Epidemiology of extended spectrum β-lactamase, AmpC and class A carbapenemases-producing organisms isolated at San Camillo Hospital of Treviso (Italy) between April 2012 and March 2014

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Summary

The indiscriminate use of broad-spectrum cephalosporins of the last years has favoured the selection of extended spectrum β-lactamases (ESBLs), AmpC and class A carbapenemases (KPC)-producing *Enterobacteriaceae* strains, representing a real health emergency. At San Camillo Hospital of Treviso, Italy, between April 2012 and March 2014, we isolated 263 suspected ESBL-producing strains from various specimens, including urine (76.4%), wound swabs (9.9%), blood cultures (4.6%), vaginal swabs (2.7%), fragments of bone (1.5%) and other materials (4.9%). The majority of the isolated bacteria were represented by *Escherichia coli* (43.3%), followed by *Klebsiella pneumoniae* (34.2%), *Proteus mirabilis* (15.2%), *Enterobacter* spp. (3.8%), *Morganella morganii* (1.1%), *Serratia* spp. (0.8%), *Proteus vulgaris* (0.4%), *Citrobacter freudii* (0.4%), *Providencia* spp. (0.4%) and *Pseudomonas aeruginosa* (0.4%). Using confirmatory phenotypic tests, 89.4% of the isolated resulted ESBL producer, 15.3% of which were also AmpC-producers, 1.5% were ESBL negative and AmpC positive, 4.2% were ESBL negative and AmpC negative, and 4.9%, consisting solely of *K.pneumoniae*, were confirmed as KPC positive. ESBL-mediated resistance to cephalosporin is not always clearly evident using susceptibility

testing performed by agar diffusion-disc or dilution methods, for this reason it is strictly recommended to use specific tests able to reveal important mechanisms of resistance. The optimal use of diagnostic tools in microbiology is necessary to fight the spreading of pathogens with multiple antibiotic resistance mechanisms and in order to avoid giving useless antibiotic therapies to the patients.

Introduction

Beta-lactamases are bacterial enzymes that inactivate beta-lactamic antibiotics and those that are able to inactivate most penicillins and cephalosporins, including the extended spectrum cephalosporins, are termed extended spectrum beta-lactamases (ESBLs). Production of ESBLs is one of the most important antimicrobial resistance mechanisms of such bacterial species and, given that their prevalence is clearly increasing in many parts of the world, hampering the antimicrobial treatment of infections caused by *Enterobacteriaceae* and representing one of the leading causes either of death among elderly and immunocompromised individuals either of the increased hospitalization time for many patients, the ESBL-producing organisms represent a challenge for microbiologists and clinicians. The first ESBL-producing strains were identified in 1983 and the clonal expansion of producer organisms caused their distribution. The vast majority of ESBLs are acquired enzymes, encoded by plasmids and today there are approximately 500 different ESBLs. By far the most clinically important groups of ESBLs are CTX-M enzymes, followed by SHV- and TEM-derived ESBLs (1,21). The acquired ESBLs are expressed at various levels, and differ significantly in biochemical characteristics such as activity against specific β-lactams (*e.g.* cefotaxime, ceftazimide, aztreonam). The level of expression and properties of an enzyme, and the co-presence of other resistance mechanisms (other β-lactamases, efflux, altered permeability) result in the large variety of resistance phenotypes observed among ESBL-positive isolates (18,21). ESBL detection and characterization is mandatory for infection control purposes and the recommended strategy is based on non-susceptibility to indicator oxymino-cephalosporins, followed by phenotypic confirmatory test (12). AmpC beta-lactamases differ from ESBLs in that they are cephalosporinases and are resistant to beta-lactamase inhibitors. They hydrolyze the cephamycins, but not the fourth generation cephalosporins (*e.g.* cefepime). AmpC is normally produced in low levels by many organisms and is not associated with resistance, but it can be produced at high levels and cause resistance. The AmpC gene is found on the chromosome of many organism species, including *Morganella morganii, Citrobacter freundii, Serratia mercescens, Pseudomonas aeruginosa,* etc. and chromosomal AmpC beta-lactamases can be produced inducibly or constutively. Laboratories should

be able to detect AmpC β-lactamases because they have been associated with false cephalosporin susceptibility, so there is a potential to falsely report them as ESBL negative organisms (20,23). The problem of dissemination of carbapenemases (KPCs) in Europe dates to around 2000 in several Mediterranean countries. KPCs confer resistance to essentially all β-lactamases, for this reason they are source of concern: they idrolyze penicillins, in most cases cephalosporins, and to varying degrees carbapenems and monobactams. The vast majority of carbapenemases are acquired enzymes, encoded by plasmids or other mobile genetic elements (25). Decreased susceptibility to carbapenems in *Enterobacteriaceae* may, however, also be caused by either ESBL or AmpC enzymes combined with decreased permeability due to alteration or down-regulation of porins (6). Strains producing carbapenemases frequently possess resistance mechanisms to a wide-range of antimicrobial agents, and infections with KPC-producing *Enterobacteriaceae* are associated with high mortality rates (28). For this reason each clinical laboratory should be able to efficiently detect carbapenemases-producer organisms.

Materials and Methods

Between April 2012 and March 2014, at San Camillo Hospital in Treviso (Italy) we isolated 263 consecutive and non-replicate strains suspected to be, according the screening test criteria, potentially ESBLs producers. These bacteria were collected from various specimens, including blood-cultures, vaginal swabs, fragments of bone, wound swabs and urine, collected either from outpatients and inpatients of different Wards of the hospital (medicine, diabetic foot surgery, angiology and rehabilitation). All the samples were collected aseptically from patients, transported to the microbiology department of the hospital and processed immediately. Each sample was cultured on MacConkey agar and Trypticase Soy Agar II with 5% sheep blood (TSA-S) plates and incubated at 37°C for 24 hours. The colonies grown were identified based on morphology and Gram negative bacilli isolated were characterized performing Gram staining, motility and standard biochemical tests.

After isolation, the strains suspected to be potentially ESBLs producers (*Enterobacteriaceae* and non-fermenting bacteria) were tested for antimicrobial susceptibility using dehydrated broth micro-dilution panels consisting of 96 wells (Sensitire, Trek Diagnostic Systems, Independence, OH, USA). The procedure consisted in taking 3 to 5 colonies grown on a plate after 24 h of incubation, suspending them in normal saline or sterile water till to have a solution of 0.5 McFarland turbidity, then transferring 10 µL of this solution in Muller Hinton Broth. The final solution containing the microorganism that had to be tested for antimicrobial susceptibility was distributed into each well of the panel by an automatic dispenser, finally the panel was incubated at 34-36°C and, after 18 h, subjected to automatic reading. Minimum inhibitory concentrations (MICs), Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were utilized for analysis of testing (3,12). According to CLSI (3) and Study Committee of Antimicrobials of Amcli (CoSA) guidelines (5) *Enterobacteriaceae* strains showing MICs ≥2 g/mL for third generation cephalosporins (Ceftazimide, Cefotaxime, Ceftriaxone) were considered potential ESBL producers and were tested further for the presence of ESBLs by phenotypic confirmatory disc diffusion test. Mueller-Hinton agar plates were inoculated with the strain to be tested and ceftazidime disc (30 g) and the combination disc ceftazidime + clavulanic acid (30 g + 10 g) were placed with 25 mm apart. An increase of ≥5 mm in zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime alone was confirmed as ESBL producing strain. Antimicrobial agent discs were obtained from ROSCO diagnostica and *E.coli* ATCC 25922

ESBL negative and *K.pneumoniae* ATCC 700603 ESBL positive were used as controls throughout the study. All the strains suspicious of possessing plasmid-mediated AmpC beta-lactamases were subjected to confirmatory phenotypic test. A Cefoxitin MIC >8 g/mL combined with a ceftazidime and/or cefotaxime MIC >1 g/mL were used as phenotypic criteria for investigation of AmpC production in group 1 *Enterobacteriacea*e according the EUCAST guidelines (12) AmpC production was confirmed phenotypically by the combination disk diffusion test using cefotaxime and ceftazidime combined with boronic acid or cloxacillin as inhibitor (Rosco Diagnostica, Taastrup, Denmark). A positive test was considered when the zone of inhibition was ≥5 mm larger than the zone generated without inhibitor. *E.coli* ATCC 25922 AmpC negative and *E. coli* CCUG 58543 acquired CMY-2 AmpC were used as controls throughout the study (15). Detection of reduced sensitivity to carbapenemases by diffusion disk method was made whenever we had *Enterobacteriaceae* with a MIC ≥0.5 g/mL to meropenem, according to epidemiological cut-off (ECOFF) values as defined by EUCAST (12) and CoSA guidelines (5). KPC-producers strains were confirmed using a combined disk test (KPC + MBL Confirm ID kit-Rosco diagnostica) that consisted into apply Meropenem, Meropenem + Dipicolinic acid (DPA), Meropenem + Boronic, Meropenem + Cloxacillin on a Muller Hinton Agar or MacConkey Agar plate inoculated with rectal swab. A Meropenem + Boronic inhibition zone ≥5 mm then Meropenem, Meropenem + DPA and Meropenem + Cloxacillin indicated a presence of a KPC enzyme (or other class A). *E.coli* ATCC 25922 was used as carbapenemase-negative control and *K. pneumonia* NCTC 13438 as KPC positive (5,12,22).

Results

During the two years period April 2012-March 2014, N=263 enterobacterial strains were collected at the Microbiology Department of San Camillo Hospital in Treviso as suspected ESBL producers (on the bases of screening tests criteria). The isolates were obtained from various specimens, including urine (76.4%), wound swabs (9.9%), blood-cultures (4.6%), vaginal swabs (2.7%), fragments of bones (1.5%) and other materials (4.9%). 44.5% of the 263 strains were isolated from patients attending medicine department, 32.4% from rehabilitation unit, 16.3% derived from out-patients, 3.4% from diabetic foot department and 3.4% from angiology unit.

The organism most commonly isolated was *E. coli* (43.3%), followed by *K. pneumoniae* (34.2%), *P. mirabilis* (15.2%), *Enterobacter spp*. (3.8%), *Morganella morganii* (1.1%), *P. vulgaris* (0.4%), *Serratia spp*. (0.8%), *C. freudii* (0.4%), *Providencia spp*. (0.4%) and *P. aeruginosa* (0.4%). Confirmatory tests showed that 235 out of 263 isolates were really ESBL producers (89.4%), 32 of which were also AmpC producers (13.6%) and 203 were AmpC negative (86.4%). 4 out of 263 (1.5%) were ESBL negative and AmpC positive, 11 (4.2%) were ESBL negative and AmpC negative, and 13 (4.9%), consisting only of *K. pneumoniae*, were KPC producers. The species distribution of the ESBL-producers strains is shown in Figure 1.

Discussion and Conclusions

Our work confirm the high prevalence of ESBL producers among *Enterobacteriaceae*, mainly in *E. coli* and *K.pneumoniae*. Antibiotic resistance is an important issue affecting public health, for this reason a rapid detection in clinical laboratories is essential in order to minimize the spread of antimicrobial-resistant organisms and to help the selection of more appropriate antibiotics. This is particularly true for

ESBL-producing bacteria, in fact, the infections caused by multidrug-resistant strains of *Enterobacteriaceae*, as well as those caused by not fermenting bacterial species carbapenemase-positive, are increasing worldwide and represent the main cause of higher mortality among immunocompromised, advanced age or with severe diseases patients (13). The production of ESBLs is a relevant problem not only for nosocomial infections, but it is becoming an important public health issue also regarding community-acquired infections (27). Most commonly, *K. pneumoniae* and *E. coli* are the bacterial species incriminated, but outbreaks have been observed also due to *Enterobacter* spp, *Pseudomonas* spp, *Citrobacter* spp, *Salmonella* spp, *Serratia* spp and *Morganella* sp. Among the risk factors for acquiring infections caused by ESBL-producing *Enterobacteriaceae* there are severity of illness, length of hospital stay, invasive procedures, intravascular devices, administration of total parenteral nutrition, mechanical ventilatory assistance, urinary catheters, haemodialysis, decubitus ulcers, poor nutritional status, antibacterial administration (*e.g.* extended-spectrum cephalosporins, aztreonam, fluoroquinolones, cotrimoxazole, aminoglycosides, metronidazole) (14,17). Because numerous studies indicate that the use of extended-spectrum cephalosporins in particular, and other antibacterials in general, are associated with the spread of ESBL-producing Enterobacteriacea, restriction of use of these antibiotics is the most common antibacterial-restriction measure employed in controlling outbreaks (16). Accordingly, in order to prevent the main modes of patient-to-patient transmission of ESBL-producing organisms in the hospital setting (19,24), a key issue in hospital infection control against ESBL pathogens is represented by the identification of colonized patients. Reporting consistently the presence of ESBLs detected in bacterial strains isolated from clinical samples is very important for several reasons: the relatively high number of strains falsely reported to be susceptible (without interpretation or therapeutic correction), the increased risk of therapeutic failure and the increased potential risk of cross-transmission. For this reason, in all at-risk units (intensive care, burn, oncology-haematology, haemodialysis and organ transplant units) it is recommended to screen patients on admission and regularly during the period of stay (4). Data obtained from our study are in line with the observations at the national level (9,10,11) and showed as the problem of multi-drug resistant bacteria represents an emerging issue in our reality. The selection of proper antibiotic therapy is a key factor relating to the effectiveness of infection control. In this context, clinical laboratory data provide clini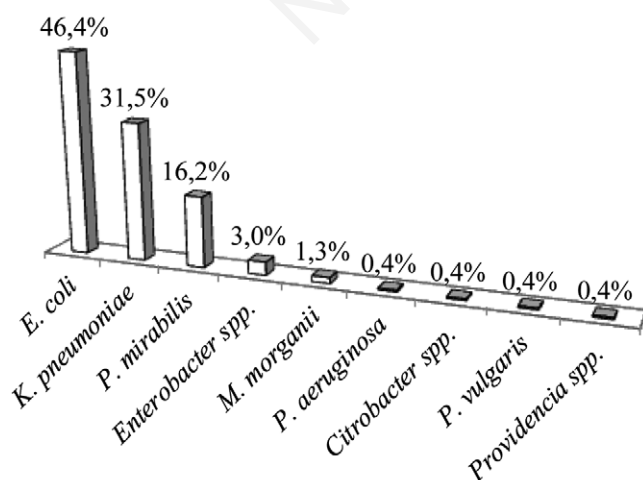cians with helpful information and are important for detection of outbreaks or clusters of cases caused by multi-resistant bacteria. Limiting the institutional use of third generation cephalosporins has been shown to help the reduction of the prevalence of ESBL-producing organisms (1,16) but, obviously, further research is required on appropriate strategies to limit the emergence and spread of resistant organisms, both in the community and the hospital settings, as well as to evaluate the available therapeutic agents and identify new ones (8). The future development of novel beta-lactams resistant to hydrolysis by these versatile enzymes and the discovery of highly potent beta-lactamase inhibitors are widely awaited (2,7,26).

Figure 1. Prevalence of the 235 strains confirmed as extended spectrum β-lactamases positive.

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