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Investigations into the Role of Zoo Animals in Transmitting the Extended Spectrum Beta Lactamases (ESBL) *E. coli* in the Environment

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ABSTRACT

Extended-spectrum beta-lactamases producing *E. coli* (ESBL *E. coli*) have emerged as an important human and veterinary medicine issue. Zoo animals may act as a reservoir of ESBL *E. coli* responsible for transmission of ESBL *E. coli* to humans, animals, and the environment. A total of one hundred fecal samples were collected from zoo animals of two different zoos and analyzed for presence of ESBL *E. coli*. *E. coli* isolates (n=52) were recovered from 100 samples. The combination of CTX-M and TEM genes (32/52; 61.5%) was commonly observed in ESBL *E. coli* isolates followed by TEM (10/52; 19.2%) and CTX genes (5/52; 9.6%). Antimicrobial susceptibility profiling of ESBL *E. coli* isolates showed that 100% of the isolates were resistant to more than three antibiotics. The study indicated that zoo animals may act as reservoirs for transmission of multidrug resistant ESBL *E. coli* to humans, animals, and environment.

Key words: Antimicrobial Drug Resistance, *Escherichia Coli*, Beta-Lactamases, Zoo Animals, Primate.

INTRODUCTION

Antimicrobial resistance (AMR) has become a major challenge in human and veterinary medicine due to unrestricted use of commonly available antibiotics. Antibiotic resistance is directly related to the decrease in number of effective antibiotics available for the treatment of bacterial infections (Dobiasova et al. 2013; Conrad et al. 2017). Resistance to 1st to 4th generation cephalosporins is due to the production of extended spectrum beta-lactamases (ESBL), inhibited by clavulanic acid, sulbactam and tazobactam (Sanjukta et al. 2019; Raheel et al. 2022). The ESBLs are plasmid-mediated enzymes which hydrolyze and inactivate a range of β-Lactam antibiotics including aztreonam, penicillin & broad-spectrum cephalosporin (Al-Muharrmi et al. 2008). ESBLs mainly include *bla* CTX-M (cefotaximase-firstly isolated in Munich), *bla* SHV (sulfhydryl variable) and *bla* TEM (Temoneira). Since 2000, *bla* TEM and *bla* SHV were predominantly present, after this date *bla* CTX-M has become dominant all over the world (Sakin et al. 2018).

Zoo provides a high density of wild captivated animals belonging to different species, which are in close contact to other animals and humans (veterinarians and visitors) (Furlan et al. 2019). Zoo animals can colonize multidrug-resistant bacteria, having the potential to act as a reservoir of zoonotic pathogens and may transfer them to humans and environment either by direct or indirect contact. Emerging infectious diseases are increasingly reported as a significant hazard to human lives with the 75% zoonotic in origin and out of them, 70% reported from wildlife (Robinette et al. 2017).

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Data regarding ESBL *E. coli* prevalence has been reported in various studies regarding clinical settings in Pakistan (Rehman et al. 2017; Abbas et al. 2019; Umair et al. 2019). Wild migratory birds and migratory avian species have been discussed as a potential carrier for the dissemination of ESBL *E. coli* in Pakistan (Mohsin et al. 2017). But there are limited reports regarding the ESBL *E. coli* prevalence and their antimicrobial resistance in zoo animals especially in Pakistan. The study was designed to explore the occurrence and molecular characterization of ESBL *E. coli* in zoo animals. The study would help in understanding the role of zoo animals in transmission of AMR bacteria (ESBL *E. coli*) to food animals, humans and the environment.

MATERIALS AND METHODS

Sampling Area

Fecal samples (n=100) were collected from two different zoos located in Potohar region Rawalpindi and Islamabad with no previous history of excessive use of antibiotics except the usage of amoxicillin with clavulanic acid in critical conditions. These zoos were maintaining 500 wild animals of different species. The average number of visitors to these zoos is 2000 persons per day which increases to 50,000 to 60,000 persons in public holidays.

Sample Collection, Enrichment, and Isolation

Fresh fecal samples (n=100) were taken from zoo animals with the help of zookeepers. These samples were collected from twenty different zoo animal's species irrespective of their age and sex, placed into zip lock bags and transferred to laboratory within 2hr of its collection. The animals sampled were blue bull, hog deer, spotted deer, white fallow deer, red deer, chinkara deer, urial, black buck, zebra, mouflon sheep, Australian sheep, miniature horse, vervet monkey, rhesus monkey, baboon, emu, ostrich, lion, tiger and bear. Enrichment of all the samples was carried out in Buffer Peptone Water (BPW), i.e., 2gm of fecal material into 10mL of BPW, at 37°C for 24hr and cultured onto MacConkey agar (MAC-CEF) containing cefotaxime (0.8gm/L). One lactose positive colony per sample was selected and confirmed as *E. coli* by their colony morphology and biochemical analysis using Indole, Methyl Red, Voges-Proskeur, Citrate (IMVIC) and Triple Sugar Iron test (TSI) (Radhouani et al. 2014; Alonso et al. 2017).

Phenotypic Confirmation of ESBL *E. coli*: Double Disk Diffusion Test (DDDT)

ESBL producing *E. coli* were confirmed phenotypically using double disk diffusion test (DDDT) as recommended by CLSI (Clinical and Laboratory Standards Institute (CLSI), 2013). About 2-3 colonies of *E. coli* confirmed isolates were dispensed into normal saline. Turbidity of the suspension was compared to 0.5 McFarland standard. The suspension was used to swab on Muller Hinton agar (MHA) plates, evenly. Antibiotic discs of ceftazidime (CAZ, 30µg) and ceftazidime with clavulanic acid (CAL, 40µg), cefotaxime (CTX, 30µg) and cefotaxime with clavulanic acid (CTL, 40µg) were applied at the distance of 30mm apart. Isolates were considered positive for ESBL detection if zone of inhibition of ceftazidime and cefotaxime with clavulanic acid is greater than 5mm than ceftazidime and cefotaxime alone.

Double Disk Synergism Test (DDST)

Synergism was checked between amoxycillin + clavulanic acid (AMC) to disks including cefepime (FEP), ceftriaxone (CRO), cefixime (CFM), aztreonam (ATM) and ceftazidime (CAZ). Briefly, inoculum of confirmed *E. coli* isolates was prepared into normal saline and confirmed the turbidity of the inoculum to McFarland standard. The preparation was evenly spread on MHA plates. Antibiotics disks were placed 30mm apart each other and from central disk. Synergism effect was considered if any other antibiotic disk gave the zone of inhibition towards amoxicillin + clavulanic acid.

Antibiotic Sensitivity Testing of ESBL *E. coli*

Phenotypic antibiotic sensitivity profile based ESBL *E. coli* isolate was assessed for 17 antibiotics including imipenem (IPM 10µg), ciprofloxacin (CIP 5µg), enrofloxacin (ENR 5µg), Augmentin (AMC 30µg), gentamycin (CN 10µg), neomycin (N 10µg), streptomycin (S 10µg), cephradine (CE 30µg), cefoxitin (FOX 30µg) cefixime (CFM 5µg), cefepime (FEP 30µg), oxytetracycline (OT 30µg), doxycycline (D 30µg), penicillin G (P 10µg), ampicillin (AMP 10µg), aztreonam (ATM 30µg), and lincomycin (MY 30µg).

Antibiotic resistance profiles were determined on MHA plates using disc diffusion method as per described in CLSI guidelines. A suspension of each phenotypically confirmed ESBL *E. coli* isolate was prepared using normal saline. Turbidity of the suspension was compared to 0.5 McFarland standards. This suspension was evenly spread on MHA plates with the help of swab. All the above-mentioned antibiotic discs were placed on MHA plates with the help of sterilized forceps at the distance of 20mm apart each other. The plates were incubated at 37℃ for 24hr aerobically. After incubation, MHA plates were observed for zones of inhibition around each antibiotic disc. A clear zone (zone of inhibition) was measured against each antibiotic disc and compared with CLSI standards. Resistance against three or more than three different classes of antibiotics was considered as Multidrug Resistance (Abbas et al. 2019).

DNA Extraction

DNA of ESBL *E. coli* confirmed isolates was extracted by boiling method. About 1-2 colonies of ESBL *E. coli* confirmed isolates were mixed with 250µl of nuclease-free water in an Eppendorf tube and heated at 94°C for 10min. The mixture was cool down and centrifuged at 10,000g for 10min. Supernatant containing DNA was shifted to a new tube while the pellet was removed (Dashti et al. 2009). The yielded DNA was stored at -20°C for future use.

Molecular Detection of ESBL Encoding Genes

Phenotypic ESBL positive *E. coli* isolates were analyzed for the presence of ESBL encoding genes i.e. *bla* CTX-M and *bla* TEM using Polymerase Chain Reaction (PCR) (Gangoué-Piéboji et al. 2005; Kaftandzieva et al.

2011). The volume of each reaction and conditions for PCR are described in Table 1.

RESULTS

Identification of ESBL *E coli*

In total, 52 ESBL *E. coli* isolates were obtained from 100 fecal samples collected from various zoo animals of two different zoos of Islamabad and Rawalpindi. The recovery of ESBL *E. coli* was more from Zoo-2 (72%) compared to Zoo-1 (45.3%) (Table 2).

Antibiotic Resistance Pattern

All the isolates showed resistance against lincomycin, streptomycin, penicillin G, enrofloxacin, cefixime, cephradine and ampicillin. The antimicrobial resistance ranging from 21.1% to 90.3% was observed against remaining nine antibiotics (Fig. 1). Moreover, all the isolates were susceptible to Imipenem (a last resort antibiotic).

Frequency of ESBL Encoding Genes

The most prevalent ESBL encoding gene detected was *bla* TEM, followed by *bla* CTX-M. However, *bla* SHV was not spotted in any of the ESBL *E. coli* isolate identified in the study. The combination of *bla* CTX-M and *bla* TEM genes was commonly observed in ESBL *E. coli* isolates (32/52; 61.5%) followed by *bla* TEM (10/52; 19.2%) and *bla* CTX genes (5/52; 9.6%).

Table 1: PCR condition for detection of CTX, TEM and SHV genes in ESBL *E. coli* isolates recovered from fecal samples of zoo animals of two different zoos of Rawalpindi and Islamabad, Pakistan. All the PCR reactions were carried out in PCR system Veriti (Applied Biosystems, Waltham, USA)

Gene	PCR conditions	PCR reaction volume
CTX gene	Initial denaturation at 94°C for 5min; 35 cycles of 95°C for 30s, 54°C for 1min, & 72°C for 1min. Final extension at 72°C for 8min.	2.5µL of 10X PCR buffer, 1.5mM $MgCl_2$, 0.2mM of each dNTP (ThermoScientific, Waltham, USA), 0.4µM of each primer, 1 unit of *Taq* DNA Polymerase (ThermoScientific, Waltham, USA), 5µL of template DNA, Final volume of 25µL with nuclease free water
TEM gene	Initial denaturation at 94°C for 5min; 35 cycles of 95°C for 30s, 56°C for 30s, & 72°C for 1min. Final extension at 72°C for 10min.	2.5µL of 10X PCR buffer, 2mM $MgCl_2$, 0.2mM of each dNTP (ThermoScientific, Waltham, USA), 0.5µM of each primer, 1 unit of *Taq* DNA Polymerase (ThermoScientific, Waltham, USA), 5µL of template DNA, and Final volume of 25µL with nuclease free water
SHV gene	Initial denaturation at 94°C for 2min; 35 cycles of 94°C for 20s, 56°C for 1min, & 72°C for 1min. Final extension at 72°C for 5min.	2.5µL of 10X PCR buffer, 1.5mM $MgCl_2$, 0.2mM of each dNTP (ThermoScientific, Waltham, USA), 1µM of each primer, 1 unit of *Taq* DNA Polymerase (ThermoScientific, Waltham, USA), 5µL of template DNA, and Final volume of 20µL with nuclease free water

Table 2: The proportion of fecal samples positive for extended spectrum beta-lactamases producing *E. coli* (ESBL *E. coli*) isolates collected from 100 zoo animals of various species from two different zoos of Islamabad and Rawalpindi, Pakistan.

Specie of animal	Scientific Name	Zoo-1		Zoo-2		Total samples positive for ESBL *E. coli*
		Samples tested	Samples positive for ESBL *E. coli*	Samples tested	Samples positive for ESBL *E. coli*	
Blue bull	*Boselaphus tragocamelus*	02	01 (50)	0	0	1/2 (50)
Hog deer	*Axis porcinus*	05	05 (100)	08	05 (62.5)	10/13 (76.9)
Spotted deer	*Axis axis*	10	04 (40)	02	02 (100)	6/12 (50)
White fallow deer	*Dama dama*	05	04 (80)	04	03 (75)	7/9 (77.7)
Red deer	*Cervus elapus*	0	0	03	01 (33.3)	1/3 (33.3)
Chinkara deer	*Gazella bennettii*	03	01 (33.3)	0	0	1/3 (33.3)
Urial	*Ovis vignei*	04	03 (75)	04	03 (75)	6/8 (75)
Black buck	*Antilope cervicapra*	08	02 (25)	04	04 (100)	6/12 (50)
Zebra	*Equus quagga*	01	0	0	0	0
Mouflon Sheep	*Ovis orientalis*	04	02 (50)	0	0	2/4 (50)
Australian Sheep	*Ovis aries*	07	0	0	0	0
Miniature horse	*Equus caballus*	05	03 (60)	0	0	3/5 (60)
Vervet monkey	*Chlorocebus pygerythrus*	02	02 (100)	0	0	2/2 (100)
Rhesus Monkey	*Macaca mulatta*	03	02 (66.6)	0	0	2/3 (66.6)
Baboon	*Papio ursinus*	02	01 (50)	0	0	1/2 (50)
Emu	*Dromaius novaehollandiae*	03	01 (33.3)	0	0	1/3 (33.3)
Ostrich	*Struthio camelus*	03	01 (33.3)	0	0	1/3 (33.3)
Lion	*Panthera leo*	02	01 (50)	0	0	1/2 (50)
Tiger	*Panthera tigris*	04	01 (25)	0	0	1/4 (25)
Bear	*Ursidae*	02	0	0	0	0
Total		75	34 (45.3)	25	18 (72)	52/100 (52)

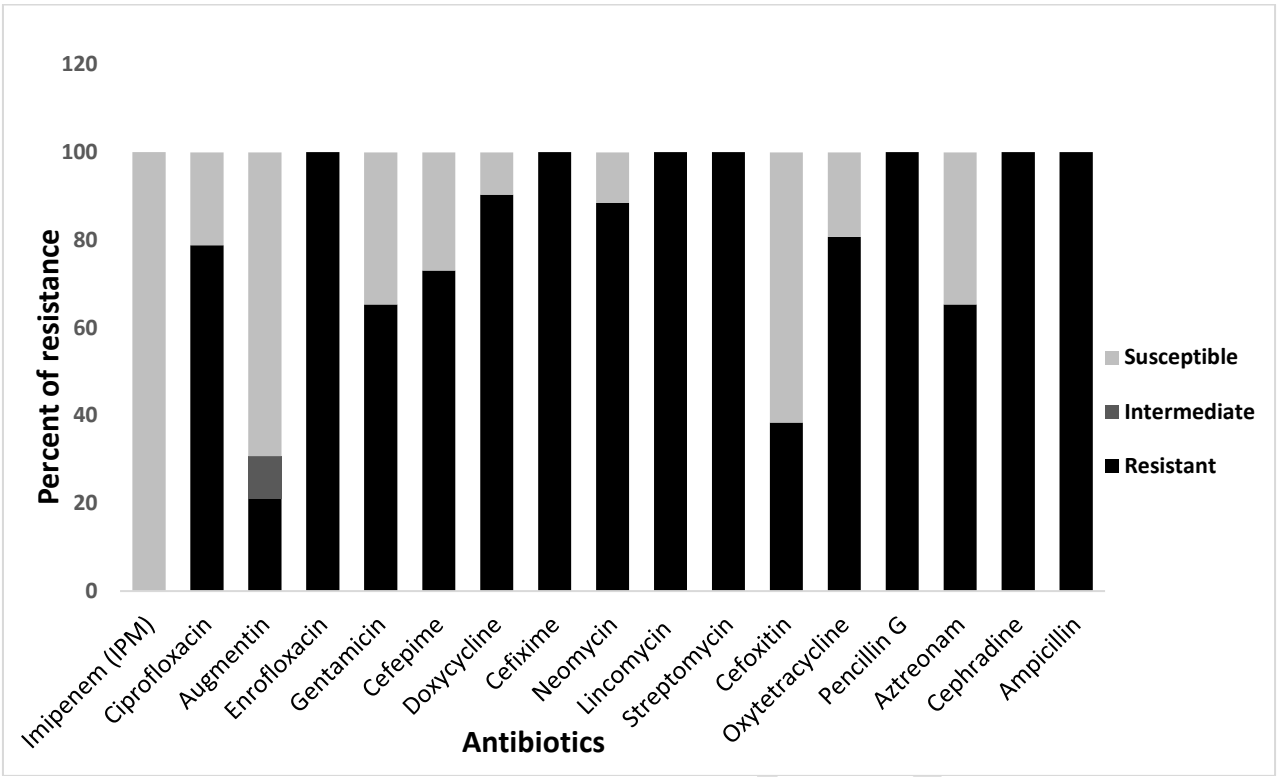

Fig. 1: Antibiotic susceptibility profile of extended spectrum beta-lactamases producing *E. coli* (ESBL *E. coli*) isolates recovered from zoo animals of two different zoos of Islamabad and Rawalpindi, Pakistan.

DISCUSSION

Zoological gardens are the animal parks (confining the wild animals for public display) has led to the dissemination of AMR bacteria into the environment (Hall et al. 2011). Zoo animals have also been reported to be involved in the spread of ESBL *E. coli* to humans, and to the environment i.e. soil and water contamination resulting into the dissemination of resistant bacteria into the ecosystem (Wang et al. 2012). Keeping in view, study was conducted to see the occurrence of ESBL *E. coli* in these animals to know their role in transmission of AMR bacteria to human population. The study indicated that from 100 fecal samples collected from various zoo animals of two different zoos of Islamabad and Rawalpindi 52 ESBL *E. coli* isolates were recovered indicating the possible role of zoo animals in spread of AMR bacteria to humans and environment (Table 2). Already it was reported that zoo animals, being the reservoir of the zoonotic pathogens results into dissemination of AMR bacteria not only to humans but also to food-producing animals i.e. chicken, cattle, birds and pigs (Zhang et al. 2017; Umair et al. 2019). More ESBL *E. coli* were recovered from Zoo-2 (72%) compared to Zoo-1 (45.3%) (Table 2). The difference of recovery of ESBL *E. coli* isolates in two different zoos may be due to differences in antibiotic usage, managemental practices and the environmental variations.

The combination of CTX-M and TEM genes was commonly observed in ESBL *E. coli* isolates (32/52; 61.5%) followed by TEM (10/52; 19.2%) and CTX genes (5/52; 9.6%). In contrast to our study previous studies have suggested CTX-M as the most prevalent genotype of ESBL in *Enterobacteriaceae* recovered from humans and non-human primates (Smet et al. 2010; Wang et al. 2012). A study carried out in China reported CTX-M to be the most prevalent gene (86% of the isolates) in ESBL *E. coli* isolates recovered from zoo animals (Wang et al. 2012). A study carried out in Pakistan detected high rate of CTX-M gene (82.6% of isolates) in ESBL *E. coli* isolates recovered from pets (dogs and cats), pet owners and veterinarians (Abbas et al. 2019). Similar to our study co-existence of various β-lactamase genes within the same isolate recovered from humans, food and zoo animals have also been reported (Dobiasova et al. 2013; Gundran et al. 2019; Jena et al. 2017). For example, analysis of 49 ESBL *E. coli* isolates recovered from zoo animals maintained at Ostrava Zoological Garden, Czech Republic indicated presence of combination of CTX-M and SHV genes in one isolate (Dobiasova et al. 2013). The treatment of infection due to ESBL *E. coli* isolates having more than one β-lactamase genes may be difficult as the probability of ESBL expression is more likely in these isolates.

The antimicrobial susceptibility profiling of all the isolates was carried out for 17 antibiotics of six classes. The findings showed that all the isolates were sensitive to Imipenem (52/52; 100%). Imipenem is expensive and considered as last resort antibiotic therefore it is rarely used in animals for treatment purposes. All the isolates showed resistance against lincomycin, streptomycin, penicillin G, enrofloxacin, cefixime, cephradine and ampicillin. These antibiotics are commonly used for treatment of infections in animals (Ur Rahman and Mohsin, 2019). Previous studies have reported AMR to be associated with excessive use of antibiotics (Burow et al. 2014; Chantziaras et al. 2014). The antimicrobial resistance ranging from 21.1 to 90.3% was observed

against remaining nine antibiotics. Although the exposure of zoo animals under study was low to antibiotics, high level of AMR was observed in isolates recovered from these animals. There is a possibility that these animals have acquired AMR bacteria from environment. The environmental bacteria may acquire AMR through three main pathways such as de-novo mutation, selection pressure of antibiotics or through gaining of evolved plasmids over time (Wellington et al. 2013). The results also showed that all the isolates were resistant to more than three antibiotics indicating that zoo animals may be responsible for transmission of multidrug resistance *E. coli* to environment.

According to our information, this is the pioneer study reporting the identification of ESBL *E. coli* and AMR genes in zoo animals of Pakistan. Although it was a small-scale study limited to two zoos of Islamabad and Rawalpindi however, it provided evidence that zoo animals may act as carrier of ESBL *E. coli* with clinically important resistant genes. Since, zoo animals are at a close contact to humans, so these resistant pathogens might get transfer to humans and to other healthy animals. Furthermore, there is a need for more epidemiological surveys to monitor the microbial resistance patterns and ESBL genotypes for prevention and disease control both in human and veterinary medicines.

Authors Contribution

Asma Riaz: Sample analysis in laboratory and writing of original manuscript draft. Muhammad Armaghan Shahzad: Collection of data and samples, Sample analysis in laboratory, Editing and Formatting of the manuscript. Aitezaz Ahsan: Conceptualization, project administration, and assistance of sample and data analysis in laboratory. Rizwan Aslam: Visualization and supervision of laboratory work. Muhammad Usman: Collection of data and samples. Basit Rasheed: Collection of data and samples. Munib Hussain: Visualization and proofreading the manuscript. Maryam Irtash: Review and editing of the original manuscript draft. Muhammad Kashif Saleemi: Data analysis. Abdul Ali: Assisted in sample analysis. Sajid Mahmood Sajid: Assisted in sample collection. Hamid Irshad: Conceptualization, Project administration, Supervision, review and editing of the manuscript.

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