Molecular identification of *blaCTX-M* and *blaTEM* genes among multi-drug resistant Enteropathogenic *Escherichia coli* isolated from children

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Abstract: In this cross sectional study (June 2016 to June 2017), we studied the isolation and molecular characterization of multi-drug resistant *Escherichia coli* (MDR-*E. coli*) from children suffering from diarrhea. For this purpose, a total of 100 fecal samples were collected with the consent of the parents/ guardians on a prescribed form. The bacterial isolation was done by employing conventional and standard microbiological procedures. Subsequently, all the isolates were identified on the basis of biochemical tests and were further characterized by amplification of 16S rRNA gene followed by di-deoxy sequencing of the amplified product. Afterwards, the isolates were subjected to antimicrobial susceptibility profiling using Kirby Bauer disc diffusion method. A total of 87 *E. coli* isolates were identified in the current study and majority of the isolates were found sensitive to all or few antimicrobials. However, 14 *E. coli* isolates were found resistance to multiple drugs including amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, cefoperazone and ofloxacin, hence termed as MDR-*E. coli*. All of the 14 isolates were further analyzed for the identification of *blaCTX-M* and *blaTEM* genes through PCR using specific primers. This resistant was found to be associated with the presence of plasmid encoded beta lactamases genes including *blaCTX-M* (13/14 *E. coli* isolates) and *blaTEM* (9/14 *E. coli* isolates). Altogether, it was found that ESBLs harboring *E. coli* is potential source of diarrhea among pediatric diarrheal patients. Therefore, molecular identification and characterization of bacterial pathogens along with antimicrobial susceptibility are critical to understand MDR- *E. coli* infections.

Keywords: Enteropathogenic *E. coli*, children, multi-drug resistant, diarrhea.

INTRODUCTION

The members of the family *Enterobacteriaceae* have a substantial impact in the microbiology of gastrointestinal tract (GIT) in humans as well as in animals (Moura *et al*., 2009; Jandhyala *et al*., 2015). While, *Escherichia coli* is one of the top ranked bacterium in this family which is responsible for several disorders including diarrhea in infants, children and adults throughout the world (Allard *et al*., 2017; Gallardo *et al*., 2017). *E. coli* is classified into different groups including enterohemorrhagic, enteropathogenic, enteroinvasive, enterotoxigenic, diffusely adherent, enteroaggregative and necrotoxic *E. coli* on the basis of pathogenicity and the mechanism of virulence as described previously in different studies (Berings *et al*., 2017).

Among these groups the enteropathogenic *E. coli* (EPEC) is foremost important and is responsible for acute and chronic diarrhea in infants and children in developing as well as in developed countries. This is also a well-established fact that EPEC associated diarrhea is the second highest cause of mortalities among infants following the rotavirus infections (Lanata *et al*., 2013). EPEC do not produce any toxins and exert its pathogenic effects by attachment/effacement (A/E) lesions in the intestines. The attachment is mediated through an outer membrane protein of the bacterium termed as intimin which is encoded by *eae* gene of the bacterium (Das *et al*., 2013). The lesions are characterized by bacterial attachment to the intestinal wall of the host and disturbing the cell surface which ultimately lead to thinning and shortening of microvilli of intestine (effacement). EPEC is predominantly transmitted through fecal-oral route by contaminated hands, foods and by fomites (Rhouma *et al*., 2016; Mourand *et al*., 2017). EPEC strains are a potential cause of acute to chronic diarrhea with high morbidity and mortality rates in developed countries (Moura *et al*., 2009).

Similarly, the antimicrobial resistance among different *E. coli* isolates has been reported in different regions of the world. Few of the studies described that *E. coli* is responsible for diarrhea in almost 200 million infants and children annually resulting in huge numbers of deaths in developed as well as in developing countries. Antimicrobial resistance or multi-drug resistance is also high particularly in developing countries (Das *et al*., 2013). The diagnosis of EPEC could be performed either

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on phenotypic or on genotypic basis including 16S rRNA or *eae* gene amplification followed by sequencing of the amplified PCR product (Tariq *et al*., 2012; Abriouel *et al*., 2017). However, the multi drug resistance is detected by molecular identification and characterization based on *blaCTX-M* or other beta lactamase genes. Experimental animal models could also be used to investigate the type of *E. coli* strains as described recently (Fayyaz *et al*., 2018). Therefore, in this study, we have focused on isolation, identification and molecular characterization of multi-drug resistant enteropathogenic *E. coli* from children suffering with diarrhea along with antibiogram studies of different *E. coli* isolates.

MATERIALS AND METHODS

Isolation of Enteropathogenic Escherichia coli (EPEC)

In the present study, a total of 100 fecal samples were collected from 1-5 years children for isolation of *E. coli* during June 2016-June 2017. For this purpose small amount of fecal material was dissolved in 2 ml of normal saline and directly inoculated on MacConkey's agar (Oxoid™, UK) followed by an incubation at 37°C for 24-48 hours (Ahmed *et al*., 2009). Initial identification was performed on the basis of standard microbiological and biochemical procedures as described recently (Fayyaz *et al*., 2018).

Antibiogram studies of EPEC

The antibiogram profiles of all isolates of *E. coli* was investigated against routinely used antimicrobial agents including amoxicillin-clavulanic acid, ciprofloxacin, imipenem, gentamicin, cefoperazone, vancomycin, amikacin, meropenem, levofloxacin, ofloxacin, teicoplanin, piperacillin-tazobactam and ceftriaxone. The antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method on Muller Hinton agar (Oxoid™, UK) according to the guidelines of Clinical Laboratory Standards Institute (CLSI-2016) as described (Berings *et al*., 2017; Fayyaz *et al*., 2018).

Molecular Identification of multi-drug resistant EPEC, blaCTX-M and blaTEM

For the molecular characterization of EPEC, the bacterial DNA was extracted using commercially available DNA extraction kit (Favorgen® Biotech Corp. Taiwan) according to manufacturer's instructions. DNA amplification was carried out by targeting the 16S rRNA gene of the bacterium using universal set of primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) as described (Dupont *et al*., 2016). DNA amplification was also performed targeting the *blaCTX-M* and *blaTEM* genes for identification of beta lactamase resistance using CTX-M and TEM forward/reverse primers as described (Ghorbani-Dalini *et al*., 2015). The amplification was carried out in total volume of 20 µL which was consisted of 1 µL of bacterial DNA template, 1 µL of each forward and reverse primers and Master Mix (12.5 µL). Whereas negative control contained water instead of template DNA, while *E. coli* genomic DNA was used as positive control. Finally, the amplified product was subjected to electrophoresis using 1.5% agarose gel stained with ethidium bromide and visualized under UV light. After the gel purification the amplified products were dispatched to Macrogen, Korea for di-deoxy Sanger sequencing method. The obtained sequences were analyzed with Chromas Pro® (Technelysium, Technelysium Pty Ltd, Australia) to assess the sequencing quality and contigs were formed and sequences were aligned using BLASTn online program. The aligned sequences were compared with the available bacterial 16S rRNA sequences in NCBI database as described (Dupont *et al*., 2016).

RESULTS

A total of 87 *E. coli* were identified out of 100 samples on the basis of the biochemical profiles. The antibiogram data indicated that majority of *E. coli* were sensitive to several antibacterial agents. However, a total of 14 (16%) *E. coli* isolates were found resistant to different groups of antibiotics including amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, cefoperazone and ofloxacin, hence termed as MDR *E. coli* strains as shown in table 1.

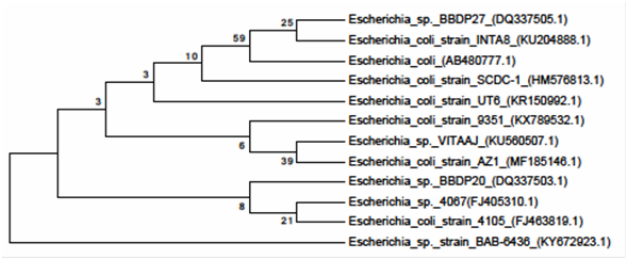

Fig. 1: Phylogenetic position of multi drug resistant *E. coli AZ1* strain

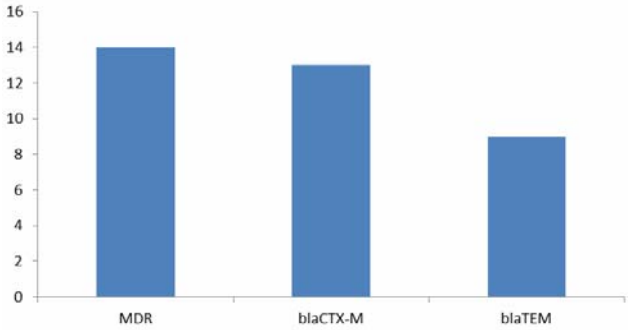

Fig. 2: Molecular identification of *blaCTX-M* and *blaTEM* genes among MDR- *E. coli*

The genomic amplification of MDR *E. coli* resulted in 1500 bp product, the sequencing data and homology search showed that isolated bacteria were

Table 1: Antimicrobial Susceptibility Profiles of MDR-*E. coli* Isolates

	Isolates														
Antibiotics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	*Resistance %*
AMC	R	R	R	R	R	R	R	R	R	R	S	R	R	R	93
CIP	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
IMP	S	S	I	S	S	S	S	S	I	S	S	S	S	S	0
GEN	R	R	S	R	I	R	I	S	S	R	I	S	R	S	43
CFP	I	S	S	R	S	S	S	S	I	S	S	S	R	S	14
VAN	No Zone of Inhibition														
AMK	R	R	R	S	R	R	I	I	R	R	R	S	S	R	64
MEM	S	I	S	S	S	S	R	I	S	S	R	S	S	I	14
LVX	R	R	R	R	R	R	R	R	R	R	R	R	I	I	86
OFX	R	R	R	R	R	R	R	R	R	R	R	R	R	I	93
TEC	No Zone of Inhibition														
TZP	R	S	R	R	S	S	R	I	R	S	I	R	S	S	43
CRO	I	S	S	R	R	I	S	S	R	S	I	R	I	S	28

AMC=Amoxicillin-Clavulanic Acid (20/10µg), Cip=Ciprofloxacin (5µg), Imp= Imipenem (10µg), GEN= Gentamicin (10µg), CFP= Cefoperazone (75µg), VAN= Vancomycin (30µg), AMK= Amikacin (30µg), MEM= Meropenem (10µg), LVX= Levofloxacin (5µg), OFX= Ofloxacin (5µg), TEC=Teicoplanin (30µg), TZP= Piperacillin-Tazobactam (100/10µg) and CRO= Ceftriaxone (30µg).

enteropathogenic *E. coli*. One sequence was submitted to GenBank and named as multidrug resistant *E. coli* AZ1 strain (GenBank Accession Number: MF185146). The phylogenetic position of this strain was shown in fig. 1. Molecular identification of *blaCTX-M* and *blaTEM* was identified among 13/14 (93%) and 9/14 (64%) *E. coli* isolates, respectively as shown in fig. 2.

DISCUSSION

The impact of enteropathogenic *Escherichia coli* (EPEC) could not be neglected as it continued as major etiological agent of diarrhea and significant morbidities and mortalities in infants and children throughout the world (Gallardo *et al*., 2017). The actual duration and progression of EPEC infection is not clearly defined. In the present study, we characterized the enteropathogenic *E. coli* on the basis of sequence analysis of 16S rRNA gene as already reported in previous studies (Dupont *et al*., 2016). In previous studies, the molecular characterization and phylogenetic analysis was performed for EPEC strains which were originated from different sources using same gene amplification and sequence analysis (Saeed *et al*., 2009; Saravia *et al*., 2017).

In the present study, antimicrobial susceptibility data showed 14 MDR- *E. coli* (16%) whereas beta lactamase enzyme *blaCTX-M* gene was identified among 93% (13/14) of MDR-*E. coli*. One of the recent studies described that extended spectrum beta lactamases are enzymes encoded by different genes on bacterial plasmids and are responsible for degradation of antimicrobials (Mourand *et al*., 2017). Overall an increased occurrence of MDR- *E. coli* was found in the current study. Many of the previous studies described *E. coli* as primary pathogen of gastrointestinal tract causing diarrhea and gastric disturbances by eruption of intestinal epithelium and imbalance of fluids (Fayyaz *et al*., 2018). An increased occurrence of *E. coli* was observed in previous studies indicating the significance of antimicrobial resistance as an alarming situation to children health in developing as well as in developed countries.

CONCLUSION

This study showed that the occurrence of diarrhea is contributed to enteropathogenic *E. coli* which could be multi-drug resistant, therefore we suggested the empirical antimicrobial therapy to combat the EPEC infections particularly among children to control diarrhea. Further, molecular identification and characterization is also critical to confirm the accurate cause of the infection.

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