Post-surgical wound infections involving Enterobacteriaceae with reduced susceptibility to β-lactams in two Portuguese hospitals

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ABSTRACT
The post-surgical period is often critical for infection acquisition. The combination of patient injury and environmental exposure through breached skin add risk to pre-existing conditions such as drug or depressed immunity. Several factors such as the period of hospital staying after surgery, base disease, age, immune system condition, hygiene policies, careless prophylactic drug administration and physical conditions of the healthcare centre may contribute to the acquisition of a nosocomial infection. A purulent wound can become complicated whenever antimicrobial therapy becomes compromised. In this pilot study, we analysed Enterobacteriaceae strains, the most significant gram-negative rods that may occur in post-surgical skin and soft tissue infections (SSTI) presenting reduced β-lactam susceptibility and those presenting extended-spectrum β-lactamases (ESBL). There is little information in our country regarding the relationship between β-lactam susceptibility, ESBL and development of resistant strains of microorganisms in SSTI. Our main results indicate Escherichia coli and Klebsiella spp. are among the most frequent enterobacteria (46% and 30% respectively) with ESBL production in 72% of Enterobacteriaceae isolates from SSTI. Moreover, coinfection occurred extensively, mainly with Pseudomonas aeruginosa and Methicillin-resistant Staphylococcus aureus (18% and 13%, respectively). These results suggest future research to explore if and how these associations are involved in the development of antibiotic resistance.

Keywords: Enterobacteriaceae• Extended-spectrum β-lactamases• Nosocomial infections• Post-surgical infections• Skin and soft tissue infections

Key Points
• when the skin is compromised infection may occur
• several microorganisms were found among post-surgical wounds infections
• we analysed the infectious gram-negative rods found in post-surgical wounds for the presence of extended-spectrum β-lactamases (ESBLs)
• ESBLs are plasmid-encoded enzymes that confer resistance to penicillins, cefalosporins (up to fourth generation), cephemycins and aztreonam
• ESBLs are encoded to plasmids that may also confer resistance to aminoglycosides and quinolones
• among the enterobacteria isolates we have found 70% of ESBL-producers in two Portuguese hospitals with different genotypic and phenotypic pro-files
• the post-surgical wounds were also coinfected with Pseudomonas aeruginosa (18% incidence) and Methicillin-resistant Staphylococcus aureus (MRSA) (13% incidence)
• if further research identifies a causal relationship between ESBL production and the development of antibiotic resistance in microorganisms, these findings may help provide the basis for nationwide strategies to prevent drug resistance

INTRODUCTION
Wound is any physical damage to the body that exposes subcutaneous tissue (1). When the skin, the primary barrier between the environment and the human body, is compromised, infection may occur (2). Damage to the skin tissue is because of any physical trauma or injury that may be accidental or intentional. Medical procedures often induce skin continuity loss in smaller (intravenous medical devices)
or greater extension (surgery). Hospitals are although important environments that may lead to the development of an infection. Although not intentionally induced, hospital-acquired wounds can also be because of the local ischemia caused by the pressure of the body on the bed surface, referred as decubitus or pressure ulcers. When such wounds become infected, they are often colonised by multiple bacterial species. Many studies refer staphylococci members as among the most prevalent agents found in skin and soft tissue infections (SSTI) along with enterococci, streptococci and gram-negative members (3). The prevalence of multidrug resistance (MDR) continues to increase among many pathogens largely because of overuse and misuse of antimicrobial agents (1). Such use not only adds to the cost of medical care, but also needlessly exposes the patient to potential toxicity and risks that promote the development and spread of antimicrobial resistance in healthcare facilities (4).

Antimicrobial resistance is a major contemporary issue (5). Mechanisms underlying the development of drug resistance are complex and varied. One example is the antimicrobial target modification exhibited by *Staphylococcus aureus*. Penicillin-binding proteins (PBP), the target for β-lactams, may be altered by mutation in *S. aureus*. This type of mutation are on the etiological course for the development of penicillin and other β-lactams resistance (6,7) such as methicillin-resistant *S. aureus* (MRSA). Resistance to the β-lactams may also occur because of the acquisition of enzymatic machinery such as β-lactamases, especially the ESBL (6). Little epidemiological studies have focused on the involvement of enterobacteria causing SSTI. In this study, the antimicrobial susceptibility of enterobacteria as etiological agents in the SSTI, the prevalence of ESBL among these pathogens and the most common coinfections associated in the SSTI in post-surgery complicated wounds in Portugal for a period of 2 years are described. The β-lactamases (bla) genes, *blaTEM*, *blaSHV* and *blaCTX-M* were screened by means of polymerase chain reaction (PCR) and sequencing methods; furthermore, it was also used a molecular fingerprinting technique designed for Enterobacteriaceae members using the enterobacterial repetitive intragenic consensus (ERIC) sequences (8) in order to understand/establish genetic relations.

**MATERIALS AND METHODS**

**Bacterial strains, identification and susceptibility**

All isolates had been provided from two Portuguese Clinical Pathology Laboratories; all patients with positive culture results of Enterobacteriaceae from surgical site infections and that include of the following findings: (a) purulent incisional drainage; (b) positive results of culture of aseptically obtained fluid or tissue from the superficial wound; (c) local signs and symptoms of pain or tenderness, swelling, and erythema, with the incision opened by the surgeon; strains were collected by swab and transported in the same day to the laboratory during the period comprised from September 2007 and August 2009; bacterial identification and preliminary antimicrobial susceptibility were determined by a microdilution method using automated Vitek II (bioMe´rieux, Marcy-l’Etoile, France) system and double checked with the disc-diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee of Clinical Laboratory Standards) guidelines (9,10) for minimum inhibitory concentration (MIC) determination, ESBL production was assessed with two Epsilometer test (E-test, AB Biodisk, Solna, Sweden) strips and confirmed as positive whenever a ≥ threefold-dilution difference between ceftazidime and cefotaxime/clavulanic acid or/and ≥ threefold-dilution difference between cefotaxime and cefotaxime/clavulanic MICs according to the manufacturer instructions.

**Conjugation experiments**

Transmissibility of resistance has been tested by matting clinical isolates to F− strains of azide-resistant *E. coli* J53 AziR on tryppticase soy broth (TSB) according to (11).

**Analytical IEF**

Crude preparations of β-lactamases from clinical strains transconjugants were obtained by sonicating the cells in phosphate buffer, pH 7.0 (12). Then, 20 μl of crude protein concentrated extract was added to 50 μl of
nitrocefin (OXOID, Hampshire, UK) solution (50 mg/l in 1% glycine plus 50% glycerol). Samples converted from yellow to dark pink in 30 to 60 seconds; 10 µl of each sample were run in precast isoelectric focusing (IEF) minigels, pH 3–10 (Bio-Rad, Milano, Italy), in a Mini-Protean II unit (Bio-Rad, Milano, Italy) according to the manufacturer’s instructions.

Genetic molecular characterisation of bla genes and genetic profiling

PCR primers were designed for blaTEM (Fw-ataaatctttgaagacgaa and Rv-caggtagcaggt aatctca) (13), blaSHV (Fw-gcgggttaattttgcg and Rv-gcgttccgatgcccggcagac) (14), blaCTX-M1 (Fw-atgcttacaattctgggattcac and Rv-taatattccttgaagacgaaa) (15), blaCTX-M2 (Fw-atgatgactcagagcattcagc and Rv-taatattcttgaagacgaaa) (16) blaCTX-M9 (Fw-gtcgacaggtgaaccg and Rv-atgattctc gccgtgaacgcc) (17) and blaCTX-M8 and blaCTX-M25 which share the same reverse primer (Rv-aacccagatgcacgtgatatca) but have different forward primers (Fw8-tcgcttacgatgcagc and Fw25-gcgcctacgatgcggtgac) (18), and conditions were performed as described. PCR amplicons were run in 1:2 agarose (Bioron GmbH, Ludwigshafen, Germany) and bands were cut off from the gel and purified for subcloning. The recovered bands from agarose were subcloned in TOPO TA CloningTM kit (Invitrogen Corporation, San Diego, CA) for further sequencing. The nucleotide sequences of both ends of the insert were determined with M13 sequencing primers specific for the cloning vector (19). Regarding molecular fingerprinting it was performed with PCR reaction according the description of Versalovic et al. (8) using the primers ERICIR-atgatgacgctgggtcagc and ERIC2-aagtaagtgacccgacgc. ERIC profiles were analysed with software, FPQuest® version 4.5, Fingerprinting II (Bio-Rad Laboratories, Hercules, CA, US).

RESULTS AND DISCUSSION

Ninety-seven enterobacteria isolates harvested during the 2-year period expressed resistance (MIC ≥ 16 µg/ml) to at least to second generation cephalosporins. Escherichia coli was the most representative member (n = 44; 46%) followed by Klebsiella pneumoniae (n = 28; 29%) and then by Enterobacter aerogenes (n = 7; 7%), Morganella morganii (n = 7; 7%), Enterobacter cloacae (n = 5; 4%), Serratia marcescens (n = 2; 2%), Citrobacter freundii (n = 1; 1%), Klebsiella oxytoca (n = 1; 1%), Proteus vulgaris (n = 1; 1%) and Serratia liquefaciens (n = 1; 1%). These findings are in agreement with most of the studies that described that among post-surgical procedures, there is an increased risk to acquire a nosocomial infection and, that among the gram-negative rods, E. coli, Klebsiella spp. and Enterobacter spp. are the most frequent (3,20,21).

MIC tests show that SSTI isolates were mostly resistant β-lactams. Concerning penicillins, they were 98% resistant to ampicillin and 89% resistant to amoxicillin/clavunate combination. Strains were also 100% resistant to all first and second generation cephalosporins, including cefalotin, cefazolin, and both sodium-cefuroxime and axetil-cefuroxime, and presented high resistance levels to third and fourth generation cephalosporins. So, to cefazidime, SSTI isolates show 97% of resistance, 88% to cefotaxime and 70% to the fourth generation cefepime. Nevertheless, regarding to carbapenems, these isolates present a general susceptibility to carbapenems, 100% to both imipenem and meropenem. The antimicrobial susceptibility pattern is variable among the aminoglycosides as SSTI isolates are 98% susceptible to amikacin and 60% resistant to gentamicin. In what concerns to quinolones, they are 70% resistant to ciprofloxacin. Finally, as regards sulfonamides such as the trimethoprim-sulphamethoxazole 70% of the strains in the study presented a susceptibility pattern.

As reported in previous Portuguese (22–25), Spanish (26,27) and other European (28) studies, E. coli and K. pneumonia are the species where ESBL is the most frequently identified. In the present study from the 97 isolates 70 (72%) carried an ESBL. Again, E. coli was the organism presenting ESBL more often (43/44; 98%) and then K. pneumoniae (24/28; 86%). E. aerogenes and K. oxytoca (n = 2 and n = 1, respectively) also produced ESBL.

Regarding ESBL enzymes, the TEM type was the most frequent (29/70; 41%) followed by the CTX-M type (23/70; 33%) and finally the SHV type (18/70; 26%). Among TEM enzymes it was found the TEM-24, TEM-52, TEM-10 and TEM-116 varieties (20%, 14%, 6% and 1%, respectively). CTX-M type is a heterogeneous group constituted by five phylogenetic branches (29). In this study it
was found representatives from two branches, the CTX-M-1 and CTX-M-9. Members of the CTX-M-1 group included the CTX-M-15 and CTX-M-1 enzymes (13% and 3%, respectively). CTX-M-9 group was represented by CTX-M-9 and CTX-M-15 enzymes (10% and 7% respectively). Finally in what concerns to the SHV enzymes it was found the SHV-5 and SHV-12 (14% and 12%, respectively).

Coinfections were also relatively common among patients from which these isolates were recovered. Among the E. coli and K. pneumoniae isolates from SSTI it was found other 91 microorganisms coinfecting the wounds (Figure 1). The most frequent coinfected microorganism was Pseudomonas aeruginosa (17/97; 18%) followed by S. aureus (15/97; 15%), E. coli (13/97; 13%), Klebsiella spp. (11/97; 11%), Enterobacter spp. (10/97; 10%) and Enterococcus faecalis (7/97; 7%).

From staphylococci members coinfecting post-surgical wounds 87% (13/15) were MRSA and only 13% (2/15) were methicillin susceptible. A total of 46% (6/13) of E. coli isolates coinfecting post-surgical wounds were also ESBL positive but 54% (7/13) did not carried any β-lactamase. Similarly, in what concerns to Klebsiella spp. coinfecting post-operative wounds 45% (5/11) harbours another ESBL, whereas 55% (6/11) does not.

In a single long-term patient it was possible to isolate five ESBL-producing E. coli and K. pneumoniae from different infection episodes. The patient, a 51-year-old male, first developed a urinary-tract infection (UTI) in April 2005 caused by two distinct ESBL-producing E. coli; one of them harbouring a TEM-10 enzyme and the other a CTX-M-15. Later in May of the same year, the same patient developed a second UTI, and this time caused by an SHV-2 producing K. pneumoniae. One month later, in July, a pressure ulcer developed possibly as a result of friction, pressure or shear and became contaminated with a SHV-5 producing K. pneumoniae. Finally, on September of the same year, this patient experienced another UTI with the same SHV-5 producing K. pneumoniae (genetic similarity ≥ 90%) found first in the purulent wound (Figure 2).

![Figure 1. Occurrence of bacteria and yeast (Candida albicans) coinfecting skin and soft tissue infections (SSTI) caused by extended-spectrum β-lactamases (ESBL)-producing Escherichia coli and ESBL-producing Klebsiella pneumoniae (white bars). The diagram shows the number (left side) of microorganism (right side) that occurred simultaneously with ESBL-producing E. coli (black bars) and with ESBL-producing K. pneumoniae (white bars).](image-url)
In another long-term patient with an oncological issue, from another hospital, experienced a rarely described situation, an injury caused by radiotherapy. This patient, a 55-year-old female, presented two SSTI episodes. The first one in August 2005 was caused by a CTX-M-14 producing *E. coli*. A few weeks later in September after apparent recovery the infection reappeared and became complicated with *P. aeruginosa* along the same CTX-M-14 producing *E. coli* (genetic similarity $\geq 90\%$; data not shown) that was present in the first episode in August.

Reduced susceptibility to formerly effective antibiotics is increasing worldwide among the SSTI causing agents (3). This is most certainly as a result of overuse, misuse and abuse of antimicrobial agents' sensu lato. Examples of abusive antimicrobial usage include the exaggerated prophylactic measures, self-medication or even use of commercial product with antiseptic/antimicrobial agents and also the use for non human purposes such as in livestock production (5,30,31). Centers for Disease Control and Prevention (CDC) and policy makers are very committed to the implementation of educational strategies not only for health professional (32,33) but also for general population.

In general, this pilot study in only two hospitals point to an important issue, that is the emergence of ESBL among gram-negative rods involved in nosocomial wounds in our country. These findings extend beyond the original purpose, of this study, suggesting exploration of interactions between ESBL and development of antibiotic resistance in *P. aeruginosa* and gram-positive bacteria such streptococci, enterococci and staphylococci with special attention to MRSA (34,35).

In conclusion, the emergence of ESBL among gram-negative rods and possibly related antibiotic-resistant strains of other pathogens (35) merits further research and epidemiologic study as well as immediate development of policies monitor drug delivery in hospital and ambulatory pharmacies and the implementation of public health defence strategies towards health promotion and drug resistance prevention.

**REFERENCES**


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