Content list available at http://epubs.icar.org.in, www.kiran.nic.in; ISSN: 0970-6429

Indian Journal of Hill Farming

2017, Special Issue, Page 88-92

Occurrence and Characterization of *Escherichia coli* Isolated from Diarrhoeic Piglets in Meghalaya

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ARTICLE INFO

Article history:
Received 7 December 2017
Revision Received 17 December 2017
Accepted 30 December 2017

Key words:
piglet, diarrhea, virulence genes, pathotype, Meghalaya

ABSTRACT

The present study was conducted to investigate the occurrence of *Escherichia coli* carrying virulence genes and antibiotic resistance associated with piglet diarrhea in Meghalaya. We analysed 294 faecal samples randomly collected from the piglets with history of diarrhoea for the isolation of *E. coli* followed by screening of virulence genes among the isolates by specific and multiplex PCR. Further *E. coli* isolates were subjected to Kirby–Bauer disc diffusion method to study phenotypic antibiotic resistance. The overall occurrence rate of *E. coli* was 51.7% (152/294). Among 152 isolates virulence factor genes detected were *stx1* (0.6%), *stx2* (7.2%), *lt* (1.97%), *stx2e* (3.9%), *eae* (15.1%), STaP (0%), K99 (0%), $fliC_{H7}$ (0.6%) and *hlyA* (15.1%). Based on multiplex PCR, out of these 152 isolates ,18 (11.8 %), 2 (1.3%) and 23 (15.1%) isolates were recorded as Shiga-toxin-producing *E. coli* (STEC), atypical enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. Coli* (ETEC), respectively. Antibiotic resistance percentages for Metronidazole, Sulphafurazole, Tetracycline, Streptomycin, Trimethoprim, Neomycin, Ampicillin, Co-trimoxazole, Ceftazidime, Enrofloxacin, Chloramphenicol, Ceftriaxone, Ciprofloxacin and Gentamicin were found to be 100, 70, 68.2, 61.7, 44.8, 35.5, 34.6, 29, 25.2, 14, 10.3, 10.3, 6.5 and 3.7, respectively. In conclusion, this study showed the frequency of virulence genes, pathotype and antimicrobial resistance in *E. coli* strains isolated from diarrhoeic piglets in Meghalaya.

1. Introduction

Escherichia coli is one of the major causative agents of diarrhoea in neonatal piglets worldwide (Toledo *et al.,* 2012). Being a genetically diverse group, most strains of *E. coli* are innocuous commensals of mammals but some are competent to cause either intestinal or extra intestinal disease and the acquisition of virulence genes is thought to endow with an evolutionary pathway to pathogenicity (Selander *et al.,* 1987; Orskov and Orskov, 1992). Manifestation of diarrhoea or disease appears to be strongly linked with the possession of putative virulence genes in *E. coli* (Grauke *et al.,* 2002). Diarrhoegenic E. coli strains are classified based on their virulence properties as enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), and enteroaggregative (EaggEC) (Nataro and Kaper, 1998). *E. coli* isolates from animals with diarrhoea usually shelters various virulence factors that have a synergistic effect in developing pathogenicity and also responsible for challange in combating diarrhoea in young pigs. Enterotoxigenic, shiga toxigenic and enteropathogenic (ETEC, STEC and EPEC, respectively) pathotypes of *E. coli* are the major pathogens causing neonatal diarrhoea in pigs (Bessone *et al.,* 2017). Indeed, simplex and multiplex PCR analysis has been useful in cost effective and speedy investigation of different virulence factors (Francis, 2004).

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Understanding the frequency of virulence genes might be more useful in developing polyvalent vaccines to stimulate immune responses against the common virulence factors for preventing enteric *E. coli* infection in piglets. Intensive farming has led farmers to incorporate antibiotics to feed, as a growth promoters and chemo prophylactic strategy (Mathew *et al.,* 2007). Humans might acquire antibiotic resistance from these practices (Okello *et al.,* 2015). Therefore it is imperative to know the prevailing antibiotics resistance profiles in order to manage the dissemination of antibiotic resistant pathogens. With this background the present study was envisaged to determine the distribution of virulence genes and antibiotic resistance associated with *E. coli* strains isolated from diarrhoeic piglets in Meghalaya.

2. Materials and Methods

2.1 Isolation, identification and confirmation of E. coli

A total of 294 faecal samples were randomly collected from the piglets with history of diarrhoea in different parts of Meghalaya from March, 2011 to March, 2013. The diarrhoeic faeces were collected in a sterile container and transported under chilled condition to laboratory. Approximately 1g of sample was suspended into 9 ml of MacConkey broth (Himedia, Mumbai, India) and incubated overnight at 37°C. The enriched broth was streaked onto the MacConkey agar and incubated at 37°C for 24 h. Further suspected pale pink colonies were randomly picked and transferred to eosin methylene blue (EMB) agar (Himedia, Mumbai, India) and incubated (37°C, 24 h). The suspected colonies were picked and further confirmed by subjecting to Gram staining and biochemical tests (IMViC).

2.2 Determination of frequency of virulence genes and Pathotyping

To extract bacterial DNA, pure cultures were grown in nutrient broth (Hi-media) at 37°C overnight. Organisms from 1.5 ml growth were pelleted by centrifugation at 1200xg for 10 min. The pellet was resuspended in 100 µl of nuclease free water (Thermoscientific, USA). The bacteria were lysed by boiling for 10 min in a water bath. The supernatant after centrifugation was directly used as template (Wani *et al.,* 2003). PCR for *stx1, stx2, eae, hlyA* was done as per Paton and Paton, (1998). PCRs for the virulence factors LT (Dallas and Falkow, 1980), STaP (So *et al.,* 1980), *stx2e* (Weinstein *et al.,* 1988) and K99 (Roosendaal *et al.,* 1987) were performed as previously described. PCR for $fliC_{H7}$ gene was performed as per Gannon *et al.* (1997). Multiplex PCR was employed to pathotype (ETEC, aEPEC & STEC) by targeting four genes (*est1, elt1, stx1*& *eaeA)* as per prior protocol (Rajkhowa *et al.,* 2015).

All PCRs were performed in the Eppendorf Thermal Cycler (Eppendorf, Germany). PCR products were separated and visualized by gel electrophoresis in 1.5% agarose (Amresco, USA) containing ethidium bromide, in Tris- Acetate-EDTA (TAE) buffer (Thermoscientific, USA). The electrophoresis was carried out at 5 volts/cm and a 100 bp DNA ladder (Sigma-Aldrich, USA) was included in each agarose run. The resolution of amplified fragment in the gel was visualized by a UV transilluminator and digitally recorded by gel documentation system (DNR MiniLumi, Israel). Materials contaminated with ethidium bromide were disposed according to local guidelines.

3. Results and Discussion

Out of the 294 samples obtained from diarrhoeic piglets screened for the presence of *E. coli*, 152 (51.7%) samples yielded characteristic dark colonies with a green metallic sheen in EMB agar. Gram negative rods were observed under the microscope. Under biochemical tests, isolates revealing positive for indole and methyl red tests, negative for Voges-Proskauer (VP) and citrate utilization tests were confirmed as *E. coli.* Screening of virulence factors/genes (*stx1, stx2,* LT, *stx2e, eae,* STaP, K99, H7and *hlyA*) employing PCR showed amplification of *stx1* in one isolate (Fig. 1), *stx2* (Fig. 2) in 11 isolates, *stx2e* in 6 isolates, LT in 3 isolates (Fig. 3) and H7 in 1 isolate. Virulence genes *hlyA* and *eaeA* were also observed in 23 isolates (Fig.4). K99 and STaP virulence factor/gene could not be observed in any of the isolates. Multiplex PCR targeting 4 genes (*estI, eltI, stx and eaeA*) to pathotype also revealed similar results. On screening 152 *E. coli* isolates for the presence of these genes by using multiplex PCR as well as simplex specific PCRs, it was confirmed that, 18 isolates (11.8%) belonged to STEC, 23 isolates (15.1%) belonged to ETEC and only 2 isolates (1.3%) belonged to aEPEC.

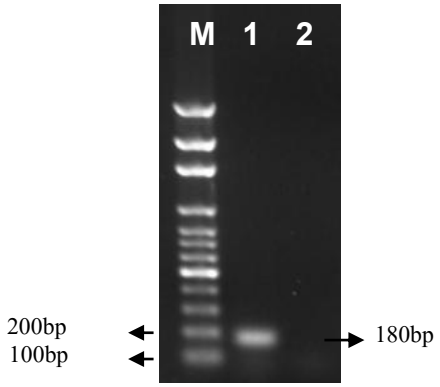

Figure 1. PCR amplification of *stx*1 gene in *E. coli*. Lane M: 100bp DNA ladder; Lane 1: *stx*1 positive strain; Lane 2, NTC

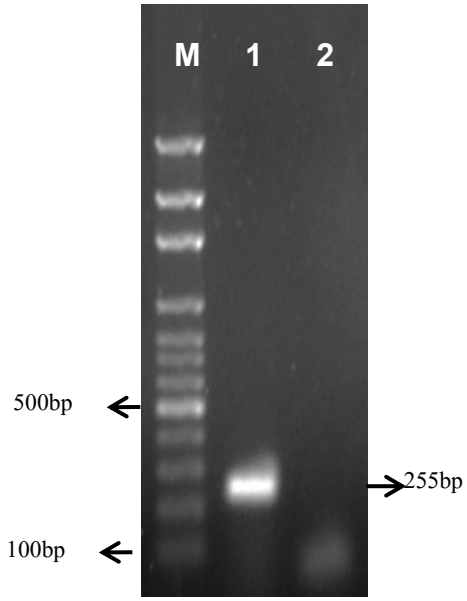

Figure 2. PCR amplification of *stx2* gene in *E. coli*. Lane M: 100bp ladder; Lane 1: *stx*2 (255bp) gene positive strain; Lane 2: NTC

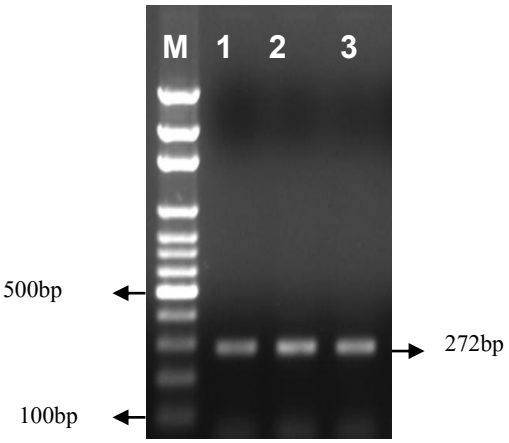

Figure 3. PCR amplification of LT gene in *E. coli*. Lane M: 100 bp DNA ladder; Lanes (1-3): LT gene positive strains

Rest isolates carried none of our targeted genes. Antibiogram profiles of the isolates are presented in Table 1. Antibiotic resistance percentages for Metronidazole, Sulphafurazole, Tetracycline, Streptomycin, Trimethoprim, Neomycin, Ampicillin, Co-trimoxazole, Ceftazidime, Enrofloxacin, Chloramphenicol, Ceftriaxone, Ciprofloxacin and Gentamicin were found to be 100, 70, 68.2, 61.7, 44.8, 35.5, 34.6, 29, 25.2, 14, 10.3, 10.3, 6.5 and 3.7, respectively. Among 107 isolates, 90 (84.1%) showed resistance to more than 2 antimicrobials and are found to be multidrug resistant (MDR).Our study was found to be in concordant with a previous Indian study on diarrhoeic piglets, where 29.93% ETEC and 2.54% aEPEC prevalences were recorded (Rajkhowa *et al.,* 2014). In our study, the majority pathotype isolated was ETEC. Diarrhea due to enterotoxigenic *E. coli* (ETEC) is one of the most frequent diseases in young piglets. Among 23 ETEC isolates 12 carried *estI*, 9 carried *eltI* and 2 carried both. In previous studies also, *estI* was the most frequently encountered toxin among ETEC (Osek and Truszczynski, 1992; Frydendahl, 2002). EPEC, capable of causing attaching effacing (A/E) lesions on the surface of the host's intestinal epithelium is classified into "typical" and "atypical" subtypes depending on the existence of attaching effacing (*eae*), and also genes encoding bundle-forming pili (*bfp*) (Trabulsi *et al.,* 2002). However, as in our study, recent data signifies that infections with aEPEC (only *eae*) surpass those with tEPEC (*eae* and *bfp*) in both developing and developed countries (Hernandes *et al.,* 2009). In the present study STEC prevalence was 11.8%. Similar kind of data is also available from a previous study where they reported, 14.4 % STEC prevalence in piglets (Rajkhowa and Sarma, 2014). In summary, among the 14 antibiotics examined *in vitro* in this study, least resistance ($\leq$ 8%) was seen against ciprofloxacin and gentamicin among the *E. coli* isolates tested. In conclusion, this study recorded the frequency of virulence genes and antimicrobial resistance in *E. coli* strains isolated from diarrhoeic piglets in Meghalaya. The most prevalent pathotype in diarrhoeic piglets was found to be ETEC. This information will be useful for scheming lucid preventive actions, including vaccination, for enteric *E. coli* infections in pigs and management of rational antibiotic use.

Acknowledgement

The authors are thankful to the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, New Delhi for sanctioning the project and providing financial support. The authors are also grateful to the Director, ICAR RC for NEH Region, Umiam for providing necessary facilities to conduct this research work.

Table 1. Antibiogram profile of *E. coli* isolates

Sl. No	Antibiotic (mcg)	No. of Resistant isolates (%)	No. of Intermediate isolates (%)	No. of Sensitive isolates (%)
1	Metronidazole (5)	107 (100)	0	0
2	Sulphafurazole (300)	75 (70)	5 (4.7)	27 (25.2)
3	Tetracycline (30)	73 (68.2)	21 (19.6)	13 (12.1)
4	Streptomycin (10)	66 (61.7)	20 (18.7)	21 (19.6)
5	Trimethoprim (30)	48 (44.8)	4 (3.7)	55 (51.4)
6	Neomycin (30)	38 (35.5)	59 (55.1)	10 (9.3)
7	Ampicillin (10)	37 (34.6)	40 (37.4)	30 (28)
8	Co-trimoxazole (25)	31 (29)	0	76 (71)
9	Ceftazidime (30)	27 (25.2)	30 (28)	50 (46.7)
10	Enrofloxacin (10)	15 (14)	1 (0.9)	91 (85)
11	Chloramphenicol (25)	11(10.3)	7 (6.5)	89 (83.1)
12	Ceftriaxone (30)	11 (10.3)	8 (7.5)	88 (82.2)
13	Ciprofloxacin (10)	7 (6.5)	3 (2.8)	97 (90.6)
14	Gentamicin (10)	4 (3.7)	12 (11.2)	91 (85)

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