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Note

A comparative study for detection of extended spectrum β-lactamase (ESBL) production by Enteroaggregative *Escherichia coli* (EAEC) strains using double disc, nitrocefin and PCR assays

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ABSTRACT

We explored and evaluated for the first time colorimetric nitrocefin assay in conjunction with the double disc test and PCR assay. We suggested the use of nitrocefin assay for rapid screening of ESBL-production by Enteroaggregative *Escherichia coli.*

Enteroaggregative *Escherichia coli* (EAEC) are a heterogeneous pathotype of diarrhoeagenic *E. coli* (DEC), which were initially identified in the year 1987 (Croxen et al., 2013; Jensen et al., 2014) and are now considered as an important emerging pathogen for endemic as well as epidemic diarrhoeal episodes globally (Croxen et al., 2013; Rogawski et al., 2017). Moreover, the EAEC pathotype has also been associated with food-borne outbreaks in the industrialized world (Harrington et al., 2006). The pathogen causes chronic inflammation and damage to the intestinal epithelium, thereby causing malnutrition as well as intellectual deficits in infants (Jensen et al., 2014; Rogawski et al., 2017) while, intestinal changes and diarrhoeal episodes are notable features in animal EAEC infections (Villaseca et al., 2005; Kolenda et al., 2015). In diagnostic settings, EAEC is reported to exhibit a typical 'stacked-brick' formation on human epithelial cell-lines, namely HEp-2 cells, which is employed as the gold standard tool (Nataro and Kaper, 1998; Estrada-Garcia and Navarro-Garcia, 2012; Jensen et al., 2014).

Of late, resistance to a wide range of antibiotics has become a common feature among the identified bacterial isolates worldwide (Aslani et al., 2011; O'Neill, 2016). Earlier workers have reported perturbing trends of multi-drug resistance (MDR), especially towards the quinolones and extended spectrum beta–lactams (ESBL) among the EAEC strains (Bangar and Ballal, 2008; Oundo et al., 2008; Aslani et al., 2011; Imuta et al., 2016). MDR pathogens producing ESBLs are reported to spread worldwide, mainly as nosocomial pathogens, in community as well as in hospital settings (Hrabák et al., 2014). In this context, patients infected with ESBL-producing organisms should additionally be placed under contact precautions in order to avoid nosocomial transmission (Siegel et al., 2007), which necessitated an urgent requirement for the rapid laboratory detection of ESBL-producing pathogens.

On the diagnostic front, the routine ESBL detection utilize the protocols recommended by US Clinical and Laboratory Standards Institute (CLSI) as well as the UK Health Protection Agency (HPA) guidelines (HPA, 2008; CLSI, 2018), which comprises of phenotypic detection methods such as combined disc test (cefotaxime, ceftazidime with or without clavulanic acid) and E-test. Besides, certain automated systems, genotypic detection methods employing PCR amplification of different groups of CTX-M (Batchelor et al., 2005), duplex PCR (Pitout et al., 2007), multiplex PCR (Woodford et al., 2006), real-time PCR (Birkett et al., 2007), pyrosequencing (Naas et al., 2007) and reverse-line hybridization (Ensor et al., 2007) have also been reported for detection of ESBL producing bacteria. Though the rapid screening as well as detection of β-lactamase-producing organisms employing nitrocefin (3-(2,4-dinitrostyryl)-(6*R*, 7*R*)-7-(2-thienylacetamido)-ceph-3-em-4 carboxylic acid, E-isomer), a chromogenic cephalosporin substrate has been reported (O'Callaghan et al., 1972; Coudron et al., 1997; Creighton, 2015), its practical utility in clinical settings does not appear to be exploited widely. Earlier studies were either restricted to

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detection of ESBL producing pathogens by either comparing nitrocefin assay with double-disc test (Tristram et al., 2005; Mansouri et al., 2012) and/or double-disc test with PCR assays (Lin et al., 2012; Yesmin et al., 2015) and/or nitrocefin assay with PCR assays targeting ESBL production genes (Jiang et al., 2006; Papanicolas et al., 2014). All three assays together have been compared for the detection of ESBL producing *S. aureus* (Papanicolas et al., 2014) and *P. aeruginosa* (Jiang et al., 2006) wherein, double disc testing revealed a better ESBL-identification than the other two assays (nitrocefin and PCR). However, such studies have never been reported for EAEC isolates. Hence, the present study was envisaged to perform an evaluation of EAEC isolates recovered from various sources using colorimetric nitrocefin test, double disc test as a phenotypic test vis-à-vis detection of *bla*AmpC, *bla*TEM, *bla*CTX-M-1 and *bla*CTX-M-9 genes by PCR assay and to suggest a rapid screening tool for detection of ESBL-production by EAEC isolates.

EAEC isolates ($n = 74$) confirmed by PCR (Vijay et al., 2015) as well as HEp-2 adherence assay (Cravioto et al., 1979) recovered from diarrhoeal samples of human ($n = 38$), canine ($n = 16$), bovine ($n = 5$), caprine ($n = 3$) and porcine ($n = 3$); food samples ($n = 8$) and sewage ($n = 1$) were screened by the nitrocefin assay, double disc test and PCR to detect ESBL production by EAEC isolates. The details of the primers used in the present study are given in Table 1.

Initially, all the EAEC isolates ($n = 74$) were subjected to double disc phenotypic testing method using antibiotic discs of cefotaxime and ceftazidime with clavulanic acid (BD Difco, USA) as per the Clinical and Laboratory Standards Institute (CLSI) (2018) guidelines. *E. coli* ATCC 25922 was used as reference control. The results revealed resistance among 39.19% (29/74) EAEC isolates by the said assay. Simultaneously, all the identified EAEC isolates ($n = 74$) were subjected to PCR amplification of the *bla*AmpC, *bla*TEM, *bla*CTX-M-1 and *bla*CTX-M-9 genes to understand genotypic profile of ESBL-producing EAEC strains. In the PCR assay, 39.19% (29/74), 62.16% (46/74), 22.97% (17/74) and 24.32% (18/74) of the EAEC isolates revealed positivity for the *bla*AmpC, *bla*TEM, *bla*CTX-M-1 and *bla*CTX-M-9 genes, respectively. Overall, a diverse PCR profile for ESBL-producing genes was observed among the EAEC isolates tested (Table 2).

Further, the chromogenic properties of the nitrocefin degradation were explored for detection of β-lactamase production by EAEC isolates. The presence of a highly reactive β-lactam ring enables nitrocefin to undergo an immediate colour change (orange to red) when hydrolysed by β-lactamase produced by the bacteria. Moreover, the test is sufficiently sensitive to indicate the presence of β-lactamases even in small quantities (O'Callaghan et al., 1972; Creighton, 2015). In brief, a working solution of nitrocefin (Cayman Chemical, USA) at a concentration of 1.0 mg mL^{-1} was prepared in sterile Phosphate Buffered Saline (PBS), of which 3–5 drops were added to each overnight grown (1 mL) EAEC isolates ($n = 74$) in nutrient broth (BD Difco, USA). This inoculated broth suspension was then incubated at 37 °C for 30 min to observe change in colour. EAEC isolates revealing change in colour within 30 min were considered as ESBL producing EAEC strains. Of the 74 tested EAEC isolates, 31 (41.89%) were found positive by the nitrocefin assay.

Our study appears to be the first of its kind to explore the utility of the colorimetric nitrocefin assay in conjunction with the double disc test as well as genotypic PCR assay. On comparative analysis of all the three assays (Table 2) for detection of ESBL producing EAEC strains, a strong positive correlation (Pearson 'r^2' value = 0.9913) was observed between the nitrocefin assay and double disc testing method for detection of ESBL producing EAEC isolates (Table 2). The EAEC isolates namely, EAHM8, EAHU37 and EAHU44 though found negative in double disc test were found positive in the nitrocefin assay for ESBL. On the contrary, EAEC isolate (EAHU30) which was found positive in double disc testing was found negative by the nitrocefin assay. False-positive or negative results employing phenotypic confirmatory tests have been reported earlier (Paterson and Bonomo, 2005). For example, some of the *Klebsiella pneumoniae* isolates were found to harbour plasmid mediated AmpC-type β-lactamases as well as other ESBL enzymes (Tzouvelekis et al., 1999). Such co-existences of both enzyme types in the same isolate might result in false-negative detection of ESBLs (Paterson and Bonomo, 2005). Further on comparing genotypic (PCR) profile either with results of double disc testing method and nitrocefin assay, a perfect correlation could not be observed for all the four ESBL genes targeted in the present study (Table 2). Such diverse genotypic pattern has been observed in earlier studies while using molecular-based ESBL detection assays (Bradford, 2001; Paterson and Bonomo, 2005). Moreover, both 'overt' as well as 'covert' phenotypic resistance observed among certain ESBL-producing bacterial strains could justify this diverse genotypic pattern (Patterson et al., 2000).

Further, in the present study, the relative sensitivity, specificity, positive and negative predictive values were determined for the nitrocefin assay as well as genotypic PCR assay by using double disc test as a reference method (Table 3). The relative sensitivity (96.55%) and relative specificity (93.33%) of the nitrocefin assay were found to be better than genotypic PCR assay (only for the spectrum of ESBL genes studied). Similar observations had been reported earlier wherein sensitivity and specificity of phenotypic methods were found to be superior to genotypic methods for ESBL detection (Paterson and Bonomo, 2005).

In view of the above observations, the nitrocefin assay could be an ideal method for rapid ESBL detection as it could detect ESBL-producing pathogens within 30 min, as compared to the PCR assay (4 h) and double disc test (18–24 h). Besides, in developing countries, where the infection rates due to ESBL bacteria are higher, rapid test such as the nitrocefin assay could serve as a method of choice for affordable screening (US$ 0.50/reaction) as compared with phenotypic double disc test (US$ 1.50/reaction) and genotypic PCR assay (US$ 9.50/reaction). Moreover, the nitrocefin assay has been advised as a rapid screening approach for ESBL producers, as in the case of *Haemophilus influenzae* (Parr and Bryan, 1984), *Enterobacter cloacae* and *Enterobacter aerogenes* (Tzelepi et al., 2000; Aibinu et al., 2003), *Pseudomonas aeruginosa* (Jiang et al., 2006) and *Staphylococcus saprophyticus* (Creighton, 2015). Also, CLSI (2018) guidelines suggest that bacterial pathogens resistant to penicillin or ampicillin due to production of β-lactamase enzyme are not reliably detected with routine disc or dilution methods, but are effectively detected using nitrocefin-assay.

In conclusion, considering the growing menace of antimicrobial resistance worldwide and elevated prevalence of ESBL among EAEC

Table 1
Oligonucleotides used in the present study.

Sl no.	Primer	Oligonucleotide sequence	Amplicon size (bp)	Reference
1	*blaTEM*	F-5′CATTTCCGTGTCGCCCTTATTC3′ R-5′CGTTCATCCATAGTTGCCTGAC3′	800	Dallenne et al., 2010
2	*blaCTX-M-1*	F-5′TTAGGAARTGTGCCGCTGYA3′ R-5′CGATATCGTTGGTGGTRCCCAT3′	688	
3	*blaCTX-M-9*	F-5′TCAAGCCTGCCGATCTGGT3′ R-5′TGATTCTCGCCGCTGAAG3′	561	
4	*blaAmpC*	F-5′CACCTCCAGCGACTTGTTAC3′ R-5′GTTAGCCAGCATCACGATCC3′	346	

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