Molecular Detection and Epidemiology of Extended-Spectrum Beta-lactamase Genes Prevalent in Clinical Isolates of Klebsiella pneumonias and E coli from Trinidad and Tobago

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ABSTRACT

Objective: The epidemiology of Extended-spectrum beta-lactamase (ESBL) producing E coli and K pneumonias is complex and varies among hospitals and countries. This study aimed at describing the molecular detection and epidemiology of ESBL subtypes prevalent in clinical isolates of K pneumonias and E coli in Trinidad and Tobago.

Methods: Over 36-months, isolates of E coli and K pneumonias from clinical specimens of patients processed at a regional tertiary hospital in the country were identified using standard microbiological methods. MicroScan System (Siemens, USA) was used to determine MIC values while E-test (AB Biodisk, Sweden) assays phenotypically confirmed ESBL production. K pneumonias (n = 65) and E coli (n = 25) isolates confirmed as ESBL producers were further subjected to multiplex PCR and PFGE tests to determine the ESBL subtypes and clonal relatedness.

Results: Female patients (67.8%) and urine samples (65%) yielded most ESBL isolates, with over 90% recovered from the hospital’s medicine and surgery facilities. All ESBL isolates including all K pneumonias producing ESBLs were 100% susceptible to carbapenems and amikacin antimicrobials. Polymerase Chain Reaction detected 100% blaTEM genes, 4.1% blaSHV and 37.5% blaCTX-M genes among E coli isolates. Similarly, 84.3% blaTEM, 34.5% blaSHV and 58.8% blaCTX-M genes were detected in K pneumonias. Pulsed-field gel electrophoresis (PFGE) results showed diverse and unrelated clones.

Conclusions: In this the first report of molecular characterization and epidemiology of ESBL subtypes in E coli and K pneumonias isolates in Trinidad and Tobago, the CTX-M, mainly phylogenetically group 1 type, was most predominant. Most ESBL isolates were still susceptible to carbapenems and aminoglycosides and their spread appears to be polyclonal and clonally unrelated.

Keywords: Molecular diagnostics, Multiplex Polymerase Chain Reaction, Extended-spectrum beta-lactamase.
The epidemiology of ESBL-producing *E coli* and *K pneumoniae* is complex and varies among institutions (4, 5). Several clinical microbiology tests that presumptively identify the presence of an ESBL have been reported but the task of identifying which specific ESBL is present in a clinical isolate is more complicated (11). Although the presence of ESBL in clinical isolates of *Salmonella enteritidis*, *E coli* and *K pneumoniae* have phenotypically been previously detected and reported in Trinidad and Tobago (12−13), yet none of the different types of ESBL genes have been described. This study is aimed at giving the molecular detection of the several ESBL gene types prevailing in clinical isolates of *E coli* and *K pneumoniae* and describing their epidemiology in Trinidad and Tobago.

**MATERIALS and METHODS**

**Bacterial isolates**

Included in this study were all phenotypically confirmed ESBL producers of consecutive non-duplicate clinical *K pneumoniae* and *E coli* isolates recovered over a 3-year (May 2005 – April 2008) period from specimens processed at the microbiology laboratory of the Eric Williams Medical Sciences Complex (EWMSC), a tertiary ambulatory regional hospital.
hospital in Trinidad and Tobago, as previously reported (13). Automated microdilution MicroScan WalkAway-96 System (Siemens, USA) was used to obtain the MIC values at concentrations and breakpoints recommended by the CLSI for antimicrobial susceptibility and ESBL screening (14). All isolates primarily indicated by the MicroScan system as possible ESBL producers with MIC breakpoints interpreted as resistant or intermediate by the system were regarded as having decreased susceptibility for the purpose of this study.

**Confirmation of ESBL phenotypes**

E-test strips (AB Biodisk, Solna Sweden) were used in accordance with the protocols from the manufacturer to phenotypically determine ESBL production in the isolates. Extended-spectrum beta-lactamase production was determined if the microbial isolate had a MIC µg/ml of ≥ 0.5 for CT, ≥ 1 for TZ; and MIC µg/ml ratio of ≥ 8 for CT/CTL or TZ/TZL. Extended-spectrum beta-lactamase production was also identified by the presence of phantom zone or a deformation of the ceftaxime or ceftazidime ellipse. A result was considered indeterminate when the MICs were outside the range of the MICs of the respective E-test ESBL test strip and a MIC ratio could therefore not be calculated. ESBL producing isolates were expected to be resistant to all penicillins, cephalosporins and aztreonam. The E-test method is very sensitive and convenient to use despite being expensive. Confirmed positive ESBL- producing isolates were stored at -70°C in trypticase soy broth (Difco Laboratories, Detroit MI, USA) supplemented with 5% glycerol until shipped to McMaster University, Hamilton, Ontario, Canada, for further molecular studies. The control strain for ESBL producing isolates were expected to be resistant to all penicillins, cephalosporins and aztreonam.

**DNA Electrophoresis**

Pulsed-field gel electrophoresis (PFGE) was used as the genotyping method in this study to compare the DNA of the ESBL producing *K pneumoniae* and *E coli* isolates and was performed as previously described (19) with some modifications. Restriction analysis of chromosomal DNA with *Xba*1 (New England BioLabs, Beverly, MA) was carried out and separation of the DNA was performed using 1% pulsed-field gel agarose (Bio-Rad Laboratories, La Jolla, CA). The pulsed-field gel electrophoresis was performed using a contour-clamped homogeneous electric field apparatus (CHEF DRIII, Bio-Rad Hercules, CA, USA). Gel images were captured on the Gel Doc imaging system using Quantity One software version 4.4.1 (Bio-Rad Laboratories, Hercules CA, USA). The results were analysed by following previously established criteria (20).

**RESULTS**

**Bacterial isolates**

The first ESBL-producing organism in the country was *Salmonella enteritidis* in 1999. Since then and from December 2004 to April 2008, 602 *K pneumoniae* and 1016 *E coli* recovered from the clinical specimens were identified as ESBL producers. A 15.2% ESBL rate among the *K pneumoniae* isolates and 9.3% among the *E coli* isolates has previously been reported in this hospital (13).

Ninety isolates comprising 65 *K pneumoniae* and 25 *E coli* isolates from patients with ages ranging from 3-days old to 82 years were selected based on higher E-test MIC values for further characterization. Results of representatives of the used isolates for the study are summarized in the Table below. Generally, most of the isolates (67.8%) were from female patients and the specimens from which the organisms were isolated were as follows: urine (65%), wound materials or pus (24%), blood (4%), respiratory tracts (1.5%) and others (5.5%). For the ESBL producers, the majority were recovered from patients seen in medical and surgical facilities of the hospital accounting for 47% and 44%, respectively. Other ESBLs were from ICU (4%), paediatrics (2%), accident and emergency (1%) and others (2%).
Ceftazidime and ceftazidime plus clavulanic acid MIC ratio of ≥ 8 for *E coli* isolates was 44.3% (11/25) and the corresponding figures for the cefotaxime/cefotaxime plus clavulanic acid was 80% (20/25). Similarly, among the 65 *K pneumoniae* strains, 67.7% and 86.2% had MIC ratio of ≥ 8 for the combinations of ceftazidime/ceftazidime plus clavulanic acid and cefotaxime/cefotaxime plus clavulanic acid respectively. Despite having a high E-test MIC ratio in the range of 7.8 – 1391, all isolates were susceptible to carbapenems (meropenem and imipenem) and all *Klebsiella pneumoniae* isolates were susceptible to amikacin but showed varied susceptibility to gentamicin and tobramycin. The isolates were not tested against ertapenem because it has only been recently introduced into the country while amikacin though in the National formulary, is rarely available or used in the country.

**Multiplex PCR gene detection**

The multiplex PCR assay detected 100% *bla*<sub>TEM</sub> genes, 4.1% *bla*<sub>SHV</sub> and 37.5% *bla*<sub>CTX-M</sub> genes among the *E coli* isolates. Similarly, 84.3% *bla*<sub>TEM</sub>, 34.5% *bla*<sub>SHV</sub> and 58.8% *bla*<sub>CTX-M</sub> genes in the *K pneumoniae* isolates were detected (Fig. 1).
All CTX-M genes were identified as alleles belonging to the phylogenetic group I.

**Pulsed-field gel electrophoresis**

The PFGE typing of the ESBL-producing isolates revealed various different and diverse DNA banding profiles among the isolates. There was no major clonal similarity or relatedness of either the *K. pneumoniae* or *E. coli* producing ESBL isolates regardless of patient or specimen source (Fig. 2).

![PFGE Typing](Fig. 2: Showing patterns generated by PFGE of *Sma*I-digested chromosomal DNA obtained from *bla*TEM, SHV and CTX-M genes produced by *K. pneumoniae* and *E. coli* isolates. Lane λ, bacteriophage lambda ladder PFGE marker (New England Biolabs), lanes 1 – 6, 7 – 12 *E. coli* isolates; and lanes 13 – 18 and 19 – 24, *K. pneumoniae* isolates.

**DISCUSSION**

To our knowledge, this is the first study to document the molecular detection of the types of ESBL genes in isolates of *K. pneumoniae* and *E. coli* in any health institution in Trinidad and Tobago. Multiplex PCR amplification assay for the detection of the *bla*TEM, *bla*SHV and *bla*CTX-M genes in clinical isolates of *E. coli* and *K. pneumoniae* was used in this study because this assay has been shown to have the advantage of rapidly screening large numbers of clinical isolates in addition to the fact that the isolated DNA was suitable for further molecular epidemiological studies if required (15). This first molecular characterization study to report ESBL types – TEM, SHV and CTX-M genes in *Enterobacteriaceae* isolates from Trinidad and Tobago revealed that TEM ESBL is highly prevalent among the isolates and the CTX-M ESBL is predominant in the country.

All the CTX-M genes detected in the present study were identified to be similar and belonged to the group 1 type. Such high predominance of CTX-M group 1 alleles have also been previously reported among clinical isolates (18). Woodford *et al.* have reported that 86.4% of 633 CTX-M producing *Enterobacteriaceae* isolates have alleles encoding group 1 CTX-M enzymes. The CTX-M enzymes are recognized as an increasingly serious public health concern worldwide and group 1 has been particularly noted to be the cause of outbreaks as reported elsewhere (21, 22). Despite the high prevalence of CTX-M ESBLs in this study, there has not been any evidence in the hospital or country of a real epidemic (caused by single bacterial clones) or endemic (maintenance of a single clone during extended period of time) report of outbreaks of infections caused by these organisms producing these enzymes as has been reported elsewhere (21, 22).

The *bla*TEM genes were amplified from 100% of ESBL producing *E. coli* and 84.3% *K. pneumoniae* isolates tested. Although we have no sequence data of the different types of TEM prevailing in our area, but as has been reported, TEM derived beta-lactamases may not play an important role in resistance to ESBL among *K. pneumoniae* in this area despite the predominance of TEM. The majority of the isolates came from female patients and from urinary tract specimens. This was not surprising since females are more vulnerable to urinary tract infection. ESBL-producing isolates also appeared to be an important cause of infection among patients attended to in the medical and surgical facilities of the hospital.

As reported previously, all the ESBL producing isolates were resistant to beta-lactam and third generation cephalosporins (13). This is most likely as a result of pressure on the use of these agents in the hospital and country as a whole. There is a high rate and extensive inappropriate use of cephalosporins in the country as reported by Pinto Pereira *et al.* (23). In Trinidad and Tobago, there is the practice of sale of some antibiotics as over the counter drugs in some places as well as the indiscriminate practice of antibiotic prescription for patients with viral infections in the community and in the country. The result of this is the selective pressure of use and overuse of antibiotics in the treatment of patients that leads to the selection for new variants of the beta-lactamases. This selective pressure created by the use of third generation cephalosporins has also been described as one of the most important factors elsewhere (24, 25).

The PFGE typing of ESBL-producing isolates showed various DNA banding profiles. This clonal diversity suggests that most of the strains have been unable to be maintained or spread in the different facilities of the hospital as is the experience in other places (26). This observation may challenge the many conventional thoughts about the nosocomial epidemiology of antibiotic resistance. A retrospective review of laboratory records revealed that these isolates did not significantly share patient demographics and occurrence periods. Despite being isolated mostly from urine of patients treated in the medical and surgical facilities of the hospital sharing significant patient demographics and isolate characteristics yet these ESBL enzymes differed. These clearly indicate that most ESBL-producing isolates were not sporadic but that multiple clones were widespread in the hospital supporting our suggestion that antibiotic use pressure could be the only major cause. This calls for serious continuous active surveillance measures and effective antibiotic use and controls practices in the hospital.

Although there is still the need for sequencing of these ESBL producers, yet we report the first attempt to study the molecular characterization of ESBL subtypes and the epidemiology of ESBL-producing *E. coli* and *K. pneumoniae* isolates in Trinidad and Tobago. This study clearly shows that CTX-M, mainly CTX-M phylogenetically group 1 ESBL-
producing E. coli and K. pneumoniae was highly prevalent in a tertiary regional hospital in Trinidad and Tobago. The good news is that most of these ESBL producers were still susceptible to some carbapenems and aminoglycosides. The spread of ESBL-producing bacteria appeared to be polyclonal and none of the bacterial strains were clonally related.

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REFERENCES