Epidemiology of extended spectrum \( \beta \)-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 \( \beta \)-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 freundii (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 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 producers 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 \( \beta \)-lactams (e.g. cefotaxime, ceftazidime, aztreonam). The level of expression and properties of an enzyme, and the co-presence of other resistance mechanisms (other \( \beta \)-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 oximino-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 cephemycins, 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 morganti, Citrobacter freundii, Serratia mercescens, Pseudomonas aeruginosa, etc. and chromosomal AmpC beta-lactamases can be produced inducibly or constitutively. Laboratories should
be able to detect AmpC β-lactamases because they have been associat-
ed with false cephalosporin susceptibility, so there is a potential to
cor rectly report them as ESBL negative organisms (20,23). The prob-
lem of dissemination of carbapenemases (KPCs) in Europe dates to around
2000 in several Mediterranean countries. KPCs confer resistance to
essentially all β-lactames, for this reason they are source of concern:
they idrolyze penicillins, in most cases cephalosporins, and to varying
degrees carbapenens and monobactams. The vast majority of car-
bp enemases are acquired enzymes, encoded by plasmids or other
mobile genetic elements (25). Decreased susceptibility to carbapen-
emases in Enterobacteriaceae may, however, also be caused by either ESBL
or AmpC enzymes combined with decreased permeability due to alter-
ation or down-regulation of porins (6). Strains producing carbapen-
emases 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. This 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 dif-
ferent 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 inocu-
bated at 37°C for 24 hours. The colonies grown were identified based on
morphology and Gram negative bacilli isolated were characterized per-
forming Gram staining, motility and standard biochemical tests.

After isolation, the strains suspected to be potentially ESBLs produc-
ers (Enterobacteriaceae and non-fermenting bacteria) were tested for
antimicrobial susceptibility using dehydrated broth micro-dilution pan-
els consisting of 96 wells (Sensititre, 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 Amci (CoSA) guidelines (5) Enterobacteriaceae
strains showing MICs ≥2 g/mL for third generation cephalosporins
(Cefazidime, Cefotaxime, Ceftriazone) 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 pos-
sessing 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
Enterobacteriaceae according the EUCAST guidelines (12) AmpC pro-
duction was confirmed phenotypically by the combination disk diffu-
sion 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 sen-
sitivity to carbapenemases by diffusion disk method was made whenev-
er 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 inoculat-
ed 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 enter-
obacterial 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-cul-
tures (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 depart-
ment 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. freundii (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
KPC negative and AmpC positive, 11 (4.2%) were ESBL negative and
AmpC negative, and 15 (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 mini-
imize 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 Enterobacteriaceae, 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 clinicians 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 setting, 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).

References
5. Documento CoSA-AMCLI. Indicazioni per la conferma fenotipica della produzione dicarbapenemasi nelle Enterobacteriaceae 2012.

![Figure 1. Prevalence of the 235 strains confirmed as extended spectrum β-lactamases positive.](image-url)