

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

Methods: Over 36-months, isolates of *E coli* and *K pneumoniae* 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 pneumoniae* ($n = 65$) 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 pneumoniae* producing ESBLs were 100% susceptible to carbapenems and amikacin antimicrobials. Polymerase Chain Reaction detected 100% *bla*_{TEM} genes, 4.1% *bla*_{SHV} and 37.5% *bla*_{CTX-M} genes among *E coli* isolates. Similarly, 84.3% *bla*_{TEM}, 34.5% *bla*_{SHV} and 58.8% *bla*_{CTX-M} genes were detected in *K pneumoniae*. 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 pneumoniae* 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.

Detección Molecular y Epidemiología de Genes de Beta-lactamasas de Espectro extendido Prevalentes en los Aislados Clínicos de *Klebsiella pneumoniae* y *E coli* en Trinidad y Tobago

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RESUMEN

Objetivo: La epidemiología de *E coli* y *K pneumoniae* productores de beta-lactamasas de espectro extendido (BLEE) es compleja y varía de un hospital o país a otro. Este estudio tiene por objeto describir la detección molecular y la epidemiología de los subtipos de BLEE prevaletentes en aislados clínicos de *K pneumoniae* y *E coli* en Trinidad y Tobago.

Métodos: Por más de 36-meses, se identificaron aislados de *E coli* y *K pneumoniae* a partir de especímenes clínicos de pacientes procesados en el hospital universitario regional del país, utilizando métodos microbiológicos estándar. Se utilizó el sistema MicroScan (Siemens, USA) para determinar los valores MIC, en tanto que la prueba del epsilómetro o E-test (biodisco AB, Suecia) confirmó fenotípicamente la producción de BLEE. Los aislados de *K pneumoniae* ($n = 65$) y *E coli* ($n = 25$) confirmados como productores de BLEE, fueron posteriormente sometidos a pruebas PCR múltiplex y PFGE con el propósito de determinar los subtipos BLEE y la relación clonal.

Resultados: Las pacientes (67.8%) y las muestras de orinas (65%) arrojaron el mayor número de aislados de BLEE, siendo más del 90% tomados de las instalaciones de medicina y cirugía del hospital. Todos los aislados de BLEE, incluyendo todas las *K pneumoniae* productoras de BLEE resultaron 100% susceptibles a los agentes antimicrobianos carbapenem y amikacina. La reacción en cadena de la polimerasa detectó 100% de genes bla_{TEM} , 4.1% de genes bla_{SHV} y 37.5% de genes bla_{CTX-M} entre los aislados de *E coli*. De manera similar, 84.3% de genes bla_{TEM} , 34.5% bla_{SHV} y 58.8% bla_{CTX-M} fueron detectados en *K pneumoniae*. Los resultados de la electroforesis en gel de campos pulsados (PFGE) mostraron clones diversos y no relacionados.

Conclusiones: En este primer reporte de caracterización molecular y epidemiología de subtipos de BLEE en aislados de *E coli* y *K pneumoniae* en Trinidad y Tobago, el tipo principalmente filogenéticamente CTX-M grupo 1 fue el de mayor prevalencia. La mayoría de los aislados de BLEE eran todavía susceptibles a los carbapenems y los aminoglucósidos, y su extensión parece ser policlonal y no hallarse clonalmente relacionada.

Palabras claves: Beta-lactamasas de espectro extendido, diagnóstico molecular, reacción en cadena de la polimerasa múltiplex.

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INTRODUCTION

Extended-spectrum beta-lactamases (ESBL) are enzymes produced by some *E coli* and *Klebsiella* species (and sometimes other Enterobacteriaceae) that inactivate penicillins, expanded-spectrum cephalosporins, monobactams including older beta-lactam antimicrobial agents and are inhibited by clavulanic acids, sulbactam or tazobactam (1). The spread of ESBL-producing bacteria occurs rapidly and has been widely reported worldwide. Continuous monitoring systems and effective infection control measures are required to curtail their spread. Prevalence of ESBL producing *K pneumoniae* and *E coli* among clinical isolates varies from country to country, institution to institution and therapeutic choices for infections caused by ESBL-producing bacterial strains remain limited (2–6).

Common ESBL genes coding for *K pneumoniae* and *E coli* have been designated TEM (described in the early 1980s from a patient named Teminora from Greece), SHV (for sulphydryl variable, first found in a single strain of *Klebsiella ozaenae* isolated in Germany) and CTX-M [cefotaximase that preferentially hydrolyze cefotaxime] (5, 7, 8). These genes mediated by chromosomes, plasmids or transposons have all been increasingly described worldwide (5, 9, 10).

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 has 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 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 $\mu\text{g/ml}$ of ≥ 0.5 for CT, ≥ 1 for TZ; and MIC $\mu\text{g/ml}$ 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 cefotaxime 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 all the phenotypic testing were *E. coli* ATCC 25922 (negative control) and *K. pneumoniae* ATCC 700603 (ESBL positive).

Multiplex PCR amplification

Detection of gene sequences coding for the TEM, SHV and CTX-M enzymes were performed by the multiplex PCR as previously described with some modifications (15). The oligonucleotide primer sets specific for the SHV, TEM and CTX-M genes and the cycling conditions used in the PCR assays have been described previously: 5'-ATG CGT TAT ATT CGC CTG TG-3' and 5'-TGC TTT GTT ATT CGG GCC AA-3' for amplification of a 747-bp sequence of *bla*SHV (16), 5'-TCG CCG CAT ACA CTA TTC TCA GAA TGA-3' and 5'-ACG CTC ACC GGC TCC AGA TTT AT-3' for amplification of a 445-bp sequence of *bla*TEM; and 5'-ATG TGC AGY ACC AGT AAR GTK ATG GC-3' and 5'-TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3' for amplification of a 593-bp sequence of *bla*CTX-M (17). A Multiplex PCR method previously described by Woodford *et al* (18) for detection of *bla*CTX-M alleles was also used to identify the CTX-M phylogenetic group of positive isolates.

All PCR reactions were carried out using 2 μl bacterial cell suspension (density of 70% T in Vitek Colorimeter) as the DNA template. The Master Mix consisting of 2.5 mM

MgCl₂, 1 x PCR buffer, 250 μM each of dATP, dGTP, dCTP, 200 μM dTTP, and 150 μM of UTP, 10 pmol each of gene-specific primers, 0.25 U of UNG (N-uracyl-glycosylase), and 0.25 U per reaction AmpliTaq Gold DNA polymerase. Polymerase chain reaction amplification conditions were as follows: initial denaturation step at 95°C for 10-minute; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30-seconds for TEM/SHV/CTX-M multiplex method and 52°C for 30-seconds for CTX-M grouping method, extension at 72°C for 2-minute, followed by a final extension step at 72°C for 10-minute. Respective genes were detected by the size separation PCR amplicons by agarose gel electrophoresis.

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*I (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%).

Table 1: E-test phenotypic characterization (MIC ratios) and multiplex PCR genotyping results of selected *K pneumoniae* and *E coli* isolates from Trinidad and Tobago

Species	Specimen	Ward	MIC Ratio		Multiplex PCR		
			CT/CTL	TZ/TZL	TEM	SHV	CTX-M
EC112	Urine	Surg	1255	7.8	+	+	+
EC113	Urine	Med	500	64	+	-	+
EC116	Urine	Med	695.5	340.4	+	-	+
EC120	Urine	Surg	500	127.7	+	-	+
EC121	Urine	Med	500	128	+	-	+
EC126	Urine	Paed	250	84.2	+	-	+
EC128	Urine	Surg	500	96	+	-	+
EC131	Urine	Med	500	84.2	+	-	+
EC132	Urine	Med	500	64	+	-	+
KP140	Blood	Med	256	84	-	+	+
KP142	Urine	Surg	500	128	+	+	-
KP145	W/s	Surg	1391	128	+	+	+
KP146	Blood	Paed	250	256	+	+	+
KP147	Sputum	Med	500	128	+	-	+
KP149	Urine	Med	250	256	+	+	+
KP150	Urine	Surg	500	128	+	+	+
KP152	Urine	Med	15.6	8	+	+	-
KP153	W/s	Med	1000	128	+	-	+
KP174	Urine	Med	500	128	+	-	+
KP176	Urine	Med	500	64	+	-	+
KP178	W/s	Surg	1000	340.4	+	-	+
KP181	Urine	Med	500	68	+	+	+
KP189	Urine	Med	1000	8	+	-	+
KP190	Urine	Surg	15.6	8	+	+	-
KP192	W/s	Surg	16	8	+	-	+
KP196	W/s	Surg	16	8	-	+	+

EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; W/s = wound swab; Med = medicine; Surg = surgery; Paed = paediatrics

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*_{TEM} genes, 4.1% *bla*_{SHV} and 37.5% *bla*_{CTX-M} genes among the *E coli* isolates. Similarly, 84.3% *bla*_{TEM}, 34.5% *bla*_{SHV} and 58.8% *bla*_{CTX-M} genes in the *K pneumoniae* isolates were detected (Fig. 1).

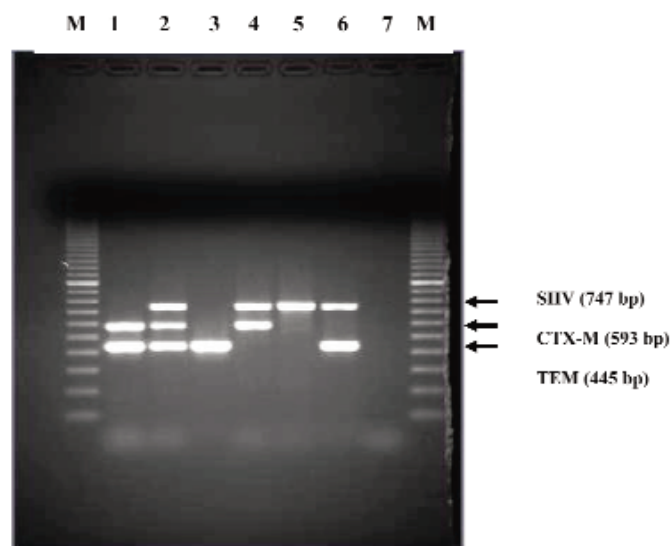


Fig. 1: Multiplex PCR detection of TEM, SHV, and CTX-M beta-lactamase genes in *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KPN). Lanes M, 100 bp ladder molecular size marker (Bio Rad); Lane 1 and 3, EC isolates; Lanes 2, 4-6, KP isolates; and Lane 7, No template (water).

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).

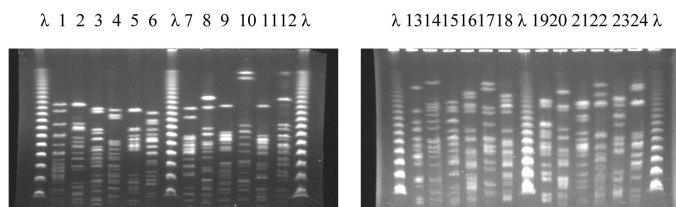


Fig. 2: Showing patterns generated by PFGE of *Sma*I-digested chromosomal DNA obtained from *bla*_{TEM}, *SHV* and *CTX-M* genes produced by *K pneumoniae* and *E coli* isolates. Lane λ , bacteriophage lambda ladder PFGE marker (New England Biolabs), lanes 1 – 6, 7 – 12 *E coli* isolates; and lanes 13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