The Pharma Innovation Journal 2019; 8(9): 34-38

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(9): 34-38

www.thepharmajournal.com
Received: 15-07-2019
Accepted: 18-08-2019

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Characterization and antibiogram profile of Enteroaggregative *Escherichia Coli* isolated from calves with diarrhoea and environmental sources in Ernakulam, Kerala

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Abstract
Enteroaggregative *E. coli* (EAEC) is an emerging enterovirulent pathogen that causes infection in humans and animals. The present study focussed on the occurrence of EAEC in calves and its associated environmental sources in Ernakulam. A total of 180 samples (60 calves' diarrhoeic faecal samples, 60 water and 60 soil samples) were collected from two panchayats of Ernakulam. All the samples were subjected to isolation of *E. coli* through conventional culture techniques. Out of 180 samples, *E. coli* was isolated from 101 samples (51 calves, 19 soil and 31 water). Further *E. coli* isolates were subjected to PCR for the detection of EAEC virulence genes *viz., astA, Pic, aggR* and *fimA* genes. On analysis 21.57, 5.89, 5.89 and 15.69 per cent of the *E.coli* isolates from calves carried the *astA, pic, aggR* and *fimA* genes. From water and soil samples of calves rearing areas seven *E.coli* isolates carried virulence genes. To study the biofilm forming ability, EAEC isolates were subjected to quantitative biofilm assay through which it was inferred that more than 85 per cent of the isolates were low biofilm producers. On antibiotic sensitivity study, from different sources showed more than 80 per cent sensitivity to nitrofurantoin and meropenem. Cent per cent sensitivity against imipenem and >75 per cent of the isolates showed resistance against ampicillin, tetracyclin, cefotaxime and azithromycin. From the study it was concluded that EAEC is an emerging threat to the public health. because of its ability to form biofilm and increasing antibiotic resistance towards commonly used antibiotics.

Keywords: Enteroaggregative *Escherichia coli*, Biofilm assay and Antibiotic sensitivity test

1. Introduction
Infectious calf diarrhoea is a syndrome with complex pathogenesis causing huge losses to the diary industry either directly through mortality or indirectly through prophylactic costs and reduced growth rates in affected calves (Radostits *et al.*, 2007) [12]. According to the 2007 National Animal Health Monitoring System for U.S dairy, 57 per cent of calf mortality was due to diarrhoea mostly seen in calves of less than one month old (USDA Dairy 2007) [16]. In India, 80-85 per cent of the total mortality is due to neonatal calf diarrhoea during the first month of age (Singh *et al*., 2009) [15]. Several studies reported that *E. coli* is the leading cause of diarrhoea, haemorrhagic colitis and dysentery in calves which are weak, malnourished, debilitated and immunosuppressed (Ellaithi, 2004; Mohamed, 2009 and Malik *et al*., 2013) [7, 9]. *Escherichia coli* is a gram-negative, rod-shaped, flagellated, non-sporulating and facultative anaerobic bacterium of the family Enterobacteriaceae. The *E.coli* strains are classified into six main pathotypes based on their distinct virulence determinants and pathogenic features, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusively adherent *E. coli* (DAEC) (Xia *et al*., 2010) [21].
Enteroaggregative *Escherichia coli* (EAEC) are characterized by the ability to aggregate intimately with each other, adhere to Human epithelial type-2 (HEp-2) cells, and forming stacked-brick like aggregates (Weintraub, 2007) [20]. The adherence pattern of EAEC is mediated by aggregative adherence fimbriae (AAF) coded on a large virulence pAA plasmid. In addition, EAEC form mucoid biofilms and secrete cytotoxins (e.g., plasmid encoded toxin-Pet) that are toxic to epithelial cells. The enterotoxins *viz.*, enteroaggregative heat stable enterotoxin (EAST1), *Pic* protein has mucinase activity, fimbrial subunit protein (*fimA*) and transcriptional activator *aggR* genes were associated with EAEC (Croxen and Finlay, 2010) [5]. With the increasing incidence in humans, EAEC has gained importance.

Enteroaggregative *Escherichia coli* forms thick biofilm on the intestinal mucosa which in turn promotes its persistent colonization, perhaps by presenting a barrier to the penetration of antibiotics and host antibacterial factors. Though HEp-2 cell culture assay is a gold standard test, complexity of procedure and time consuming nature reduced the usage in diagnosis. So biofilm assay can be used for its simple procedure and cost effectiveness. As EAEC adhere to abiotic surfaces and their ability to form biofilms, help in performing the assay using an ELISA plate reader at 570 nm.

Antibiotics are playing a very crucial role in protecting the health of both humans and animals. Indiscriminate usage and improper dosage lead to increased selection pressure among the pathogens leading to development of resistance to such organisms. The *E. coli* infections are very common among the animals and humans which are gaining rapid resistance to most commonly used antibiotics in the recent times. The EAEC pathotype has an ability to form biofilm which further aggravates the resistance by transfer of plasmids among the organisms within the biofilm. To understand the antibiotic resistance patterns among the isolates obtained in the present study disc diffusion method of antibiotic sensitivity test was performed as per CLSI, (2018) [4] guidelines. To find the sources of infection and antibiotic resistance patterns, the present study was aimed at identifying the occurrence of EAEC in calves and also their associated environment in Ernakulam district of Kerala.

2. Materials and Methods

2.1 Sample collection area of study

Calf diarrhoeic faecal samples were collected from 30 households each from two panchayats namely Kalady (P1) and Malayattoor (P2) of Ernakulam district. To know the association with the environment, soil and water samples from the every individual household was also collected. Soil samples of about 100g and water about 500ml were collected. All the samples were collected in suitable aseptic containers. All the samples were transported to a laboratory under chilled conditions and were processed within 24h of collection.

2.2 Microbiological assay

In the present study, a total of 60 calf diarrhoeic faecal samples and environmental sources *viz.*, soil (60) and water (60) samples were initially screened for 'presumptive' colonies of *E. coli* by overnight enrichment of samples in *E. coli* enrichment broth, followed by streaking on selective agars *viz.*, MacConkey (MCA) agar and Eosin Methylene Blue (EMB) agar and incubation of plates at 37^0C for 24 h.

2.3 PCR assay

From the isolates, DNA was isolated using modified phenol-chloroform method from overnight broth culture. Later the EAEC virulence factors were identified using specific primer as explained in Table.1. The PCR assay was performed in a 25 µl reaction volume containing 50ng/µl of DNA template, 200m*M* PCR buffer (10X), 10m*M* dNTPs, 25m*M* of $MgCl_2$, 10p*M*/µl of each primer and 5 units of Taq DNA polymerase. The cycling conditions for the genes *astA, Pic, aggR* and *fimA* were included in Table.2. The PCR products were visualized by gel electrophoresis in 1.5% agarose (Hi Media, India) containing SYBR green, in Tris–acetate–EDTA (TAE) buffer.

Table 1: Primers used for the identification of Enteroaggregative *E. coli*

Genes	Primers sequence	Size (bp)	Ref.
astA F	5'-GCCATCAACACAGTATATCC-3'	106	Vijay *et al.* (2015) [14, 17].
astA R	5'-GAGTGACGGCTTTGTAGTCC-3'		
Pic F	5'-AGCCGTTTCCGCAGAAGCC -3'	1111	Chandra *et al.* (2013) [3].
Pic R	5'AAATGTCAGTGAACCGACGATTGG-3'		
aggR F	5'-GTATACACAAAAGAAGGAAGC-3'	254	Ratchtrachenchai *et al.* (1997) [13].
aggR R	5'-ACAGAATCGTCAGCATCAGC-3'		
fimA F	5'-TTAGTAGCCTGATGTTGCTGGGCA-3'	342	Rawool *et al.* (2015) [14].
fimA R	5'-ATGTGCCTGCCGCAGTTTCAATAC-3'		

Table 2: PCR conditions for detection of virulence genes of EAEC

Genes	Initial denaturation (^{0}C/min)	Denaturation (^{0}C/sec)	Annealing (^{0}C/sec)	Extension (^{0}C/sec)	Final Extension (^{0}C/min)	No. of cycles
astA	95/5	95/45	59.4/30	72/30	72/5	35
Pic	94/5	94/30	62/30	72/60	72/10	30
aggR	94/5	94/60	57/60	72/60	72/15	35
fimA	94/5	94/60	56/60	72/60	72/10	35

2.4 Quantitative biofilm assay

Biofilm formation test was performed according to a method described by Wakimoto *et al.* (2004) [18] with some modifications using a 96-well micro titre plate. Biofilm formation was quantified using an enzyme linked immunosorbent assay plate reader at 570 nm. Strains with optical density (OD) at 570 nm of more than 0.2 were regarded as biofilm producers (biofilm-positive strains).

2.5 Antibiotic resistance profiling

All the EAEC isolates were subjected to antibiotic resistance profiling against 20 different antibiotics by disc diffusion method, as per the guidelines provided by clinical laboratory standard institute (CLSI, 2018) [4].

3. Results

An overall occurrence of *E. coli* was noticed in 51 out of 60 calves diarrhoeic samples (Table 3). The occurrence of *E. coli* in water and soil sources associated with calves was found to be 51.66 per cent and 31.66 per cent respectively. Statistical analysis revealed no significant difference between the panchayats in occurrence of *E.coli* ($p< 0.05$). A total of 51 isolates were found to be carrying the virulence genes associated with EAEC on subjecting to uniplex PCR. The presence of virulence genes among the isolates from calves (Fig. 1) was found to be *astA* gene (18.33%), *Pic* (5%), *aggR* (5%) and *fimA* (13.33%) genes respectively. Occurrence of multiple virulence genes *viz.*, *astA* and *pic, astA* and *fimA, pic* and *fim A* was observed in two isolates each respectively.

Among the 7 isolates from the environmental sources of calves sheds (Table 4), *astA* gene was encoded by 2 (6.66 per cent) water samples. The *fimA* gene was detected from soil (1) and water (4) samples. Overall occurrence of *astA* and *fimA* gene was found to be 1.66 and 4.16 per cent whereas *pic* and *aggR* could not be isolated from any of the environmental sources. None of the isolates from environmental sources during the study carried multiple virulence genes. Out of all the samples subjected, EAEC associated virulence genes were observed among 26 samples. On statistical analysis there was no significant difference in occurrence of virulence genes within the panchayat as well as between the panchayats.

On performing quantitative biofilm assay all the obtained isolates were classified into three major categories as high biofilm producers, moderate biofilm producers and weak biofilm producers. The criteria were adopted based on OD 570 nm values, by considering both positive and negative control optical density (OD) values. Thus, in the present study those EAEC isolates showing an average values of 1.55 and above (OD 570 nm) were considered as high biofilm producers, 0.78 to 1.54 (OD 570 nm) as moderate biofilm producers and 0.77 and below (OD 570 nm) were considered as low biofilm producers (Mohamed *et al.*, 2006). Among the isolates from calves 84 per cent were low biofilm producers whereas only 10.5 per cent showed high biofilm forming ability. From the environmental sources none of the isolates were high biofilm producers where as 85 per cent of the isolates were low biofilm producers (Fig. 2).

Isolates obtained from calf diarrhoeic faecal samples were subjected to antibiotic resistance profiling. Out of the 19 isolates, ampicillin showed highest resistance (84 per cent) followed by cefotaxime (57.89 per cent), tetracycline (57.8 per cent), streptomycin and azithromycin (52.63 per cent) respectively. All the isolates were sensitive to imipenem followed by meropenem (89.47 per cent), chloramphenicol (84.2 per cent) and kanamycin (84.21 per cent). Isolates from the environment showed sensitivity towards imipenem, followed by meropenem (77.81 per cent) and nitrofurantoin (66.6 per cent). Maximum resistance among the isolates was observed towards norfloxacin (78 per cent), tetracycline and cefotaxime (77.7 per cent). Resistance towards cephalosporins was the significant finding of the present study.

Table 3: Occurrence of *E. coli* in calf diarrhoeic faecal samples of two panchayats in Ernakulum district

Sl. no.	Panchayats	Samples analysed	*Escherichia coli* positive samples	
			No.	Per cent
1	P 1	30	27	90.00[a]
2	P 2	30	24	80.00[a]
	Total	60	51	85.00
P				0.069

Figures bearing common superscript do not differ significantly.

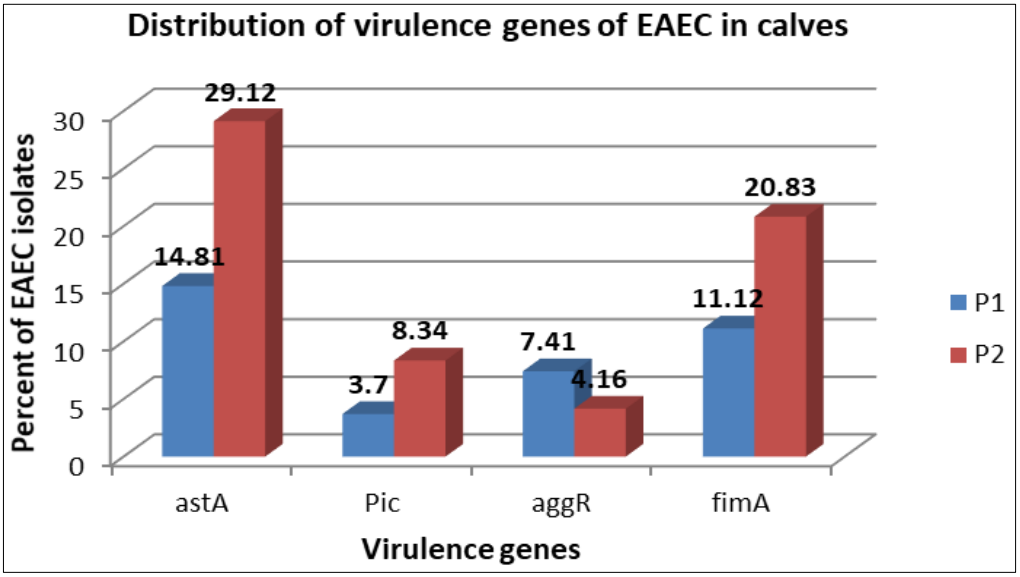

Fig 1: Distribution of virulence genes in EAEC isolates from calves diarrhoeic faecal samples

Table 4: Distribution of virulence genes in EAEC isolates from calves associated environmental sources

Panchayat	No. of *E.coli* isolates	Distribution of virulence genes of EAEC							
		astA		*Pic*		*aggR*		*fimA*	
		No.	%	No.	%	No	%	No	%
P1	Soil (11)	0	0	0	0	0	0	1	9.10[a]
	Water (18)	2	11.11[a]	0	0	0	0	3	16.67[a]
P2	Soil (8)	0	0	0	0	0	0	0	0
	Water (13)	0	0	0	0	0	0	1	7.69[a]
Total	50	2	4.00	0	0	0	0	5	10.00
P value		0.150		1		1		0.305	
χ^2		2.034		0		0		1.054	

Figures bearing common superscripts do not differ significantly (p>0.05

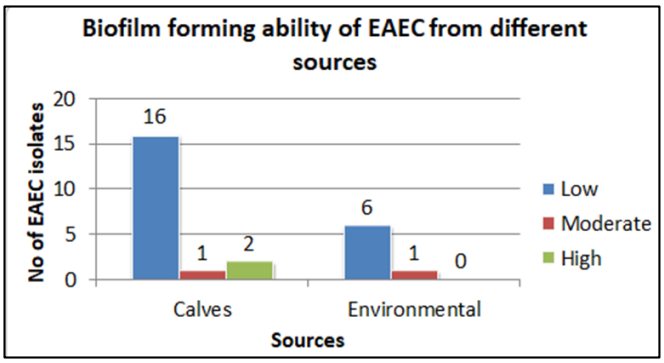

Fig 2: Number of calves and environmental EAEC isolates displaying degree of biofilm formation.

4. Discussion

Escherichia coli is the most commonly isolated organism among the calves suffering from infectious diarrhoea, (Ansari *et al.*, 2014) [1]. In calves out of 51 culture positive isolates subjected to PCR for detection of EAEC virulence genes, 21.57, 15.69, 5.89 and 5.89 per cent of the isolates carried genes *viz.*, *astA*, *fimA*, *Pic* and *aggR* respectively. Occurrence of multiple virulence genes like *astA* and *Pic*, *astA* and *fimA*, *Pic* and *fimA* genes was observed in two isolates each respectively. Similar results were obtained by Vijay *et al.* (2015) [14, 17] in Bareilly, UP. Another study conducted by Wani *et al.* (2013) showed no occurrence of EAEC among the calves both by cultural and molecular techniques which indicate the diversity in the identification of the pathotypes. In a UK based study where a large number of cattle at slaughter house were examined for the presence of EAEC. This study revealed no EAEC from the intestinal contents of the animals (Enne *et al.*, 2008) [8]. So the present study cannot be compared to any of the above mentioned studies. The EAEC strains were heterogeneous and diversified with a variety of virulence factors. The heterogeneity was the result of presence of a heavy molecular weight plasmid carrying many genes responsible for pathogenesis. This plasmid plays a role in acquisition or deletion of the virulence genes leading to heterogenecity with in the pathotype.

Biofilm helps in survival of organism and become a persistent infection in the host. They also protect themselves against host immune mechanisms. Studies revealed that EAEC causes damage to the intestinal mucosa (Mohamed *et al.*, 2007) [10]. Based on above criterion in the present study majority of the EAEC strains from calves isolates were low biofilm producers (84.21 per cent), followed by high biofilm producers (10.53 per cent) and moderate biofilm forming ability (5.26 per cent) respectively. Similar results were obtained by Vijay *et al.* (2015) [14, 17] where majority of the animal isolates were low biofilm producers. Biofilm formation by EAEC contributes to its persistent infections. Due to diversity in distribution of virulence factors differences in the biofilm forming ability cannot be ruled out through the study

Isolates from calves exhibited antibiotic resistances towards cefotaxime and tetracycline with greater than 50 per cent of isolates showing resistance. In calves 84 per cent of the isolates were resistant to ampicillin. Similar pattern of resistance was reported by Dhaka *et al.*, 2016 [6], showed highest resistance against ampicillin (96 per cent), tetracycline (88 per cent), cefotaxime (84 per cent), Co-trimoxazole (80 per cent) and ciprofloxacin (52 per cent) from 25 isolates from Bareilly, India. The resistance to tetracycline might be plasmid mediated among the pathogens. Resistance to cefotaxime is contrary to the findings of (Rawool *et al.*, 2015) [14] where the isolates (92.85 per cent) were sensitive towards the antibiotic. All the isolates were resistant to a minimum of two antibiotics and multidrug resistance was observed among 4 isolates from the animals. The isolates were sensitive towards imipenem (100 per cent) followed by gentamicin and meropenem (78.57 per cent).

In conclusion the present study affirms that cattle and associated environments remain a source of infections to humans. Utility of the PCR was established for identifying virulence genes of EAEC. Genetic basis for biofilm production and antibiotic resistance yet to be addressed.

5. Acknowledgements

The authors thank the DEAN, College of Veterinary and Animal Sciences and faculty of Department of Veterinary Public Health, CVAS, Mannuthy for providing necessary facilities for the research.

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