RESEARCH ARTICLE

Characterization of Extended Spectrum β-lactamase and AmpC Producing Organisms Isolated from a Third Level Hospital Patients in Dominican Republic

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ABSTRACT

Extended-spectrum β-lactamases (ESBLs) producing Enterobacteriaceae have become a challenge for clinicians as they frequently show co-resistance to other antibiotic classes. AmpC-type β-lactamases (AmpCs), tend to be more difficult to treat as they could be induced during antibiotic therapy. To characterize ESBLs and AmpCs producing organisms, we collected clinical samples identified through MicroScan® as members of the Enterobacteriaceae family and ESBLs producers, following Clinical Laboratory Standards Institute indications, from January through March 2021, in a third level hospital in Santiago de Los Caballeros, Dominican Republic. Samples underwent manual confirmation via ESBL + AmpC screen discs kit (Liofichelm® srl, Italy) and a genotype analysis was done by DNA extraction for detection of specific genes related with ESBL (blaSHV, blaTEM, blaCTX, and blaCMY-4) or AmpC (CIT, MOX, DHA and FOX) expression. 54 samples were confirmed as ESBL and/or AmpC producers with Escherichia coli as the most frequent (33/61%) followed by Klebsiella sp. (9/17%). blaCTX was detected in 29 (67%) isolates, followed by blaTEM (23/53%) and blaSHV (18 /40%). Among the AmpC encoding genes, DHA and CIT gene pools were detected in 3 (7%) and 2 (5%) of the samples, respectively. Two or more genes were detected in 19 (44%) samples. All the AmpC genes were found in an isolate which already had, at least, two other β-lactamase encoding genes. In this cohort, ESBLs and AmpCs -producing organisms with multiple resistance genes were detected. Efforts to curb antibiotic availability and to establish antimicrobial stewardship programs are needed.
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Introduction

The diversification of new β-lactamas, which recognize and inactivate almost all hydrolysable β-lactams, poses a major challenge to clinicians as it renders most first-line antibacterial drugs ineffective to treat common infections. The widespread use of broad spectrum cephalosporins to fight infections caused by gram negative organisms, such as the Enterobacteriaceae family, have instigate this phenomenon.

Extended-spectrum β-lactamas (ESBLs), which belong to Ambler class A, hydrolyze oxyimino-β-lactams (ceftaxime, ceftriaxone, ceftaxidime, and the monobactam aztreonam) but are inhibited by common β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam). The most commonly reported ESBLs are CTX-M, SHV, and TEM.1 Frequently, ESBL producing Enterobacteriaceae show co-resistance to other antibiotic classes, such as aminoglycosides, trimethoprim/sulfamethoxazole, and quinolones. AmpC-type β-lactamas, on the other hand, belong to Ambler class C and confer resistance to the same cephalosporins as the ESBLs but are not affected by most of the commercially available inhibitors. Moreover, chromosomally AmpC-mediated resistance can be induced after β-lactam exposure, while plasmid-mediated ampC genes (blaCMY, blaDHA, blaMOX, etc.) are constitutively expressed.2

Globally, 16% of Klebsiella pneumoniae and 21% of Escherichia coli isolates collected form blood samples have been found to produce ESBL3 CTX-M is reported as the predominant enzyme, with blaCTX-M-14 and blaCTX-M-15 as the most common encoding genes.4 AmpC has an overall lower prevalence than ESBL. In Egypt, 5.6% of isolates collected from bloodstream infections were recently reported as AmpC producers,5 while in Portugal only 2.6% of the non-susceptible to third-generation cephalosporins Enterobacteriaceae were found to be AmpC producers, with DHA and CMY as the most widespread enzymes.6 In Latin America, regional studies have reported a prevalence of 53% ESBL-producing K. pneumoniae and 25% E. coli among isolates collected from clinical infections,7,8 and CTX-M-2 and CTX-M-1 groups have been the most reported in this region.9,10,11 AmpC coding genes have been detected in Latin America countries, for example, in Colombia.12 In Dominican Republic (DR), our group reported recently that 54.4% of isolates identified as members of Enterobacteriaceae family collected form patients showed resistance to third-generation cephalosporins,13 but there is no information whatsoever about the spread of β-lactamas, as DR lacks surveillance programs for bacterial resistance mechanisms. The aim of this study, thus, was to assess the prevalence and genotypic characteristics of ESBLs and AmpC producing Enterobacteriaceae in a tertiary referral center in DR, in order to have a better idea of the incidence of those microorganisms in the region.

Methods

Clinical samples identified as members of the Enterobacteriaceae family and resistant to at least one third-generation cephalosporin, following the Clinical Laboratory Standards Institute indications for ESBL identification,1,4 were collected from January through March 2021. The species identification and the routine automated susceptibility testing were performed via MicroScan® in the microbiology laboratory at Hospital Metropolitano de Santiago (HOMS), a tertiary care teaching hospital located in Santiago de los Caballeros, second largest city in DR and fourth in the Caribbean. The isolates were collected regardless of their origin, so isolates from urine, secretions, and feces, from hospitalized or from the ambulatory setting, were included in the study. Depending on the source of the sample, different antibiotics are tested on the automated system, thus, not all the samples have been tested for susceptibility to all the antibiotics.

PHENOTYPIC CONFIRMATION

The collected samples, with phenotypic susceptibility patterns consistent with ESBL production, subsequently underwent manual confirmation via ESBL + AmpC screen discs kit (Liofichelm® srl, Italy). The isolate was plated on Mueller-Hinton agar and claxacillin discs, ceftaxime discs with and without clavulanic acid, and discs carrying the three components simultaneously, were placed at distances of 20 mm, following the manufacturer recommendations. After an overnight incubation, the inhibition areas were measured and ESBL and/or AmpC production was determined based on the manufacturer indications. Klebsiella pneumoniae ATCC 700603 and Escherichia coli ATCC 25922 were used as positive and negative controls, respectively.

GENOTYPIC ANALYSIS

DNA extraction of the isolates considered ESBL and/or AmpC producers was carried out using a microwave.1,5 Briefly, two colonies of each isolate were dissolved in 500 μl distilled water, heated for 10 seconds, followed by centrifugation for two minutes at 13000 rpm. The supernatant was used for the PCR detection of ESBL and AmpC encoding genes. Invitrogen Platinum Hot Start PCR 2X Master
Mix (Thermo Fisher Scientific) was used and reactions were prepared as follows: for \( \text{blaSHV} \), \( \text{blaTEM} \), \( \text{blaCTX} \), and \( \text{blaCMY-4} \), 12.5 \( \mu l \) of the master mix, 2.5 \( \mu l \) of DNA, 1 \( \mu l \) of each the forward and reverse primers, and 8 \( \mu l \) of ultra-pure water, were used. A multiplex with 25 \( \mu l \) of the master mix, 10 \( \mu l \) of DNA, and 2 \( \mu l \) of each oligonucleotide for the gene pools CIT, MOX, DHA, and 1.5 \( \mu l \) for FOX was performed. The target genes, primer sequences, PCR conditions, and source of the primers are listed in Table 1.

### Table 1. Sequences of primers and thermal conditions used in PCR amplifications

<table>
<thead>
<tr>
<th>Target genes</th>
<th>PCR Product size</th>
<th>Primers used (5’ - 3’)</th>
<th>Thermal cycling conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{blaSHV} )</td>
<td>867 bp</td>
<td>( \text{SHV}<em>\text{fwd} ) GGGATGGCTTATATCGCC&lt;br&gt;( \text{SHV}</em>\text{rev} ) TTAGGCTTACGCAGGCTC (17)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 60ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td>Sadegh et al (16)</td>
</tr>
<tr>
<td>( \text{blaTEM} )</td>
<td>800 bp</td>
<td>( \text{TEM}<em>\text{fwd} ) TAATCAGTGAGGCACCTATCTC&lt;br&gt;( \text{TEM}</em>\text{rev} ) GAGTATTACACACTTCCG (17)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 45ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td></td>
</tr>
<tr>
<td>( \text{blaCTX} )</td>
<td>593 bp</td>
<td>( \text{CTX}<em>\text{fwd} ) TTTGGATGTGACAGTACCAGT&lt;br&gt;( \text{CTX}</em>\text{REV} ) CGATATCGTTGGTGGCATA (17)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 54ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td></td>
</tr>
<tr>
<td>MOX-1, MOX-2, CMY-1, CMY-8 - CMY-11</td>
<td>520 bp</td>
<td>( \text{MOX}<em>\text{fwd} ) GCTGCTCAAGGAGCACAGGAT&lt;br&gt;( \text{MOX}</em>\text{rev} ) CACATTGACACAGGCTTCG (18)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 54ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td>Pérez-Pérez &amp; Hanson (17)</td>
</tr>
<tr>
<td>LAT-1, LAT-4, CMY-2, CMY-3, CMY-5 - CMY-7, BIL-1</td>
<td>462 bp</td>
<td>( \text{LAT}<em>\text{fwd} ) TGGCCAGAACTGACAGGCAAA&lt;br&gt;( \text{LAT}</em>\text{rev} ) TTTCCTCGTACAGGCTG (18)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 64ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td></td>
</tr>
<tr>
<td>DHA-1, DHA-2</td>
<td>406 bp</td>
<td>( \text{DHA}<em>\text{fwd} ) AACTTTCACAGGTGCTGGGT&lt;br&gt;( \text{DHA}</em>\text{rev} ) CCGTATCGCATCTGGC (18)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 64ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td></td>
</tr>
<tr>
<td>FOX-1 - FOX-5b</td>
<td>190 bp</td>
<td>( \text{FOX}<em>\text{fwd} ) AACATGGGGTATCAGGGAGATG&lt;br&gt;( \text{FOX}</em>\text{rev} ) CAAAAGCCCTGTAACCGGATT (18)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 55ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td>Stapleton et al (18)</td>
</tr>
<tr>
<td>CMY-4</td>
<td>1850 bp</td>
<td>( \text{CMY}<em>\text{fwd} ) GATTCCTTGGACTCTTCAG&lt;br&gt;( \text{CMY}</em>\text{rev} ) TAAAACCAGGTTCAGATG (18)</td>
<td>95ºC 5 min, 30x (95ºC 30s, 55ºC 1 min, 72ºC 1 min)→72ºC 10 min</td>
<td></td>
</tr>
</tbody>
</table>
Characterization of Extended Spectrum β-lactamase and AmpC Producing Organisms Isolated from a Third Level Hospital Patients in Dominican Republic

STATISTICAL ANALYSIS
Statistical analysis was carried out using the SPSS 26v statistical software. We used frequency and percentage for data distributions at frequency tables; for data comparison we applied Chi-squared analysis.

Results
During the three months of sample collection, a total of 119 reports with isolates identified as members of the Enterobacteriaceae family and phenotypic characteristics consistent with ESBL production, according to the automated system, were analyzed. Of those, 39 were discarded due to suspicion of contamination or mixed culture. A total of 54 out of the 80 remaining samples (67.5%) were phenotypically confirmed as ESBL and/or AmpC producers (Figure 1). Of those, 33 (61%) were harvested from urine, 12 (22%) from pus aspirates, and 9 (17%) from feces. The majority of the samples included in the study were collected from outpatients (34 out of 54, 63%). According to the automated system, Escherichia coli was the most frequently isolated pathogen (33, 61%), followed by Klebsiella sp. (9, 17%), Serratia marcescens (3, 6%), and Raoultella ornithinolytica (3, 6%) (Table 2). After manual confirmation, phenotypic detection of ESBL and AmpC production combined was found in 46 out of the 54 (85%) isolates. Six (11%) were identified as ESBL but no AmpC positive, and two (4%) as AmpC, but no ESBL, positive (Table 3).

Figure 1. Samples collected

Figure 2. Distribution of SHV, TEM, CTX codifying genes and CIT and DHA gene pools detected in clinical isolates
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Table 2. Distribution of ESBL/AmpC producing Enterobacteriaceae after double discs confirmation

<table>
<thead>
<tr>
<th>Specie (no. isolates)</th>
<th>Outpatients % (no. isolates)</th>
<th>Inpatients % (no. isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ESBL+AmpC</td>
<td>ESBL</td>
</tr>
<tr>
<td>E. coli (n=33)</td>
<td>58 (19)</td>
<td>9 (3)</td>
</tr>
<tr>
<td>Klebsiella sp (n=9)</td>
<td>33.3 (3)</td>
<td>22.2 (2)</td>
</tr>
<tr>
<td>S. marcescens (n=3)</td>
<td>67 (2)</td>
<td>0</td>
</tr>
<tr>
<td>R. ornithinolytica (n=3)</td>
<td>67 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Klyvera ascorbata (n=2)</td>
<td>0</td>
<td>50 (1)</td>
</tr>
<tr>
<td>Providencia sp (n=2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter sp. (n=1)</td>
<td>100 (1)</td>
<td>0</td>
</tr>
<tr>
<td>P. mirabilis (n=1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (54)</td>
<td>50 (27)</td>
<td>11 (6)</td>
</tr>
</tbody>
</table>

The 54 isolates were subjected to genotypic analysis by PCR. ESBL/AmpC encoding genes were detected in 43 of the 54 isolates (80%). The most frequent ESBL-encoding gene was blaCTX, detected in 29 (67%) of the isolates, followed by blaTEM, detected in 23 (53%), while 18 (40%) contained blaSHV. Among the AmpC encoding genes, only a signal from the DHA and CIT gene pools was detected in 23 (7%) and 2 (5%) of the samples, respectively. The distribution between outpatients / inpatients is shown in table 2. Twenty-four (56%) isolates harbored only one of the analyzed genes, distributed as follows: blaCTX was detected in 14 (58%), blaTEM in 6 (25%) and blaSHV was present in 4 (17%) isolates. Two or more genes were detected in 19 (44%) samples. blaCTX + blaTEM was the most frequent combination, found in 13 (68%) isolates. Three isolates contained 4 different genes, two of them (Escherichia coli and Providencia stuartii) with the combination blaCTX + blaTEM + CIT + DHA and the third (Raoultella ornithinolytica) with blaCTX + blaTEM + blaSHV + DHA. Thus, all the AmpC genes were found in an isolate which already had, at least, two other β-lactamase encoding genes. The two isolates (Escherichia coli and Proteus mirabilis) that were phenotypically identified as AmpC producers, but no ESBL, by the manual confirmation kit, did not contain any of the analyzed AmpC encoding genes. Instead blaTEM was the only gene detected in both isolates.

Table 3. Prevalence of ESBL/AmpC codifying genes detected in clinical isolates

<table>
<thead>
<tr>
<th>ESBL/AmpC codifying genes in Enterobacteriaceae</th>
<th>1 gene % (no. isolates)</th>
<th>2 genes % (no. isolates)</th>
<th>3 genes % (no. isolates)</th>
<th>4 genes % (no. isolates)</th>
<th>Non-detected % (no. isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outpatients (n=34)</td>
<td>53 (18)</td>
<td>15 (5)</td>
<td>9 (3)</td>
<td>3 (1)</td>
<td>20 (7)</td>
</tr>
<tr>
<td>Inpatients (n=20)</td>
<td>30 (6)</td>
<td>20 (4)</td>
<td>20 (4)</td>
<td>10 (2)</td>
<td>20 (4)</td>
</tr>
<tr>
<td>Total (n=54)</td>
<td>44 (24)</td>
<td>17 (9)</td>
<td>17 (7)</td>
<td>2 (3)</td>
<td>20 (11)</td>
</tr>
</tbody>
</table>
All the analyzed ESBL/AmpC producing isolates exhibited multidrug resistant profiles, according to the automated system, with resistance to three or more antibiotic classes (Table 4). Among non-β-lactam drugs, the highest resistance rates were detected against trimethoprim/sulfamethoxazole (95%) and ciprofloxacin (93%), followed by gentamicin (63%). However, amikacin showed much more susceptibility, only 11% of the tested isolates were reported resistant. For β-lactam antibiotics, all isolates were resistant to ceftaxime, but ceftazidime was more active (59% resistance rate). Combination of β-lactams and β-lactamase inhibitors showed activity only against 7% of the isolates in the case of ampicillin-sulbactam but up to 69% with piperacillin-tazobactam. The cefamycin cefoxitin showed a resistance rate of 46%, while fourth generation cephalosporins (cefpime and aztreonam) were barely active against the ESBL producing isolates (98% resistance rate). Carbapenems (imipenem, meropenem, and ertapenem) were the most active β-lactam agents (14%, 14%, and 30% resistance rates, respectively).

Table 4. Antimicrobial resistance profile of ESBL / AmpC producing Enterobacteriaceae

<table>
<thead>
<tr>
<th>no. isolates tested</th>
<th>AMK: n=37</th>
<th>GEN: n=38</th>
<th>STX: n=42</th>
<th>CIP: n=54</th>
<th>CTX: n=54</th>
<th>SAM: n=54</th>
<th>FOX: n=54</th>
<th>CAZ: n=46</th>
<th>FEP: n=54</th>
<th>ATM: n=54</th>
<th>TZP: n=36</th>
<th>MER: n=29</th>
<th>IPM: n=37</th>
<th>ETP: n=30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance (%)</td>
<td>11</td>
<td>63</td>
<td>95</td>
<td>93</td>
<td>100</td>
<td>93</td>
<td>46</td>
<td>59</td>
<td>98</td>
<td>98</td>
<td>31</td>
<td>14</td>
<td>14</td>
<td>30</td>
</tr>
</tbody>
</table>

AMK: amikacin; GEN: gentamicin; STX: trimethoprim-sulfamethoxazole; CIP: ciprofloxacin; CTX: ceftaxime; SAM: ampicillin-sulbactam; FOX: cefoxitin; CAZ: ceftazidime; FEP: cefepime; ATM: aztreonam; TZP: piperacillin-tazobactam; MER: meropenem; IPM: imipenem; ETP: ertapenem

The number of isolates tested for each antibiotic is shown (n).

DISCUSSION

Antimicrobial resistance in Latin America is evolving, as shown by observational studies such as those from the Study for Monitoring Antimicrobial Resistance Trends (SMART) Program or the SENTRY Antimicrobial Surveillance Program. According to SENTRY, Latin America has the greatest increase in ESBL-phenotype rates worldwide, with a 22.4% increase from the 1997-2000 to the 2013-2016 periods.19 However, further studies are needed to understand these trends and their connection to circulating resistance genes worldwide, as well as to determine the local epidemiology, which is necessary to optimize treatments to common infections. DR is lacking reliable data about this topic, consequently, treatment recommendations are based on international guidelines instead of locally designed protocols.

One of the few previous studies with molecular characterization of resistance genes in DR was carried out by the SMART program from 2008-2012 on Klebsiella pneumoniae isolated from intra-abdominal infections.20 This study showed an increasing rate of ESBLs producing Enterobacteriaceae in DR, ranging from 15% in 2009 to 60% in 2012, with a decline in 2013 to 40%. A total of only 8 isolates from DR were subjected to molecular characterization, and all of them contained only the blaCTX-M-15 gene. Similarly, in our study, we identified an elevated prevalence of ESBL producing Enterobacteriaceae.

Of the tested isolates, selected because of their resistance to third generation cephalosporins, 67.5% were confirmed to be ESBL by phenotypical detection (54 out of 80), and 53.75% (43 out of 80) by gene detection. The most prevalent ESBL encoding gene was blaCTX (67%), followed by blaTEM (53%). blaCTX+blaTEM (68%) was the most frequent combination. These results are shared across the world, where blaCTX genes (blaCTX-M mainly) are booming, with frequent and simultaneous expression of enzymes SHV and TEM.21, 22, 23, 24 Latin America shows a similar trend, for instance, in Chile, blaCTX-M-1 was the most frequent ESBL gene detected in isolates collected from patients in 2015 (85%), followed by blaSHV (81%), blaTEM (73%), and blaCTX-M-2 (20%), with more than 90% of the isolates exhibiting multiple production of ESBL.11 In Colombia, ESBLs have expanded quickly, specially CTX-M (25) and Brazil also reported CTX-M as the most prevalent ESBL enzyme, where CTX-M-2 and CTX-M-15 were the dominant variants.10

On the other hand, genes encoding AmpC β-lactamases are often carried in plasmids with multiple resistance genes.26 We detected only two clinical isolates harboring AmpC encoding genes (Fig 2), which simultaneously carried 2 or more ESBL encoding genes. The two isolates carried a gene encoding CIT where one of the samples carried both, DHA and CIT gene. In Spain, CMY-2 and DHA-1 where detected in 75% of E. coli isolates
harboring AmpC genes collected from clinical samples in 2010-2011. Presence of these enzymes in the community is worrisome, as production of AmpC β-lactamases, specifically DHA-1, significantly increases the minimal inhibitory concentration of carbapenems.

Finally, 11 (20%) of the phenotypically positive ESBL and/or AmpC strains lacked TEM, SHV, CTX-M or AmpC encoding genes. Exhaustive analysis should be carried out to identify other possible ESBL-encoding genes or alleles not included in this study.

All the analyzed isolates showed high rates of resistance to multiple drugs, not only extended spectrum cephalosporins. A significant percentage of the isolates were resistant to non-β-lactam antibiotics such as cotrimoxazole, ciprofloxacin, and gentamicin. This is a common phenomenon among ESBL producing organisms due to the acquisition of plasmids carrying genes encoding different resistant mechanisms. This results in a very high percentage of multi-drug resistant (MDR) microorganisms, which are responsible for many of the hospitals acquired infections and usually involve higher costs, morbidity and mortality than infections caused by non-resistant organisms. Resistance to carbapenems was also high (14-30%), leaving clinicians with fewer treatment options. Access to drugs such as carbapenems, tigecycline, ceftolozane/tazobactam and ceftazidime/avibactam may be limited by local supply chains and cost, raising the stakes for communities and patients.

Further studies are needed in order to include isolates from other hospitals in DR. But, more important, we need to sequence the resistance genes and identify the specific alleles circulating locally. Easy access to antibiotics may contribute to resistance in DR. Knowledge of local resistance genes may help guide surveillance efforts and the deployment of rapid diagnostic testing for antimicrobial stewardship programs.

Conclusions
In DR, ESBL producing microorganisms are prevalent, and ESBL and AmpC-producing organisms commonly contain multiple resistance genes. blaTEM and blaCTX were the most frequent in this study. Co-resistance to oral alternatives such as fluoroquinolones is high among this microorganisms, making parenteral options necessary to treat infections caused by ESBL producing pathogens. Efforts to curb antibiotic availability and to establish antimicrobial stewardship programs are needed in order to tackle antimicrobial resistance in the country.
References


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