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Antimicrobial assay of shigatoxigenic *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) isolated from diarrhoeic faecal samples of piglets and infants in Mizoram

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ABSTRACT

Antimicrobial resistance is a common problem which is accelerating day by day in veterinary medicine. The main reason is believed to be the indiscriminate and irrational use of antibiotics. Diarrhoeic faecal samples (584: 320 from piglets and 264 from infants) were collected from different farms and hospitals located in different districts of Mizoram. Out of 1,260 *E. coli* isolates, 65 (5.15%) *E. coli* isolates were found positive for at least 1 virulence gene (stx_1, stx_2, *eaeA, hlyA*) under the study, of which 40 (3.17%) and 25 (1.98%) were recorded as STEC and EPEC, respectively, when screened by multiplex PCR. All the 65 *E. coli* isolates were subjected to antimicrobial sensitivity test against 12 commonly used antimicrobial agents. Among the isolates from piglets, highest sensitivity was exhibited by chloramphenicol (89.58%) and highest resistance by nalidixic acid (85.41%). On the other hand, enrofloxacin exhibited 100% sensitivity while amoxicillin, polymyxin B and kanamycin exhibited 100% resistance among the infant isolates. It may be concluded that 5.15% isolates were positive for virulence and the isolates showed increased tendency of resistance to many of the commonly used antibiotics reflecting a greater threat to treat the commonly occurring diseases with antibiotics.

Key words: Antimicrobial sensitivity, Enteropathogenic *Escherichia coli*, Infant diarrhoea, Piglet diarrhoea, Shigatoxigenic *Escherichia coli*

Acute diarrhoea, a leading cause of mortality in developing countries, is considered as one of the main causes of morbidity and mortality especially in children. Diarrhoeagenic *E. coli* (DEC), important etiological agents of diarrhoea (Asadi *et al.* 2010, Moyo *et al.* 2007), includes enteropathogenic *E. coli* (EPEC) and shigatoxin-producing *E. coli* (STEC) (Nataro and Kaper 1998, Begum *et al.* 2014). STEC, an important food-borne pathogen (Mekata *et al.* 2014), is implicated in outbreaks of food-borne illness and can cause diarrhoea, especially among the infants. STEC is associated with piglet diarrhoea in India (Mandakini *et al*. 2015). *E. coli* O157: H7 can be regarded as prototype of STEC causing disease in humans, and most outbreak cases of bloody diarrhoea and hemolytic-uremic syndrome (HUS) are attributed to strains of STEC serotype O157: H7 (Pennington 2010). EPEC is a major cause of infantile diarrhoea among children in developing countries (Dhanashree and Mallya 2008, Ghosh and Ali 2010). EPEC is often grouped together and also known as attaching and effacing *E. coli,* adheres to the intestine and produces attaching and effacing (AE) lesions (Dhanashree and Mallya 2008), which are characterized by dissolution of brush border membrane and destruction of microvilli. It lacks fimbriae; produces heat stable (ST) and heat labile toxins (LT); possess *eaeA* gene that encodes intimin protein involved in the intimate adhesion of bacteria to the enterocytes and production of AE lesion on intestinal mucosa (Bhat *et al.* 2008, Duda-Madej *et al.* 2013). *E. coli* resistance to commonly used antibiotics was highest in the isolates from chickens (74.0%), followed by pigs (64.8%) and cattle (61.3%) (Kikuvi *et al.* 2013). The single main factor contributing the increase in the antibiotics resistance is irrational use of antibiotics (Raji *et al*. 2008, Laxminarayan *et al*. 2013). As these organisms are ubiquitous in nature, transmission can occur in every possible way causing diseases in both man and animals.

Begum *et al.* (2014) reported that STEC and EPEC seem to be associated with diarrhoea in piglets and infants in Mizoram. Therefore, the present study was conducted to

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investigate and rule out the emergence of antibiotic resistance as well as for identification of most effective antibiotic for treatment of STEC and EPEC associated problems in Mizoram.

MATERIALS AND METHODS

Sampling and isolation of E. coli isolates: Faecal samples (584) were collected from piglets (320), 20–60 days old and infants (264), 2–24 months old with the history of diarrhoea from different districts of Mizoram during June 2010 to April 2011. Samples were collected by dry rectal swabbing using sterilized absorbent cotton swab for closer locations and sterilized swab dipped in nutrient broth for distance locations. Each swab containing the collected sample were inserted into separate sterilized test tubes plugged with nonabsorbent cotton plug and carried to the laboratory under proper cold chain for further processing. Details of the sample collection are depicted in Tables 1, 2. The *E. coli* isolates were isolated from the fecal samples as dealt previously (Begum *et al.* 2014).

Table 1. Details of diarrhoeic faecal samples collected from piglets from 5 different districts of Mizoram**.**

Time of collection	Place of collection (Districts)	No. of samples collected
June 2010	Aizawl	21
July 2010	Aizawl	52
August 2010	Kolasib	40
September 2010	Lunglei	32
October 2010	Aizawl	22
November 2010	Champhai	20
December 2010	Aizawl	25
January 2011	Serrchip	24
February 2011	Aizawl	21
March 2011	Aizawl	26
April 2011	Aizawl	37
		320

Procurement of standard cultures: The standard cultures of *E. coli* (positive for stx_1, stx_2, *eaeA* and *hlyA* gene) were kindly provided by Dr S A Wani, Division of Veterinary Microbiology and Immunology, Sher-e-Kashmir University of Agricultural Sciences and Technology. *Salmonella enteritidis* ATCC 13076 was procured commercially.

Multiplex PCR for detection of STEC and EPEC isolates: A multiplex PCR was carried out in a thermal cycler for all *E. coli* isolates exhibiting positive morphological and biochemical test using four sets of oligonucleotide primers for stx_1, stx_2 (STEC) and *eaeA*, *hlyA* (EPEC) genes as per Paton and Paton (1998) with suitable modifications. Details of the primers used in this study are given in Table 3 (Begum *et al.* 2014).

The amplified PCR products were visualized under UV trans-illuminator after agarose gel electrophoresis (1.5% agarose in 1X TAE at 80 V/cm for 45 mins) and documented by a gel documentation system. A known molecular weight marker (100 bp DNA ladder) was used for each run to

Table 2. Details of the diarrhoeic faecal samples collected from infants from various hospitals located at Aizawl district

Month of collection	Place of sample collection				Total no. of samples collected
	Civil Hospital, Aizawl	Private Hospital 1 Bawngkawn	Private Hospital 2 Chanmari	Private Hospital 3 Durtlang	
June 2010	5	2	1	15	23
July 2010	4	4	5	6	19
August 2010	6	4	4	10	24
October 2010	0	0	5	10	15
November 2010	5	7	0	8	20
December 2010	8	0	5	7	20
January 2011	5	0	8	4	17
February 2011	10	0	2	20	32
March 2011	8	5	7	13	33
April 2011	0	10	5	12	27
Total	60	32	53	126	264

Table 3. Details of the oligonucleotide primers used in the present study

Primer name	Sequences (5' - 3')	Expected amplicon size (bp)	References
stx_1 Fstx_1 R	ATAAATCGCCATTCGTTGACTACAGAACGCCCACTGAGATCATC	180	Paton and Paton (1998)
stx_2 Fstx_2 R	GGCACTGTCTGAAACTGCTCCTCGCCAGTTAATCTGACATTCTG	255	Paton and Paton (1998)
eaeA FeaeA R	GACCCGGCACAAGCATAAGCCCACCTGCAGCAACAAGAGG	384	Paton and Paton (1998)
hlyA FhlyA R	GCATCATCAAGCGTACGTTCCAATGAGCCAAGCTGGTTAGCT	534	Paton and Paton (1998)

Table 4. List of the antibiotics used in the present study

Serial No.	Antimicrobial agent	Conc./disc
1	Amoxycillin (Am)	30µg
2	Gentamicin (G)	10 µg
3	Ofloxacin (Of)	5 µg
4	Enrofloxacin (Ex)	10 µg
5	Chloramphenicol (C)	30 µg
6	Cefixime (Cfx)	5 µg
7	Tobramycin (Tb)	10 µg
8	Nalidixic acid (Na)	30 µg
9	Polymyxin B (Pb)	300 µg
10	Kanamycin (K)	30 µg
11	Ceftriaxone (Cl)	30 µg
12	Cefhotaxime (CE)	30 µg

compare the amplicon size. The PCR reaction was performed thrice to ensure the repeatability of the techniques and to make sure that strains were correctly assigned to respective patterns.

Antibiotic sensitivity test: All STEC and EPEC isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method against 12 commonly used antibiotics as per method of Bauer *et al.* (1966). *Salmonella enteritidis* (ATCC 13076) was used as standard culture. Following antimicrobial agents were used in the present study (Table 4).

Each isolate was grown in brain heart infusion (BHI) broth overnight, under constant shaking at 37°C. One hundred microliter of overnight broth culture (100 µl) was spread uniformly over Mueller Hinton (MH) agar No. 2 plate. The plate was allowed to dry for 10–15 min to absorb the liquid and antibiotic discs were placed on inoculated agar surface at about 2 cm apart by using flamed forceps. The plates were incubated at 37°C overnight and diameter of the zones of inhibition was measured. The measurements were compared with zone size interpretative chart furnished by the manufacturer and the zones were graded as sensitive, intermediate and resistant.

RESULTS AND DISCUSSION

As the antimicrobial resistance has become a global health issue, the major goal of this study was to document antimicrobial drug resistance of historical bacteria, *E. coli* from humans and animals to associate emergence of resistance. The study was carried out with the samples collected form diarrhoeic piglets and infants in the Northeastern state of India, Mizoram. All of the diarrheic samples were examined using culture and PCR techniques for confirmation (Figs 1, 2). To help characterize evolution of drug resistance in *E. coli* for antimicrobial drugs, we tested 65 *E. coli* isolates from human and swine sources for susceptibility to a common panel of 12 antimicrobial agents.

Of the 48 *E. coli* isolates from piglets, majority exhibited varied level of resistance to different antibiotics. Isolates exhibited highest resistance to nalidixic acid followed by

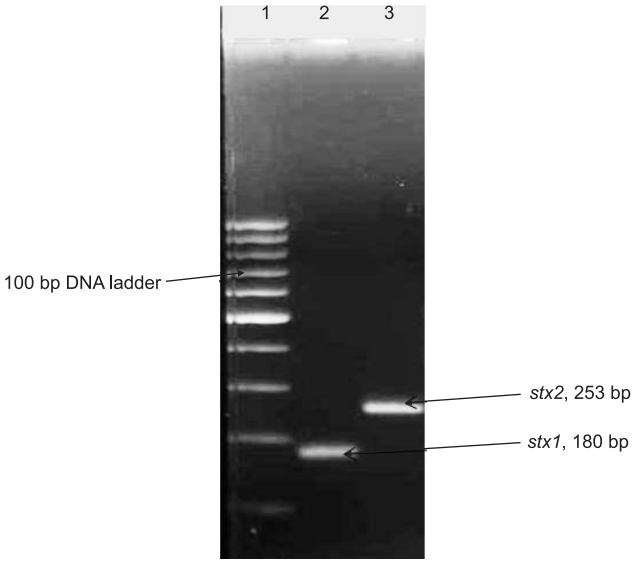

Fig. 1. Multiplex PCR analysis for *E. coli* isolates positive for STEC targeting *stx*1 and *stx*2 genes. Lane 1. 100 bp DNA ladder; lane 2. *E. coli* isolate positive for *stx*1 gene; lane 3. *E. coli* isolate positive for *stx2* gene.

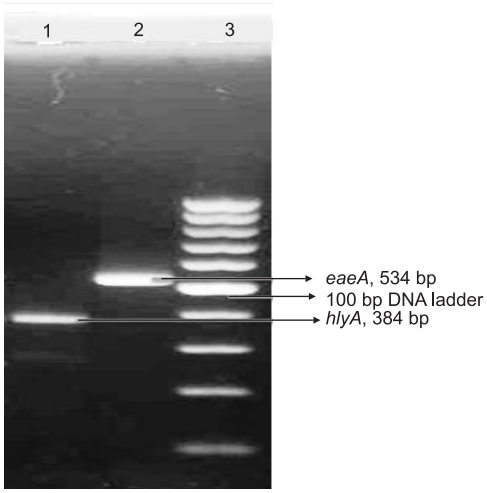

Fig. 2. Multiplex PCR analysis for *E. coli* isolates positive for EPEC targeting *eaeA* and *hlyA* genes. Lane 1. *E. coli* isolate positive for *hlyA* genes; lane 2: *E. coli* isolate positive for *eaeA* gene; lane 3: 100 bp DNA ladder.

polymyxin B, amoxicillin, cefixime, kanamycin, cephotaxime, ceftriaxone, ofloxacin, enrofloxacin, tobramycin, gentamicin and chloramphenicol, in contrast, highest sensitivity was exhibited to chloramphenicol followed by ofloxacin, tobramycin, ceftriaxone, enrofloxacin, gentamicin, polymyxin B, cefhotaxime, amoxycillin, cefixime, kanamycin and nalidixic acid (Table 5).

Name of different antibiotics

This study adds to existing knowledge about resistance in commensal *E. coli* in an Indian community setting. In 17 *E. coli* isolates from infants, highest resistance (100%) was exhibited to amoxycillin, polymyxin B and kanamycin followed by nalidixic acid. On the contrary, highest

Table 5. Details of antimicrobial sensitivity / resistance pattern of *E. coli* isolated from piglets

Antimicrobial agents	Concentration per disc	Sensitive	Intermediate	Resistant
Amoxycillin (Am)	30µg	14 (29.16%)	6 (12.5%)	28 (58.33%)
Gentamicin (G)	10 µg	2 (43.75%)	23 (47.91%)	4 (8.33%)
Ofloxacin (Of)	5 µg	37 (77.08%)	3 (6.25%)	8 (16.66%)
Enrofloxacin (Ex)	10 µg	32 (66.66%)	9 (18.75%)	7 (14.58%)
Chloramphenicol (C)	30 µg	43 (89.58%)	2 (4.16%)	3 (6.25%)
Cefixime (Cfx)	5 µg	10 (20.83%)	21 (43.75%)	17 (35.41%)
Tobramycin (Tb)	10 µg	35 (72.9%)	7 (14.58%)	6 (12.5%)
Nalidixic acid (Na)	30 µg	4 (8.33%)	3 (6.25%)	41 (85.41%)
Polymyxin B (Pb)	300 µg	19 (39.58%)	0	29 (60.41%)
Kanamycin (K)	30 µg	4 (8.33%)	28 (58.33%)	16 (33.33%)
Ceftriaxone (Cl)	30 µg	34 (70.83%)	4 (8.33%)	10 (20.83%)
cefhotaxime (CE)	30 µg	17 (35.41%)	18 (37.5%)	13 (27.08%)

Table 6. Details of antimicrobial sensitivity / resistance pattern of *E. coli* isolated from infants

Antimicrobial agents	Concentration per disc	Sensitive	Intermediate	Resistant
Amoxycillin (Am)	30µg	0	0	17(100%)
Gentamicin (G)	10 µg	2 (11.76%)	15 (88.23%)	0
Ofloxacin (Of)	5 µg	0	17 (100%)	0
Enrofloxacin (Ex)	10 µg	17 (100%)	0	0
Chloramphenicol (C)	30 µg	0	17 (100%)	0
Cefixime (Cfx)	5 µg	15 (88.2%)	2 (11.76%)	0
Tobramycin (Tb)	10 µg	14 (82.3%)	3 (17.64%)	0
Nalidixic acid (Na)	30 µg	2 (11.76%)	0	15 (88.2%)
Polymyxin B (Pb)	300 µg	0	0	17 (100%)
Kanamycin (K)	30 µg	0	0	17 (100%)
Ceftriaxone (Cl)	30 µg	16 (94.1%)	1 (5.88%)	0
cefhotaxime (CE)	30 µg	0	17 (100%)	0

sensitivity was exhibited to enrofloxacin (100%) followed by ceftriaxone, cefixime, tobramycin and least sensitivity to gentamicin and nalidixic acid. Infant isolates exhibited 100% intermediate sensitivity to ofloxacin, cefhotaxime and chloramphenicol (Table 6).

E. coli isolates (256) from piglets (suckling and weaned piglets) in Croatia showed the highest rate of resistance to oxytetracycline, streptomycin and ampicillin (98%, 91% and 85% of isolates, respectively) and whereas 87% of the isolates were resistant to 4 or more antimicrobials including nalidixic acid (80%). Further, in parallel to results of our study, their isolates were sensitive to enrofloxacin (50%) and chloramphenicol (54%) (Habrun *et al.* 2010). In another study, animal *E. coli* isolates including pigs showed increased resistance to 11/15 antimicrobial agents, including ampicillin, sulfonamide and gentamicin. A much smaller number of isolates were resistant to antimicrobial drugs introduced for clinical use since 1980, such as amoxicillin/clavulanic acid (5.6%), ceftriaxone (2.4%), ceftiofur (2.3%). Even the resistance to chloramphenicol is also small percentage from isolates of *E. coli*. This indicates that the isolates are sensitive to newer antimicrobials (Tadesse *et al.* 2012).

Pig isolates showed increased resistance to 11/15 antimicrobial agents, including ampicillin, sulfonamide and gentamicin (Tadesse *et al.* 2012). The majority of the swine isolates were resistant to tetracycline (79.57%), nalidixic acid (78.49%), trimethoprim-sulfamethoxazole (73.12%) and kanamycin (55.91%) (Meng *et al.* 2014). Further, pig isolates were sensitive to ceftazidime, ceftriaxone, cefotaxime cefepime, and amikacin (Tian *et al.* 2012) of which some findings are true with our study.

Abea *et al*. (2009) reported that among 72 EPEC tested against 12 antibiotics, 56.9% showed resistance to at least 1 antimicrobial agent and resistance was observed against tetracycline (36.1%), trimethoprim-sulfamethoxazole (27.8%), streptomycin (23.6%), cephalothin (16.7%), amoxicillin-clavulanic acid (2.8%) and ceftazidime (1.4%). All the strains were susceptible to ciprofloxacin, chloramphenicol, ceftriaxone, gentamicin and nalidixic

acid. It is reported that *E. coli* is resistant to 12 commonly used (ceftiofur, colistin, amoxicillin, amoxicillin-clavulanic acid, enrofloxacin, gentamycin, penicillin, oxytetracycline, lincospectin, tiamulin + tetracycline, erythromycin, doxycicline) tested antimicrobials (Herman *et al.* 2010).

E. coli isolates from children were resistant to nalidixic acid (45%), tetracycline (37%), ampicillin (37%), sulfamethoxazole/trimethoprim (29%) and amoxicillin/ clavulanic acid (29%). No isolates were resistant to imipenem. Altogether, 72% of isolates were resistant to at least one antibiotic and 33% were multi-drug resistant. High rates of cross-resistance were found for the 15 antibiotics studied. Ciprofloxacin was found effective as only 8% of isolates were found resistant to this antimicrobial agent (Shakya *et al.* 2013) which agrees with the results obtained from its congener agent, enrofloxacin in our study. Rivero *et al*. (2010) reported that most of the verotoxxigenic *E. coli* (VTEC) isolates recovered from children with acute diarrhoea exhibited sensitivity to all antimicrobials tested (cefotaxime, ciprofloxacin, gentamicin, imipenem, streptomycin exhibited 100% sensitivity) except one O157: H7 strain exhibited resistance to amoxicillin / clavulanic acid and two O145 : H^- and one O157: H7 strain resistant to cephalothin.

More than 80% of *E. coli* pathotypes from the samples of young children with diarrhea were resistant to ampicillin, amoxycillin, metranidazole and co-trimoxazole (Sudershan *et al.* 2014). A study conducted in Bolivia indicated that more than 67% of the ETEC strains from children with diarrhoea were resistant to one or several antimicrobial agents like ampicillin, ampicillin sulbactum, tetracycline and chloromphenicol (Rodas *et al*. 2011). Diarrhoeic samples (68%) from children exhibited multiple resistance to antibiotics tested, viz. ciprofloxacin, ampicillin, amoxicillin clavulanic acid and trimethoprim-sulfamethoxazole (Iseri *et al.* 2011).

The large number of antibiotics included in the study provides a comprehensive overview of resistance pattern in commensal *E. coli*. The result obtained in this study reflects decrease in the sensitivity of chloramphenicol, tobramycin, gentamycin and ofloxacin, which exhibited 100% sensitivity in the past indicating the increase threat to antibiotic resistance in swine as well as human isolates with the exception of enrofloxacin which is still in accordance with the past record exhibiting 100% sensitivity in infant isolates. But there is no doubt that sensitivity to enrofloxacin will be decreasing in the near future if indiscriminate use of antibiotics is not discontinued. Amoxycillin, polymyxin B and kanamycin exhibited 100% resistance in infant isolates

The variation in the sensitivity pattern might be due to acquiring of drug resistance gene through horizontal gene transfer as a result of indiscriminate use of antibiotics. Multiple antimicrobial resistance in STEC and EPEC may partly result from the spread of genetic elements including plamids, transposons and or integrons (Schroeder *et al.* 2002). Because, antimicrobial resistant bacteria from food animals may colonize the human population via food chain, contact through occupational exposure or waste run off from animal production facilities (Van den Bogaard and Stobberieng 1999), it is possible that the resistance bacteria may be readily transferred from food animals to humans (Schroeder *et al.* 2002).

Our result also indicated the emergence of multiple drug resistance STEC and EPEC isolates in piglets and infants in this region. This may further complicate the future therapeutic options that are being developed for treatment of hemorrhagic colitis (HC), HUS and various other diseases caused by STEC and EPEC strains which in turn indicates emergence of potential zoonotic threat to human population. The problem of antimicrobial resistance knows no boundaries. Drug-resistant microbes of all kinds can move among people and animals, from one country to another without notice. From the early stages of identifying and discovering antibiotic resistance, the problem was clearly severe in developing countries where drug availability was limited and resistance was high. However, the developed world with its abundant resources, has more vigorously studied the resistance. Continued surveillance of emerging antimicrobial resistance in pig and human is required to ensure public health due to close association of pigs and human in this region. Furthermore, antimicrobial resistance should be taken into account as to and when the control and management strategies are to be developed to reduce the burden associated with antimicrobial resistance for animal and environmental health.

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