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Research Article

MOLECULAR CHARACTERIZATION OF ENTEROPATHOGENIC *E. COLI* (EPEC) AND SHIGA-TOXIN PRODUCING *E. COLI* (STEC) FROM DOMESTIC ANIMALS: PREVALENCE, VIRULENCE, COLONIZATION FACTORS AND THEIR ANTIMICROBIAL RESISTANCE

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ABSTRACT: To ascertain the presence and molecular characterization of enteropathogenic and Shiga toxin *E. coli*, 200 fecal samples were collected from rabbits (24), slaughtered pigs (22), calves (39), poultry (41), dogs (38), sheep and goats (36). All the 200 isolates were screened using m-PCR for the presence of the eae, stx1, and stx2 genes. Additionally, the confirmed EPEC isolates were screened concerning their virulence factors (bfpA, astA and ecpA genes) and serogroup by PCR and antibiotic resistance. Of the 200 samples, 38 (19.00%) and 28 (14.0%) were found to be STEC and EPEC, respectively. It was discovered that 11 (30.55%), 12 (33.33%) isolates from sheep and 17 (43.58%), 5 (12.82%), isolates from calves, respectively, were STEC and EPEC 6 (25.0%), 10 (45.45%), and 5 (13.15%) isolates were found to be EPEC in rabbits, pigs, and dogs, respectively. The two most common EPEC serogroups were O118 (18.41%) and O88 (36.84%). Of the 38 EPEC isolates, 100% carried the ecpA gene, while 18 isolates (47.36%) took the astA gene. Among 38 EPEC isolates, only 5 (13.15%) dogs were found to have the bfpA gene, making them typical EPEC, while 33 (86.84%) isolates were classified as atypical EPEC. Thirty-eight EPEC isolates were tested for antibiotic sensitivity, and the results indicated that they were resistant to ciprofloxacin (13.15%), kanamycin (42.10%), streptomycin (42.10%), doxycycline hydrochloride (28.94%), and nalidixic acid (55.26%).

Keywords: EPEC, STEC, bfpA, astA, ecpA, m-PCR.

INTRODUCTION

Enteropathogenic *Escherichia coli* (ETEC) and Shiga toxin-producing *E. coli* (SPEC) are frequently found in the feces of food animals and pose greater threats to the health of human beings and livestock [1]. Although healthy sheep, cattle, and pigs appear to be important EPEC reservoirs, they have also been linked to diarrhea in several different animal species, including dogs, pigs, rabbits, and cattle [2]. STEC strains may also have additional virulence genes for intimin and enterohaemolysin in addition to carrying genes producing Shiga toxins [3]. EPEC isolates are described as intimin-containing diarrhoeagenic *E. coli* isolates that own the capacity to shape attaching and effacing (AE) lesions on intestinal cells and lack the genes that code for Shiga toxin. The eae gene encodes a protein known as "intimin," which is utilized in the molecular diagnosis of EPEC *E. coli* strains. Producing the distinctive A/E histology involves several processes, the first of which is localized adherence, which is reliant on the existence of a plasmid known as the EPEC adherence factor (EAF) plasmid.

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This plasmid codes for the type IV pilus known as ‘‘bundle-forming pilus (BFP),’’ whose structural gene is called bfpA. Typical EPEC have the EPEC adherence factor (EAF); whereas atypical EPEC carry the locus of enterocyte effacement (LEE) but not the EAF plasmid. EPEC contains other possible virulence factors. A low-molecular-weight entero-aggregative heat-stable toxin 1 (EAST1), which is encoded by the astA gene, is produced by certain EPEC strains [4]. The ecpA gene encodes a large pilin subunit, which is the basis of the *E. coli* common pilus (ECP), one of the numerous fimbrial structures produced by EPEC [5]. Among EPEC strains, more than 200 O serogroups have been found. The World Health Organisation initially identified 12 serogroups as EPEC, also known as the traditional EPEC, and they are O26, O55¸ O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 [6]. Reviews of EPEC and STEC detections from various animal species in India are numerous.

However, there hasn't been enough in-depth research done on their colonization and virulence variables. Characterizing the EPEC isolates in terms of their sero-group, accessory virulence traits such as the ecp, bfpA, and astA genes, and antibiotic resistance becomes crucial.

MATERIALS AND METHODS

Sample collection

Fecal samples from two hundred distinctive animals inclusive of calves (39), slaughtered pigs (22), rabbits (24), poultry (41), dogs (38), sheep, and goats (36) were collected from the Jammu region. Rectal swabs from live animals and intestinal swabs from animals that had been slaughtered were carried within a 2 to 3-hour period to the lab on ice where the experiments were conducted. The collected samples were either processed straightaway or kept at 4ºC for further processing.

Isolation of *E. coli*

MacConkey's agar plates were used to inoculate all of the samples, which were then cultured for 24 hours at 37°C. From each plate, at least three well-isolated pink colonies were chosen at random and sub-cultured on eosin methylene blue (EMB) agar (HiMedia, India) in order to see the distinctive green metallic sheen of *E. coli*. Pure cultures of well-separated colonies from EMB agar plates were streaked on nutrition agar slants and subjected to conventional morphological and biochemical characterization following the guidelines provided by earlier researchers [7]. All *E. coli* isolates from nutrient agar slants had been characterized on the premise of biochemical tests with a HiMotility *E. coli* test kit (HiMedia, India Cat#KBM001). Following standard techniques, an IMViC test was performed to identify a single *E. coli* isolate that was chosen at random from each fecal sample [29, 32].

Extraction of bacterial DNA

Molecular characterization was performed on all presumed *E. coli* isolates. The snap-and-chill approach was used to extract the DNA. To liberate the DNA, the colonies were boiled in distilled water for ten minutes, cooled on ice for ten minutes, and centrifuged at 10,000×g for one minute. A volume of roughly 2µL was utilized as the polymerase chain reaction template from the supernatant [8, 29, 32].

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

Multiplex PCR (m-PCR) for the stx1, stx2, and eae genes was performed on each confirmed *E. coli* isolate using particular primers (Table 1) [8]. 10 microlitres of the PCR product were electrophoresed with a standard molecular weight marker for one hour at 5 V/cm on a 2% (w/v) agarose gel.

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

All of the *E. coli* isolates with the eae gene and without the stx1 or stx2 were classified as EPEC and subjected to primer-specific screening for bfpA [10], ecpA [5], and astA [9]. The amplified products of bfpA, ecpA, and astA were electrophoresed for one hour at 5 V/cm in a 1% (w/v) and in a 2% (w/v) agarose gel respectively using a Standard molecular weight marker.

Sero-grouping of *E. coli* isolates

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

Antimicrobial susceptibility testing

In accordance with criteria published by the Clinical and Laboratory Standards Institute (CLSI) in 2010, the EPEC isolates were subjected to a disc diffusion method test to determine their antimicrobial resistance to 12 antibiotics [11]. The following antimicrobial

discs (HiMedia Pvt. Ltd., India) were used ampicillin (Amp 10 µg); cefixime (Cfm 5 µg); ciprofloxacin (Cip 5 µg); chloramphenicol (C 30 µg); doxycycline hydrochloride (Do 30 µg); kanamycin (K 30 µg); minocycline (MI 30 µg); streptomycin (S 10 µg); nalidixic acid (NA 30 µg); norfloxacin (NX 10 µg); tetracycline (TE 30 µg); and trimethoprim (TR 5 µg).

RESULTS AND DISCUSSION

Isolation of presumptive *E. coli*

Out of 200 Faecal samples, a total of 560 presumptive *E. coli* isolates were attained (Fig 1a and 1b) and only one isolate of these 560 presumptive bacterial isolates was chosen for additional processing from each sample (200) that demonstrated the biochemical traits of *E. coli* on the HiMotility *E. coli*

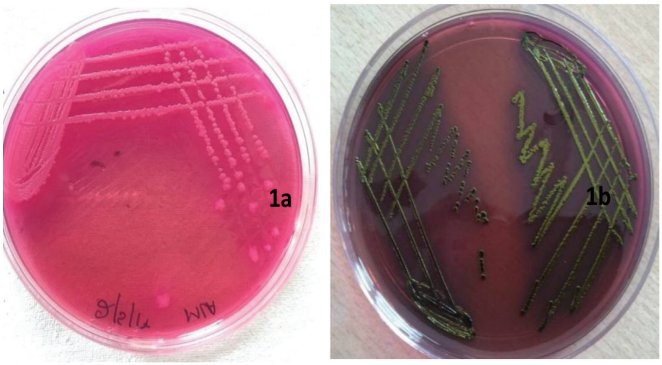

Fig. 1. Isolation of ESBL producing *E. coli* on (a) MLA and (b) EMB agar.

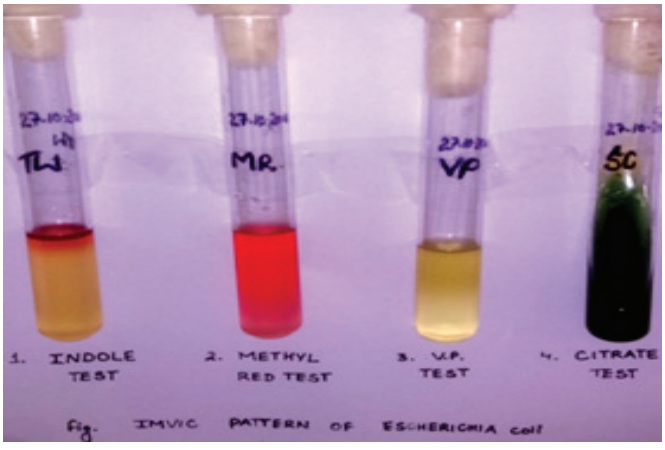

Fig. 2. IMViC pattern of *E. coli* isolates from faecal samples. The isolates show (from left to right) positive test results (a) indole production, (b) methyl red test while negative results for (c) Voges-Proskauer and (d) citrate utilization test.

test kit. The isolates that were chosen for further confirmation were identified using the +ve, +ve, -ve, and -ve IMViC patterns, as presented in Fig. 2.

Detection of EPEC and STEC

Molecular screening for the eae, stx1 and stx2 genes was done using multiplex PCR on all 200 isolates of *E. coli*; one from each fecal sample. Amplifications of 180 bp, 255 bp, and 384 bp were obtained from isolates that had the stx1, stx2, and eae genes, respectively. Table 2 represents the relative distribution of these genes among species, and Fig. 3 shows the representative gene profiles.

Out of the 39 *E. coli* isolates from calves studied in this investigation, only 5 (12.82%) had the eae gene alone. As a result, the carriage of 12.82% EPEC is greater than 5.83% reported in Dublin, Ireland [13] and 2.7% in Sao Paulo, Brazil [12], but in line with 10.4% in Germany [2]. However, Waltner-Toewset *et al.* [14] from Ontario reported a higher percentage (41%). In the case of sheep out of 36 *E. coli* isolates, 12 (33.33%) contained the eae gene alone. According to earlier research, a sizable portion of sheep were colonized with EPEC [2, 15]. In the current study, 33.33% of the sheep harboured EPEC, compared to fewer than 17.7% of the sheep investigated by Aktan *et al.* [16] in England. Prior reports, however, indicated a higher percentage (55%) [17].

Of the 24 *E. coli* isolates obtained from rabbits, 6 (25.0%) were found to be EPEC. However, their carrying rate in healthy animals is lacking. In comparison, 74% and 83% of rabbits in the USA [19] and Spain [18] reported having EPEC in diarrhoeal rabbits, respectively, compared to 25% of rabbits in our study. Porcine EPEC has been linked to post-weaning diarrhea in pigs, and 10 (45.45%) of the 22 isolates in pigs were identified as such [20]. However, in Hungary, Malik *et al.* [21] found no difference between the frequency of eae+ *E. coli* appearance in diarrhoeal and non-diarrhoeic pigs. EPEC was found in 45.45% of the healthy pigs in the current study. Although pigs have not been extensively studied as an EPEC reservoir; however, in one research 89% of the pig samples had eae+ [17]. Compared to the 45.45% share in our analysis, this is a relatively high figure. However, the results of De la Fuente *et al.* [15], Aktan *et al.* [16] from England and Wales reported 0.75%, and Krause *et al.* [2] from Germany reported 17.6%, all of which were lower than the results of our investigation. Similarly, out of 38 *E. coli* isolates from dogs, 5 (13.15%) had the eae gene alone. EPEC

Table 1. List of primer sequences and predicted amplicon length.

Primer	Sequence (5'-3')	Target gene	PCR Conditions	Amplicon size (bp)	Reference
Stx1 F Stx1R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	*stx*$_1$	Initial Denaturation at 95°C for 2 min, Denaturation at 95°C1 min; Annealing at 65°C for 2 min;Elongation at 72°C for 1.5 min.	180	[8]
Stx2 F Stx2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	*stx*$_2$	Initial Denaturation at 95°C for 2 min, Denaturation at 95°C1 min; Annealing at 65°C for 2 min;Elongation at 72°C for 1.5 min.	255	[8]
eae F *eae* R	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	*eae*	Initial Denaturation at 95°C for 2 min, Denaturation at 95°C1 min; Annealing at 65°C for 2 min;Elongation at 72°C for 1.5 min.	384	[8]
EAST11a EAST11b	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	*astA*	Initial Denaturation at 95°C for 5 min, Denaturation at 95°C for 30 sec; Annealing at 55°C for 30 sec; Elongation at 72°C for 40 sec.	111	[9]
ecpA F ecpA R	GCAACAGCCAAAAAAGACACC CCAGGTCGCGTCGAACT	*ecpA*	Initial Denaturation at 94°C for 5 min, Denaturation at 94°C for 45 sec; Annealing at 62°C for 1 min; Elongation at 72°C for 1 min.	477	[5]
EP1 EP2	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	*bfpA*	Initial Denaturation at 94°C for 5 min, Denaturation at 94°C for 30 sec; Annealing at 56°C for 1 min; Elongation at 72°C for 2 min.	326	[10]

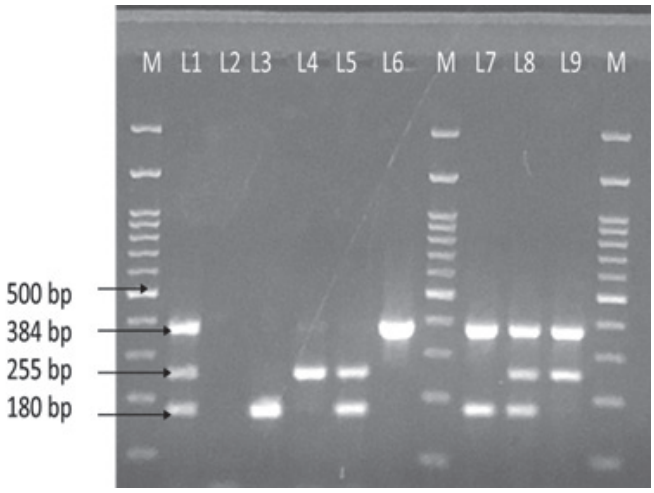

Fig. 3. Representative stx1, stx2 and eae genes profile of *Escherichia coli* isolates using multiplex polymerase chain reaction (m-PCR). Lane M: 100bp DNA ladder. Lane 1: positive control. Lane 2: negative control. Lane 3: stx1 positive. Lane 4: stx2 positive. Lane 5: stx1, stx2 positive. Lane 6: eae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

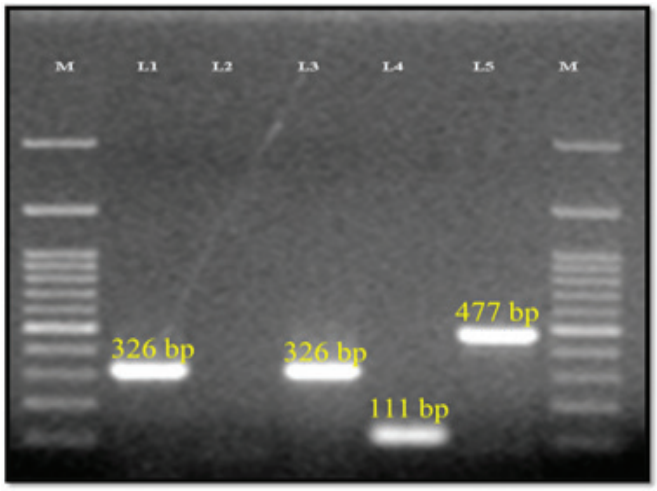

Fig. 4. Representative bfpA, ecpA and astA genes profile of *Escherichia coli* isolates using multiplex polymerase chain reaction (m-PCR). Lane M: 100bp DNA ladder; Lane 1: positive control; Lane 2: negative control; Lane 3: bfpA positive; Lane 4: astA positive. Lane 5: ecpA positive.

strains with virulence features similar to human strains are often isolated from diarrhoeic and healthy dogs [22]. While it is greater than the 7.2% reported from Germany [2] and the 5.5% reported by Mainil [24], the shedding rate of 13.15% of EPEC in dogs in the current study is comparable to the 12.6% recorded from Brazil [23]. Screening for the presence of those three genes revealed that none of the 41 isolates from chickens carried any gene.

Of all the domestic animals examined in this investigation, only 17 (43.58%) and 11 (30.55%) isolates of calves and sheep, respectively, yielded STEC. The results of previous studies, which showed that calves are a possible source of STEC in their GIT,

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

Sl. No.	Species	No. of Isolates	*stx*$_1$	*stx*$_2$	*eae*
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

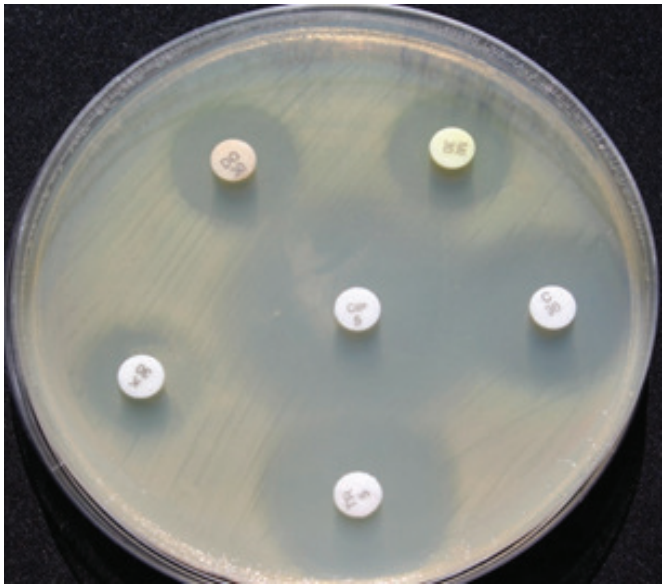

Fig. 5. Antimicrobial sensitivity pattern of EPEC isolates against chloramphenicol (C 30µg), ciprofloxacin (Cip 5 µg), doxycycline hydrochloride (Do 30 µg), kanamycin (K 30 µg), minocycline (MI 30µg), trimethoprim (TR 5µg).

were supported by the carriage of 43.58% STEC in calves [25]. The frequency found in this experiment is similar to the 44% and 46% of calves reported in Brazil [26] and Japan [27]. Geographical variances are probably the reason for these variations. Research has demonstrated that the environment could affect a calf's

Table 3. Virulence gene profile and sero-group of EPEC from animals.

Species	Sero-group	No. of Strains	Virulence genes		
			bfpA	*ecpA*	*astA*
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

ability to shed STEC [28]. The current investigation indicated that sheep had a 30.55% STEC carriage rate, which is greater than the 12.32% reported for lambs in the state of Jammu and Kashmir [29] but lower than the 68% and 88% recorded in lambs in Germany and Spain [30, 31].

Sero-grouping and virulence gene profile of EPEC

All the 38 EPEC isolates were sero-group for somatic 'O' antigen at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, H.P. O188 (36.84%) and O118 (18.42%) were the most common serogroups, followed by O149 (7.89%), O22 (5.26%), and O11 (5.26%). The different serogroups that were investigated during this study are listed in Table 3. The serogroup O88 was also isolated from cattle in Jammu [32] and in pigs from Switzerland [17], in contrast to our investigation. On the other hand, there has been no record of the serogroups discovered in sheep or rabbits. Nakazato *et al.* [23]

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

from Brazil reported the isolation of eae+ *E. coli* belonging to serogroup O11 from dogs. All the EPEC isolates were screened for the presence of bfpA, ecpA and astA genes by PCR as presented in Fig. 4 and their virulence profile is summarized in Table 3. In the present study, the entire dog EPEC isolates harbour the bfpA gene which matches the study reported by previous researchers [22, 23]. According to an Iranian study, both healthy and diarrheal dogs may serve as EPEC reservoirs and this suggests that canines may be a risk factor for the spread of diarrhea to humans [33]. The ecpA gene was reported from all the EPEC isolates in contrast to Hernandes *et al.* [5]. Several reports have previously shown that this gene is highly conserved among the different intestinal and extraintestinal *E. coli* pathotypes, including commensal strains [34, 35, 36]. The presence of ecpA was found in all strains that displayed an LAL pattern on HEp-2 cells, according to a recent gene search investigation using Brazilian EPEC strains [37, 38]. This present work focused on the astA gene distribution in a wide set of EPEC strains that were recovered from dogs, sheep, pigs, cattle, and rabbits in Jammu because EPEC carriage by companion animals could be a public health issue, the astA gene, has been found in several categories of human diarrhoeagenic *E. coli* including EPEC and is thought to be an additional factor in the pathophysiology of *E. coli* diarrhea [39]. Gene astA was detected in 80% of atypical EPEC isolates in cattle, which is similar to the 87% of Canadian isolates reported by De Sousa and Dubreuil

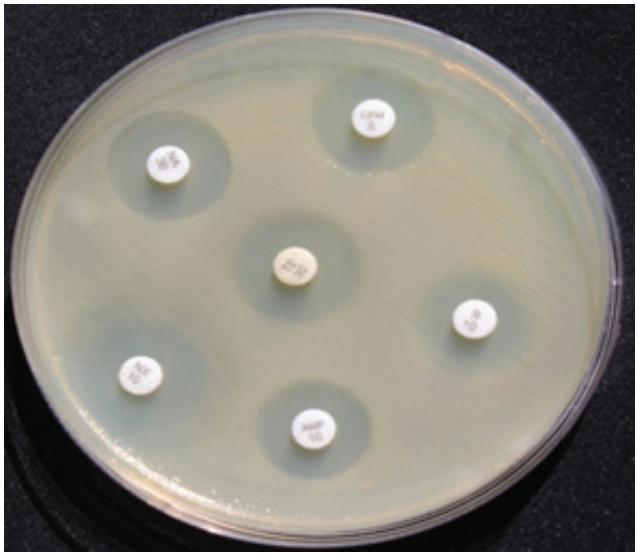

Fig. 6. Antimicrobial sensitivity pattern of EPEC isolates ampicillin (Amp 10 µg), cefixime (Cfm 5 µg), nalidixic acid (N\A 30 µg), norfloxacin (NX 10 µg), streptomycin (S 10 µg), tetracycline (TE 30 µg).

[39]. Gene astA was detected in 16.6% of atypical EPEC isolates in sheep, which is much more than the 1.6% discovered by Yuste and co-worker [40] in Spain but less than the 26.4% reported by Frohlicher and co-worker [17] in Switzerland. Similarly, in pig atypical isolates, the astA gene was found in 80% (eight out of ten), which is 8% reported from Switzerland [17]. Of the EPEC isolates in dogs, 80% had the astA gene. Since we lack a reference to compare our results to, this is most likely the first study of its kind.

Antimicrobial susceptibility testing

As illustrated in Fig. 5 and Fig. 6, the prevalence of antibiotic resistance in the EPEC strains obtained from animals is presented in Table 4. Overall, 26 (68.42%) of the isolates were resistant to at least one of the 12 antibiotics tested.12 (31.57%) of the isolates exhibited an intermediate susceptibility to the 12 antibiotics tested, while no isolate was pan-sensitive. Tetracycline resistance was not widely present in the EPEC isolates used in this investigation. This is contrary to the results of Meyer *et al.* [41], who found that animal-isolated *E. coli* had a greater rate of tetracycline resistance. To our surprise, all the isolates were sensitive to ampicillin. This runs counter to the observations made by Hoyle *et al.* [42], who found widespread ampicillin resistance among, *E. coli* isolates received from animal feces dwelling in natural farms even if the use of antimicrobial drugs is restricted. In line with Rehman and co-workers [32], every EPEC isolate of calves in our study was susceptible to ciprofloxacin, norfloxacin, and chloramphenicol. However, in this study antibiotic kanamycin is resistant to all isolates of calves and dogs, consistent with those of researchers Bolton *et al.* [14] from Ireland and Banik *et al.* [43] from the Southern part of Bengal, respectively. A variety of antibiotics, including ampicillin, chloramphenicol, ciprofloxacin, and nalidixic acid, were shown to be effective against the ovine *E. coli* isolates in the in vitro sensitivity experiment used to determine their antibiotic resistance profile. On the other hand, intermediate resistance to drugs such as kanamycin was observed. Similar findings were reported previously stating that *E. coli* isolates from sheep were susceptible to ciprofloxacin, nalidixic acid, and chloramphenicol [44].

CONCLUSION

In this study, the bfpA and astA genes in dogs and EPEC serogroups O88, and O118 from healthy sheep and rabbits in India are isolated and characterized for the first time. Healthy animals normally appeared to be reservoirs of atypical EPEC. However, dogs and cats carried typical EPEC. In our study, all animals except dogs carried atypical EPEC while all the isolates from dogs were typical. These typical EPECs were isolated from healthy dogs indicating the possibility of dogs being the reservoir of typical EPEC.

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