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Short Communication

Epidemiology of extended-spectrum β-lactamase-producing Enterobacteriaceae among healthcare students, at the Portuguese Red Cross Health School of Lisbon, Portugal

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ABSTRACT

Objective: The aim of the present study was to prospectively evaluate the prevalence of intestinal carriage by extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae among Portuguese students attending a Bachelors' course in healthcare, and to determine the molecular features of ESBL-producing isolates.

Methods: One-hundred and eleven faecal samples recovered from Portuguese healthcare students were screened for either ESBL-producing, carbapenem-resistant, colistin-resistant or pan-aminoglycoside-resistant Enterobacteriaceae, using respective screening media. All recovered isolates were tested for antimicrobial susceptibility and characterised by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Results: A total of 17 ESBL-producing Enterobacteriaceae (16 *Escherichia coli* and a single *Klebsiella pneumoniae*) were recovered from 16 students, representing a prevalence of 14.5%. The *E. coli* isolates were distributed into three sequence types (STs) and seven PFGE types. The most common ESBL identified was CTX-M-1 ($n = 13$; 76%), followed by CTX-M-15 ($n = 3$; 18%) and CTX-M-8 ($n = 1$; 6%). The majority of the strains were resistant to sulfonamides (88%) and fosfomycin (71%). Resistance to aminoglycosides was observed at a low rate, that is 12% for both tobramycin and kanamycin. No colistin-, carbapenem- or pan-aminoglycoside-resistant isolates were recovered. A major clone, ST10-$bla_{CTX\text{-}M\text{-}1}$, included 12 *E. coli* isolates. The $bla_{CTX\text{-}M\text{-}1}$ gene was always located on an IncFIA/FIB plasmid type, co-harbouring genes encoding resistance to tetracycline, sulfonamides, trimethoprim–sulfamethoxazole and fosfomycin.

Conclusion: The most commonly identified ESBL gene in *E. coli* was $bla_{CTX\text{-}M\text{-}1}$, usually identified among ESBL-producing isolates recovered from animals. A high prevalence of faecal carriage of ESBL-producing *E. coli* was found among healthy healthcare students, underlying this population as an important reservoir.

1. Introduction

The spread of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae has been considered as a global threat worldwide for the last 20 years [1]. ESBLs confer resistance to most β-lactam antibiotics, including penicillins and broad-spectrum cephalosporins, with only carbapenems being spared by those enzymes [2].

ESBL-producing *Escherichia coli* are a major cause of community and nosocomial acquired infections [3], whereas ESBL-producing *Klebsiella pneumoniae* are mainly hospital-acquired pathogens [4]. Whereas up to the 1990s, ESBLs were mainly encoded by the bla_{TEM} and bla_{SHV} genes, currently the most prevalent ESBL enzymes recovered from humans belong to the CTX-M family, with CTX-M-15 being the most frequent ESBL determinant identified worldwide [5–7].

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In Portugal, the proportion of ESBL-producing *E. coli* and *K. pneumoniae* isolates causing invasive infections was estimated in 2017 at 15.7% and 44.9%, respectively [8]. Although several studies on ESBL-producing Enterobacteriaceae have been done in Portugal, most have only focussed on clinical isolates (recovered from hospitals or nursing homes). These actually showed, similarly to many places worldwide, a predominance of CTX-M-15 and the occurrence of CTX-M-1 at a low rate [3,9–12]. A single study performed more than 10 years ago evaluated the carriage of ESBL producers in the community. The prevalence of ESBL-producing *E. coli* among healthy children was 2.7% (three isolates producing CTX-M-1, TEM-52 and SHV-12 only, respectively) [13]. Another study conducted in 2014 among healthy adults identified four *E. coli* ESBL-positive isolates, producing CTX-M-14 and CTX-M-27 [14]. However, no study had been designed to prospectively evaluate the prevalence of the carriage of ESBL producers in the community.

The aim of the present study was to prospectively evaluate the prevalence of intestinal carriage by ESBL-producing Enterobacteriaceae as well as other multidrug-resistant isolates among a group of Portuguese students attending a Bachelors' course in healthcare, and to determine the epidemiological and antimicrobial resistance features of those ESBL-producing isolates.

2. Methods

2.1. Sampling

Between March and May 2018, 111 rectal swabs were collected from 111 students at Escola Superior de Saúde da Cruz Vermelha Portuguesa (ESSCVP) in Lisbon, Portugal. Students were attending either the first, second, third or fourth year of their degrees. Of note, half of the students ($n = 55$) degrees included a clinical internship, and subsequently had contact with hospitals, long-term care facilities or community patients.

The study was explained orally in the classroom and a sterile swab with transport medium and a questionnaire were distributed to all the students. The questionnaires included demographic data, and risk factors for colonisation with multidrug-resistant bacteria such as hospitalisation during the last 6 months, antibiotherapy at the time of sampling or during the previous 2 months, attendance at the clinical internship, contact with isolation patients, contact with animals and travelling abroad. All individuals who accepted to participate returned the rectal swab sample and the completed questionnaire. The protocol was approved by the Direction of ESSCVP and a written informed consent was obtained from each participant (all were aged over 18 years).

2.2. Bacterial isolates

Rectal swabs were enriched overnight in 5 mL Luria–Bertani (LB) broth. Ten microlitres of each sample were plated onto five different selective media: ChromID ESBL® (bioMérieux, Balme-La-Grotte, France), SuperPolymyxin, SuperAminoglycoside, Super-Carba and CarbaSmart® (bioMérieux) in order to respectively select for ESBL producers, colistin-resistant, pan-aminoglycoside-resistant and carbapenem-resistant Enterobacteriaceae. Species identification was performed on all selected isolates using the API20E system (bioMérieux).

2.3. Susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion method on Mueller–Hinton agar plates (Bio-Rad, Cressier, Switzerland) for amoxicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanic acid, temocillin, cephalotin, ceftazidime, cefotaxime, cefepime, cefoxitin, aztreonam, ertapenem, imipenem, meropenem, amikacin, gentamicin, tobramycin, kanamycin, fosfomycin, nalidixic acid, chloramphenicol, tetracycline, colistin, sulfonamides, trimethoprim–sulfamethoxazole (SXT), ciprofloxacin and tigecycline, following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Colistin resistance was evaluated by broth microdilution according to EUCAST. Identification of ESBL producers was confirmed with the Rapid ESBL NP test [15].

2.4. Molecular characterisation

Identification of ESBL encoding genes was performed by polymerase chain reaction (PCR), as previously described [16], followed by sequencing of the amplicons (Microsynth, Balgach, Switzerland).

The clonal relationship of the isolates was evaluated by pulsed-field gel electrophoresis (PFGE) [17], and multilocus sequence typing (MLST), as previously described [18,19]. Sequence types (STs) were assigned using the MLST databases for *K. pneumoniae* and *E. coli* (https://cge.cbs.dtu.dk/services/MLST-2.0/).

2.5. Transformation experiments and plasmid characterisation

Plasmids carrying $bla_{\text{CTX-M}}$ and bla_{TEM} genes were extracted from the original isolates using the Kieser method [20] followed by electrotransformation. *Escherichia coli* TOP10 was used as the recipient and transformants were selected on LB agar plates supplemented with cefotaxime (1 μg/mL). PCR amplification of $bla_{\text{CTX-M}}$ and bla_{TEM} genes was performed using the *E. coli* transformants as templates to further assess the presence of the expected gene. Plasmid size was evaluated from the transformants using the Kieser method followed by electrophoresis. An incompatibility group of the plasmids was determined by PCR-based replicon typing (PBRT) on each transformant [21].

2.6. Statistical analysis

Risk factors were assessed using SPSS software, version 21.0 (IBM Portugal, Lisbon, Portugal). The χ^2 and Fisher tests were used to identify variables associated with the carriage of ESBL producers and colistin-resistant isolates (gender, age, schooling year, previous hospital admission, current or previous antibiotherapy, attendance at the clinical internship, contact with isolation patients, contact with animals and travelling abroad). *P*-values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Study population

Among the 111 students screened, 85 attended the Bachelor's in nursing and 26 were studying physiotherapy. They were distributed over the 4 years of schooling (Table 1). Most of the students were female ($n = 97$; 87%), and the mean age was 25.7 years. Only a few participants ($n = 3$) had been admitted to a hospital during the previous 6-month period, and 11 (10%) were under current or previous (last 6 months) antibiotherapy. Half of the students had already attended the clinical internship, and 44% had had contact with patients. Regular contact with animals (cat, dog, rabbit, sheep, goat) was observed for 17% of the individuals and travelling abroad was recorded in 30%.

Table 1
Characteristics of students enrolled in the prospective study and risk factors for intestinal carriage of ESBL-producing Enterobacteriaceae.

	Nursing (n = 85)	Physiotherapy (n = 26)	Total (n = 111)	Carriage of ESBL producers		P-value
				Yes (n = 17)	No (n = 94)	
Gender						
Male	13	1	14	3	11	0.422
Female	72	25	97 (87%)	14 (82%)	83 (88%)	
Age (years)						
Mean	26.5	23.2	25.7			
Schooling year						
First	40	7	47 (42%)	14 (82%)	33 (35%)	0.009
Second	18	0	18 (16%)	1 (6%)	17 (18%)	
Third	11	19	30 (27%)	1 (6%)	29 (31%)	
Fourth	16	0	16 (14%)	1 (6%)	15 (16%)	
Previous hospital admission						
Yes	2	1	3 (3%)	0	3 (3%)	0.455
No	83	25	108	17	91	
Current or previous antibiotherapy						
Yes	9	2	11 (10%)	2 (12%)	9 (10%)	0.781
No	76	24	100	15	85	
Clinical internship attendance						
Yes	37	18	55 (50%)	4 (25%)	44 (46%)	0.034
No	48	8	56	12	51	
Contact with isolated patients						
Yes	45	4	49 (44%)	4 (25%)	45 (47%)	0.096
No	40	22	62	12	50	
Contact with animals						
Yes	11	8	19 (17%)	3 (19%)	16 (17%)	0.851
No	74	18	92	13	79	
Travel abroad						
Yes	23	10	33 (30%)	3 (19%)	30 (32%)	0.299
No	62	16	78	13	65	

ESBL, extended-spectrum β-lactamase.

3.2. Prevalence of ESBL producers and risk factors

Of the 111 samples collected from the healthcare students, 17 ESBL-producing enterobacterial isolates were recovered, of which 16 were *E. coli* and one was *K. pneumoniae*, corresponding to a prevalence rate of 14.5%. No carbapenem-resistant, pan-aminoglycoside-resistant or colistin-resistant Enterobacteriaceae were isolated.

Among the different variables analysed (Table 1), attending the first year of schooling of nursing or physiotherapy was significantly associated with intestinal carriage of ESBL producing Enterobacteriaceae (P = 0.009).

3.3. ESBL determinants

The most commonly identified ESBL among *E. coli* was CTX-M-1 (n = 13, 76%), followed by CTX-M-15 (n = 2) and CTX-M-8 (n = 1) (Table 2). The single *K. pneumoniae* isolate recovered produced CTX-M-15. This latter isolate, as well as the only CTX-M-15-producing *E. coli*, co-produced the narrow-spectrum β-lactamase, TEM-1.

PBRT analysis showed that the $bla_{\text{CTX-M-1}}$ gene was either located on IncFIA/FIB (n = 11), IncFIC (n = 1) and IncI1 (n = 1) plasmid types, the $bla_{\text{CTX-M-8}}$ gene on the FIA/FIB plasmid type and the $bla_{\text{CTX-M-15}}$ gene either on FIA/FIB, FIC and P plasmid types. In most cases (59%), plasmids carrying the $bla_{\text{CTX-M-1}}$ gene co-harboured genes encoding resistance to tetracycline, sulfonamides, fosfomycin and SXT.

3.4. Susceptibility testing

Antimicrobial susceptibility testing showed that a majority of the ESBL-producing isolates were resistant to cefotaxime (94%), ceftazidime (94%), cefepime (100%), tetracycline (76%), sulfonamides (88%), SXT (82%) and fosfomycin (71%). Resistance to aminoglycosides was observed at a low rate, that is 12% for both tobramycin and kanamycin. All isolates remained susceptible to carbapenems, colistin and chloramphenicol.

3.5. Clonal relationship

PFGE analysis distributed the 16 ESBL-positive *E. coli* isolates into seven PFGE types (Fig. 1) and three STs were identified by MLST (Table 2), namely ST10 (n = 13), ST130 (n = 2) and ST38 (n = 1). The unique ESBL-producing *K. pneumoniae* isolate belonged to ST348.

4. Discussion

The present study identified a prevalence of 15% ESBL-producing Enterobacteriaceae among the healthcare students at a teaching university in Lisbon, Portugal. This rate may be considered very high, owing to the screened population. Indeed, these individuals were for the most part young, healthy people, who did not present (for the majority) significant well-established risk factors (travelling in endemic countries, hospitalisation, antibiotic selective pressure). This rate is twice as high when compared with a recent study performed in Spain, a neighbouring country) which focussed on hospitalised patients screened at admission (7.7%), despite the fact that those individuals presented additional risk factors, being unhealthy and older (mean age of 69 years old) [22]. This high rate might be in part related to the spread of a single clone among those students, possibly resulting from cross-contaminations or contamination from a unique and unknown source. This prevalence rate was much lower than that observed in India with 34% of the healthy population attending hospital for regular check-ups being colonised by ESBL-producing Enterobacteriaceae [23].

Over the 17 ESBL-producing isolates, all but one corresponded to *E. coli*, with only a single *K. pneumoniae* being identified. CTX-M-

Table 2
Genetic and phenotypic features associated with the ESBL-producing isolates.

Isolate	Species	ST	Resistance determinants	PFGE	Plasmid incompatibility	Plasmid size (kb)	Resistance phenotype transformants[a]
AA1	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA2	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA3	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA5	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA6	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA8	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	B	Inc FIA/FIB	84	TET, KMN, TMN, SUL, SXT, FOS
AA10	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA11	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AA13	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
AB3	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	C	Inc I1	84	TET, SUL, SXT, FOS
AB14	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
BB8	*E. coli*	ST131	$bla_{CTX\text{-}M\text{-}1}$	D	Inc FIC	76	No co-resistance
CA1	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}1}$	A1	Inc FIA/FIB	56	TET, SUL, SXT, FOS
DA11	*E. coli*	ST 131	$bla_{CTX\text{-}M\text{-}8}$	A2	Inc FIA/FIB	56	SUL, SXT
AB13	*E. coli*	ST10	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$	E	Inc FIA/FIB	61	TET, KMN, TMN
KA1	*E. coli*	ST38	$bla_{CTX\text{-}M\text{-}15}$	F	Inc FIC	23	SUL, SXT
KA7	*K. pneumoniae*	ST348	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$	Kp	Inc P	319	SUL, FOS

ESBL, extended-spectrum β-lactamase; PFGE, pulsed-field gel electrophoresis; ST, sequence type.
Underlined terms indicate those resistance determinants being co-localized together with ESBL encoding gene on a same plasmid.
[a] Antibiotic abbreviations: FOS, fosfomycin; KMN, kanamycin; SUL, sulfamide; SXT, trimethoprim–sulfamethoxazole; TET,- tetracycline; TMN, tobramycin.

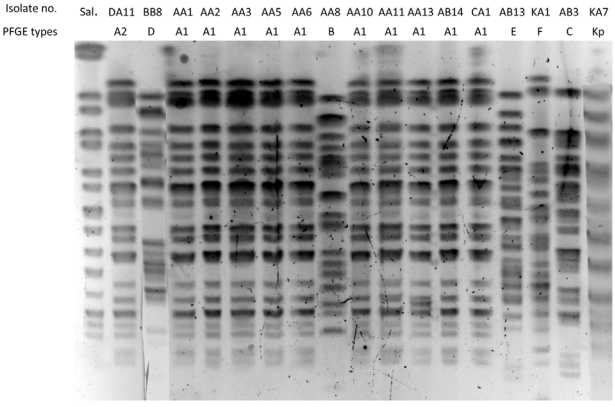

Fig. 1. Pulsed-field gel electrophoresis of the ESBL-positive *E. coli* isolates. Sal, *Salmonella braenderup* as molecular size standard; AXX, first-year nurse student; BXX, second-year nurse student; CXX, third-year nurse student; DXX, fourth-year nurse student; KA, first-year physiotherapy student.

1 was the most commonly identified ESBL (76%) whereas CTX-M-15 (18%) was much less prevalent. This situation is surprising as what is observed in most European countries is actually the opposite, with CTX-M-15 producers over-predominating [24]. Conversely, CTX-M-1 producers are commonly observed among animals in many European countries, being the most commonly identified ESBL in many different food-producing animals (poultry, cattle, swine) [25–28].

Sequence typing identified three different STs among the recovered *E. coli* isolates, namely ST10, ST131 and ST38. Those STs are commonly identified among human isolates, with ST10 being a commensal lineage that is also often recovered from animals. By contrast, ST131 is mainly found in humans and related to extra-intestinal infections but also in carriage in healthy individuals [29].

Among the two students colonised with an ST131 *E. coli,* there were different particularities that could be noted; one of the students colonised with the *E. coli* $bla_{CTX\text{-}M\text{-}1}$-ST131 was a vegetarian, had travelled in India, Italy and Ireland during the 6-month period prior to the study, was attending their second year of schooling, and had contact with hospitalised patients during their clinical internship. The second individual colonised with an ST131 *E. coli* (being a CTX-M-8 producer) was a fourth-year student who also had contact with patients.

Regarding the CTX-M-15-producing *E. coli* isolates, they were of the ST38 type, which is not surprising considering that this clonal background has been reported to be the second most prevalent one producing CTX-M-15 after ST131 [30]. The CTX-M-15-producing *K. pneumoniae* was an ST348, an ST that was shown to be the source of urinary tract infections in both humans and companion animals in Portugal [31].

The most commonly identified clone was ST10-$bla_{CTX\text{-}M\text{-}1}$, corresponding to 12 *E. coli* isolates. Surprisingly, 11 out of those 12 isolates were recovered from 11 students in their first year of study, with only a single isolate from a student attending the third year. This result was very surprising, as our primary hypotheses were that either no difference would be observed among all the students during all the student years, or an increased ESBL rate might be observed along the years in case a difference would be noticed. This latter hypothesis would have correlated with some previous studies showing that healthcare workers in contact with patients being colonised with ESBL producers are at higher risk of being colonised with ESBL producers [32]. Here the opposite was observed, with most students in contact with patients (ESBL colonisation status unknown) being negative for ESBL producers.

The reason why such a high proportion of first-year students are significantly colonised remains intriguing. Such a high proportion of colonisation with ESBL producers may be also identified in the general and the healthy population in Portugal. It is important to highlight that those first-year students were for the most part colonised with a single clone, belonging to ST10, which is a very common background, *E. coli* being found not only in humans, but also in animals and in food. Therefore, we might speculate that the source of colonisation of students by the ST10-$bla_{CTX\text{-}M\text{-}1}$ could be food-related, considering they actually share some common meals when eating at the university cafeteria.

The $bla_{CTX\text{-}M\text{-}1}$ gene of this major clone was located on a 56-kb IncFIA/FIB plasmid that co-harboured genes encoding resistance to tetracycline, sulfonamides, SXT and fosfomycin. Besides the worldwide dissemination of *E. coli* CTX-M-1 in animals, an increasing prevalence of $bla_{CTX\text{-}M\text{-}}$1-positive *E. coli* in human communities has lately been described in Mediterranean countries including Spain (2% in 2008 [33]) and Portugal (1% in 2009 [34]). We might therefore hypothesise that at least part of what we have shown is representative of what is happening in the community. Of note, data collected about antibiotic consumption among the subjects studied showed that little antibiotic selective pressure

existed ruling out such an explanation for a putative colonisation by resistant isolates.

In conclusion, our study revealed a very high rate of colonisation by ESBL-producing *E. coli* (15%) within a young healthy population. A surprising finding was the high rate of the detection of CTX-M-1 producers identified, contrasting with the low rate of CTX-M-15 producers usually identified among healthy populations worldwide.

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Competing interests

None to declare.

Ethical approval

A consent form was completed by each participant.

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