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RESEARCH ARTICLE
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Multi-drug resistance among Shiga toxin producing Escherichia coli isolated from bovines and their handlers in Jammu region, India

Majueeb U Rehman[1], Mohd Rashid[1], Javeed Ahmad Sheikh[1], Shakil Ahmad Wani[2] and Shaheen Farooq[2]

1. Division of Veterinary Public Health and Epidemiology, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu (SKUAST-J), J&K, 181102, India; 2. Division of Veterinary Microbiology and Immunology, Sher-e-Kashmir University of Agricultural Sciences and Technology- Kashmir (SKUAST-K), J&K, 190001, India.
Corresponding author: Majueeb U Rehman, email: mujeebvpe@gmail.com
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Abstract

Aim: The objective of this study was to determine the antibiotic resistance pattern of Shiga-toxin producing *Escherichia coli* (STEC) in bovines and their handlers.

Materials and Methods: Of the total of 126 *E.coli* isolates screened by multiplex PCR for the presence of Shiga-toxin genes, 15 STEC isolates were obtained comprising of 9 isolates from cattle, 3 from buffaloes, and 3 from bovine handlers, which were tested for their antibiotic sensitivity/ resistance pattern to various antibiotics.

Results: Twelve of the 15 STEC isolates (80%) showed resistance to three or more antibiotics. Chloramphenicol was the most effective with 86.6% sensitivity, followed by Norfloxacin (80%), Ciprofloxacin (73.3%), and Co-trimoxazole (73.3%). Whereas 66.6% of the STEC isolates were resistant to Amikacin and Ampicillin, the other 60% were resistant to Amoxycillin, Cefixime, and Kanamycin.

Conclusion: Multiple drug resistance patterns among the STEC in the present study, especially high resistance to the frequently used antibiotics in both bovines and their handlers, implies that antibiotic resistance is often acquired due to their indiscriminate use; thereby, creating a need for rational and judicious use of antibiotics in the field.

Keywords: antibiotic sensitivity pattern, bovines, bovine handlers, multi-drug resistance, STEC,

Introduction

Antimicrobials have served variety of purposes, including treatment of infections, prophylaxis, and growth promotion. However, their widespread use in both animals and humans has led to several undesirable consequences; the most important among these is the antibiotic resistance in bacteria [1]. The indiscriminate and uncontrolled use of antimicrobial agents in animals exerts a selection pressure and encourages the proliferation of drug resistant strains of food borne pathogens in animal populations which may be transmitted to humans from the environment or through food [2]. Due to poor environmental sanitation and low personal hygiene, the situation could endanger public health. Since the last decade Shiga toxin producing *E. coli* (STEC) strains have emerged as important food-borne zoonotic pathogens, particularly the serotype O157: H7, which is the most common cause of haemorrhagic colitis and haemolytic uraemic syndrome in humans [3]. Ruminants, especially cattle, have been identified as the major reservoirs of STEC and also serve as the major source of human infections [4]. STEC exhibit antibiotic resistance due to the presence of plasmid carrying genes for drug resistance. Class 1 integrons located on mobile plasmids have facilitated the emergence and dissemination of antimicrobial resistance among STEC in humans and food animals [5]. These antimicrobial-resistant Shiga toxin-producing *E.coli* in food-producing animals can pose a global public health hazard because of potential transmission from animals to humans. In view of the importance of STEC as an emerging zoonotic pathogen, it has been the subject of several studies including their resistance to antibiotics. In India antibiotic resistance of STEC has been reported including multi drug resistance in both animals [6,7] and humans [8,9] from various parts of India, but there is paucity of information on this aspect in Jammu region. Relatively less information exists about antibiotic resistance of STEC in cattle and buffalo handlers of the region.

With this concern, this study was undertaken to determine the antibiotic resistance pattern of Shiga toxin-producing *E.coli* isolated from bovines as well as their handlers of the area.

Materials and Methods

Sample collection: The study samples comprised of a total of 103 faecal samples from bovines collected per rectally which included 60 samples from cattle and 43 from buffaloes collected from Cattle farm, Belicharana and Cattle farm, Faculty of Veterinary Science,

Table-1. List of primers (5'-3') used in the mPCR reaction.

Primer	Sequence (5'-3')	Amplicon size	Reference
stx1-F	ATAAATCGCCATTCGTTGACTAC	180 bp	Paton and Paton [8]
stx1-R	AGAACGCCCACTGAGATCATC		
stx2-F	GGCACTGTCTGAAACTGCTCC	255 bp	
stx2-R	TCGCCAGTTATCTGACATTCTG		
eaeA-F	GACCCGGCACAAGCATAAGC	384 bp	
eaeA-R	CCACCTGCAGCAACAAGAGG		
hlyA-F	GCATCATCAAGCGTACGTTCC	534 bp	
hlyA-R	AATGAGCCAAGCTGGTTAAGCT		

R.S.Pura, as well as from the villages of Sidher, Khanachak, and Kotli, R.S.Pura in Jammu region. From bovine handlers a total of 70 samples were taken comprising of 27 stool samples and 43 fingertip rinse samples. Stool samples and fingertip rinses were collected from the persons who handled the cattle and buffaloes at these places.

Multiplex polymerase chain reaction for the isolation of STEC: A total of one hundred twenty six *E. coli* isolates comprising of fifty one *E. coli* isolates from cattle, thirty five isolates from buffaloes and fourty isolates from bovine handlers were subjected to multiplex PCR (mPCR) for the detection of Shiga-toxin producing (*stx*) genes [10]. Primers used in the study are listed in Table-1.

The template DNA was prepared from *E. coli* isolates first revived in Mac Conkey agar to obtain fresh isolates. 100 µl of nuclease free water was taken in a separate micro centrifuge tube and a loopful of each isolate was mixed with it thoroughly. The suspended isolates in the micro centrifuge tubes were then subjected to heat lysis by keeping in boiling water for 10 minutes. Then the micro centrifuge tubes were quickly placed in ice for 10 minutes and centrifuged at 10000 rpm for 10 minutes. Two µl of the supernatant was then taken as template DNA for mPCR. The mPCR was carried out in a final reaction volume of 25µl using 0.2 ml thin wall sterile and nuclease free PCR tubes (Eppendorf, Germany). The PCR mixture contained a final concentration of 2 mM $MgCl_2$, 0.6 mM concentrations of each 2`-deoxynucleoside 5`-triphosphate (dNTPs), 5 µl of 5X assay buffer, 0.5µl of forward and reverse primers, 2.0µl template DNA and 1.0 U of GoTaq DNA Polymerase (Promega Corporation, Madison, U.S.A). PCR was performed in a Thermocycler (Applied Biosystems Gene Amp PCR System 2400) with heated lid using the steps and cycle conditions as: initial denaturation at 95°C for 2 minutes followed by 15 cycles; each cycle consisting of denaturation at 95°C for 1 minute, annealing at 65°C for 2 minutes and extension for 1.5 minutes at 72°C. A second phase of 20 cycles was followed with each cycle consisting of denaturation for 1 minute at 95°C, annealing at 60°C for 2 minutes and extension for 2 minutes at 72°C. A final extension was done at 72°C for 5 minutes. The PCR product was analysed by agarose gel electrophoresis for the amplicon sizes of 180 bp, 255 bp, 384 bp and 534 bp.

Antibiotic sensitivity test of STEC isolates: All the STEC isolates obtained from cattle, buffaloes and bovine handlers were tested for their antibiotic sensitivity/ resistance pattern to various antibiotics by disc diffusion technique [11]. The antibiotic discs (Hi Media Laboratories Pvt. Ltd. Mumbai) used were Amikacin (AK) 30 µg, Ampicillin (AMP) 10µg, Amoxicillin (AMX) 10µg, Cefotaxime (CTX) 30µg, Cefixime (CFM) 5 µg, Chloramphenicol (C) 30µg, Cefuroxime (CXM) 30µg, Ciprofloxacin (CIP) 5µg, Co-trimoxazole (COT) 25µg, Gentamicin (GEN) 10µg, Nalidixic acid (NA) 30µg, Norfloxacin (NX) 10µg, Tetracycline (TE) 30µg, Kanamycin (K) 30µg and Streptomycin (S) 10µg. Inoculum for culture and sensitivity test (CST) was prepared by inoculating 3-4 colonies of STEC in 5 ml nutrient broth and incubated at 37°C for 4 hours till light to moderate turbidity develops. Plates of Mueller Hinton Agar (MHA) (Hi-Media, Mumbai) were seeded with about 100 µl of inoculum using sterile cotton swabs. The inoculated plates were allowed to dry. Antibiotic discs were placed on inoculated agar surface about 2 cm away from one another. The plates were incubated at 37°C for 16-18 hours and diameter of the zones of inhibition were measured and interpreted based on the manufacturer's interpretative chart.

Results and Discussion

Of the total 126 isolates subjected to mPCR (Figure-1), a total of fifteen *E. coli* isolates revealed the presence of Shiga-toxin producing (*stx*) genes; nine isolates were obtained from cattle, three from buffaloes and three from bovine handlers. Twelve of the 15 STEC isolates (80%) showed resistance to three or more antibiotics (multi drug resistance). Chloramphenicol was the most effective with 86.6% sensitivity followed by Norfloxacin with 80% sensitivity observed among the isolates. Further, 73.3% of the STEC isolates were sensitive to Ciprofloxacin and Co-trimoxazole, 60 % to Gentamicin, 53.3% to Nalidixic acid and 46.6% to Cefuroxime (Table-2).

Among STEC isolates from cattle, Chloramphenicol was the most effective with 88.8% sensitivity while Amikacin was the most resistant with 88.8% resistance observed among the isolates. Further, 77.7% of the STEC isolates were sensitive to Co-trimoxazole and Norfloxacin, 66.6% to Ciprofloxacin and 55.5% to Gentamicin and Nalidixic acid. Three of the four STEC isolates from cattle possessing stx_1, stx_2, and *hlyA* genes

Table-2. Antibiotic sensitivity/sesistance pattern of STEC isolates (n=15) from bovines and their handlers.

Sr.No.	Antimicrobial Agent	No. of STEC isolates		
		Sensitive	Intermediate	Resistant
1	Amikacin, 30µg	2 (13.3)	3 (20)	10 (66.6)
2	Ampicillin, 10 µg	1 (6.6)	5 (33.3)	9 (60)
3	Amoxicillin, 10µg	1 (6.6)	4 (26.6)	10 (66.6)
4	Cefotaxime, 30µg	2 (13.3)	9 (60)	4 (26.6)
5	Chloramphenicol, 30µg	13 (86.6)	1 (6.6)	1 (6.6)
6	Ciprofloxacin, 5µg	11 (73.3)	1 (6.6)	3 (20)
7	Co-trimoxazole, 25µg	11 (73.3)	0 (0)	4 (26.6)
8	Cefixime, 5 µg	4 (26.6)	2 (13.3)	9 (60)
9	Cefuroxime, 30µg	7 (46.6)	4 (26.6)	4 (26.6)
10	Gentamicin, 10 µg	9 (60)	5 (33.3)	1 (6.6)
11	Nalidixic acid, 30µg	8 (53.3)	2 (13.3)	5 (33.3)
12	Norfloxacin, 10 µg	12 (80)	0 (0)	3 (20)
13	Tetracycline, 30µg	3 (20)	5 (33.3)	7 (46.6)
14	Kanamycin, 30 µg	1 (6.6)	5 (33.3)	9 (60)
15	Streptomycin, 10 µg	3 (20)	8 (53.3)	4 (26.6)

Figures in parenthesis indicate percentage out total number of STEC isolates

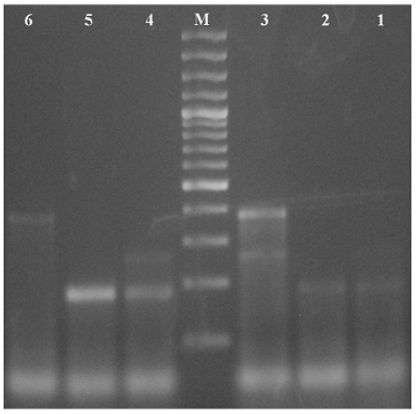

Figure-1. Agarose gel showing mPCR amplification products of stx_1, stx_2 and *eaeA* genes.
Lane M: 100 bp molecular weight marker,
Lane1 & 2: Amplified product of stx_1 gene,
Lane 3: Amplified products of stx_2 and *eaeA* genes,
Lane 4: Amplified products of stx_1 and stx_2 genes,
Lane 5: Amplified product of stx_1 gene,
Lane 6: Amplified product of *eaeA* gene

were resistant to three or more antibiotics while the fourth isolate was resistant only to Amoxicillin. One isolate carrying only stx_1 gene was resistant only to Amikacin. Further, all the other four STEC isolates; two isolates carrying stx_1, stx_2, *eaeA* and *hlyA* genes and the other two with stx_2, *eaeA* and *hlyA* genes were multi-drug resistant. Two of the three STEC isolates from buffaloes with virulence gene profile as stx_1, stx_2, *hlyA* and stx_1, stx_2 showed sensitivity to Chloramphenicol, Ciprofloxacin, Co-trimoxazole, Gentamicin and Norfloxacin. However, the former showed resistance only to Ampicillin while as the latter was resistant to Ampicillin, Amoxycillin, Cefotaxime, Streptomycin and Cefuroxime. Also one isolate bearing stx_1, stx_2, *eaeA* and *hlyA* genes was resistant to most of the antibiotics tested. All the three STEC isolates from bovine handlers were resistant to Ampicillin, Amoxycillin and Amikacin and sensitive to Chloramphenicol, Ciprofloxacin and Norfloxacin. Two of these isolates one with stx_2 gene and another possessing stx_2 and *eaeA* genes were sensitive to Gentamicin also, and the isolate with stx_1, stx_2 and *hlyA* genes was sensitive to Nalidixic acid.

Multi drug resistance of STEC isolates including high resistance to Amikacin (80%), Ampicillin (73%) and Tetracycline (63%) from calves has earlier been reported from Gujarat state of India [6]. Similar resistance to Tetracycline (32%) and Streptomycin (29%) was also observed in Spain both from cattle and humans, however, lower resistance was observed with Ampicillin (10%), Co-trimoxazole (8%) and Kanamycin (7%) [12]. High resistance among STEC isolated from diverse sources has also been reported in other studies [13,14]. However, resistance of STEC to Ampicillin, Gentamicin, Kanamycin, Nalidixic acid, Tetracycline, Streptomycin and Cefuroxime isolated from humans [15] and Ampicillin, Gentamicin and Kanamycin isolated from cattle in Japan [16] were lower compared to our results. Lower resistance of STEC to Amikacin and Gentamicin from cattle and with Ampicillin, Amoxicillin, Cefuroxime, Gentamicin and Tetracycline from humans was also recorded in Brazil and Switzerland respectively [17,18]. These differences could be due to varied genetic mechanisms that lead to bacterial resistance and their spread in bacterial population enabled by highly efficient transfer system of mobile genetic elements [19]. Also, during the recent years, the importance of integrons for the dissemination of resistance in *E. coli* has been established [5].

Resistance to ampicillin and amoxycillin observed in the present study might be attributed to the production of β-lactamase group of penicillin destroying enzymes. These enzymes are chromosome or plasmid borne, and may be constitutive or inducible [20]. Resistance to aminoglycosides is due to aminoglycoside modifying enzymes e.g., amikacin

resistance in *E.coli* is associated with aminoglycoside phosphotransferase coded by a transferable plasmid-borne gene [21]. Rapid transferability of extended spectrum β-lactamase (ESBL) genes among *E. coli* including STEC by conjugation may account for resistance to cephalosporins [22]. High resistance of STEC isolates especially to ampicillin, amikacin, cefixime and tetracycline observed in the present study may be due to extensive use of these antibiotics in farm animals or humans which exerts a selection pressure on microbes encouraging the growth of resistant ones.

Conclusion

In the present study, multi-drug resistance pattern (resistance to atleast three antibiotics) was found among eighty percent of the STEC isolates. However, high sensitivity of STEC isolates to Chloramphenicol, Norfloxacin and Gentamicin was observed. This resistance pattern among the STEC especially high resistance to the frequently used antibiotics in both bovines and their handlers implies that resistance is often acquired due to their indiscriminate use, thereby, creating a need towards rational and judicious use of antibiotics in the field.

Authors' contributions

MUR designed the study, drafted and revised the manuscript. MR and JAS contributed in collection, analysis and processing of samples. SAW and SF assisted in molecular work. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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