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Abbreviation and Symbol	Elaboration
%	Percent
et al.	And his associate
CVASU	Chittagong Veterinary and Animal Sciences University

PRTC	Poultry Research and Training Centre
MC	MacConkey agar
EMB	Eosin Methylene Blue agar
PBW	Phosphate Buffer Water
IUCN	International Union for Conservation of Nature
Hrs	Hours
mm	Millimeter

Abstract

The aim of this study was to determine the resistance of different antimicrobial agents to *Escherichia coli* isolated from nonhuman primates at a wildlife-human interface. Bacterial isolates from fecal samples of wild non-human primates at Bangabandhu Sheikh Mujib Safari Park, Cox's Bazar (Dulhazra Safari Park) were screened for the presence of *Escherichia coli*. A total of 13 samples were tested during March, 2017 to May, 2017 at Poultry Research and Training Centre, Chittagong Veterinary and Animal Sciences, where 12 (92.3%) samples were positive for *E. coli* in bacteriological tests. Isolated *E. coli* were tested for resistance with five different antimicrobial agents (Amoxicillin, Ampicillin, Ciprofloxacin, Sulfamethoxazole, Colistin sulphate) which were carried out by the Kirby-Bauer disc diffusion method as per recommendation of CLSI (Clinical and Laboratory Standards Institute) and efficacy of antibiotics was determined by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. The *E. coli* were found 100% resistant to Amoxicillin, Ampicillin, Sulfamethoxazole. Conversely, 100% sensitivity was observed in Colistin sulphate followed by Ciprofloxacin (41.67%). All of the isolates showed multiple antimicrobial resistances in non – human primates may be a continuing threat to the effectiveness of antibiotic therapeutic agents. Therefore, it is important to monitor the occurrence of antimicrobial resistance in wild non-human primates at Bangabandhu Sheikh Mujib Safari Park, Cox's Bazar which is very essential for public health context.

Key words: Non-human primates, *Escherichia coli*, Antibiogram.

Chapter 1: Introduction

Escherichia coli naturally exist as a commensal in the gut of humans and animals where it enjoys synergetic association with other members of the bacterial family *Entebacteriaceae*. It also plays a beneficial role in the prevention of potentially pathogenic organisms in the gut. Recent studies revealed that *E. coli* which form part of the normal intestinal flora of humans and animals are capable of inhibiting the growth of other toxigenic strains of *E. coli* that often associated with food-borne diseases in humans (Nataro and Kaper, 1998). However, some strains of *E. coli* occasionally emerge as pathogens due to the presence of certain pathogenic features and virulence genes, which are located on transmissible genetic elements and these distinguish them from ordinary commensal strains (Ronsengren *et al*., 2009). *Escherichia coli* strains that produce cytotoxic necrotizing factors (CNFs) belong to the pathotype necrotoxigenic E.coli (NTEC) and are associated with intestinal and extraintestinal infections in both humans and animals (Kaper et al., 2004).

The use of antibiotic mostly fluoroquinolones and third generation cephalosporin have considered a common sub therapeutic agent in animals. Such antibiotics are commonly incorporated as animal food and drink supplements to cure and prevent disease. The sub therapeutic use of the antibiotic has generated a selective pressure which leads to emergence of antibiotic resistance bacteria.

Antibiotic resistance in pathogenic bacteria from human, animal and environmental sources is recognized as a global problem in public health. Since it was first ascertained in the early 1980s (Riley and Remis, 1983), the enterohemorrhagic *Escherichia coli* (EHEC) strain is a subset of Shiga toxin-producing *E. coli* strains that have been adjoining with animals and human diseases. In humans including self-limited watery diarrhea, hemorrhagic colitis, and the hemolytic-uremic syndrome (HUS) (Griffin and Tauxe, 1991), this syndrome occurred in 2–7% of people with *E. coli* 0157:H7.This infection causes bloody diarrhea (Easton , 1997), in many areas of the world (Parry and Palmer, 2000, Trevena *et al*., 1996).

Among the EHEC serotypes, O157:H7, which expresses somatic (O) antigen 157 and flagellar (H) antigen 7, causes serious morbidity and large disease outbreaks, making this bacterium one of the most serious food-borne and waterborne pathogens worldwide (Bonetta *et al*., 2011*).* In 1995, an outbreak of *E. coli* O157:H7 infections in people were traced to jerky made from deer meat (Keene *et al*., 1997). The attackable sectors of the community (children and the elderly) are the most chance of developing severe infection, making a very pathetic issue in public health and across the food and agricultural industries (Heuvelink *et al.,* 1998).

Cattle seem to be principal source for verotoxin-producing *E. coli* O157 (Blanco *et al*., 1996, Jackson *et al*., 1998, Johnsen *et al*., 2001). Although it has also been found in sheep, goats, heifer, birds, deer, geese, turkey, seabirds, dogs, cat, gull, chicken, pig, monkey, reptiles, llama, and horses, as well as on flies (Kudva *et al*., 1996, Heuvelink *et al*., 2002, Synge, 2000, Oswald *et al*., 2000, DebRoy Roberts, 2006). However, the extent to which these animal species play a role in the epidemiology of O157 infection remains to be established. Although most infections of O157 in humans have been linked to exposure to a food vehicle or water, person-to-person transmission of O157 and transmission by direct contact with animals or animal manure have also been reported. (Swerdlow et al., 1992)

Zoos and Safari parks visit are popular leisure activities and also has become an important feature of education for children. Such visits are highly advantageous to children in helping them to learn about different aspects of animal husbandry and farm produce. Several outbreaks recorded of *Escherichia coli* O157 infections occurred among agricultural fair, festival, and zoo visitors in farm visits occurred in Pennsylvania, Washington (Cruelly and Baysinger, 2001), Canada, and North Wales (Payne *et al*.,2003). During 2003 to 2004 the capacity of STEC O157 to persist and multiplicative in the farm environment (animal feces, straw, soil, water) (Davies et al., 2005) and their natural occurrence in several wild animal species from which interspecies transmission to domestic animals may occur,(Hancock et al ., 1998) preventing the introduction of the infection, routine testing of brought-in replacement animals, culling infected animals, and closing infected zoos, all do not appear to be feasible or effective control measures. *Escherichia coli* O157:H7 has been detected in the feces of white-tailed Deer (*Odocoileus virginianus*), but the extent of direct or indirect zoonotic risk of this source of *E. coli* O157:H7 has yet to be determined (Sargeant *et al*., 1999). The morbidity and the mortality associated with outbreaks of

gastrointestinal illnesses caused by STEC have highlighted the threat they pose to public health. Therefore, monitoring the presence of *E. coli* in animals will assure promote diagnosis and identify the source of infection that may assist in risk management.

Epidemiological investigation of *E.coli* in animal populations has focused mainly on the bovine reservoir, whether the prevalence in monkeys is not well known. The aim of this study was to determine the prevalence of *E. coli* in fecal samples collected from monkeys at the Bangabandhu Sheikh Mujib Safari Park, Cox's Bazar (Dulhazra Safari Park) in Bangladesh.

Seven species (14 subspecies) of macaques are known in South Asia (Molur *et al.*) and five of them are found in Bangladesh. *Macaca nemestrina*, *M. fascicularis*,*M. arctoides*, and *M. assamensis* occur only in the north-easternand south eastern hill areas. The rhesus macaques are distributed throughout the country. The rhesus macaque (*Macaca mulatta*) is one of the best-known species of Old World monkeys. It is listed as Least Concern in the IUCN Red List of Threatened Species in view of its wide distribution, presumed large population, and its tolerance of a broad range of habitats. Native to South, Central, and Southeast Asia, rhesus macaque troops inhabit a great variety of habitats, from grasslands to arid and forested areas, but also close to human settlements (Timmins et al., 2008).

Rhesus macaque populations in Bangladesh can be divided into two major categories:1) those living close to human settlements (generally known as urban monkeys); and 2) those living in forested habitats. A total 1528 into 37 groups of rhesus macaques were identified among 16 urban populations, besides a total 5313 into 176 groups were identified in natural habitat. Among these groups 49 were identified in northeastern, (Satchari, WBFR, Rema-Kalenga, Adampur, Borolekha, Juri, Harinchara, Khadimnagar and Tea gardens) 68 in southeastern, (Sitakunda, Hazarikhil, Fashiakhali, Himchari, Kaptai, Rangamati, Bandarban and Khagrachari Hill Tracts) 18 in central region (Bhawal and Madhupur deciduous forest) and 41 in Sundarbans (Southwestern). (Kamrul et al., 2013). My study area, Bangabandhu Sheikh Mujib Safari Park (Dulhazra Safari Park) is located at southeast part of Bangladesh.

Currently, there is little information regarding the molecular basis of antibiotic resistance in *E. coli* isolated from the free range monkeys, and therefore this study was carried out to screen and analyze the antimicrobial resistance in *E. coli* isolated from a free range of Rhesus monkeys at Bangabandhu Sheikh Mujib Safari Park, Cox's Bazar (Dulhazra Safari Park) in Bangladesh.

OBJECTIVES

1. To isolate and identify *E. coli* from fecal samples of free range Rhesus monkey at Safari Parks.
2. To determine antibiotic resistance pattern of *E. coli* isolated from Rhesus monkey.

Chapter 2: Methods and Materials

2.1: Study area and animals:

A cross-sectional survey was undertaken by collecting feces from free range monkeys (Figure 1) (Indian Rhesus monkey, *Macaca mulatta*) of Bangabandhu Sheikh Mujib Safari Park, Cox's Bazar (Dulhazra Safari Park) of different ages and sexes during 8th March to 11th March of 2017. In total, 13 fecal samples were collected from individual animals. Fecal samples were collected immediately after voiding.

2.2: Sample collection and processing:

Fecal samples were firstly collected in separate clean plastic zipper bag (Figure 2) immediately after voiding. Than Fecal samples were transferred scientifically in a clean sterile screw capped falcon tube containing buffer peptone water. Soon after collection, the samples were kept into a cool box with ice for ceasing the growth and activity microorganism. Each sample was labeled with date of sampling, type of sample and the place of origin. The samples were transported to the Department of Microbiology and Veterinary Public Health or to the Poultry Science and Research Centre, Chittagong Veterinary and Animal Sciences University (PRTC-CVASU) as early as possible and stored at 4°C until bacteriological investigation.

2.3: Study Design

This study was done by using the following study design:

Bangabandhu Sheikh Mujib Safari Park, Cox's Bazar (Dulhazra Safari Park)

↓

Free range monkeys

↓

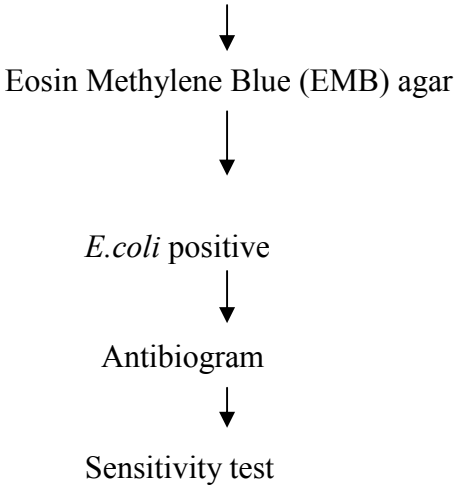
Collection of fecal sample into plastic zipper bags after voiding (Collection of fecal sample after removing of upper portion of feces)

↓

Peptone broth

↓

MacConkey agar



2.4: Method of Bacterial isolation:

E. coli from samples were isolated and identified according to the techniques recommended by OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition, 2008 with some modifications (OIE, 2008). For initial detection of *E. coli* from samples, it was enriched into BPW. Samples were transferred to falcon tube containing five ml of BPW (figure 3) that was prepared according to the manufacturer's instructions (Oxoid, England) for enrichment. Then it was incubated at 37°C for 24 hours**.** After the enrichment step, 0.1ml enriched broth were inoculated into Maconkey agar (Figure 5) (Oxoid, England) and incubated at 37˚C for 24-48 hours (Figure 6). After incubation, plates were examined for the presence of characteristic colonies suspected to be *E. coli*. Suspected positive samples were inoculated to the EMB agar (MERCK, Mumbai) (selective media for *E. coli*) where characteristic metallic sheen indicated positive one. At the same time, each of the enriched samples was inoculated onto a blood agar plate to observe characteristic growth. Characteristic colonies yielded on the *E.coli* selective medium and colonies on the blood agar plate.

Table-1: Methods on culture test

SN	Agar/ test	Incubation time and temperature	Observations
1.	MacConkey agar	Incubated at 37^0C temperature for 24 hours.	Dark pink colored raised colony. (Figure 7)
2.	Eosin Methylene Blue (EMB) agar	Incubated at 37^0C temperature for 24 hours.	Characteristic Metallic sheen. (Figure 8)
3.	Blood agar	Incubated at 37^0C temperature for 24 hours	Large, gray, moist colonies Colonies are without hemolysis

2.5: Method of Antimicrobial susceptibility test:

Antibiotic resistance was determined by a disk diffusion method on Mueller-Hinton agar (Oxoid) according to the Clinical Laboratory Standards Institute (formerly National Committee of Clinical Laboratory Standards, NCCLS) standards. Bauer-Kirby disk-diffusion procedure (Bauer *et al.,* 1966) was used to determine the microbial resistance. Muller-Hinton (MH) agar was prepared according to the manufacturer`s instructions (Oxoid, UK). A bacterial turbidity equivalent to 0.5 McFarland standards was used as inoculum for each isolate. The antibiotic resistance pattern for the isolates tested against the antibiotic was determined considering the zones of inhibition (Figure 9) as "resistant (R)", "intermediately sensitive (I)", and “sensitive (S)” as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2009), shown in table 2. For an isolate to test, a sterile swab was dipped into the inoculum and rotated against the side

Figures

Figure 1: Free range Monkey of Safari park

Figure 2: Fecal sample in Plastic zipper bags

Figure 3: Fecal sample on Felcon tube with PBW

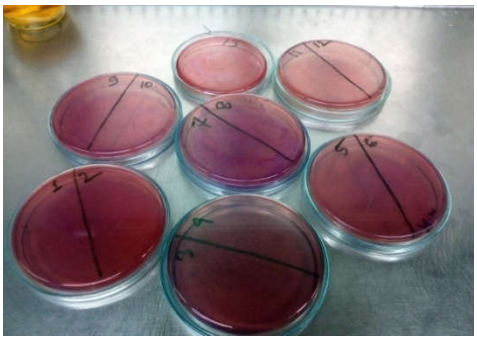


Figure 4: Prepared Agar plates

Figure 5: Inoculation of Sample for microbial growth

Figure 6: Incubation of inoculated agar plates

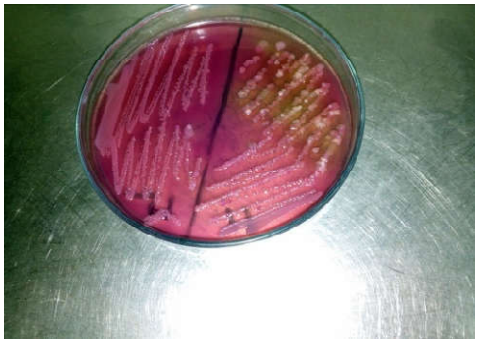


Figure 7: Pink, small drop shape colonies on Macconkey agar plate

Figure 8: Metallic green sheen color coloies on EMB agar plate

Figure 9: Zone of Inhibition on Mueller-Hinton agar plate

of the tube with firm pressure. Then after removing the excess fluid from the swab the dried surface of a MH agar was inoculated by streaking the swab three times over the entire agar surface rotating the plates approximately at 60 degrees for each time to ensure an even distribution of the inoculums. The antimicrobial disk was then placed on the surface of the inoculated agar. Disk was placed carefully on the surface of the agar with a gentle pressure to make a complete contact. Then, the agar plate was incubated at 35^{o}C for 16 to 18 hours. At the end of incubation the size of zone of inhibition around a micro-disk was measured and the result was recorded.

Table 2: Standard measurement of diameter of zone of inhibition.

SL	**Name of Antimicrobial agents**	**Diameter of zone of inhibition (millimeter)**		
		Resistant	**Intermediate**	**Sensitive**
1	Ampicillin	≤13	14-16	≥17
2	Amoxicillin	≤13	14-17	≥18
3	Ciprofloxacin	≤15	16-20	≥21
4	Colistin sulphate	≤10	11-13	≥14
5	Sulfamethoxazole	≤10	11-15	≥16

Source: CLSI, 2007; Seol et al., 2005; LO-Ten-Foe et al., 2007

R'=resistance, 'I'=intermediate, 'S'=sensitive

2.6: Data analysis

Data was stored in MS excel (Microsoft Word 2007) and descriptive analysis was done in this study.

Chapter 3: Results

Among 13 samples of feces of Rushes monkeys 12 (92.3%) fecal sample were positive and individual colonies of *E.coli* were isolated through different tests.

3.1: Results of cultural examination:

E. coli was cultured on MacConkey and EMB medium for morphological characterization. After 24 hrs, 92.3% samples were found positive and formed two types of colonies were isolated under microscopic examination. All the isolated colonies were pink on MacConkey agar, while metallic sheen on EMB agar. In the microscopic examination of Gram's staining, all the positive samples are found as Gram-negative, pink colored, rod shaped bacteria which are arranged in single or in pairs.

Table-3: The examination results were done for isolation of *E. coli*

SL	Name of the media /test	Total no. of sample	No. of positive sample	Percentage (%)
1.	MacConkey agar	13	12	92.3%
2.	Eosin Methylene Blue (EMB) agar	13	12	92.3%
3.	Gram 's staining	13	12	92.3%

3.2: Result of antibiogram test

All the 12 positive isolates were subjected to do antibiotic sensitivity test to five different antimicrobial agents. . From the isolates 100% samples were sensitive to Colistin Sulfate which was the highest in sensitivity. 41.67% samples showed sensitive to Ciprofloxacin. From the isolates 100% samples showed resistance to Amoxicillin, Ampicillin and Sulfamethoxazole.

Table-4: Antimicrobial resistance pattern against *E. coli* isolates

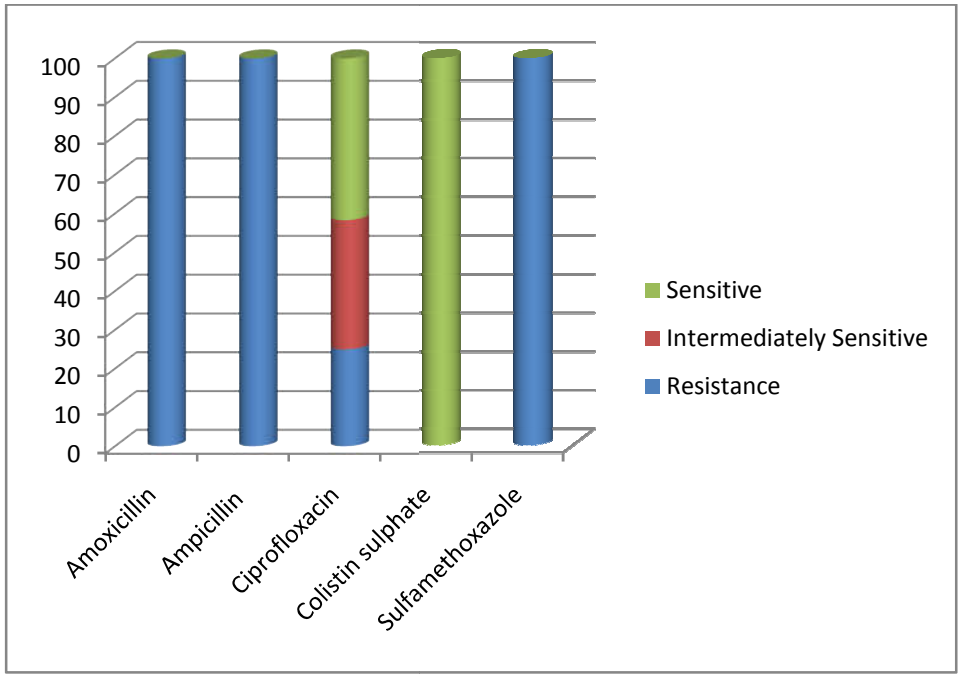
Sample NO	Amoxicillin (AMX)	Ampicillin (AMP)	Ciprofloxacin (CIP)	Colistin Sulphate (CT)	Salphamethazole (SXT)
Sample 1	R	R	S	S	R
Sample 2	R	R	R	S	R
Sample 3	R	R	R	S	R
Sample 4	R	R	S	S	R
Sample 5	R	R	I	S	R
Sample 6	R	R	S	S	R
Sample 7	R	R	I	S	R
Sample 8	R	R	S	S	R
Sample 9	R	R	R	S	R
Sample 10	R	R	I	S	R
Sample 11	R	R	S	S	R
Sample 12	R	R	I	S	R

R= Resistance; I= Intermediately sensitive; S= Sensitive;

Table-5: Prevalence of antimicrobial resistance pattern against E. coli isolates

Antimicribial agents	No.of isolates	Resistance (%)	Intermediately Sensitive (%)	Sensitive (%)
Amoxicillin	12	12(100)	0%	0%
Ampicillin	12	12(100)	0%	0%
Ciprofloxacin	12	3(25)	4(33.33)	5(41.67))
Colistin sulphate	12	0%	0%	12(100)
Sulfamethoxazole	12	12(100)	0%	0%

Graphical representation of prevalence of antimicrobial resistance:



Graph 1: Prevalence of antimicrobial resistance

Chapter 4: Discussion

To our knowledge, this is the first reported isolation of E. coli in wild rhesus monkeys in Bangabandhu Sheikh Mujib Safari Park (Dulhazra Safari Park), Bangladesh. The study was conducted with the aim of isolation and identification of *Escherichia coli* present in fecal sample of wild rhesus monkeys. Antibiogram was also done to know the sensitivity and resistance pattern against different antibiotics. Here our study observed that 92.3% *E.coli* present in fecal sample of wild monkeys. This result partially agreed with the findings of Gagandeep et al., (2001) where he reported that after infection, stool samples from 17 of the 22 infected monkeys (Adult *Macaca radiata)* were isolated 16 (72.72%) monkeys had diarrhea and excreted *E. coli* O157 in bacteriological examination. Enteropathogenic E.coli was also reported as a common opportunistic pathogen causing diarrhoea and wasting in rhesus macaques infected with simian immunodeficiency virus (Mansfield et al., 2001). Our results also different from some other findings. Hayashimoto et al., (2016) reported that A total of 74(32.17%) stool or rectal swab samples were positive for Enteropathogenic *E.coli* in Common Marmosets (*Callithrix jacchus*) where EPEC was detected in 10 of 98 clinically healthy samples (10.2%), 17 of 85 diarrhea samples (20%), and all 47 bloody stool samples (100%). Martin et al., (2009) reported that, Twenty-five (27%) of 92 clinically normal macaques (*Macaca mulatta and Macaca fascicularis*) were found to have b-haemolytic *Escherichia coli* isolated from their faeces. Sample inoculation and cultural characteristics of the bacteria in different cultural media as recorded in the study were almost similar as reported by Aseel et al., (2013).

In our results E. coli isolated from feces of rhesus monkeys showed variation in sensitivity to different antibiotics. Colistin sulfate was showed 100% effective. This antibiotic generally used in poultry practices. It is seldom used in mammals and large ruminants. Therefore it may be a cause that this drug is 100% effective against isolated E. coli from wild monkeys. Most of the isolates showed resistance against resistances against Amoxicillin (100%), Ampicillin (100%) and Sulfamethoxazole (100%). Almost every isolates of this study exhibited multiple resistances to more antibiotics which is similar with few previous reports. Present study was agreed with the

previous study where monkeys isolate found resistances to Amoxicilin and Sulfamethoxazole; and also found sensitive to Colistin sulphate. (Nobuhito et al., 2016).

We found high level of resistance against Amoxicillin, Amoxicillin and Sulfamethoxazole. It is indicating that these antibiotics were used very frequently in treating large animal. Wild monkeys get antibiotic treatment very rare and in safari park. But still shows the isolated *E. coli* had resistance against antibiotic. It indicates antibiotic resistance is transferred in the environment and/or antibiotic resistance genes transfer to monkey through food chains. Antibiotic resistance is an increasingly serious threat to global public health now days. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements. Similar to our study, prevalence of antibiotic resistance in *E. coli* isolates were found biological and environmental sources such as human feces, human urine, cattle, sheep, goat, chicken, duck, pigeon, drain sewage and soil (Zinnah *et al*., 2008). During our sample collection we showed, wild monkeys of Dulhazra Safari Park were taking wastage feed, drain water, and stay contact with other wild and captive animals in Safari park. We also showed that visitors of Safari park offered food to monkeys and they were taking those feeds from visitors. So we hypothesized that drug resistancy may transfer through *E. coli* contaminated feed and water through those sources.

Ruminants are considered to be reservoir of *E. coli* O157 infections, wild bird may play a key role in emergence by providing a "zoonotic pool" of the infectious agents mainly *E. coli* O157:H7; wild bird play an important role in dissemination of *E. coli* O157:H7; could be the main reservoir for *E. coli* O157:H7 especially gull that spreads *E. coli* O157:H7 to cattle and other animals (Wallace *et al*., 1997, Foster *et al*., 2006). Other researcher (Warshawsky *et al*., 2002) found that manure, rail and environmental of petting zoo animal that causes human infected cases with *E. coli* O157 so that different isolated rate among animal species could be related with manure, animal food, water supply (Bonetta *et al*., 2011) contaminated with *E. coli* O157 that may contaminated with feces of wild bird.

Chapter 5: Conclusion

It can be concluded that out of 13 fecal samples 12 (92.3%) were positive for E.coli in our experiment. From the antimicrobial sensitivity test, it can be said that Colistin sulphate is the best drug of choice to treat E coli infection in wild monkeys like Bangabandhu Sheikh Mujib Safari Park (Dulhazra Safari Park). Present research findings showed that, the source of single and multiple antimicrobial- resistant of *E. coli* isolates is the frequently found in different antimicrobials including Ampicillin, Amoxicillin, Sulfamethoxazole. Therefore, it is important to monitor the occurrence of resistance among bacteria from animals and food, as these bacteria are able to spread through food products to human. Finally to determine the prevalence of various strains, serotypes of *E. coli* and to identify the pattern of antimicrobial resistance, comprehensive study or research should be needed.

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Biography

I am Pronesh Dutta, son of Mr. Nani Gopal Dutta and Mrs. Shika Biswas. I passed Secondary School Certificate examination in 2009 (G.P.A-5.00) from Chittagong Governtment High School, Chittagong followed by Higher Secondary Certificate examination in 2011 (G.PA-5.00) from Hazera Taju University College, Chittagong. Now I am an intern veterinarian under the Faculty of Veterinary Medicine. I enrolled for Doctor of Veterinary Medicine (DVM) degree in Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. I have immense interest to work in the field of Wildlife Health and conservation.