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Characterization of shiga-toxin producing *E.coli* (STEC) and Enteropathogenic *E. coli* (EPEC) from domestic animals for virulence and colonisation factors and their antimicrobial resistance

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Abstract

Aim: To determine the occurrence and molecular characterization of Shiga toxin-producing *E. coli* (STEC) and Enteropathogenic *E. coli* (EPEC) from different domestic animals for serogrouping, virulence factors and their antibiotic resistance from Jammu region.

Methodology: Faecal samples were collected from 200 different animals including calves (39), rabbits (24), dogs (38), slaughtered pigs (22), poultry (41), sheep and goat (36). All the 200 strains were screened for the presence of *stx1*, *stx2* and *eae* genes by m-PCR and screening of confirmed EPEC isolates with respect to their serogroup, virulence factors (*bfpA*, *astA* and *ecpA* genes) by PCR and antibiotic resistance.

Results: Of total 200 *E. coli* isolates, 38 (19.00%) were detected as EPEC and 28 (14.00%) as STEC. 5 (12.82%), 17 (43.58%) isolates from calves and 12 (33.33%), 11 (30.55%) isolates from sheep were found as EPEC and STEC, respectively. In rabbits, pigs and dogs, 6 (25.0%), 10 (45.45%) and 5 (13.15%) isolates were detected as EPEC, respectively. The most predominant EPEC serogroups were O88 (36.84%), O118 (18.42%). All 38 EPEC (100%) isolates carried *ecpA* gene and 18 (47.36%) isolates carried *astA* gene. Of 38 EPEC, only 5 (13.15%) isolates from dog carried *bfpA* gene, therefore considered as typical EPEC and 33 (86.84%) isolates were designated as atypical EPEC. Antimicrobial sensitivity test (AST) of 38 EPEC isolates showed resistance to nalidixic acid (55.26%), kanamycin (42.10%) followed by streptomycin (42.10%), doxycycline hydrochloride (28.94%), ciprofloxacin (13.15%), tetracycline (13.15%). Surprisingly, all the 38 isolates were sensitive to ampicillin.

Keywords: AST, EPEC, STEC, *bfpA*, *astA*, *ecpA*

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) are commonly recovered from the faeces of food-producing animals and pose threats to health of humans and livestock [29]. Healthy cattle, sheep, and pigs appear to be important reservoirs of EPEC but they have also been implicated as causative agent of diarrhoea in number of animal species, including rabbits, pigs, cattle and dogs [23]. STEC strains carry genes encoding Shiga toxins and may possess other virulence genes for intimin and enterohemolysin [40]. EPEC isolates are defined as intimin-containing diarrhoeagenic *E. coli* isolates that possess the ability to form attaching and effacing (AE) lesions on intestinal cells and do not possess genes coding for Shiga toxin. This protein called 'intimin' is encoded by *eae* gene which is used for the molecular diagnosis of EPEC *E. coli* strains. Multiple steps are involved in producing the characteristic A/E histopathology first of which is localized adherence, which is dependent on the presence of a plasmid designated the EPEC adherence factor (EAF) plasmid. This plasmid encodes a type IV pilus called the 'bundle-forming pilus (BFP)' whose structural gene is referred as *bfpA*. All those EPEC which carry EPEC adherence factor (EAF) are called typical EPEC, whereas, atypical EPEC contain the locus of enterocyte effacement (LEE), but do not contain the EAF plasmid. There are additional potential virulence factors present in EPEC. Some EPEC strains produce a low-molecular-weight Enterotoxigenic heat-Stable Toxin 1 (EAST1) encoded by *astA* gene [38]. Similarly, there are reports of additional fimbrial structures produced by EPEC, one among them is *E. coli* common pilus (ECP), which is

composed of a major pilin subunit encoded by the *ecpA* gene [20]. Over 200 O serogroups have been identified among EPEC strains. The 12 serogroups originally recognized by the World Health Organization as EPEC or the classical EPEC are O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 [19].

There are several reports of detection of EPEC and STEC from different animal species in India. But detailed study for their virulence factors and colonization factors is lacking. So it becomes essential to characterize the EPEC isolates with respect to their serogroup, accessory virulence attributes like *bfpA*, *astA* and *ecpA* genes and antibiotic resistance.

Materials and methods

Sample collection

Faecal samples were collected from 200 different animals including calves [39], rabbits [24], dogs [38], slaughtered pigs [22], poultry [41], sheep and goat [36] from Jammu region during the period from March 2016 to April 2017. The samples included rectal swab from live animals and intestinal swabs from slaughtered animals. These samples were transported to the laboratory on ice, where experimental works were carried out. The samples were processed immediately or stored at 4°C to be processed later on.

Isolation of *E. coli*

All the samples were inoculated on MacConkey's agar plates (HiMedia, India) and incubated for 24 hrs at 37°C. At least 3 well isolated pink colonies were randomly picked from each

plate and sub-cultured on eosin methylene blue (EMB) agar (HiMedia, India) to observe the characteristic green metallic sheen of *E. coli*. Well separated colonies from EMB agar plates were streaked on nutrient agar slants as pure culture and subjected to standard morphological and biochemical characterization as described by Buchanan and Gibbon (1994). All *E. coli* isolates from nutrient agar slants were characterized on the basis of biochemical tests with HiMotility *E. coli* test kit (HiMedia, India Cat#KBM001). One *E. coli* isolate selected randomly from each faecal sample were further subjected to IMViC test following the standard procedures to confirm their identity.

Extraction of bacterial DNA

All the presumptive *E. coli* isolates were subjected to the molecular characterization. The DNA was isolated by snap and chill method which includes boiling of colonies suspended in distilled water for 10 min to release DNA, cooled on ice for 10 min and centrifuged at 10,000×g in a for 1 min. About 2 µL of the supernatant was used as the template for polymerase chain reaction.

Molecular screening of *E. coli* isolates for *stx1*, *stx2* and *eae* genes

All the confirmed *E. coli* isolates were subjected to molecular screening by multiplex PCR (m-PCR) for *stx1*, *stx2* and *eae* genes using specific primers (Table 1). About 10 microlitres of PCR product was electrophoresed in a 2% (w/v) agarose gel for 1 hr at 5 V/cm with a Standard molecular weight marker

Table 1: List of primer sequences and predicted amplicon length

Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
Stx1 F Stx1R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCCACTGAGATCATC	<i>stx1</i>	180	(30)
Stx2 F Stx2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	<i>stx2</i>	255	(30)
<i>eae</i> F <i>eae</i> R	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	<i>eae</i>	384	(30)
EAST11a EAST11b	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	<i>astA</i>	111	(42)
<i>ecpA</i> F <i>ecpA</i> R	GCAACAGCCAAAAAAGACACC CCAGGTCGCGTCAACT	<i>ecpA</i>	477	(20)
EP1 EP2	AATGGTGCTTGCGCTTGCTGC GCCGCTTATCCAACCTGGTA	<i>bfpA</i>	326	(18)

Molecular screening of EPEC for virulence factors *bfpA*, *astA* and *ecpA*

All the *E. coli* isolates carrying *eae* gene and devoid of *stx1* and *stx2* were declared as EPEC and screened for *bfpA* as per Gunzburg *et al.* (1955), *ecpA* as per Hernandez *et al.* (2011) and *astA* as per Yamamoto and Echeverria (1996) using specific primers (Table 1). The amplified product of *bfpA* and *ecpA* was electrophoresed in a 1% (w/v) agarose gel and *astA* amplicons in a 2% (w/v) agarose gel for 1 hr at 5 V/cm with a Standard molecular weight marker.

Serogrouping of *E. coli* Isolates

All the *E. coli* isolates carrying one or more of the virulence genes screened were serogrouped on the basis of their O antigen by the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (H.P) - 173204 (India).

Antimicrobial susceptibility testing

The EPEC isolates were tested for antimicrobial susceptibility

against 12 antibiotics using disc diffusion technique [5] following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). Antimicrobial discs (HiMedia Pvt. Ltd., India) used were ampicillin (Amp 10 µg); cefixime (Cfm 5 µg); chloramphenicol (C 30 µg); ciprofloxacin (Cip 5 µg); doxycycline hydrochloride (Do 30 µg); kanamycin (K 30 µg); minocycline (MI 30 µg); nalidixic acid (NA 30 µg); norfloxacin (NX 10 µg); streptomycin (S 10 µg); tetracycline (TE 30 µg); and trimethoprim (TR 5 µg).

Results and Discussion

Isolation of presumptive *E. coli*

A total of 560 presumptive *E. coli* isolates were obtained from 200 faecal samples (Plate 1). Out of these 560 bacterial isolates, only one isolates from each sample showing the biochemical characteristics of *E. coli* on HiMotility *E. coli* test kit were selected for further processing. The identity of the selected isolates were further confirmed by +ve, +ve, -ve and -ve IMViC pattern as depicted in plate-2.

Plate 1: Isolation of ESBL producing *E. coli* on MLA and EMB agar.

Plate 2: IMViC pattern of *E. coli* isolates from faecal samples. The isolates show positive test results for indole production and methyl red test while negative results for Voges-Proskauer and citrate utilization test.

Detection of EPEC and STEC

All the 200 *E. coli* isolates, one each from each faecal sample, were subjected to molecular screening for *stx1*, *stx2* and *eae* genes by multiplex PCR. Isolates carrying *stx1* gene produced an amplicon of 180 bp, those carrying *stx2* showed an

amplicon of 255 bp and the presence of *eae* gene was detected by the amplification of 384 bp product. The species wise relative distribution of these genes is depicted in table 2 and the representative gene profiles are depicted in plate-3.

Table 2: Virulence gene profiles of *E. coli* isolates from different animal species

S. No.	Species	No. of Isolates	<i>stx1</i>	<i>stx2</i>	<i>eae</i>
1	Rabbit	6	-	-	+
2	Pig	10	-	-	+
3	Calf	5	-	-	+
4	Calf	8	+	+	-
5	Calf	2	-	+	-
6	Calf	6	+	-	+
7	Calf	1	+	-	-
8	Dog	5	-	-	+
9	Sheep	7	+	+	-
10	Sheep	1	-	+	-
11	Sheep	1	+	+	+
12	Sheep	2	+	-	-
13	Sheep	12	-	-	+
Total		66	25	19	45

In the present study, only 5 (12.82%) isolates out of 39 *E. coli* isolates from calves carried *eae* gene alone and thus the carriage of 12.82% EPEC is comparable to the 10.4% in Germany but higher than 2.7% in Sao Paulo, Brazil [1] and 5.83% reported in Dublin, Ireland [11]. However, higher percentage (41%) was reported by Waltner-Toews *et al.* (1986) from Ontario. In case of sheep 12 (33.33%) isolates out of 36 *E. coli* isolates carried *eae* gene alone. The previous studies have shown that a high percentages of sheep were colonized with EPEC [13, 23]. Aktan *et al.* (2004) from England detected EPEC in less than 17.7% of the sheep studied, while in present study 33.33% of sheep harboured EPEC. However, higher percentage (55%) was reported by Frohlicher *et al.* (2008).

Out of 24 *E. coli* isolates from rabbits, 6 (25.0%) isolates were detected as EPEC. However their carriage rate in healthy animals is lacking. In our study 25% of rabbits carried EPEC, which is comparatively less than that of 74% and 83% as reported by Blanco *et al.* (1997) from Spain and Swennes *et al.* (2012) from USA, in diarrhoeic rabbits, respectively. Similarly in pigs, 10 (45.45%) isolates out of 22 turned out to be EPEC. Porcine EPEC are associated with post weaning diarrhoea in pigs (44). However in Hungary, Malik *et al.* (2006) found no difference in the frequency of occurrence of *eae+* *E. coli* in diarrhoeic and non-diarrhoeic pigs. In the present study, EPEC was detected in 45.45% of healthy pigs. There are few reports on pigs as a reservoir of EPEC and in one study by Frohlicher *et al.* (2008) the proportion of *eae+* *E. coli* samples in pigs was 89%. This is quite high when

compared with the proportion of 45.45% in our study. But De la Fuente *et al.* (2002) reported less than 20%; Aktan *et al.*, 2004 from England and Wales reported 0.75%, Krause *et al.*, 2005 from Germany reported 17.6%, which were lower than our study. On the same pattern, out of 38 *E. coli* isolates from dogs, 5 (13.15%) carried *eae* gene alone. EPEC strains with virulence properties similar to human strains are frequently isolated from diarrhoeic and healthy dogs [16]. The shedding rate of 13.15% of EPEC in dogs in the present study is, comparable to that of 12.6% reported from Brazil [28], but higher than 7.2%, reported from Germany [23] and 5.5% reported by Mainil *et al.* (1998). From poultry, 41 isolates were screened for these three genes and none of them carried any gene.

In the present study, out of all the domestic animals studied, STEC could be isolated from only 17 (43.58%) isolates of calves and 11 (30.55%) isolates of sheep. The carriage of 43.58% STEC in calves supported the findings of other workers, who showed that calves are possible reservoir of STEC in their gastrointestinal tract [17]. The frequency, detected in the present study is comparable to the 44% in Brazil [27], 46% in Japan [22] reported for calves. These variations are likely due to geographical differences. It has been shown that the environment may have an influence on the shedding of STEC in calves [37]. In the present study the carriage of STEC in sheep is 30.55% which is higher than 12.32% reported for lambs in Jammu and Kashmir state [7], but lower than the 68% and 88% found in lambs in Germany and Spain (6,10), respectively.

Plate 3: Representative *stx1*, *stx2* and *eae* genes profile of *Escherichia coli* isolates using multiplex polymerase chain reaction (m-PCR). Lane M 100bp DNA ladder. Lane 1, positive control. Lane 2, negative control. Lane 3, *stx1* positive. Lane 4, *stx2* positive. Lane 5, *stx1*, *stx2* positive. Lane 6, *eae* positive. Lane 7, *stx1*, *eae* positive. Lane 8, *stx1*, *stx2*, *eae* positive. Lane 9, *stx2*, *eae* positive.

Serogrouping and Virulence gene profile of EPEC

All the 38 EPEC isolates were serogrouped for somatic 'O' antigen at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, H.P. The most predominant serogroups were O88 (36.84%) and O118 (18.42%) followed by O149 (7.89%), O22 (5.26%) and O11 (5.26%). The different serogroups found in the study are depicted in table 3. In contrast to our study, the serogroup O88 was also isolated by Rehman *et al.* (2014) from Cattle in Jammu and by Frohlicher *et al.* (2008) from Pig in Switzerland. Where as the serogroups found in sheep and rabbit have not been reported yet. Nakazato *et al.* (2004) also

reported the isolation of *eae+* *E. coli* belonging to serogroup O11 from dogs in Brazil. All the EPEC isolates were screened for the presence of *bfpA*, *ecpA* and *astA* genes by PCR as depicted in plate 4 and their virulence profile is detailed in table 3. In dog all the isolated EPEC in the present study harboured *bfpA* gene which matches to the study reported by Goffaux *et al.*, 2000 and Nakazato *et al.*, 2004. A study by Salehi *et al.* (2010) from Iran indicated that not only diarrhoeic dogs but also healthy dogs act as potential reservoirs of EPEC and should be considered as a potential risk factor in transmission of diarrhoea to humans. The *ecpA* gene was reported from all the EPEC isolates in contrast to

Hernandes *et al.*, 2011. Several reports have previously shown that this gene is highly conserved among the different intestinal and extraintestinal *E. coli* pathotypes, including commensal strains [31, 34, 8, 3]. A recent gene search study with Brazilian aEPEC strains showed that *ecpA* was present in all strains displaying an LAL pattern on HEp-2 cells by Scaletsky *et al.*, 2010. In the present work, the distribution of the *astA* genes in a large collection of EPEC strains isolated from cattle, sheep, pig, dog and rabbit in Jammu was examined. In cattle *astA* gene was present in 80% of atypical EPEC isolates, which is comparable with 87% reported by De Sousa and Dubreuil (2001) from Canada. In sheep, *astA* gene was present in 16.6% of atypical EPEC isolates, this occurrence is much higher than 1.6% found by Yuste *et al.* (2006) in Spain, but lower (26.4%) as reported by Frohlicher *et al.* (2008) from Switzerland. Similarly, 80% (Eight out of Ten) *astA* gene was present in pig atypical isolates, which is 8% reported by Frohlicher *et al.* (2008) from Switzerland. In dogs *astA* gene was present in 80% (Four out of Five) EPEC isolates. Probably this is the first study of this kind as we do not have the reference to compare our results.

Table 3: Virulence gene profile and serogroup of EPEC from animals

Species	Serogroup	No. of Strains	Virulence genes		
			<i>bfpA</i>	<i>ecpA</i>	<i>astA</i>
Calves	O22	2	-	+	+
	O88	1	-	+	+
	O88	1	-	+	-
	O149	1	-	+	+
Sheep	O88	4	-	+	-
	O88	2	-	+	+
	O118	3	-	+	-
	UT	3	-	+	-
Pig	O88	4	-	+	+
	O118	4	-	+	+
	UT	2	-	+	-
Dog	O11	2	+	+	+
	UT	1	+	+	-
	UT	2	+	+	+
Rabbit	O88	2	-	+	-
	O149	2	-	+	-
	UT	2	-	+	-
Total		38	5	38	18

'+' Positive; '-' negative.

Plate 4: Representative *bfpA*, *ecpA* and *astA* genes profile of *Escherichia coli* isolates using multiplex polymerase chain reaction (m-PCR). Lane M 100bp DNA ladder. Lane 1, positive control. Lane 2, negative control. Lane 3, *bfpA* positive. Lane 4, *astA* positive. Lane 5, *ecpA* positive.

Antimicrobial susceptibility testing

The prevalence of antimicrobial resistance among the EPEC strains isolated from animals is shown in Table 4 and depicted in plate 5 and 6. Overall, 26 (68.42%) of the isolates were resistant to at least one of the 12 antibiotics tested, 12 (31.57%) had an intermediate susceptibility, and no isolate was pan-sensitive. In this study, the prevalence of tetracycline resistance in EPEC isolates was low. This is contrary to the results of Meyer *et al.* (2008), who reported higher rate of tetracycline resistance in *E. coli* isolated from animals. To our surprise all the isolates were sensitive to ampicillin. This is contrary to the findings of Hoyle *et al.* (2006), who observed wide spread ampicillin resistance among, *E. coli* isolates obtained from animal faeces living in organic farms even when the use antimicrobial agents is restricted. In our study

all of the EPEC isolates of calves were sensitive to chloramphenicol, ciprofloxacin and norfloxacin, which is in agreement with Rehman *et al.* (2014). However, in our study antibiotic kanamycin is resistant to all isolates of calves and dogs, which is comparable to the findings of Bolton *et al.* (2014) from Dublin, Ireland and Banik *et al.* (2016) from South Bengal, respectively. The in vitro sensitivity assay carried out to determine the antibiotic resistance profile of ovine *E. coli* isolates revealed that the isolates were sensitive to a range of antimicrobials like ampicillin, chloramphenicol, ciprofloxacin and nalidixic acid. However, intermediate resistance was found against antibiotics like kanamycin. Similar results were reported by Purkayastha *et al.* (2010) who reported sheep *E. coli* isolates to be sensitive to chloramphenicol, ciprofloxacin and nalidixic acid.

Table 4: Number of susceptible (S), intermediate (I), and resistant (R) strains of enteropathogenic *Escherichia coli* isolated from animals to 12 antimicrobial agents.

Antimicrobials	Calves(5)			Sheep(12)			Pig(10)			Dog(5)			Rabbit (6)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin	5	0	0	12	0	0	10	0	0	5	0	0	6	0	0
Cefixime	5	0	0	12	0	0	10	0	0	0	5	0	0	6	0
Chloramphenicol	5	0	0	12	0	0	10	0	0	5	0	0	0	6	0
Ciprofloxacin	5	0	0	12	0	0	10	0	0	0	0	5	0	6	0
Doxycycline hydrochloride	5	0	0	12	0	0	10	0	0	0	0	5	0	0	6
Kanamycin	0	0	5	0	12	0	10	0	0	0	0	5	0	0	6
Minocycline	5	0	0	12	0	0	10	0	0	0	5	0	0	0	6
Nalidixic acid	5	0	0	12	0	0	0	0	10	0	0	5	0	0	6
Norfloxacin	5	0	0	12	0	0	10	0	0	0	5	0	6	0	0
Streptomycin	0	5	0	12	0	0	0	0	10	0	5	0	0	0	6
Tetracycline	5	0	0	12	0	0	10	0	0	0	0	5	6	0	0
Trimethoprim	5	0	0	12	0	0	10	0	0	0	5	0	6	0	0

Figure in parenthesis indicates number of isolates

Plate 5: Antimicrobial sensitivity pattern of EPEC isolates against chloramphenicol (C 30 µg), ciprofloxacin (Cip 5 µg), doxycycline hydrochloride (Do 30 µg), kanamycin (K 30 µg), minocycline (MI 30 µg), trimethoprim (TR 5 µg). **Plate 6:** Antimicrobial sensitivity pattern of EPEC isolates ampicillin (Amp 10 µg), cefixime (Cfm 5 µg), nalidixic acid (N/A 30 µg), norfloxacin (NX 10 µg), streptomycin (S 10 µg), tetracycline (TE 30 µg)

Conclusion

This study concluded the role of dogs as a reservoir of EPEC and can play a crucial role in transmission of certain pathogens to humans and livestock due to faecal contamination in the environment. Meanwhile high proportion of EPEC and STEC in other healthy animal species may sow seed for future infections in humans around the globe. The study also reports, for the first time, the isolation and characterization of *bfpA* and *astA* gene in dog and EPEC serogroups O88, O118 from healthy sheep and rabbit in India.

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