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Molecular characterisation of CTX-M-type extended-spectrum β-lactamases of *Escherichia coli* isolated from a Portuguese University Hospital

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Abstract

Study objectives: The aim of this work was to study the prevalence of CTX-M-producing *Escherichia coli* isolates collected from the Hospital of the University of Coimbra (HUC) and to characterise these isolates both phenotypic and genotypically.

Methods: Between November and December 2007, 220 non-duplicate *E. coli* isolates were recovered at HUC. The extended-spectrum β-lactamases (ESBL)-producers were identified by the automatic VITEK 2 system and advanced expert system (bioMérieux, Marcy l'Étoile, France), further confirmed by the disk diffusion synergy test. The bla_{CTX-M} genes were detected by PCR, and the amplicons were sequenced. Genetic relatedness was assessed by ERIC-PCR. A CTX-M-15-producing *E. coli* isolate collected in 2004 at the same hospital was included in the study.

Results: Twenty-one isolates were identified as ESBL-producers resistant to all penicillins, first generation cephalosporins, cefotaxime and/or ceftazidime, but susceptible to imipenem. The majority of the isolates were collected from urines. Sequence analysis identified the CTX-M-15 enzyme in all isolates. All the isolates were clonally related. DNA fingerprinting was identical with the *CTX-M-15*-producing strain collected in 2004.

Conclusion: Our results showed the spread of hospital-acquired urinary tract infections caused by *CTX-M-15*-producing *E. coli,* and the prevalence of these infections in women. Also, the emergence of *CTX-M-15* in this institution is related to the spread of a clone over time. We concluded the need to upgrade the control infection measures in this hospital, as this study confirmed the presence of an endemic *E. coli* clone disseminated in different wards, a clone already identified in 2004, and according to our results, maintained at this hospital until 2007.

Keywords

CTX-M, *Echerichia coli*, ESBL

Introduction

Extended-spectrum β-Lactamases (ESBL) is the name given to some specific proteins that confer bacterial resistance to penicillins, first-, second- (except cephamicins) and third-generation cephalosporins, as cefotaxime (CTX) and ceftazidime (CAZ), and aztreonam [1, 2]. Generally, bacteria containing ESBL genes are susceptible to β-Lactamases inhibitors (clavulanate, sulbactam and tazobactam) [3]; Cephamicins and Carbapenems are not hydrolysed by ESBLs [2].

The existence of these enzymes is being documented since the introduction of third generation cefalosporins in the market [4]. ESBL gives the bacteria the ability to inactivate the previous mentioned antibiotics before they reach the penicillin-binding proteins existent in bacteria's surface [5].

ESBL codifying genes may be harboured both in chromosomal DNA and plasmids [6]. The ESBL genes can be divided in three big families: *TEM, SHV* and *CTX-M* [4]. TEM and SHV encoded proteins are reliant on key amino acid substitutions to raise their binding capacity to third generation cephalosporins, especially on CAZ. CTX-M encoded proteins have an intrinsic extended-spectrum profile, which confers them the particular characteristic of hydrolysing efficiently CTX [3, 4].

CTX-M-enzymes are a type of ESBL whose prevalence has dramatically increased worldwide in the past years [7-9].

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The name given to this ESBL type evidences the greater capacity to hydrolysecefotaxime [10] and ceftriaxone (CFX), than ceftazidime (CAZ) [3, 7]. However, some types of CTX-M β-Lactamases have emerged with a high affinity to hydrolyseceftazidime as well.

CTX-M β-Lactamases have been reported worldwide since the first isolation of an enzyme with clinical origin, CTX-M-1, in the late 1980s [11, 7]. In some specific geographical areas, CTX-M are now the most commonly found type of β-lactamase [9]. Today, more than 50 types of CTX-M ESBL have been identified and five sub-lineages have been created [12, 13]. The differences among the groups are based in single or few amino-acids residues, with minor allelic variations: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 [9, 13]. The first non-TEM, non-SHV ESBL was isolated in Japan in 1986. In 1989, in Germany, another non-TEM, non-SHV strain-producer was discovered, and named CTX-M-1 (isolated in Munich). Both CTX-M enzymes were discovered in *E. coli* [3]. In 1993 and 1996 two variants of the enzyme CTX-M-1 were discovered, CTX-M-2 and CTX-M-3, respectively [3, 14, 15]. In less than 15 years, ESBL spread has been huge, and these enzymes are spread among diverse bacterial species and worldwide.

Some types of CTX-M β-lactamases (namely CTX-M-15) have reached proportions in some countries that may be considered endemic, and is now considered an epidemic ESBL [13]. A survey was realised in 1999 in Portugal, but no CTM-X enzymes were detected in clinical isolates [8]. In July 2003, the first description of an isolate from *E. coli* producing CTX-M-14 was reported in this country [7, 8]. The first *CTX-M-15* isolated in Portugal was identified in 2005[16]. Recent studies indicate that *E.coli* *β*-lactamase-producers are widespread across the country both in nosocomial and community environments [9].

In this study, we aimed to evaluate the prevalence of CTX-M-enzymes in *E.coli* isolates in the University of Coimbra Hospital, to characterise these isolates both phenotypic and genetically, and also to track the dissemination in this hospital between 2004 and 2007, in order to assess the efficacy of infection control measures.

Materials and methods

Bacterial isolates

The 220 clinical isolates of *E.coli* were collected in the pathology laboratory service of the University of Coimbra Hospital, between November and December 2007, from different wards of this tertiary care hospital—including the Emergency Medical Service, and were sent to the Microbiology Laboratory of the Faculty of Pharmacy of the University of Coimbra. These isolates belonged to different biologic fluids, namely urine, blood and wound exudates.

Susceptibility test

The determination of susceptibility tests was performed at the hospital using the automatic commercial system VITEK 2 advanced expert system (AES) (bioMérieux, Marcy l'Étoile, France). Of the 220 isolates received, 24 were identified as ESBL-producers by VITEK 2 AES. Interpretative reading of susceptibility tests aims to analyse susceptibility patterns rather than results for individual antibiotics, so that underlying resistance mechanisms may be predicted [17].

The double disk diffusion synergy test was used to confirm the presence of ESBL, using CAZ, CTX and amoxicillin/clavulanic acid.

Samples were screened by inoculating a Mueller-Hinton agar, and the diffusion susceptibility test was realised using the following antibiotics: ceftazidime, cefotaxime, imipenem, ciprofloxacin, nalidixic acid, and gentamicin. The results were interpreted using CLSI—formerly National Committee for Clinical Laboratory Standards—criteria [18]. A disc of amoxicillin with clavulanic acid was added in a distance of 1.5 cm from ceftazidime and cefotaxime discs, in order to detect synergy between these antibiotics, and suspect of ESBL production.

CTX-M genes detection

All ESBLs isolates were submitted to PCR amplification to test for bla_{CTX}. In both cases specific primers were used to detect known sequences. DNA was extracted by suspending a couple of colonies in MilliQsterile water, and heating at 99ºC for 15 minutes. PCR occurred in the following conditions: initial denaturation of 95ºC for 7 minutes, followed by 25 cycles of 94ºC for 1 minute, 52ºC for 1 minute, 65ºC for 8 minutes, and a final extension of 65ºC for 19 minutes.

ERIC-PCR typing

The isolates were submitted to DNA-fingerprinting enterobacterial repetitive intergenic consensus (ERIC-PCR) in order to establish a genetic relationship between the isolates. The primers were used in each tube in a quantity of 15 pmol of ERIC1 primer (STABvida, Portugal)—5'ATGTAAGCTCCTGGGATTCAC3'—and the same quantity of ERIC-2 primer—3'AAGTAAGTGACTGGGGTGAGCC5'—, 6 mM of $MgCl_2$ (Qiagen Quality, Izasa, Portugal) and 3,4 μL of DMSO (Qiagen Quality, Izasa, Portugal). Amplicons were

separated on a 2% agarose gel containing ethidium bromide (10 mg/mL) with a 0,5x TBE running buffer. An isolate of *E. coli* American Type Culture Collection (ATTC) 25922 was used as a control pattern. The isolate Ec 12 from a sample collected in 2004 in the same hospital that produces the CTX-M-15 enzyme was also used as control pattern.

Nucleotide sequence determination

DNA was purified using a purification kit (Qiagen Quality, Izasa, Portugal), and was further sequenced (STABvida, Portugal). The sequences were analysed with the BLAST program at the NCBI website (www.ncbi.nlm.nih.gov/blast).

Results

Origin of clinical isolates

Twenty-seven percent of the 220 clinical isolates were collected in patients hospitalised in the internal medicine ward. The urology-renal transplant and nephrology wards provided 16% of the samples and the emergency medical service provided 15%; these isolates are considered as result of community infections. However, the isolates collected in this service did not contain any ESBL-producer-organism (data not shown). About 77% of the bacteria derived from urinary tract infections and 16% from septicemia. The remaining isolates were collected in wound's exudates, both surgical and non-surgical.

The percentage of samples collected in women was 69% (86.2% from urine) and 31% in men (55.8% from urine samples).

Antibiotic susceptibility and prevalence of ESBLs by phenotypic method

Twenty-one isolates suggested the production of CTX-M enzymes by the double disk synergy test. From these clinical isolates studied, 16 were from urine samples, three were from wound exudates, and two from blood samples. All isolates were resistant to ciprofloxacin, gentamicin and nalidixic acid, but susceptible to imipenem. The resistance towards cefotaxime and ceftazidime was different between the isolates and that led to the organisation of the samples into groups: group 1 contains bacteria resistant to CTX, but susceptible to CAZ; group 2 contains bacteria both resistant to CTX and CAZ; group 3 contains isolates that even though they are resistant to cefotaxime, they are in a less extended level than the previous mentioned bacteria, see Table 1. About 38% of the isolates were resistant to ceftazidime.

Detection and identification of *CTX-M* genes

PCR was performed using bla_{CTX-M} primers in order to identify the presence of this gene among the isolates. From the 24 bacteria identified as ESBL-producers by the hospital, 21 have confirmed the presence of *CTX-M* gene, see Table 1. Those bacteria that were not identified as producers of CTX-M β-lactamases, were excluded from the study.

ERIC-PCR

ERIC-PCR analysis was used to obtain the genetic relatedness of the studied bacteria. All bacteria shared an

Table 1: *E. coli* isolates grouped according to their resistance profile towards cefotaxime and ceftazidime

Isolate	Group	Specimen	ERIC-Profile	CTX-M type	CTX (mm)	CAZ (mm)
Ec 47	1	wound	Type 1		0	19
Ec 94		urine	Type 1		0	20
Ec 161-A		urine	Type 1		0	16
Ec 161-B		urine	Type 2	CTX-M-15	0	19
Ec 250		wound	Type 1[a]		0	19
Ec 305		urine	Type 1		0	19
Ec 314		urine	Type 1		0	14
Ec 408		urine	Type 1		0	17
Ec 467		urine	Type 1		0	17
Ec 115	2	urine	Type 1	CTX-M-15	0	8
Ec 149		urine	Type 1		0	10
Ec 378		urine	Type 1	CTX-M-15	0	11
Ec 395		blood	Type 1[a]		0	12
Ec 420		wound	Type 1		0	13
Ec 85	3	urine	Type 1		10	19
Ec 89-B		urine	Type 1	CTX-M-15	9	19
Ec 138		blood	Type 1		9	19
Ec 144-A		urine	Type 1		7.5	15
Ec 90		urine	Type 1[a]		7.5	10.5
Ec 104		urine	Type 1[a]		7.5	10.5
Ec 155		urine	Type 1[a]		7.5	11

elevated percentage of similarity but two types of DNA fingerprints were easily identified, see Figure 1, with type 1 being prevalent. Within the type 1 some bacteria presented small band variations in their profile, and were classified as being in the type 1ª. Inside the same group with common phenotypic characteristics, different types of ERIC-PCR patterns were found. Nevertheless, they were classified as very closely genetically related. The profile isolates was compared with the isolate Ec 12 collected in 2004 in the same hospital. The similarities among the profiles allow us to conclude that these bacteria share the same genetic background.

The sample Ec 161-B showed a unique ERIC profile, but its resistance phenotype was the same as other isolates studied. The other nine samples not included in the figure had the exact same pattern than type-1 samples (data not shown).

DNA sequenciation

All the isolates submitted to sequencing determination revealed the presence of a *CTX-M-15* gene.

Discussion and conclusion

This report is an insight on the dissemination of CTX-M-type β-lactamase-producers in nosocomial infections in Portugal. The prevalence of this enzyme type among clinical isolates has increased substantially in the past years [7–9], and it is surprising to find this emergence also in the number and variety of our isolates. Despite the fact that in our study no ESBL had provenience from the community environment, the truth is that the production of these enzymes is increasing outside the hospital and it is becoming an important public health issue [13]. It is possible that many of these outpatients had been already hospitalised previously, for example, those from renal transplants, suggesting that resistant bacteria with origin at the hospital are spreading in the community.

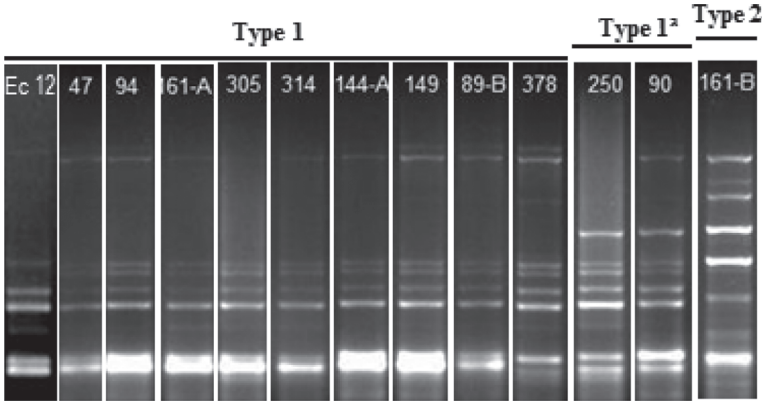
The most common microorganism ESBL-producer involved in urinary tract infections is *E. coli* [13]. This data was confirmed with our results: 77% of the bacteria derived from urinary tract infections, and 69% of the infections occurred in women. A study held in 2005 suggested that an elevated number of patients that have developed infections with ESBL-producers organisms had their gastrointestinal tract colonised previously by them [2, 13]. The infection of female urinary tract is easier if there is a previous colonisation of the gastrointestinal tract, which explains the high rates of urinary tract infections in women.

The fact that different wards were contaminated with the same bacterial clone, not only in the year of 2007, but also during the year 2004, showed that there is an endemic strain in this hospital, and that disinfection measures should be increased, and developed. Moreover, an isolate with a unique DNA fingerprint was found, suggesting that the β-lactamase is spreading to different strains (and probably bacterial species), since CTX-M enzymes are usually located in conjugative plasmids.

Despite the fact that the first detection of *CTX-M-15* in Portugal has occurred recently [16], our results show a quick spread and proliferation of this β-lactamase, since all 21 samples were identified as CTX-M-15-producers.

Our results reaffirmed the need to use multiple ESBL screening tests, and not to rely only on ceftazidime and cefotaxime inhibition patterns [19]. These bacteria had different resistance patterns, suggesting the presence of different ERIC-PCR profiles, that were not observed. Only the isolate Ec 161-B has shown a different ERIC-PCR profile, even though its inhibition pattern was the same as other bacteria; its resistance gene was identified also as being *CTX-M-15*. From the 24 isolates identified in the hospital as being ESBL-producers, only 21 were confirmed by molecular methods. The hospitals should not rely only in phenotypic methods, but also molecular methods to confirm resistance, and track resistant clones disseminated in the institution. This approach would increment effective control infection measures. Moreover, outpatients should be screened for colonisation with resistant bacteria before their admission.

Figure 1: ERIC-PCR DNA fingerprinting profiles of bacterial isolates



Lanes are labeled with sample number and with the different profiles obtained with this technique.

In conclusion, our study suggests the quick spread hospital-acquired infections of ESBL-producer *E. coli* harbouring *CTX-M-15* gene that confer high resistance to 3rd generation cephalosporins, namely cefotaxime and ceftazidime, and all the remaining β-lactam antibiotics, except cephamycins and carbapenems. This bacterial proliferation is more likely in urinary tract infections in women, but men over a certain age (usually over fifty-years-old) can also be affected. The hospital should upgrade their disinfection measures in order to avoid the proliferation of these clones between the different wards, and molecular identification should be used to characterise the spread and origin of resistant clones. Among the measures that should be introduced or reinforced, highlight should be made to the increasing number of external cleaning for all the rooms (twice a day) as well as introduce it at weekends, to give education to all the staff regarding antimicrobial resistance, and to educate patients to wash their hands before meals and after going to the toilet, and to educate clinical staff to disinfect their hands before and after seeing a patient [20].

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Conflict of interest

The authors declare no conflict of interest.

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