PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ESBL-PRODUCING THE ENTEROBACTERIACEAE FROM ANIMAL FECAL SAMPLES IN SOUTHERN PUNJAB, PAKISTAN

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ABSTRACT: *The prevalence of extended-spectrum β-lactamases (ESBL) producing members of the Enterobacteriaceae family is increasing day by day and becoming a worrisome issue around the globe. But in Pakistan, little is known about ESBL producing bacteria, especially in farm animals. Therefore, we report on the characterization and prevalence of ESBL-producing bacteria from the sheep and goat population in Southern Punjab, Pakistan. A total of 200 fecal samples collected, from sheep (n=100) and goats (n=100), were cultured and identification of Enterobacteriaceae was done by standard procedures. Molecular detection of resistant bacteria was further done by PCR (SHV, CTX-M, and TEM). Our results showed the highest percentage of E. coli (88%) followed by K. pneumonia, C. freundii, E. aerogenes, and E. cloacae. The bacterial strains showed high resistance against Cefotaxime and the highest susceptibility towards Ertapenem. PCR-based results revealed CTX-M (26%) as the predominant ESBL gene followed by SHV (14%). TEM was not detected in any bacterial isolate. Overall, our study suggests a high prevalence of ESBL producing Enterobacteriaceae members in sheep and goat farms in Southern Punjab of Pakistan.*

1. INTRODUCTION

Antibiotic resistance has been recognized as an emerging problem in human and veterinary medicines worldwide. The excessive use of antimicrobial agents is an important factor for the emergence and dissemination of antibiotic-resistant bacteria [1]. The principle behind the spread and development of resistance is that bacteria are subjected to different types and concentrations of antibiotics. Over time, resistant bacteria are selected due to selective pressure having specific fingerprints for resistance against antibiotics that have been used [2]. Bacteria gain resistant genes through mobile elements such as plasmids, integrons, and transposons [3]. Resistant genes, once acquired, can be transferred to other bacteria and this ability to transfer resistant genes is well known among members of the Enterobacteriaceae family [4]. The worldwide rise of antimicrobial resistance in bacteria, combined with the decreasing number of new antibacterial drugs, can soon lead to the failure of treating bacterial infections in animals as well as in humans. Infections with β-lactamase producing bacteria are associated with increased morbidity, mortality, and healthcare costs.

The Gram-negative bacteria achieve resistance against β-Lactam antibiotics by producing enzymes known as β-lactamases. Extended Spectrum Beta Lactamases (ESBL), are hydrolytic enzymes produced by Gram-negative pathogens. These enzymes confer resistance to many β-Lactam antibiotics [5]. The emergence of ESBL producing bacteria of the Enterobacteriaceae family has been increased in recent years, which is a major challenge for healthcare around the globe to treat bacterial infections [6]. There are more than 340 β-lactamases identified and reported so far [7]. In Enterobacteriaceae, ESBL which have been frequently encountered belongs to SHV, TEM, and CTX-M families [8]. ESBL producing members of Enterobacteriaceae have been reported in farm animals including Sheep and Goat [9]–[12]. But there is very limited information available about the prevalence of ESBL producing bacteria in farm animals in Pakistan.

The purpose of this study is to determine the occurrence and pattern of antibiotic susceptibility among Enterobacteriaceae. And to determine the prevalence of ESBLs producing Enterobacteriaceae obtained from fecal samples of farm animals including sheep and goats in Southern Punjab, Pakistan.

2. MATERIALS AND METHODS

2.1 Sample Collection

The present study was conducted on farm animals including the Sheep and Goat population in the Punjab province of Pakistan. Fecal samples were taken from November 2016 to August 2017. A total of 200 fecal samples were collected from Sheep and Goat (100 samples each).

2.2 Isolation and Identification of Enterobacteriaceae

All samples were processed for isolation of Enterobacteriaceae viz Escherichia, Klebsiella, Citrobacter, Enterobacter spp. All the samples were streaked on MacConkey agar and then incubated at 37°C for about 24 hours to get primary bacterial growth. After 24 hours, the morphology of bacterial colonies was examined and 4 bacterial colonies per sample were sub-cultured on separate media plates. Pure cultures were obtained as per the procedure described by OIE [13]. For identification purpose, Oxidase test and Gram-staining was performed on all 800 bacterial colonies as per the recommendation of Merchant and Packer [14]. Further confirmation of colonies was done by Analytical Profile Index (API) Remel RapID-One strips (Remel Co, Lenexa, USA). It is a rapid identification method for the identification of Enterobacteriaceae members.

2.3 Antibiotic susceptibility testing

The susceptibility of all identified bacterial strains to 4 different β-lactams was done by the Kirby Bauer disc diffusion method. Bacterial isolates were plated on the Mueller Hinton agar to check their susceptibility was tested according to the Clinical and Laboratory Standard Institute's guidelines (CLSI) [15]. β-lactam antibiotics include Ertapenem (10µg), Ceftazidime (30µg), Cefotaxime (30µg), and Amoxicillin/Clavulanic acid (30µg). Resistant bacterial isolates were selected and enriched in Tryptone Soya Broth (TSB) at 37ºC for 24 hours. DNA was extracted from samples by using the Phenol-Chloroform Procedure.

Table 1: Primers used in PCR for the detection of ESBLs genes

Gene or Target region	Primers	Amplicon size (bp)	Annealing Temperature	References
bla*TEM*	**TF**-ATTCTTGAAGACGAAAGGGCCT **TR**-TTGGTCTGACAGTTACCAATGC	1100	51°C	[17]
blaSHV	**SF**-ATGAGTTATATTAGAATGGT **SR**-GTTAGCGTTGCCAGTGCTCG	860	50°C	[18]
bla*CTX-M*	**CF**-CGCTTTGCGATGTGCAG **CR**-ACCGCGATATCGTTGGT	550	51°C	[19]

2.4 PCR Amplification

Polymerase Chain Reaction was performed using specific primers for bla*TEM*, bla*SHV*, and bla*CTX-M* genes described elsewhere [16] with slight modifications in cyclic conditions. The primers used in PCR assays are listed in Table 1

3. RESULTS

3.1 Prevalence of Enterobacteriaceae

A total of 200 fecal samples were collected from sheep and Goats. From each sample, 4 bacterial colonies were selected so we had 800 bacterial colonies. All bacterial isolates were Oxidase negative. In Gram's staining technique, organisms were found as Gram-negative bacilli identifying as Enterobacteriaceae members.

A total of 5 bacterial species belonging to the family Enterobacteriaceae was obtained from fecal samples. These include: *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae,* and *Citrobacter freundii* (Table 2)

Table 2: Number and percentage of Enterobacteriaceae members isolated from fecal samples of farm animals

Bacterial Isolates	Sheep Total No. (%)	Goat Total No. (%)
E. coli	352 (88)	354 (88.5)
K. pneumoniae	28 (7)	20 (5)
E. aerogenes	4 (1)	8 (2)
E. cloacae	4 (1)	8 (2)
C. freundii	12 (3)	10 (2.5)
Total isolates	400	400

3.2 Antimicrobial susceptibility testing

The antibiotic susceptibility pattern of bacterial isolates from fecal samples of farm animals is presented in Table 3. Bacterial isolates from sheep fecal samples showed high resistance against Cefotaxime and the highest susceptibility against Ertapenem. In the case of goat samples, the highest susceptibility is also seen against Ertapenem.

3.3 PCR Amplification

Resistant bacterial isolates were selected for PCR. The number of resistant bacterial isolates: Sheep n=42 and Goats n=54. PCR screening for the genes bla$_{TEM}$, bla$_{SHV}$, and bla$_{CTX-M}$ confirmed ESBLs producing bacterial strains from the Enterobacteriaceae family. CTX-M was the most common ESBL type, followed by the SHV.

Table 3: Antibiotics susceptibility profile of Enterobacteriaceae isolates (n=800) from fecal samples of farm animals.

Antibiotics	Sheep n=400			Goat n=400		
	R	I	S	R	I	S
Cefotaxime (CTX)	22	24	354	54	54	292
Ertapenem (ETP)	0	4	396	0	16	384
Amoxicillin/Clavulanic acid (AMC)	18	4	378	6	28	366
Ceftazidime (CAZ)	2	8	390	26	36	338

R: resistant, I: intermediate, S: susceptible

Table 4: Distribution of ESBLs types in resistant bacterial isolates from fecal samples

ESBLs families	Sheep No. of isolates/Total	Goat No. of isolates/Total
CTX-M	12/42	13/54
SHV	6/42	8/54
TEM	0/42	0/54

4. DISCUSSION

ESBLs producing Enterobacteriaceae of human and animal origin are increasing day by day which is a matter of concern for both medical and veterinary practitioners around the world. The emergence and spread of ESBLs producing bacteria seem to be caused by excessive use of β-Lactams. Around the world, many investigations have been conducted to investigate the number, presence, and different types of ESBLs in farm animals [9–12, 20]. But in Pakistan research on ESBLs especially in farm animals is limited. So, our study proves to be beneficial concerning the use of β-Lactam antibiotics and their consequences to the development of resistant bacterial strains in the natural environment.

In the current study, resistance is reported in Enterobacteriaceae isolates against β-Lactams. Most of the isolates showed resistances as 5 different types of bacteria: *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae* and, *Citrobacter freundii* were isolated from fecal samples of sheep and goats. Over 40% of bacterial isolates showed resistance to β-Lactams including Cefotaxime (CTX), Ertapenem (ETP), Amoxicillin/Clavulanic acid (AMC), and Ceftazidime (CAZ). In our study, we observed a resistance pattern of 12% for AMC, 38% for CTX, and 14% for CAZ. In Pakistan, a similar type of study was conducted by [21]in food-producing animals. They reported resistance patterns as 80% for AMC,

74% for CAZ, and 69% for CTX [22]. In our findings, we recovered a higher percentage of E.coli from farm animals as compared to already published data in America. A common thing between both studies was the presence of 100% Gram-negative bacteria [23].

All the isolates which show antimicrobial resistance in our study were screened to rule out the ESBLs production. ESBLs is a superbug that is creating problems for public health and the food industry. Our finding showed that almost 40% of resistant bacterial isolates were identified as ESBLs producing Enterobacteriaceae. The most prevalent ESBLs genes detected in our study were CTX-M 26% followed by SHV 14%. Other phenotypically confirmed ESBL isolates do not have ESBL encoding genes (CTX-M, SHV, and TEM). These isolate probably producing other ESBLs enzymes. [24]% prevalence of ESBLs producing Enterobacteriaceae, and among these in 28.9% isolates CTX-M ESBL was detected [24,.25]Enterobacteriaceae isolates from farm animals in Spain and found 27% isolates with ESBLs-producing enzymes [25].

In Pakistan, the frequent and excessive use of B-Lactams in commercial farming plays a major role in the occurrence of resistance in bacteria by producing Extended Spectrum Beta Lactamases. Though, we cannot generalized the results of the study as we have analyzed limited samples obtained from rural areas of Southern Punjab, Pakistan. We can assume that actual resistant bacteria might be much higher in number. In our study, CTX-M comes out as the predominant genotype. Our results are in agreement with similar studies from Pakistan [26], Indian [27], and China [28, 29].

In conclusion, our study shows that ESBLs producing Enterobacteriaceae are dominant in different farm animals in Punjab, Pakistan. Additional studies using broader populations from other areas of Pakistan should be conducted to better understand the epidemiology of ESBLs in farm animals. Furthermore, farmers and veterinary practitioners should be informed and encouraged to be prudent in the use of antibiotics for farm animals.

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