence of *E. coli* in the faecal sample of *Accipitriformes* (50.00%), *Ciconiiformes* (100.00%), *Psittaciformes* (12.50%). The orders: *Anseriformes*, *Bucerotiformes*, *Casuariiformes*, *Columbiformes*, *Galliformes*, *Gruiformes*, *Passeriformes*, *Strigiformes* and *Struthioniformes* did not harbour MDR *E. coli* (0.00%). The overall occurrence in the bird category was found at 11.11%.

The frequency MDR *E. coli* isolates based on bird's feeding habit has been mentioned in Table 22. The perusal of the table revealed that carnivores (33.33%) and omnivores (12.50%) were possessing MDR *E. coli*. There was no herbivorous bird under this study.

4.3.2.3. Multi Drug Resistance *E coli* isolates from mammals and birds

The cumulative occurrence of MDR *E. coli* among mammals and birds have been shown in Table 23. The highest in carnivores (23.80%) followed by herbivores (10.52%) and the lowest proportion of resistances in omnivores (9.90%).

Antimicrobial resistance is a complex and multifaceted problem involving humans, animals, and the environment. However, the role of wildlife in the emergence of antibacterial resistance might be underestimated. The first report of antibacterial resistance in wildlife revealed chloramphenicol resistance in *E. coli* isolates obtained from Japanese wild birds (Sato *et al*. 1978). Since then, the occurrence of resistant bacteria in wild animals has been increasingly reported within diverse animal species across different geographical areas (Tsubokura *et al*. 1995; Dobbin *et al*. 2005; Costa *et al*. 2008; Rose *et al*. 2009; Akhter *et al*. 2010; Wasyl *et al*. 2018).

4.3 Detection of ESBL producing *E. coli* in captive mammals and birds

The *in vitro* test were conducted to detect ESBL harbouring *E. coli* by Hexa G-Minus 24 kit (HiMedia, India) containing set of six antibiotic discs (Cefpodoxime, Cefpodoxime & clavulanic acid, Ceftazidime, Ceftazidime & clavulanic acid , Cefotaxime Cefotaxime & clavulanic acid) and double disc diffusion test disc method employing two discs cefotaxime (CTX) and cefotaxime-clavulanic acid (CEC). A difference of ≥ 5 mm between the zone diameters of cefotaxime and cefotaxime-clavulanic acid discs was considered as phenotypic confirmation of ESBL producing *E. coli*.

The frequency of ESBL producing *E coli* in faecal samples has been presented in Table 24 & 25. In overall prevalence, out of 73 selected *E. coli*, 54 (73.79%) isolates were suspected as ESBL producers and 11 (15.07%) were MDR.

4.4.1. Detection of ESBL producing *E coli* isolates from captive mammals

The result of ESBL producing *E. coli* from the faecal sample of mammals is shown in Table 24. Out of 73 isolates, the ESBL producing isolates from the faecal sample of mammals were 33 (76.74%). The perusal of data from Table 26, it was deduced that by phenotypic confirmation of ESBL producing *E. coli* by *Hexa* G minus 24 kit, the Orders: *Carnivora* attributed 13 (59.09%) ESBL *E. coli* isolates, *Proboscidea* 01(50.00%), *Perissodactyla* 04 (100.00%), *Artiodactyla* 09 (81.82%), *Primate* 04 (80.00%) and *Rodentia* 02 (100.00%). The phenotypic confirmation of ESBL producing *E. coli* by Combination Disc Method showed the Order-wise occurrence of ESBL *E. coli* among captive mammals that *Carnivora*, *Proboscidea*, and *Rodentia* had an isolation frequency of 50% for *E. coli* compared with *Perissodactyla* (75.00%) *Artiodactyla* (72.72%) and *Primate* (60.00%). The overall occurrence of ESBL producing *E. coli* in mammals was 71.74% and 56.82%. The Perusal of Table 28 revealed that herbivorous mammals under this study had the higher percentage (84.21%) of ESBL producing *E coli* detected by Hexa G-Minus 24 kit than by combination of disc method (63.15 %), followed by carnivorous (66.67% vs. 61.11%) and omnivores (55.56 vs. 33.33%).

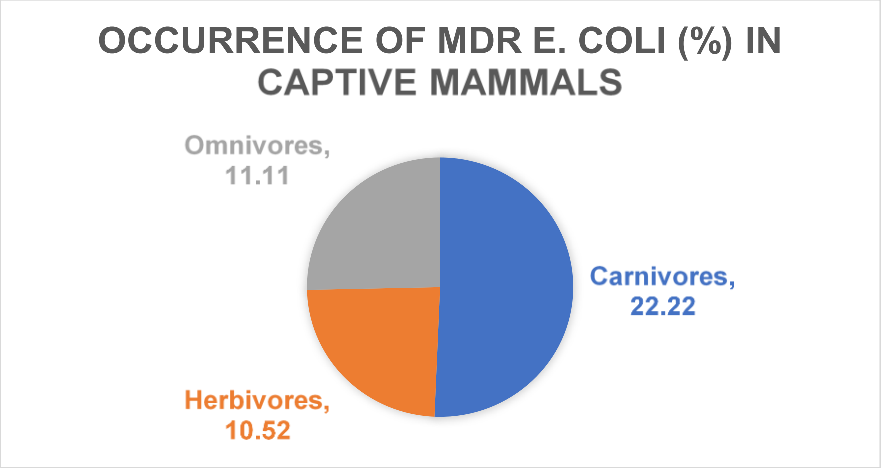


Figure 15: *Occurrence of MDR E. coli in captive mammals*

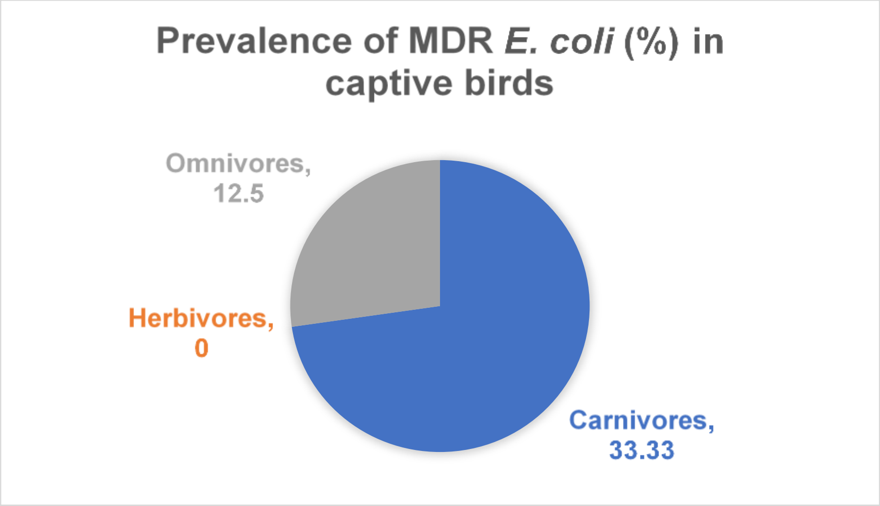


Figure 16: *Occurrence of MDR E. coli in captive birds*

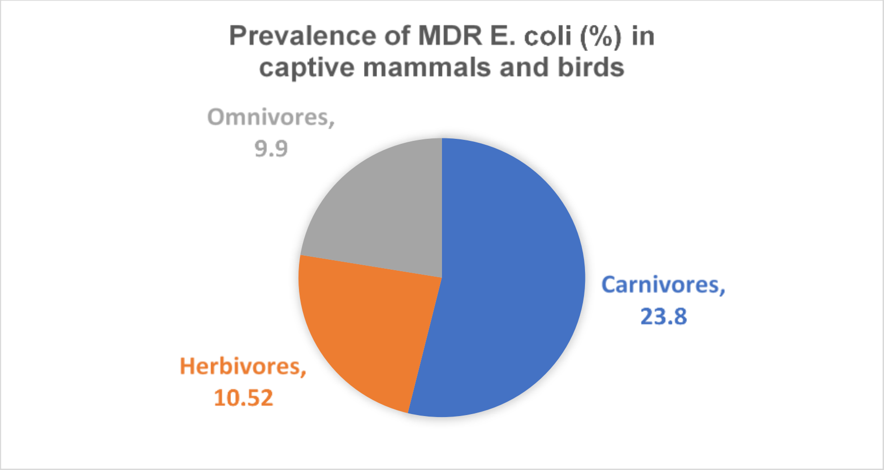


Figure 17: *Occurrence of MDR E. coli in captive mammals and birds*

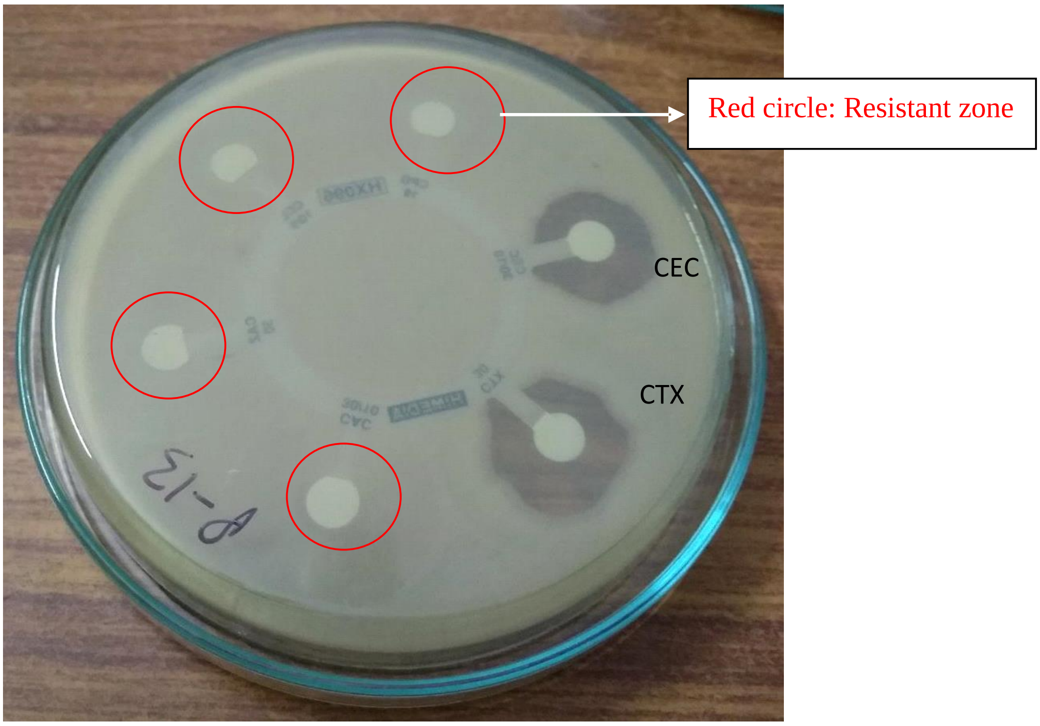


__Figure 18:__ Confirmation of ESBL producing E. coli by Hexa G-minus 24 kit for ESBL production.

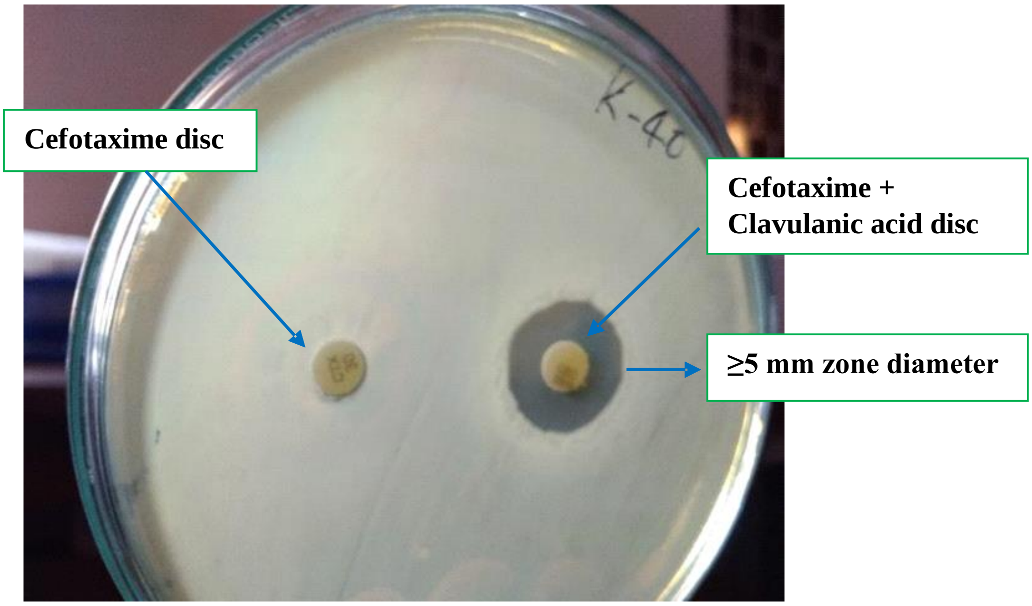


__Figure 19:__ Confirmation of ESBL producing E. coli by Combination Disc Method

4.4.2. ESBL producing *E coli* isolates from birds

In the present study, Hexa G-Minus 24 kit had detected 21 (95.54%) isolates from faecal samples of captive birds, which was higher than detected by Combination disc method (86.36%). Hexa G-Minus 24 kit found to be more sensitive than Combination disc method in our study (Table 25).

The ESBL producing *E. coli* based on bird's feeding habit has been mentioned in Table 17, Table 18 and Table 19. The perusal of the table revealed that carnivores (67.67 vs. 67.67%) and omnivores (79.17% vs. 33.33%) were possessing ESBL *E. coli* confirmed by both methods of detection.

Further, the perusal of the Table revealed trend of order wise occurrence of ESBL *E. coli* in *Accipitriformes* (100.00% & 50.00%), *Anseriformes* (100.00% & 50.00%), *Bucerotiformes* (0.00% by both method), *Casuariiformes* (100.00% & 0.00%), *Ciconiiformes* (100.00% & 0.00%), *Columbiformes* (0.00%), *Galliformes* (100.00% & 0.00%), *Gruiformes* (100.00% & 100.00%), *Passeriformes* (100.00% & 0.00%), *Psittaciformes* (71.42% & 42.86%), *Strigiformes* (0.00% & 100.00%), *Struthioniformes* (0.00%). The overall occurrence in bird category was found (77.78% & 37.03%**)**

The overall result in the case of mammals and birds showed that the ESBL producers were 73.9% and 61.74% by Hexa disc and combination disc methods, respectively.

4.4.3. ESBL producing *E coli* isolates from mammals and birds

Hexa G-Minus 24 kit detected ESBL in 54 (73.90%) isolates whereas 45 (61.64%) isolates were detected by Combination disc method, and 9 (12.32%) isolates were missed by Combination disc method. Inferentially, the Hexa G-Minus 24 kit is found to be better than Combination disc method in the detection of ESBLs (Table 30)

4.4.4. MDR *E coli* isolates from mammals and birds

In this study, multidrug resistance (≥3 antibiotic classes) pattern was prevalent among the ESBL-producing isolates.

The perusal of Table 28 shows 15.21% of ESBL-producing *E. coli* isolates from a faecal sample of mammals were showed co-resistance to tetracycline, fluoroquinolone classes of antibiotics plus beta-lactam groups of antibiotics. One isolate had additional 3.03% co-resistance to chloramphenicol. Similarly, 14.81% ESBL-producing *E. coli* isolates from the faecal sample of birds were showing co-resistance to tetracycline, fluoroquinolone classes of antibiotics plus beta-lactam groups of antibiotics (Table 29).

Similar studies focused on wild birds such as those of Alcala *et al*. (2016) and Parker *et al*. (2016) reported prevalence rates of 14% and 2.7%, respectively. Velhner *et al*. (2018) detected resistance to fluoroquinolones in five *E. coli* isolates. A multidrug resistant E. coli from a farm deer (*Cervus elaphus*) exhibiting resistance to ciprofloxacin was reported (Alonso *et al*., 2016). Lonacaric *et al*. (2016) detected an ESBL phenotype in *E. coli* isolate.

Focusing on the resistance to antimicrobial agents among our *E. coli* collection, the prevalence to at least one antimicrobial tested was low (6.7%). This percentage is in agreement with previous studies carried out on small terrestrial mammals and wild ruminants (Silva *et al*. 2010; Literak *et al*. 2010), but contrast with the higher levels of antimicrobial resistance reported in domestic (Wieler Literak *et al*. 2011) and food-producing animals (Dierikx *et al*. 2013; Ben Sallem *et al*. 2012). There are also several studies in wild environments reporting high rates of antimicrobial resistance, especially in birds (Pinto *et al*., 2010; Hasan *et al*., 2014) and large mammals (Literak *et al*. 2010; Hasan *et al*. 2014; Poeta *et al*. 2009). These differences in the prevalence of resistance among different hosts may be explained by various factors. The exposition to the selective pressure associated with the extended use of antibiotics in farming and veterinary practice and the direct contact with human seems to be in relation with the increasing prevalence of antimicrobial resistance bacteria among companion and food-producing animals (Agerso *et al*. 2013; Durso *et al*. 2014).

Wild environments are normally not exposed to clinically used antimicrobial agents but there is also number of possible exposure routes that might select for resistance (Martinez 2009). There is evidence suggesting that migratory birds can also play an important role in the spread of antibiotic resistance (Pinto *et al*. 2010; Hasan *et al*. 2014). In addition, some host factors such as diet can affect the dynamics of the microbiota and therefore, the prevalence of resistant bacteria among gut commensal bacteria (Williams *et al*. 2011). The herbivore diet of most of the animals involved in the present study might explain in part the low level of antimicrobial resistance detected among faecal *E. coli* isolates. This result is in accordance with previous studies carried on in different animals (Silva *et al*. 2010; Costa *et al*. 2008, Radhouani *et al*. 2013). Among quinolone resistant bacteria, isolates were resistant to both enrofloxacin and ciprofloxacin.

Another important aspect to highlight is that, even though most of the *E. coli* isolated in this study was susceptible to the tested antimicrobials, a multiresistant ESBL strain was also obtained from a deer faecal sample. The prevalence of ESBL-producing *E. coli* strains in this study can be considered low in comparison with other wild mammals, such as foxes or wild boars, where an ESBL-producing prevalence of 4% (Radhouani *et al.* 2013) and 10.4% (Poeta *et al.* 2009) have been reported, respectively. This might be explained by the fact that at the top of the food chain and can accumulate multi resistant bacteria derived from their diet (Radhouani *et al.* 2013).

Velhner *et al*. (2018) detected resistance to fluoroquinolones in five *E. coli* isolates. A multidrug resistant *E. coli* from a farm deer (*Cervus elaphus*) exhibiting resistance to ciprofloxacin was reported (Alonso *et al.* 2016). Lonacaric *et al.* (2016) detected an ESBL phenotype in *E. coli* isolate.

Table 24. Phenotypic confirmation of prevalence of ESBL-producing *E. coli* isolates over the species distribution among captive mammals

Sl. no	Species	No. of *E. coli* isolates	Number of MDR *E. coli*	ESBL *E. coli* confirmed by Hexa G minus 24		ESBL *E. coli* confirmed by Combination Disk Diffusion test	
				No.	**%**	**No.**	**%**
1.	Himalayan Black Bear	3	-	01	33.33	01	33.33
2.	Bengal Tiger	4	-	02	50.00	01	25.00
3.	White Tiger	2	-	01	50.00	01	50.00
4.	Asiatic Lion	6	1	04	66.67	03	50.00
5.	Hybrid Lioness	1	1	01	100.00	01	100.00
6.	Leopard	2	1	01	50.00	01	50.00
7.	Jackal	1	1	01	100.00	01	100.00
8.	Hyena	1	-	01	100.00	01	100.00
9.	Wolf	1	-	01	100.00	01	100.00
10.	Elephant	2	1	01	50.00	01	50.00
11.	One horned Rhinoceros	2	-	02	100.00	02	100.00
12.	Zebra	2	-	02	100.00	01	50.00
13.	Giraffe	1	-	01	100.00	01	100.00
14.	Indian Gaur	1	-	01	100.00	01	100.00
15.	Sangai Deer	1	-	01	100.00	01	100.00
16.	Chinkara Deer	1	1	01	100.00	01	100.00
17.	Black Buck	2	-	02	100.00	01	50.00
18.	Hog Deer	1	-	01	100.00	00	0.00
19.	Swamp Deer	1	-	01	100.00	01	100.00
20.	Sambar Deer	1	-	01	100.00	01	100.00
21.	Rhesus Monkey	1	-	01	100.00	01	100.00
22.	Chimpanzee	2	1	02	100.00	01	50.00
23.	Langur	2	-	01	50.00	01	50.00
24.	Porcupine	2	-	02	100.00	01	50.00
Total		**43**	**07**	**33**	**76.74**	**26**	**60.47**

Table 25. Phenotypic confirmation of prevalence of ESBL-producing *E. coli* isolates among captive birds

Sl. no	Species	No. of *E. coli* isolates	ESBL producing MDR *E. coli*	ESBL *E. coli* confirmed by Hexa G minus 24		ESBL *E. coli* confirmed by Combination Disk Diffusion test	
				No.	%	No.	%
1.	Budgerigar	2	1	02	100.00	01	50.00
2.	Emu	1	-	01	100.00	01	100.00
3.	Crane	2	1	02	100.00	02	100.00
4.	Swan	1	-	01	100.00	01	100.00
5.	Duck	1	-	01	100.00	01	100.00
6.	Lady Amherst's Pheasant	1	-	01	100.00	01	100.00
7.	Silver pheasant	2	-	02	100.00	01	50.00
8.	Kalij pheasant	1	-	01	100.00	01	100.00
9.	Golden pheasant	1	-	01	100.00	01	100.00
10.	Parrot	2	-	01	50.00	01	50.00
11.	Alexandrine Parakeet	1	-	01	100.00	01	100.00
12.	Peacock	1	-	01	100.00	01	100.00
13.	Pheasant grey peacock	1	-	01	100.00	01	100.00
14.	Painted stork	1	1	01	100.00	01	100.00
15.	Love bird	1	-	01	100.00	01	100.00
16.	Myna hill	1	-	01	100.00	01	100.00
17.	Vulture	1	-	01	100.00	01	100.00
18.	Eagle	1	1	01	100.00	01	100.00
Total		**22**	**6**	**21**	**95.45**	**19**	**86.36**

Table 26: Overall result of captive mammals

Sl.no.	Order	No. (%) of positive *E. coli*	ESBL producing MDR *E. coli*	No. (%) of ESBL Producing *E. coli* by *Hexa* G minus 24 kit	No. (%) of ESBL Producing *E. coli* by Combination Disc Method
1	***Carnivora***	22 (81.48%)	4 (18.18%)	13 (59.09%)	11 (50.00%)
2	***Proboscidea***	02 (100%)	1 (50.00%)	01(50.00%)	01 (50.00%)
3	***Perissodactyla***	04 (100%)	-	04 (100.00%)	03 (75.00%)
4	***Artiodactyla***	11 (91.67%)	1 (9.09%)	09 (81.82)	08 (72.72%)
5	***Primate***	05 (83.33%)	1 (20.00%)	04 (80.00%)	03 (60.00%)
6	***Rodentia***	02 (100%)	-	02 (100.00%)	01 (50.00%)
Total		**46 (86.79%)**	**7(15.21%)**	**33 (71.74%)**	**26 (56.82%)**

Table 27: Overall result of captive birds

Sl. no.	Order	No. (%) of positive *E. coli*	ESBL producing MDR *E. coli*	No. (%) ESBL *E. coli* by by *Hexa* G minus 24 kit	No. (%) ESBL *E. coli* by combination disc method
1	***Accipitriformes***	2 (66.67%)	1(50.00%)	2(100.00%)	2 (100.00%)
2	***Anseriformes***	2 (100.00%)	-	2 (100.00%)	2 (100.00%)
3	***Bucerotiformes***	0 (0.00%)	-	0 (00.00%)	0 (00.00%)
4	***Casuariiformes***	1 (50.00%	-	1 (100.00%)	1 (100.00%)
5	***Ciconiiformes***	1 (100.00%)	1(100.00%)	1 (100.00%)	1 (100.00%)
6	***Columbiformes***	1 (100.00%)	-	0 (00.00%)	0 (00.00%)
7	***Galliformes***	7 (70.00%)	-	7 (100.00%)	2 (28.57%)
8	***Gruiformes***	2 (66.67%)	1(50.00%)	2 (100.00%)	2(100.00%)
9	***Passeriformes***	1 (50.00%)	-	1 (100.00%)	0 (00.00%)
10	***Psittaciformes***	7 (53.85%)	1 (14.28%)	5 (71.42%)	3 (42.86%)
11	***Strigiformes***	1 (50.00%)	-	0 (00.00%)	1 (100.00%)
12	***Struthioniformes***	1 (100.00%)	-	0 (00.00%)	0 (00.00%)
	Total	**27 (65.85%)**	**4 (14.81%)**	**21 (77.78%)**	**19 (70.37%)**

Table 28: Overall result of captive mammals based on their feeding habit

Sl. no.	Category	Total no. of *E. coli* isolates	MDR *E. coli*	No. (%) of ESBL Producing *E. coli* by *Hexa* G minus 24 kit	No. (%) of ESBL Producing *E. coli* by Combination Disc Method
1.	**Carnivores**	18	4 (22.22%)	12 (66.67%)	11 (61.11%)
2.	**Herbivores**	19	2 (10.52%)	16 (84.21%)	12 (63.15%)
3.	**Omnivores**	09	1 (11.11%)	05 (55.56%)	03 (33.33%)
	Total	**46**	**7(15.21%)**	**33 (71.74%)**	**26 (56.52%)**

Table 29: Overall result of captive birds based on their feeding habit

Sl. no.	Category	Total no. of *E. coli* isolates	MDR *E. coli*	No. (%) of ESBL Producing *E. coli* by *Hexa* G minus 24 kit	No. (%) of ESBL Producing *E. coli* by Combination Disc Method
1.	**Carnivores**	03	1(33.33%)	02 (66.67%)	02 (66.67%)
2.	**Herbivores**	-	-	-	-
3.	**Omnivores**	24	3 (25.00%)	19 (79.16%)	17 (70.83%)
	Total	**27**	**4 (14.81%)**	**21(87.50%)**	**19 (70.37%)**

Table 30: Overall result of all captive mammals and birds based on their feeding habit

Sl. no.	Category	Total no. of *E. coli* isolates	MDR *E. coli*	No. (%) of ESBL Producing *E. coli* by *Hexa* G minus 24 kit	No. (%) of ESBL Producing *E. coli* by Combination Disc Method
1.	**Carnivores**	21	5 (23.80%)	14 (66.67%)	13 (61.90%)
2.	**Herbivores**	19	2 (10.53%)	16 (84.21%)	12 (63.15%)
3.	**Omnivores**	33	4 (12.12%)	24 (72.72%)	20 (60.60%)
	Total	**73**	**11 (15.06%)**	**54 (73.9%)**	**45 (61.64%)**

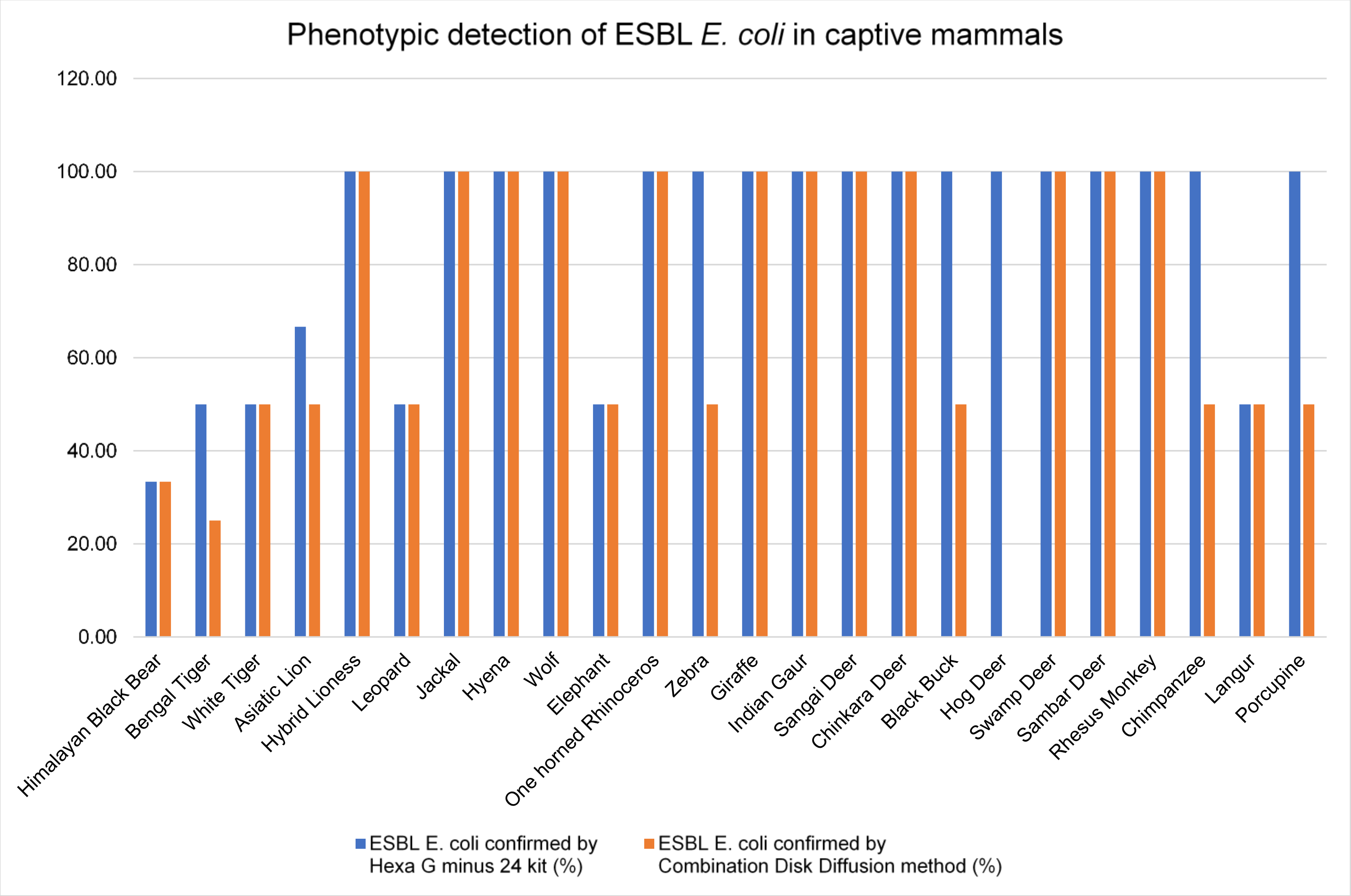


Figure 20: *Percentage of phenotypic detection of ESBL E. coli in captive mammals*

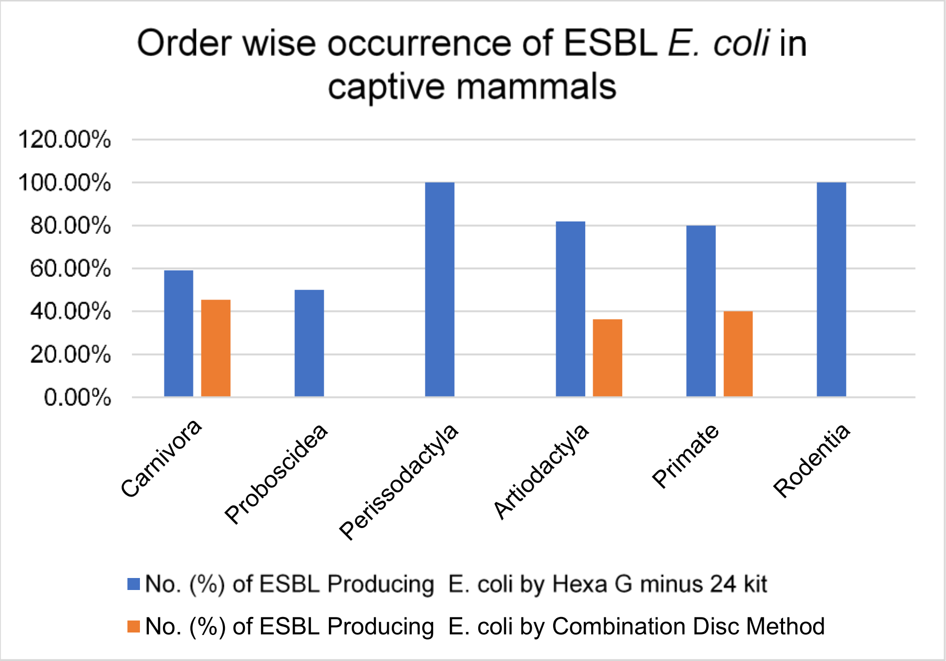


Figure 21: *Order wise occurrence of ESBL E. coli in captive mammals*

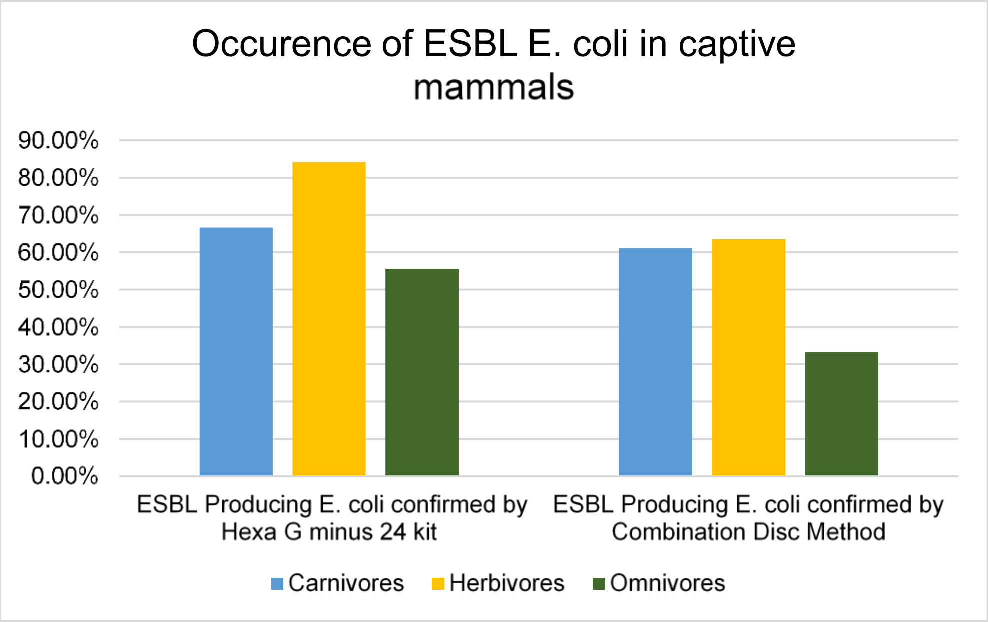


Figure 22: *Occurrence of ESBL E. coli in captive mammals*

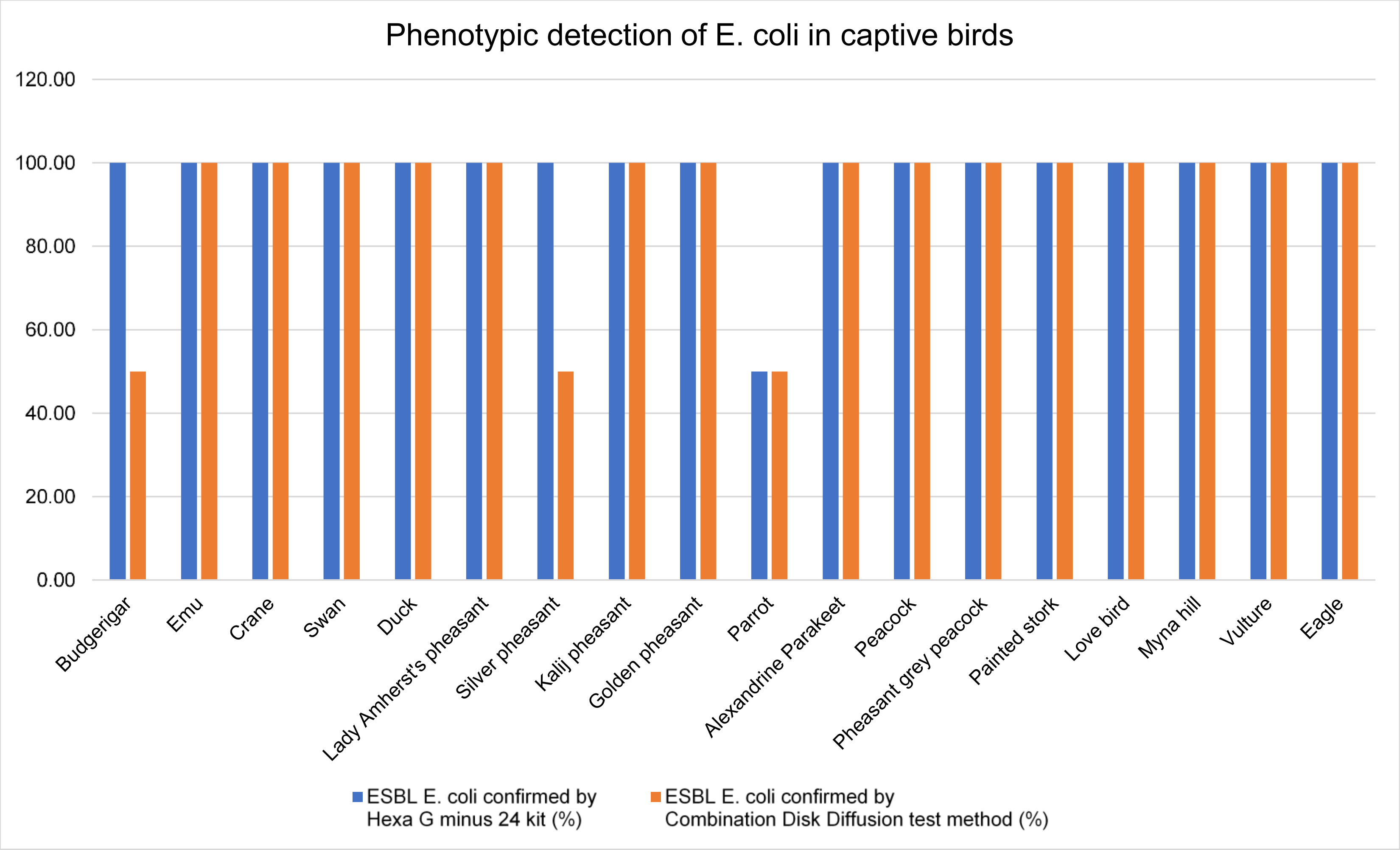


Figure 23: *Percentage of phenotypic detection of ESBL E. coli in captive birds*

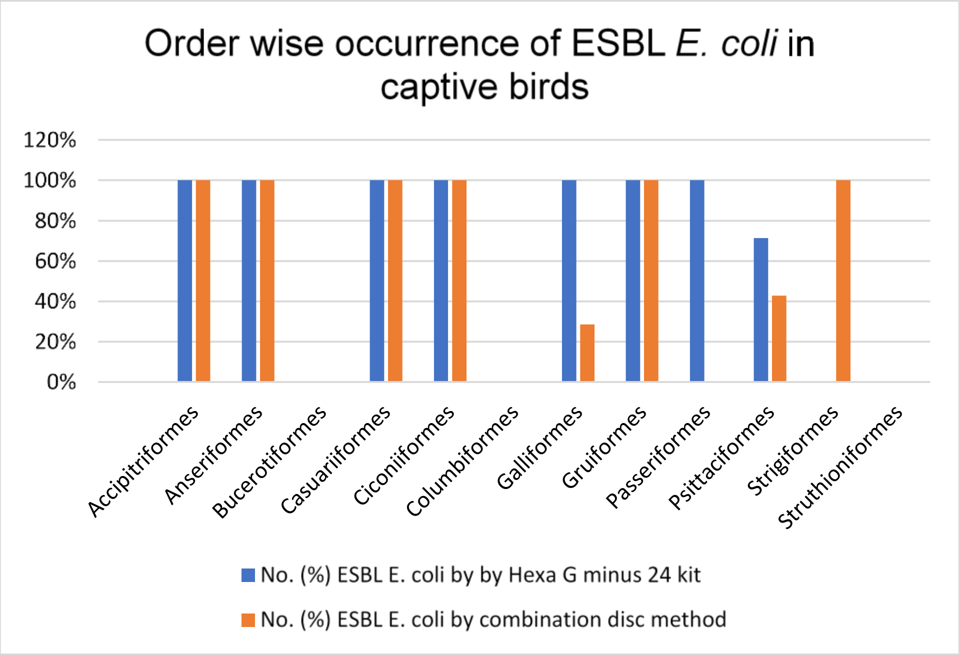


Figure 24: *Order wise occurrence of ESBL E. coli in captive birds*

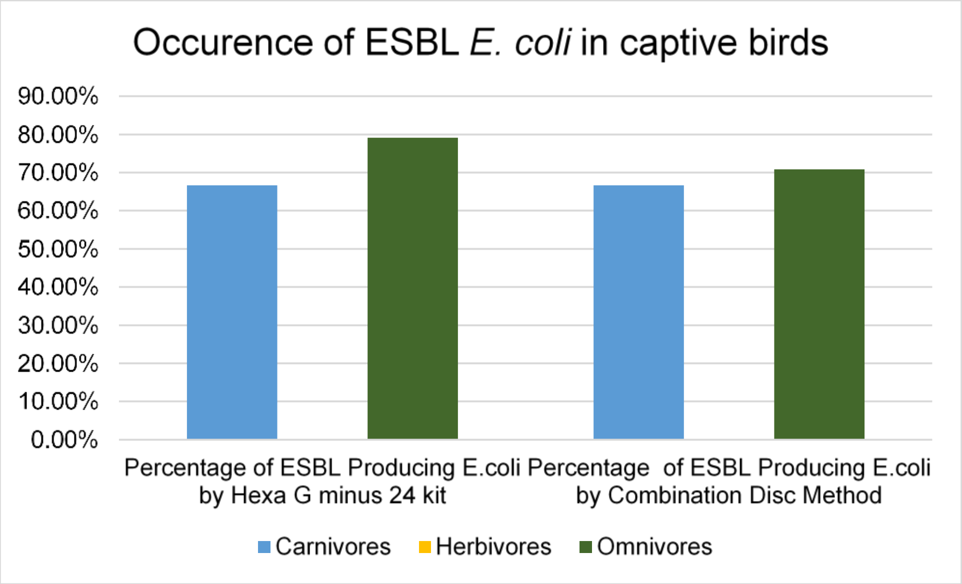


Figure 25: *Occurrence of ESBL E. coli in captive birds*

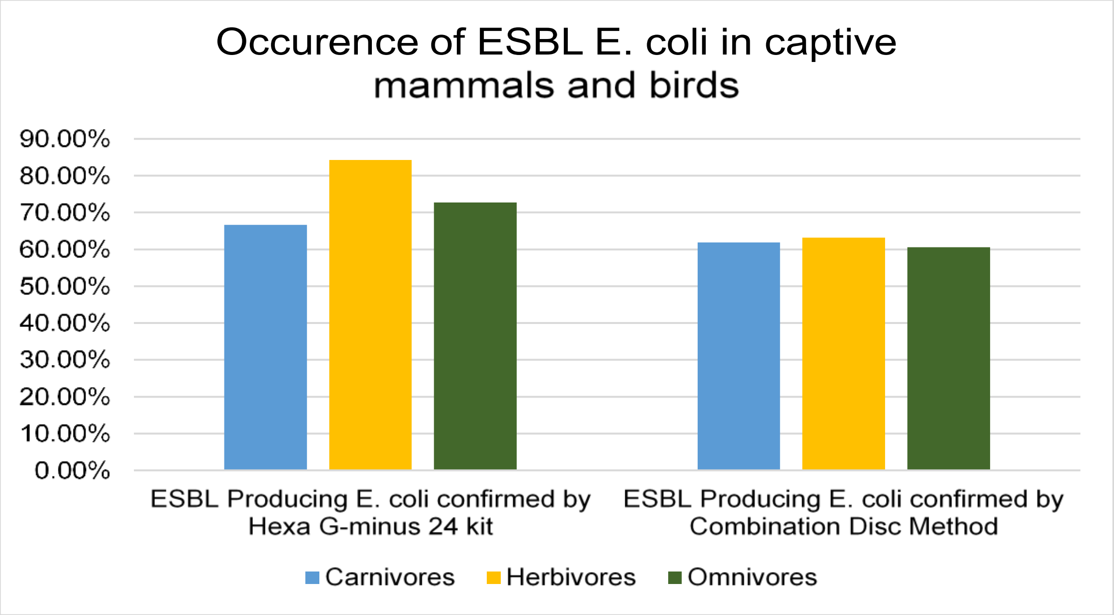


Figure 26: *Occurrence of ESBL E. coli in captive mammals and birds*

Chapter 5

SUMMARY AND CONCLUSIONS

Antibiotic resistance develops when bacteria adapt and grow in the presence of antibiotics. The escalating resistance to third generation cephalosporins, used for therapy of infections caused by members of *Enterobacteriaceae* have become a big threat to both human and animal population. In gram negative pathogens β-lactamases remain the most important contributing factor to β-lactam resistance and the increasing prevalence of ESBLs as well as their alarming evolution seems to be directly linked to the clinical use of novel sub-classes of β- lactams. Extended Spectrum β-Lactamases (ESBLs) enzymes are known to confer resistance to nearly all β-lactam antibiotics. The *Escherichia coli* also produce Extended Spectrum β-Lactamases (ESBLs) enzymes by virtue of which it confers resistance to third generation cephalosporins. Captive wild animals get exposed to human habitat directly or indirectly through environment, water etc. The antimicrobials and the in-contact resistant bacteria may facilitate the existing resistant through the exchange of genetic material between the incoming pathogens and natural inhabitants.

There is paucity of information on drug resistant *E coli* among in wild animals and ESBL producing *E. coli* in India, including Bihar. A baseline epidemiological information has been required to explore the prevalence, antibiotic resistance and ESBL production of *E. coli* in captive mammals and birds. Therefore, the study was conducted based on the prevalence, antibiotic resistance and its patterns among the *E. coli* isolates of captive wild animals and the results are summarized based on the objectives of the study.

In the present study, a total of 94 faecal samples were obtained from the captive wild mammals and birds of The Sanjay Gandhi Biological Park, Patna, Bihar, and Kanpur Zoological Park, Kanpur, U.P. Out of 94 samples 93 (98.94%) were found positive for gram negative bacterial growth.

Amongst, 93 isolates 73 were identified as *Escherichia coli* based on morphological, cultural, biochemical and 16S rRNA based confirmation.

The Order-wise occurrence of *E. coli* among captive mammals that *Proboscidea*, *Perissodactyla*, and *Artiodactyla* had an isolation frequency of 100% for *E. coli* compared with *Carnivora* and *Primate* with 84.62% and 83.33%, respectively. Feeding habit-based occurrence was the highest in mammalian herbivores (95.00%) followed by carnivores and omnivores (81.82%). The overall occurrence of *E. coli* in mammals was 86.79%.

In the case of captive birds, the occurrence of *E. coli* in the faecal sample of omnivores was 66.67% followed by carnivores (60.00%). There was no herbivores bird under this study. The highest percentage of isolate was found in *Anseriformes* (100.00%). The degree of susceptibility against various antibiotics used in this study ranged from 35.62% up to 97.26%. The isolates were highly sensitive to Chloramphenicol and Gentamicin (97.26%) followed by Amikacin (90.41%), Ciprofloxacin (71.23%), Enrofloxacin (72.60%), Amoxicillin-Clavulanic acid (58.90%), Ceftriaxone (50.68%) while the Oxytetracycline showed the least inhibition to the growth (35.62%).

The *E. coli* isolates showed a full range of resistance (19.18% up to 100.00%) for the ten antibiotics used in the study. The highest rate of resistance was seen against Ampicillin (100%) followed by Cefotaxime (76.71%) and Oxytetracycline (52.05%). Minimum resistance was observed for Chloramphenicol (2.74%) followed by Enrofloxacin (13.70%), Ciprofloxacin (15.07%) and Amoxycillin-Clavulanic acid (19.18%). None of the isolates was found resistant to aminoglycosides (Amikacin and Gentamicin).

Furthermore, among the 10 antimicrobial drugs tested, against *E. coli* isolates and multiple drug resistance ranged from five (01 isolates) up to even eight (one isolate) antibiotic compounds. The *E. coli* isolates from the captive mammals and birds were having resistant *E. coli* to at least three class of antibiotics considered as MDR (Multi drug Resistant). Resistance to three or more antimicrobial agents was found in approximately 15.07% of the isolates. Four of them displayed resistance to 6 antibiotics, 5 showed resistance to 7 antibiotics, one revealed resistance to 5 antibiotics. One isolate showed resistance to 8 antibiotics.

Overall occurrence of multi drug resistance *E. coli* isolates from captive mammals was 15.23%. The highest prevalence was recorded in *Proboscidea* (50.00%) followed by *Primate* (20.00%), *Carnivora* (18.18%), *Artiodactyla* (9.09%) (25.00%). No MDR *E. coli* was found in the faecal sample of *Perissodactyla* and *Rodentia* (0.00%).

The Order wise prevalence trend of multidrug resistance *E. coli* in the faecal sample of birds including *Psittaciformes* (12.50%), *Accipitriformes* and *Gruiformes* (50.00%), *Ciconiiformes* (100.00%). The orders: *Anseriformes*, *Bucerotiformes*, *Casuariiformes*, *Columbiformes*, *Galliformes*, *Passeriformes*, *Strigiformes* and *Struthioniformes* did not harbour MDR *E. coli* (0.00%). The overall occurrence in the bird category was found at 11.11%.

The cumulative occurrence of MDR *E. coli* among mammals and birds, highest in carnivores (23.80%) followed by herbivores (10.52%) and the lowest proportion of resistances in omnivores (9.90%).

The frequency of ESBL producing *E coli* in faecal samples, overall prevalence out of 73 selected *E. coli*, 54 (73.79%) isolates were suspected as ESBL producers and 11 (15.07%) were MDR.

Out of 73 isolates, the ESBL producing isolates from the faecal sample of mammals were 33 (76.74%). By phenotypic confirmation of ESBL producing *E. coli* by *Hexa* G minus 24 kit, the Orders: *Carnivora* attributed 13 (59.09%) ESBL *E. coli* isolates, *Proboscidea* 01 (50.00%), *Perissodactyla* 04 (100.00%), *Artiodactyla* 09 (81.82), *Primate* 04 (80.00%) and *Rodentia* 02 (100.00%). The phenotypic confirmation of ESBL producing *E. coli* by Combination Disc Method showed the Order-wise occurrence of ESBL *E. coli* among captive mammals that *Carnivora, Proboscidea*, and *Rodentia* had an isolation frequency of 50% for *E. coli* compared with *Perissodactyla* (75.00%), *Artiodactyla* (72.72%) and *Primate* (60.00%). The overall occurrence of ESBL producing *E. coli* in mammals by both methods was 71.74% and 56.82%. Herbivorous mammals under this study had the higher percentage (84.21%) of ESBL producing *E. coli* detected by Hexa G-Minus 24 kit than by combination of disc method (63.15 %), followed by carnivorous (66.67% vs. 61.11%) and omnivores (55.56 vs. 33.33%).

In the present study, Hexa G-Minus 24 kit had detected 21 (95.54%) isolates from faecal samples of captive birds, which was higher than detected by Combination disc method (86.36%). The ESBL producing *E. coli* based on bird's feeding habit, carnivores (67.67 vs. 67.67%) and omnivores (79.17% vs. 33.33%) were possessing ESBL *E. coli* confirmed by both method of detection. The trend of order wise occurrence *of E. coli* in *Accipitriformes* (100.00% & 50.00%), *Anseriformes* (100.00% & 50.00%), *Bucerotiformes* (0.00% by both method), *Casuariiformes* (100.00% & 0.00%), *Ciconiiformes* (100.00% & 0.00%), *Columbiformes* (0.00%), *Galliformes* (100.00% & 0.00%), *Gruiformes* (100.00% & 100.00%), *Passeriformes* (100.00% & 0.00%), *Psittaciformes* (71.42% & 42.86%), *Strigiformes* (0.00% & 100.00%), *Struthioniformes* (0.00%). The overall occurrence in bird category was found by both methods (77.78% & 37.03%**).** The overall result in the case of mammals and birds showed that the ESBL producers were 73.9% and 61.74% by Hexa disc and combination disc methods, respectively.

Drug resistant *E coli* are prevalent among captive birds and mammals and may become major problem in the area of infectious diseases. Early detection of changing resistance patterns is very important in preventing the dissemination of resistant bacteria and modifying the treatment strategies.

In view of the emerging drug resistance amongst bacteria, therapeutic regimen should only be advocated as far as possible after culture and sensitivity has been performed, hence suggesting that routine diagnosis of ESBL producing *E. coli* strains should be done as this would not only help in the proper treatment of the animal but also prevent further development of bacterial drug resistance. Epidemiologic surveillance and correct use of antimicrobial agents will help prevent the steady increase of antimicrobial drug resistance worldwide. The findings of the study alarms as the treatment of infections involving multiply resistant strains are often difficult.

In line with above facts the following recommendations for future research are forwarded:

- Determination the strain of *E. coli* which are possible causes of infection to both wildlife and humans after considering the zoonotic nature of the organism
- Genotypic characterization of antibiotic resistant genes in captive mammals and birds or in other species of animal kingdom
- Antibiotic resistant in other microorganisms isolated from wildlife fauna
- Comparative analysis of antibiotic resistance in different ecological niches.

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