Commensal *Enterobacteriaceae* as reservoirs of extended-spectrum beta-lactamases, integrons, and *sul* genes in Portugal

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INTRODUCTION

Antimicrobial resistance has become a global public health problem, compromising the treatment of several infectious diseases. Acquisition of integrons and beta-lactamase genes by *Enterobacteriaceae* is increasingly recognized, being associated with resistance to multiple antibiotics (Machado et al., 2007; Coque et al., 2008; Bush, 2010; Pitout, 2010). The production of extended-spectrum beta-lactamases (ESBLs) constitutes one of the currently most spread and relevant antibiotic resistance mechanisms, compromising the use of several beta-lactams. Portugal is one of the European countries with higher rates of ESBL producers in the clinical setting, with a shift from TEM or SHV variants to CTX-M—types noticed since 2003 (Machado et al., 2007; ECDC, 2011).

Bacteria colonizing the human intestine have a relevant role in the spread of antimicrobial resistance. We investigated the faecal carriage of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in healthy humans from Portugal and analyzed the distribution of *sul* genes and class 1 and 2 integrons. Faecal samples (*n* = 113) were recovered from healthy persons (North/Centre of Portugal, 2001–2004) and plated on MacConkey agar with and without ceftazidime (1 mg/L) or cefotaxime (1 mg/L). Isolates representing different morphotypes/plate and antibiotic susceptibility patterns (*n* = 201) were selected. Isolates resistant to sulfonamides and/or streptomycin, gentamicin, and trimethoprim were screened (PCR and sequencing) for *sul* genes (*sul*1, *sul*2, *sul*3) and class 1 and 2 integrons. Presence of ESBLs was inferred using the double disk synergy test (DDST) and further confirmed by PCR and sequencing. ESBL producers were selected for clonal analysis, plasmid characterization and conjugation assays by standard methods. ESBL-producing isolates were found in 1.8% (2/113) of samples, corresponding to *Escherichia coli* of phylogroups A (*n* = 1) and B1 (*n* = 1) carrying transferable *bla*CTX-M-14 and the new *bla*TEM-153, respectively. A 80kb IncK plasmid bearing *bla*CTX-M-14 was found, being highly related to that widely spread among CTX-M-14 producers of humans and animals from Portugal and other European countries. *sul* genes were found in 88% (22/25; *sul*2-60%, *sul*1-48%, *sul*3-4%) of the sulfonamide resistant isolates. Class 1 integrons were more frequently found than class 2 (7%, 14/201 vs. 3%, 6/201). Interestingly, gene cassette arrangements within these platforms were identical to those commonly observed among *Enterobacteriaceae* from Portuguese food-producing animals, although *aadA13* is here firstly described in *Morganella morganii*. These results reinforce the relevance of human commensal flora as reservoir of clinically relevant antibiotic resistance genes including *bla*ESBLs, and highly transferable genetic platforms as IncK epidemic plasmids.
the epidemiology of multidrug resistant Enterobacteriaceae in Portugal and the contribution of different settings to the burden of infections with antibiotic resistant bacteria, we investigated, during the initial spread period of CTX-M enzymes, the faecal carriage of ESBL-producing Enterobacteriaceae, class 1 and class 2 integrons, and sul genes in Portuguese healthy humans.

**MATERIALS AND METHODS**

**BACTERIAL ISOLATES**

From January 2001 to February 2004, a total of 113 nonduplicate faecal samples were recovered from randomly selected healthy humans [53% females; age ranging from 5 to 59 (n = 99) and 60 to 76 (n = 14) years old] living in the North and Centre of Portugal. Samples were from persons without previous exposure to antibiotic therapy, hospitals, or long-term care facilities at least in the 3-month period before sampling. Rectal swabs were immersed in transport medium, faeces suspended in 1 mL of saline, and aliquots of 200 μL seeded in MacConkey agar with and without ceftazidime (1 mg/L) or cefotaxime (1 mg/L). Presumptive Enterobacteriaceae (oxidase-negative facultative aerobic Gram negative rods) were selected for further studies. Enterobacteriaceae isolates recovered in the same time period from Portuguese hospitals (n = 4; 2003–2004) or marine coastal waters (n = 1; 2003) close to clandestine discharge points of water streams contaminated with faecal coliforms, and producing ESBL-types similar to the ones identified in this study, were also included for clonal investigation and/or plasmid relationships analysis (Machado et al., 2007, 2009).

**ESBL DETECTION AND ANTIMICROBIAL SUSCEPTIBILITY**

Each different morphotype growing on MacConkey agar with ceftazidime or cefotaxime was screened for ESBL production by the double disk synergy test (DDST) (Jarlier et al., 1988), and susceptibility testing to non-beta-lactam antibiotics (aminoglycosides, quinolones, sulfonamides, trimethoprim, tetracyclines, chloramphenicol) was carried out in positive isolates using the standard disk diffusion method (CLSI, 2007). Morphotypes corresponding to non-ESBL producers recovered from MacConkey agar with and without antibiotics were tested to streptomycin, gentamicin, trimethoprim and sulfonamides (CLSI, 2007).

**ESBL CHARACTERIZATION AND EPIDEMIOLOGICAL FEATURES**

Characterization of ESBLs was performed by amplification of blα genes and sequencing (Table 1), and ESBL-producing isolates were further identified by API ID 32GN (bioMérieux, Marcy l’Etoile, France). Clonal relatedness of ESBL producers was investigated by pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) (Machado et al., 2005, 2008), and multilocus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli). E. coli phylogenetic groups were identified by a multiplex PCR (Clermont et al., 2000).

The transferability of ESBL genes was assessed by filter mating assays with E. coli BM21R (nalidixic acid- and rifampicin resistant, lactose fermentation positive and plasmid-free) (Machado et al., 2008), and ESBL-encoding plasmids were identified by PCR-based replicon typing and further hybridization (bla<sub>ESBL</sub>, rep probes), as previously described (Novais et al., 2010). Plasmid relationships were established by comparison of restriction fragment length polymorphism (RFLP) patterns obtained after digestion with EcoRI, PstI, and HpaI restriction enzymes (Valverde et al., 2009).

**CHARACTERIZATION OF INTEGRONS AND sul GENES**

Isolates representing different morphotypes and resistance patterns to streptomycin, gentamicin, trimethoprim and/or sulfonamides were selected for screening of class 1 and class 2 integrons by PCR (Table 1), as these phenotypes are commonly associated with these genetic structures (Machado et al., 2005). Class 1 and class 2 integrons were further characterized by RFLP-typing and sequencing, as described (Machado et al., 2005). Integrons were designated by roman numbers and a subindex indicates the class to which each integron belongs, as previously described (Machado et al., 2005). Isolates resistant to sulfonamides were also screened for the presence of sulfonamide resistance genes (sul1, sul2, sul3) by PCR (Table 1).

**RESULTS**

**EPIDEMIOLOGICAL BACKGROUND**

A total of 201 Enterobacteriaceae isolates representing different colony morphotypes and antibiotic susceptibility patterns were obtained. Resistance to at least one antibiotic was observed in 79 isolates, and the resistance rates were higher for streptomycin (36%, 73/201) than for trimethoprim (15%, 31/201), sulfonamides (12%, 25/201), and gentamicin (4%, 9/201).

The proportion of faecal carriage of ESBL-producing Enterobacteriaceae was 1.8% (2 of 113 samples), corresponding to samples recovered in 2003 from two females, aged 21 and 76 years, respectively.

**ESBL CHARACTERIZATION**

We identified Escherichia coli isolates producing CTX-M-14 (phylogroup A, ST665) or TEM-153 (phylogroup B1), a novel TEM-type enzyme differing from TEM-1 by three amino acid changes (E104K, M182T, and G267V) (GenBank accession number KC149518). TEM-1 enzyme was also identified in the CTX-M-14 producer. The CTX-M-14-producing E. coli isolate showed a phenotype of resistance against streptomycin, sulfonamides, trimethoprim, tetracyclines, chloramphenicol, nalidixic acid, and ciprofloxacin, while the TEM-153 producer was only resistant to nalidixic acid, ciprofloxacin, and neomycin.

Both ESBLs were successfully transferred by conjugation, and resistance to streptomycin was co-transferred with the bla<sub>CTX-M-14</sub> gene. A clonal relationship was not established between CTX-M-14-producing E. coli recovered from healthy volunteers and from Portuguese hospitalized patients or marine coastal waters (Machado et al., 2007, 2009). However, the plasmid containing bla<sub>CTX-M-14</sub> (a 80kb IncK-bla<sub>CTX-M-14</sub> plasmid, was similar to that of hospitalized patients from Portugal and other European countries (Valverde et al., 2009; Cottell et al., 2011; data not shown).

**ANALYSIS OF INTEGRONS**

Class 1 and/or class 2 integrons were detected in 9% (19/201) of the isolates, being class 1 integrons more frequently found than...
Table 1 | Primers used in this study.

| Primer | Oligonucleotide sequence (5′–3′) | Gene | Reference |
|--------|---------------------------------|------|-----------|
| TEM-F  | ATG AGT ATT CAA CAT TTC CG       | blaTEM| Rasheed et al., 1997 |
| TEM-R  | CTG ACA GAA ACC AAT GCT TA       |      |           |
| SHV-1  | GGC TTA TTC TTA TTT GTC GC       | blaSHV| Rasheed et al., 1997 |
| SHV-2  | TTA GGC TTG CCA GTC CTC          |      |           |
| CTX-M1-F | TTT GGC ATG TGC AGT ACC AGT AA  | blaCTXM| Edelstein et al., 2003 |
| CTX-M1-R | CTA TAT CTG TGG TGC TGC CAT A   |      |           |
| CTXM1-F | GAC GAT GTC ACT GGC TGA GC       | blaCTXM (group I) | Pitout et al., 2004 |
| CTXM1-R | AGC CGA CGA CGC TAC A            |      |           |
| Toho1-F | GCC ACC TGG TTA ACT ACA ATC C     | blaCTXM (group II) | Pitout et al., 2004 |
| Toho1-R | CGG TAG TAT TGC CCT TAA GCC       |      |           |
| CTXM825F | GGC TTT GCC ATG TGC AGC ACC      | blaCTXM (group III) | Pitout et al., 2004 |
| CTXM825R | GCT CAG TAT GCA GCC              |      |           |
| CTXM924F | GCT GGA GAA AAG CAG CGG AG       | blaCTXM (group IV) | Pitout et al., 2004 |
| CTXM924R | GTA AGC TGA CGC AAC GTC TG        |      |           |
| CTXM-9-F | GTG ACA AAG AGA GTG CAA CGG      |     |           |
| CTXM-9-D | ATG ATT CTC GGC GCT GAA GCC      |     |           |
| IntI1-F | GGT CAA GGA TCT GGA TTT CG       | intI1| Mazel et al., 2000 |
| IntI1-R | ACA TGG GTG TGA TAC ATC ATC TGC  |     |           |
| 5’CS | GGC ATC CAA GCA GCA AG           |     | Class 1 integron variable region | Levesque et al., 1995 |
| 3’CS | AAG CAG ACT TGA CCT GA           |     |           |
| IntI2-F | GGA GCC ATC GAC TAT TTG TA       | intI2 | Mazel et al., 2000 |
| IntI2-R | GTA GCA AAC GAG TGA CGA AAT G    |     |           |
| orbitX-R | GAT GCC ATC GCA AGC AG          |     |           |
| Sul1-F | CGCCGTTGGCTACCTCAAGC             | sul1 | Kerrn et al., 2002 |
| Sul1-B | GCCATCCGGTGTTCCCG               |     |           |
| Sul2-F | GGCCTCAAGCGAGATGGCATATT         | sul2 | Kerrn et al., 2002 |
| Sul2-B | GGCGTTGATACCCGCGGACC            |     |           |
| sul3F | GAGCAAGATTGGATACG                | sul3 | Perreten and Boerlin, 2003 |
| sul3R | CATCTGCAGCTAACCTAGGCTGTAGG     |     |           |

Table 2 | Class 1 and class 2 integron types found among Enterobacteriaceae recovered from faecal samples of Portuguese healthy humans.

| RFLP type | Length of variable region (bp) | Gene cassettes and order | Resistance phenotypea | No. of isolates | Isolation date |
|-----------|--------------------------------|--------------------------|-----------------------|----------------|----------------|
| CLASS 1 INTEGRONS | | | | | |
| I1 | 1000 | aadA1 | Sm, Sp | 2 | 2001 |
| I1 | 1500 | dfrA1-aadA1 | Tp, Sm, Sp | 3b | 2003/2004 |
| III1 | 1800 | dfrA12-orfX-aadA1 | Tp, Sm, Sp | 1 | 2001 |
| VI1 | 1500 | dfrA17-aadA5 | Tp, Sm, Sp | 3 | 2001/2003 |
| XXIV1 | 1400 | aadA13 | Sm, Sp | 3 | 2001 |
| CLASS 2 INTEGRONS | | | | | |
| I2 | 1900 | dfrA1-sat2-aadA1-orfX | Tp, Str, Sm, Sp | 4 | 2001/2004 |
| III2 | 2300 | estX-sat2-aadA1-orfX | Str, Sm, Sp | 1 | 2004 |

aSm, streptomycin; Sp, spectinomycin; Str, streptothricin; Tp, trimethoprim.
b(*) One isolate corresponded to the CTX-M-14-producing E. coli.

class 2 integrons [7% (14/201) vs. 3% (6/201)]. Simultaneous presence of class 1 and class 2 integrons was found in one isolate. A low diversity of integrons and of their gene cassettes was observed (Table 2). Five different class 1 integron types were identified, with types I1 (dfrA1-aadA1, n = 3), VI1 (dfrA17-aadA5, n = 3) and XXIV1 (aadA13, n = 3) corresponding to the most prevalent. Among ESBL-producing isolates, class 1 integrons were only identified in the CTX-M-14 producer, with the integron (type I1, dfrA1-aadA1) and the blaESBL gene being co-transferred by conjugation. Two class 2 integron types were observed: type I2
The occurrence of sul genes was similar to that reported in other studies, with sul2 being more commonly found than sul1, and sul3 being scarcely observed (Enne et al., 2001; Grape et al., 2003; Infante et al., 2005; Hammerum et al., 2006; Trobos et al., 2008; Bailey et al., 2010). These high rates of sul genes are identical to those detected among food-producing animals which are highly exposed to sulfonamides, and hence an involvement of the food chain cannot be discarded (Perreten and Boerlin, 2003; Infante et al., 2005; Hammerum et al., 2006; Trobos et al., 2008; Bailey et al., 2010). The observation of sul3 in one isolate harboring intI1 but lacking sul1 suggests a replacement of sul1 by sul3, as previously reported in similar structures of Salmonella and E. coli isolates (Antunes et al., 2007; Curião et al., 2011). Although sul genes were not observed in some sulfonamide resistant isolates, we could not discard the presence of other resistance mechanisms, as mutations at the chromosomal gene (folP) for dihydropyroate synthase (DHPS) (Sköld, 2001) or acquisition of unknown genes.

Prevalence data on intestinal carriage of relevant antibiotic resistance genes and/or structures promoting gene expression
in different time periods are important to identify sources and hotspots of antibiotic resistance with relevance for the human health. In summary, data from this retrospective study reinforce the relevance of human commensal flora as reservoirs of clinically relevant antibiotic resistance genes (blaCTX-M-14 or blaTEM153; sul1)-genetic platforms (integrons and IncK plasmids). These findings impose future extensive follow-up evaluations in order to understand the trends of the antibiotic resistance genes epidemiology in the community and clinical settings over the time, and eventually anticipate the detection of microorganisms with the potential to cause pandemics in the future.

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