

High prevalence of multidrug resistant ESBL- and plasmid mediated AmpC-producing clinical isolates of *Escherichia coli* at Maputo Central Hospital, Mozambique

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Abstract

Background Epidemiological data of cephalosporin-resistant Enterobacterales in Sub-Saharan Africa is still restricted, and in particular in Mozambique. The aim of this study was to detect and characterize extended-spectrum β -lactamase (ESBL) - and plasmid-mediated AmpC (pAmpC)-producing clinical strains of *Escherichia coli* at Maputo Central Hospital (MCH), a 1,000-bed reference hospital in Maputo, Mozambique. Methods A total of 230 clinical isolates of *E. coli* (n=230) from urine (n=199) and blood cultures (n=31) were collected at MCH during August-November 2015. Antimicrobial susceptibility testing was performed by the disc diffusion method and interpreted according to EUCAST guidelines. Isolates with reduced susceptibility to 3rd generation cephalosporins were examined further; phenotypically for an ESBL-/AmpC-phenotype by combined disc methods and genetically for ESBL- and pAmpC-encoding genes by PCR and partial amplicon sequencing as well as genetic relatedness by ERIC-PCR. Results A total of 75 isolates with reduced susceptibility to cefotaxime and/or ceftazidime (n=75) from urine (n=58/199; 29%) and blood (n=17/31; 55%) were detected. All 75 isolates were phenotypically ESBL-positive and 25/75 (33%) of those also expressed an AmpC-phenotype. ESBL-PCR and amplicon sequencing revealed a majority of blaCTX-M (n=58/75; 77%) dominated by blaCTX-M-15. All AmpC-phenotype positive isolates (n=25/75; 33%) scored positive for one or more pAmpC-genes dominated by blaMOX/FOX. Multidrug resistance (resistance \geq three antibiotic classes) was observed in all the 75 ESBL-positive isolates dominated by resistance to trimethoprim-sulfamethoxazole, ciprofloxacin and gentamicin. ERIC-PCR revealed genetic diversity among strains with minor clusters indicating intra-hospital spread. Conclusion We have observed a high prevalence of MDR pAmpC- and/or ESBL-producing clinical *E. coli* isolates with FOX/MOX and CTX-Ms as the major β -lactamase types, respectively. ERIC-PCR analyses revealed genetic diversity and some clusters indicating within-hospital spread. The overall findings strongly support the urgent need for accurate and rapid diagnostic services to guide antibiotic treatment and improved infection control measures.

Background

The World Health Organization recognizes antimicrobial resistance (AMR) as a major global public health problem [1, 2]. The dissemination of multidrug-resistant (MDR), extended-spectrum β -lactamase (ESBL)-producing *Enterobacterales* is of particular concern limiting treatment options for invasive infections to last line antibiotics [3-5].

ESBLs are enzymes that hydrolyze an extended spectrum of β -lactam antibiotics including penicillins and oxyimino-cephalosporins, but not cephamycins [6]. The most widely distributed ESBL enzymes are CTX-M-type β -lactamases, which preferentially hydrolyze cefotaxime. The worldwide dissemination of CTX-M-type β -lactamases has been dramatic and greater than the impact of the TEM- and SHV-type ESBLs [5, 7].

Plasmid-mediated AmpC β -lactamases (pAmpCs) are also an important cause of broad spectrum β -lactam resistance in Gram-negative bacteria [8]. Many Gram-negative bacteria including most *Enterobacterales* have chromosomally-encoded AmpC- β -lactamases. Some AmpC-genes have been

mobilized from their chromosomal origin and may be spread by plasmids (pAmpC), with *bla*_{MOX/FOX/DHA/CMY} being the most prevalent variants [6, 8, 9]. In general, AmpC β -lactamases confer resistance to a wide range of β -lactam drugs including penicillins, cephamycin, 1st – 3rd generation cephalosporins as well as classical β -lactamase inhibitors like clavulanic acid and tazobactam to which ESBLs are sensitive [6]. Co-presence of ESBLs and pAmpCs may occur [10].

In the context of low- and medium income countries (LMICs), and sub-Saharan Africa in particular, there are knowledge gaps concerning the epidemiology of ESBL- and pAmpC-producing *Enterobacterales* in different ecological settings [11-13]. This study delineates plasmid AmpC- and ESBL-mediated resistance in clinical isolates of *E. coli* processed at the Maputo Central Hospital, Mozambique, in 2015.

Methods

Bacterial isolates. The strain collection consisted of isolates recovered from clinical specimens referred to the Department of Clinical Microbiology, Maputo Central Hospital, a 1,000-bed reference hospital in Maputo, Mozambique. We included urine and blood culture samples from mid-August through mid-November 2015. Urine samples were obtained from both in- and outpatient whereas blood cultures were from inpatients only. One-per-patient isolate was included in the study.

Bacterial species identification and antimicrobial susceptibility testing (AST). *E. coli* strains were identified by standard biochemical tests including the API-20E kit (bioMerieux, la Balme-les-Grottes, France), and AST was performed using the disk diffusion method according to EUCAST guidelines [14] including ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, meropenem, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and nitrofurantoin. MDR was defined as acquired resistance to at least one agent in three or more antimicrobial classes [15].

Quality assurance was performed weekly during the study period using the wild-type *E. coli* ATCC 25922 strain. Moreover, *K. pneumoniae* ATCC 700603 (ESBL-type *bla*_{SHV-18}) and *E. coli* A5-8 (pAmpC-type *bla*_{DHA}) were used as ESBL- and AmpC-positive controls, respectively. *E. coli* A5-8 is a well characterized strain that was obtained from the Norwegian Advisory Unit for Detection of Antimicrobial Resistance. Strains were stored in skimmed milk broth with 10% glycerol at -70°C until molecular analysis.

Phenotypic detection of β -lactamases. Isolates that showed reduced susceptibility (I or R) to cefotaxime and/or ceftazidime were examined for ESBL-production. Moreover, isolates with reduced susceptibility to cefotaxime and/or ceftazidime and cefoxitin were examined for increased AmpC-production[16]. The mechanisms of resistance were examined using ROSCO combined disc tablets (Rosco Diagnostic, Denmark) for ESBL and/or increased AmpC-production and interpreted according to manufacturer's instructions.

Genetic detection and characterization of β -lactamases. Genetic characterization was performed at the Antimicrobial Research Unit, University of KwaZulu-Natal (UKZN). Phenotypical ESBL-positive isolates

were examined for the presence of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{CMY}, *bla*_{DHA}, *bla*_{FOX} and *bla*_{MOX} genes using consensus PCRs (Table 1) as described [17-21]. Briefly, DNA extractions were performed from overnight bacterial cultures using GeneJet Purification kit (Thermo Scientific, USA). The extracted DNA was stored at -20°C until use. PCR products were visualized by agarose gel electrophoresis, and *bla*_{CTX-M} amplicons were sent for DNA sequencing at Inqaba Biotec (South Africa).

Genetic typing of ESBL-producing strains by ERIC-PCR. The clonal relatedness of ESBL-positive isolates was examined by ERIC-PCR and analysed as described [21, 22]. Briefly, PCR products were visualized by agarose gel electrophoresis and genetic variation was analysed using the GelComparII version 6.0 software package (Applied Maths, Belgium) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis to produce a dendrogram.

Results

Bacterial isolate selection, identification and AST. *E. coli* isolates were identified in urinary ($n=199/823$; 24.1%) and blood ($n=31/255$; 12.1%) culture samples. Urinary tract isolates were from in- ($n=109$) and out-patients ($n=90$). All blood culture isolates were from in-patients. A total of 58/199 (29 %) of urine isolates (51/109 (47%) and 7/90 (8%) isolates from in- and out-patients, respectively) showed reduced susceptibility (R or I) to cefotaxime and/or ceftazidime and as compared to 17/31 (55 %) of blood isolates (Additional file 1: Tables S1 and S2). The ESBL confirmation test was positive for all 75/230 (32.6%) isolates tested. The ESBL-positive rates were significantly different in isolates from out- versus in-patients, respectively 7/90 (7.7%) and 51/109 (46.8%). Reduced susceptibility to meropenem was not observed. MDR was observed in all ESBL-positive isolates.

Reduced susceptibility to ceftazidime was detected in 41/75 (54.6%) of the isolates tested and further examined for increased AmpC-production; 7/17 and 34/58 were blood and urine isolates, respectively (Tables S1 and S2). A total of 25/41 (60.9%) isolates were confirmed positive for increased AmpC-production using the ROSCO combined disc method, representing 25/75 (33%) of the total ESBL-positive population.

The presence of co-resistance to clinically important antibiotics was also examined (Tables S1 and S2) in isolates from blood: ciprofloxacin ($n= 12/17$: 71%), gentamicin ($n= 8/17$: 47%) and trimethoprim-sulfamethoxazole ($n= 17/17$; 100%), and urine: ciprofloxacin ($n=40/58$; 69%), gentamicin ($n= 27/58$; 47%) and trimethoprim-sulfamethoxazole ($n= 55/58$; 95%). Interestingly, a majority of the isolates, even pAmpC-negative, expressed resistance to β -lactamase inhibitors (amoxicillin-clavulanic acid and piperacillin-tazobactam) indicating additional resistance mechanisms.

Detection of genes encoding ESBL and plasmid-mediated AmpC. A total of 58/75 (77%), 39/75 (52%), and 1/75 (1%) ESBL-producing isolates were positive for *bla*_{CTX-M} (Figure 1), *bla*_{SHV} and *bla*_{TEM} by PCR, respectively. The *bla*_{CTX-M} amplicons were sequence-typed with the following results: CTX-M-15 ($n=24$), CTX-M-28 ($n=2$), CTX-M-117 ($n=1$), CTX-M-36 ($n=1$), CTX-M-164 ($n=1$) and CTX-M no type ($n=29$) (Figure

1). Available resources did not allow sequence typing of SHV-amplicons, but 13/17 (76.5%) of *bla*_{CTX-M}⁻ negative, ESBL-producing isolates were *bla*_{SHV}-positive, indicating a SHV-ESBL-type. The only *bla*_{TEM} positive isolate was positive for *bla*_{CTX-M-15}. A total of 25/75 (33%) isolates examined scored positive for one or more plasmid mediated AmpC-genes; *bla*_{CMY} (*n*=1), *bla*_{DHA} (*n*=13), *bla*_{MOX} (*n*=22), and *bla*_{FOX} (*n*=24). The isolates contained two (*n*=15) or three (*n*=10) pAmpC genes: *bla*_{CMY;DHA} (*n*=1), *bla*_{DHA;FOX} (*n*=2), *bla*_{MOX;FOX} (*n*=12) and *bla*_{DHA;MOX;FOX} (*n*=10). Importantly, only phenotypical AmpC-isolates were positive for *bla*_{AmpC}.

ERIC – PCR results. Distinct profiles were obtained for the 75 isolates tested using ERIC-PCR fingerprinting (Figure 1). Fragments of different molecular weights were observed and polymorphisms based on fragment length were used as a means of differentiating *E. coli* isolates. The absence or presence of a band was also noted in determining variation among the isolates. ERIC-PCR profiles comprised between 4 and 12 individual bands, varying in size from 0.5 – 10 kb (Figure 1). Amplifications of different intensities were observed and visual analysis of the ERIC profiles included primary, secondary and tertiary amplification. Primary amplification products refer to those products of high intensity, which appear extremely bright on the gels. Secondary amplification products were those products that are not as bright as the primary amplification products but more intense than the tertiary amplification products, while the tertiary amplification products were the minor amplification products of low intensity. All isolates were typeable using this fingerprinting technique and band patterns were reproducibly obtained by repeat amplification.

The ERIC-PCR profiles allowed differentiation of the 75 isolates into 50 ERIC-PCR types, which were grouped into eight main clusters (A-H), with each of the clusters further sub-divided into two or three main sub-clusters (Figure 1). The urine isolates were diverse and distributed in all eight identified clusters (clusters A-H) with characteristics as outlined below. Isolates with similar profiles demonstrated different β -lactamase gene content. The blood isolates were more clonal in nature and mostly observed in clusters F and G, but predominantly in cluster G. Although closely related, they demonstrated differences in their β -lactamase resistance gene content (data not shown).

Discussion

Literature reviews show that most studies on the presence of clinical isolates of ESBL-producing *Enterobacterales* in Africa have been conducted in Northern and Eastern Africa, with a relative lack of data in Sub-Saharan Africa [13, 23-25]. In general, and particularly in Mozambique there is very limited data from surveillance or clinical studies documenting the susceptibility pattern of common pathogens. Previous studies have revealed a great variety in proportions of ESBL-producing *Enterobacterales*, underlining the importance of surveillance studies and local data in order to guide antimicrobial therapy and infection control [23, 24].

In this study, the overall prevalence of plasmid-mediated AmpC- and/or ESBL-production in clinical isolates of *E. coli* was 10.8% (25/230) and 32.6% (75/230), respectively. Both phenotypes were present in

urinary tract and blood culture isolates and with a significant higher prevalence of ESBL in urinary isolates obtained from in-patients (8%) compared to out-patients (47%). CTX-Ms were the most dominant ESBL-type, with CTX-M-15 as the major subtype. Most of the *bla*_{CTX-M} negative, ESBL-positive isolates were negative for *bla*_{TEM}, but positive for *bla*_{SHV}, indicating an SHV-ESBL-type. This is in accordance with the international situation [5, 7] and the recent meta-analysis of ESBL-producing *Enterobacterales* in East Africa hospitals [23].

Moreover, a study of ESBL-producing *Enterobacterales* in stool samples in Mali, Niger and Cameroon showed that CTX-M was the dominant ESBL-type [25]. Finally, our results are also in line with the recent detection of a relatively high prevalence of ESBL CTX-M-type producing *Enterobacterales* in stool samples from Mozambique university students [21].

A total of 11% (25/230) of the isolates expressed an AmpC phenotype and all those were pAmpC-PCR positive. Surprisingly, all isolates contained two to three pAmpC genes of which *bla*_{FOX} was the most prevalent. These observations are in contrast with the worldwide observations of *bla*_{CMY} as the most prevalent pAmpC gene in *E. coli* populations [8, 9]. CMY-2 has the broadest geographic spread among pAmpCs and is an important cause of extended-spectrum beta-lactam resistance in *E. coli* as well as in non-typhoid *Salmonella* strains in many countries [26]. The finding of multiple pAmpC-*bla* genes in single strains has recently been reported in a Tunisian study. Briefly, a total of 11 out of 75 pAmpC positive clinical strains of *Enterobacterales* were shown to contain up to three different pAmpCs [10]. In contrast to our study, CMY-2 was the most common pAmpC-type in their study. Moreover, the combination of MOX-, FOX- and CMY-2 type enzymes was dominant in their isolates, in contrast to ours that mostly contained MOX- and FOX-types in combination with DHA.

ERIC-PCR has been a useful rapid method in various molecular epidemiological studies to describe the genetic relatedness in *Enterobacterales* strain collections [27]. Our ERIC-PCR results revealed an overall genetic diversity of pAmpC - and/or ESBL -positive *E. coli* strains at the Maputo Central Hospital. The results indicate that there is not a dominant clone of ESBL-/pAmpC positive *E. coli*. However, there are several clusters with clonal relatedness indicating minor outbreaks between patients at specific departments. This notion is supported by the isolation of CTX-M-15 producing strains with similar resistance patterns from the Pediatric department linked in time within cluster B.

The observation of multidrug-resistant pAmpC- and/or ESBL-producing *E. coli* in a high proportion of clinical isolates during a period of three months is a major concern. *E. coli* is the most prevalent cause of urinary tract infections and Gram-negative bacteremia in most countries [28, 29]. A large proportion of the ESBL-producing strains also expressed resistance to fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole, limiting treatment options to last resort antibiotics such as carbapenems, piperacillin-tazobactam, colistin or tigecycline. Those drugs are not easily available at Maputo Central Hospital and in developing countries in general. Thus, a significant proportion of clinical *E. coli* strains at the Maputo Central Hospital is in fact not treatable with the locally available antibiotics.

This is in line with the recently reported antimicrobial surveillance data of blood culture Gram-negative pathogens from Blantyre, Malawi [30].

In conclusion, our study has shown: (i) a high proportion of pAmpC- and/or ESBL-producing clinical isolates of *E. coli* from the urinary tract and blood cultures at the Central Hospital. (ii) CTX-Ms and FOX/MOX were the dominant ESBL- and pAmpC-types, respectively. (iii) All ESBL- and pAmpC-producing isolates were multidrug-resistant, only susceptible to antibiotics that are not easily available at the hospital. (iv) Studies of the genetic relatedness between ESBL- and / or pAmpC-producing isolates demonstrate genetic diversity and some clusters indicating within-hospital spread of isolates. The overall findings strongly support the urgent need for accurate and rapid diagnostic services to guide antibiotic treatment of life-threatening infections and improved infection control measures. The findings have a probable transfer value to other hospitals in Mozambique as the Central Hospital has reference functions with transfer of patients to and from other hospitals.

Abbreviations

AST: antimicrobial susceptibility testing; ATCC: American Type Culture Collection; ERIC-PCR: Enterobacterial repetitive intergenic consensus PCR; ESBL: extended-spectrum β -lactamase; ISCISA: Instituto Superior de Ciências de Saúde; MCH: Maputo Central Hospital; MDR: multidrug-resistance; pAmpC: plasmid-mediated AmpC β -lactamase; UKZN: University of KwaZulu-Natal; UPGMA: Unweighted Pair Group Method with Arithmetic Mean.

Declarations

Ethics approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN: BE 030/16) and the Institutional Bioethics Committee for Health-CIBS- Instituto Superior de Ciências de Saúde (ISCISA: TFCMCSCE 02/15). The committees waived the requirements for informed consent.

Consent of publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the first and the corresponding author on reasonable request.

Competing interests

SYE is a member of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser, UK.

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Authors' contributions

The study was designed by CELE, TFZ, GSS, BH, SYE, and AS. Collection of data and laboratory analysis were performed by CELE, JOS, UG, and HYC. All authors contributed to the interpretation of data and writing of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Primer sequences for PCR amplification of ESBL and pAmpC genes

Genes	Primers	Sequence(5' to 3')	Annealing (°C)	Reference
TEM	1	AAAATTCTTGAAGACG	60	[17]
	2	TTACCAATGCTTAATCA		
CTX-M	1	GGTTAAAAAATCACTGCGTC	57	[18]
	2	TTGGTGACGATTTTAGCCGC		
SHV	1	TAACTCCCTGTTAGCCA	56	[17]
	2	GATTTGCTGATTTGCCCC		
CMY	1	GAT TCC TTG GAC TCT TCA G	50	[19]
	2	TAA AAC CAG GTT CCC AGA TAG C		
FOX	1	CAC CAC GAG AAT AAC CAT	50	[19]
	2	ATG TGG ACG CCT TGA ACT		
MOX	1	GCTGCTCAAGGAGCA CAG GAT	50	[20]
	2	CACATTGACATAGGT GTG GTGC		
DHA	1	AACTTTCACAGGTGTGCT GGGT	50	[20]
	2	CCGTACGCATACTGGCTT TGC		

Figures

ISOLATE CTX-M

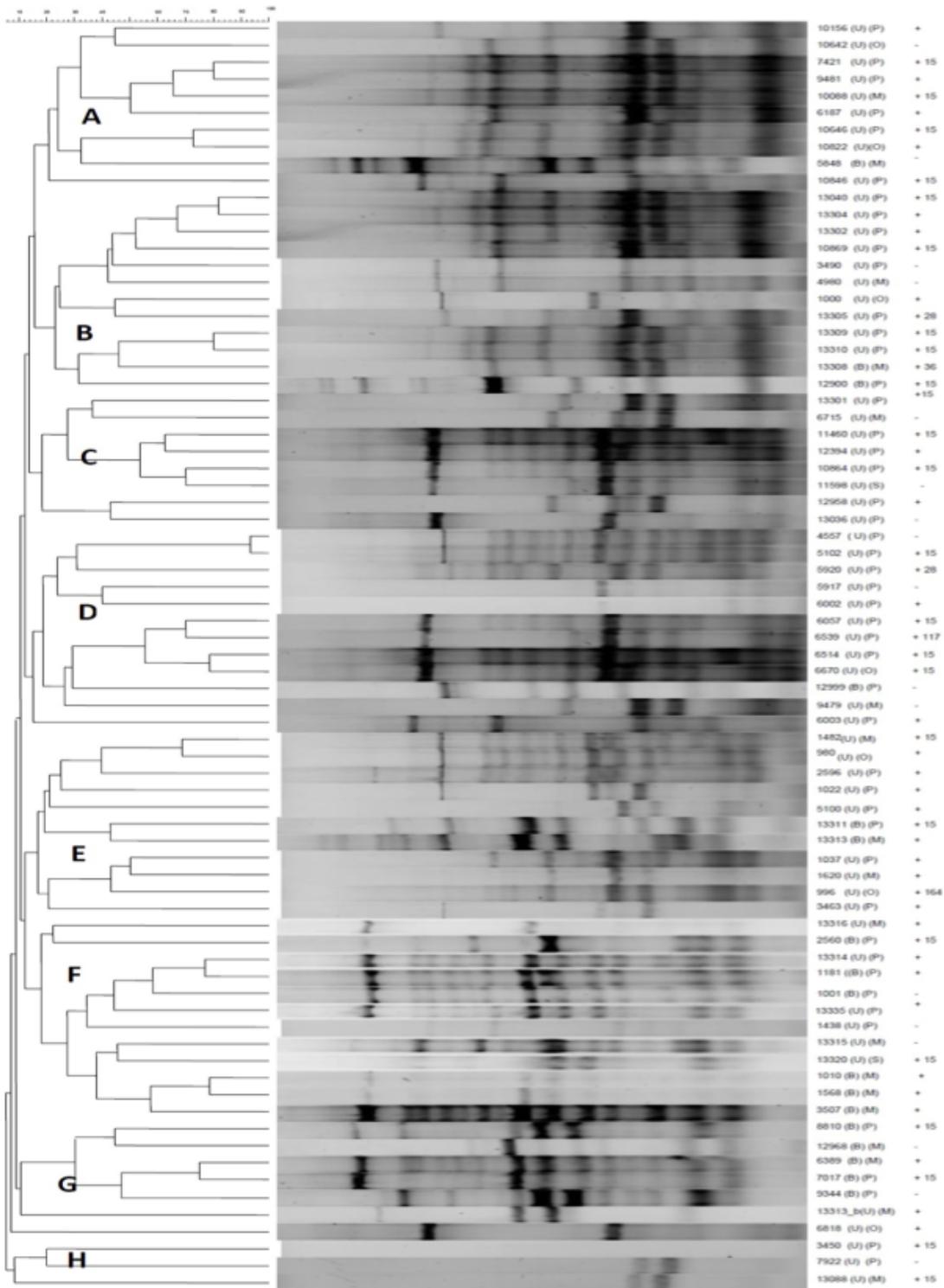


Figure 1

Cluster analysis of ESBL-positive *E. coli* (n=75) isolates based on ERIC-PCR fingerprinting patterns using Jacquard index and UPGMA clustering. The scale at the top left represents percentage similarity. Isolate columns: identification with origins in brackets: O = outpatient, P = paediatrics, M = medicine, S = surgery, U = urine and B= blood. Presence (+) or absence (-) of blaCTX-M and sequence type are also indicated for each strain.

Supplementary Files

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