Full Length Research Paper

Characterization of drug-resistance and molecular epidemiology of Enterobacter cloacae among patients with clinical infections in a teaching hospital, China

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A total of 114 non-repetitive Enterobacter cloacae isolates were collected from patients with clinical infections and tested for susceptibility to 15 antimicrobials. Enterobacter repetitive intergenic consensus (ERIC) analysis was used to determine the genetic relatedness between isolates with enzymes. Touchdown polymerase chain reaction (PCR) and single PCR were used to detecting the genes encoding beta-lactamase (single PCR rechecked) and integron, aac(6′)-Ib, respectively. Transferability of drug-resistant was studied by conjugation experiments. 41.2% strains possessed one or two AmpC β-lactamase genes, and 29.8% isolates harbored one or more broad-spectrum beta-lactamase genes. While aac(6′)-Ib was detected in six isolates. 26 isolates were detected with class1 integron and four different gene cassettes were found in these strains. None of isolate was carbapenemase producer. Patients with these bacterial infections suffered a failure of different cephalosporin treatment in clinical. The presence of producing ESBLs, AmpC β-lactamase was correlated with the reduced susceptibility to carbapenems among E. cloacae. In addition, these genes could transmit between different species by plasmids and enhanced by insertion sequences. ERIC revealed 11 unrelated profiles, which indicated that there was scattered transmission among E. cloacae with enzymes in the hospital environment, even acrosse more than three years.

Key words: Enterobacter repetitive intergenic consensus, integron, plasmid, resistance.

INTRODUCTION

In recent years, Enterobacter cloacae has emerged as an important nosocomial pathogen which could cause a wide spectrum of infections including respiratory system disease, urinary tract infections, involving mostly patients with impaired host deficiency, bacteremia (Wisplinghoff et al., 2004; Galani et al., 2005). The main antibiotic-resistant mechanism is overproducing ESBLs, AmpC β-lactamase combined with the deficiency of OmpK35/36, which also correlated with reduced susceptibility to carbapenems in E. cloacae (Bradford et al., 1997; Pasteran et al., 2010; Dora et al., 2006). And carbapenems antibiotics are still the most efficient therapies. But the emergence of carbapenem-resistant E. cloacae producing OXA-48 β-lactamases or New Delhi metallo-β-lactamase (NDM-1) is causing a serious clinical problem (Castanheira et al., 2011; Moquet et al., 2011; Amelie et al., 2010; CDC, 2010). These carbapenemase producers are difficult to detect in the clinical laboratory and may be the source of multi-drug resistance which leading to a therapeutic dead end.

Integron is a transferable genetic unit that is capable of capturing and expressing gene cassettes, and played an important role in Multi-drug resistant and the dissemination of antibiotic resistant genes among bacteria. Aminoglycoside resistance genes, such as aac, aph, and aad, are usually found among the cassette genes (Nemec et al., 2004). And recently blaVIM-1 and blaVIM-2 is also emerging in the gene cassette of class1 integron. Worsely, novel gene cassette is identified

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identified by the presence of the insertion element IS\textit{Ecp}1 upstream of \textit{bla}_{\text{CTX-M-like}} was investigated by PCR as reported previously (Eckert et al., 2004). TD-PCR amplifications were performed using a thermal cycle (Eppendorf) as follows: initial denaturation at 94°C for 5 min, annealing temperature from 60 to 50°C, 3 cycles per temperature, 2 cycles in 50°C; all together were 32 cycles, and a final elongation at 72°C for 10 min.

Sequencing

PCR products were purified, and sequenced with BigDye terminator v3.1 on an ABI PRISM 3730XL DNA Analyzers (Applied Biosystems, Foster City, CA). DNA sequences were compared to the National Center Biotechnology Information database.

Conjugation experiments

To determine whether the antibiotic-resistant gene was carried on a transferable plasmid, conjugation experiment was carried out with \textit{E. coli} 600 (EC 600) and \textit{E. cloacae} with CTX-M-type ESBLs as recipient and donor, respectively. The filter mating technique was performed as previously described (Pitout et al., 1998). And transformants were selected in Mueller Hinton agar plates containing rifampin 250 mg/ml and cefotaxime 10mg/ml. PCR for determination of drug-resistant genes among conjugons was performed as described previously.

Molecular typing by \textit{Enterobacter} repetitive intergenic consensus (ERIC)

The DNA was extracted using a genomic DNA extraction Kit. The primer design for ERIC was 5′-AAGTAGTGACTGGGTTAGCG-3′. PCR conditions were performed as follows: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 45 s, 48-52°C for 45 s, 72°C for 2 min, and a final elongation at 72°C for 10 min. Amplified products from the isolates were analysed by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide.

RESULTS AND DISCUSSION

Genes encoding \textit{\beta}-lactamases

The predominant genotype of AmpC \textit{\beta}-lactamase was MIR-3-type (40/47), followed by DHA-1-type (12/47, Figure 1), GenBank reference accession no. AY743435, AY635140. While 34 (29.8%) isolates carried broad-spectrum beta-lactamase genes. PCR amplification and sequencing analysis of strains with these genes revealed that the main ESBLs genotype was \textit{bla}_{\text{CTX-M}} (16/18) in \textit{E. cloacae}, which include twelve \textit{bla}_{\text{CTX-M-3}} and four \textit{bla}_{\text{CTX-M-14}} (GenBank reference accession no. HQ214051, DQ328959) in this study. Environments analysis of \textit{bla}_{\text{CTX-M}} genes inhibited that they were all located on IncF plasmid except for CTX-M-14. IS\textit{Ecp1} (GenBank reference accession no. GQ385318) sequence was presence upstream of the \textit{bla}_{\text{CTX-M}} gene. Two isolates were OXA-1-type ESBLs producers (GenBank reference accession no. GQ896560) mainly mediated the resistance to amino- and uramino-type penicillin, which

Figure 1. TD-PCR AmpC beta-lactamase genes electrophorogram. 5: Negative control; 1 - 4: ACT-1(302 bp) and DHA- 1(405 bp) positive; M: DNA Maker.
restricted the using of beta-lactams. Some non-ESBL-related \( \textit{bla} \) genes were also widespread including TEM-1 (27/34), SHV-11 (2/34). GenBank reference accession no. FJ668751, GU064390 (Figure 2). And isolates of the \( E. \textit{cloacae} \) producing TEM-1 and SHV-11-broad-spectrum \( \beta \)-lactamase were resistant to most of the cephalosporins antibiotics, which suggested the possibility mutation of broad-spectrum \( \beta \)-lactamase to ESBLs. \( E. \textit{cloacae} \) 62 carried VIM gene determined by TD-PCR, but negative rechecked by single PCR, which maybe caused by mismatch. False-positive result was existed in carbapenemase if detected by using TD-PCR. None of isolates was found carried with NDM-1 or OXA-48 gene. In addition, TD-PCR had almost 100% susceptibility in detection of ESBLs, AmpC \( \beta \)-lactamase and carbapenemase, respectively. This PCR method was a fast, low cost and reliable tool for the screening of common beta-lactamases. It also could be used in epidemiological surveys.

Analysis of integrons

No isolate harbored \( \textit{Int2} \). Isolates carried with class I integron genes were further investigated by PCR and sequencing of the variable regions. 26 strains with class I integron products were dived into five types by restriction analysis. And among 23 isolates of \( E. \textit{cloacae} \) with the \( \textit{intI1} \) variable regions, PCR amplification and sequencing revealed that 2 isolates contained a 709bp gene cassette array with \( \textit{dfrA15} \) (GenBank reference accession no.HQ880286). A 1087bp PCR product was obtained from 18 isolates. And sequencing confirmed the presence of gene cassettes \( \textit{aadB-aadA2} \) (GenBank reference accession no.HQ880259). 3 integron-positive isolates contained a 1009bp gene cassette array with \( \textit{aadA1} \) (GenBank reference accession no.GQ924774). Meanwhile four isolates carried these four geneb cassettes, and mainly mediated the resistance to aminoglycoside and trimethoprin. It is a challenge for the point of the reusing about old drugs in recent years.

Transferability of antibiotic-resistant genes

Fifteen to sixteen donors were successed in conjugation experiment, PCR amplification and antibiotic-susceptibility results showed that all recipients acquired antibiotic-resistant genes and antibiotic resistance possessed by donors.

Antibiotic susceptibilities

All isolates were resistant to cefoxitin, and some of them exhibited MDR phenotype. Only found three amikacin-resistant strains and six isolates were intermediate or resistant to ertapenem. No imipenem or meropenem resistant strain was found but had isolates with reduced susceptibility. Isolates with enzymes present a high level of resistance to many antibiotics commonly applied in clinical, such as Cefoperazone/sulbactam (>52%, Table 1). After conjugation and selection on MH agar containing cefotaxime and rifampicin, transfer of cefotaxime-resistance could be observed in 15 cases, which carried CTX-M-type ESBLs. It was an important alarm of hand hygiene in doctors.

ERIC analysis

For ERIC analysis, 11 distinct patterns (1 to 11) were recognized among 63 isolates with enzymes (Figure 3). The four leading ERIC patterns were as followed: type 9 (26 isolates), type 3 (9 isolates), type 7 (8 isolates), type11 (8 isolates). Type 9 was predominated and the isolates were mainly isolated from patients with wound

![Figure 2. TD-PCR ESBLs genes electrophorogram. 1: Negative control; 2 – 4: CTX-M(544 bp) and TEM (862 bp) positive meanwhile; 5: TEM-positive; 6: CTX-M-positive; 7: SHV(870 bp)-positive; 8: OXA-1(564 bp)-positive; M: DNA Maker.](image-url)
Table 1. Drug-susceptibility (%) in 114 E. cloacae with different type of enzymes.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>All isolates (n=114)</th>
<th>Integron (+,n=26)</th>
<th>ESBLs (+,n=34)</th>
<th>AmpC β-lactamase (+,n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>R</td>
<td>n</td>
<td>R</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>52</td>
<td>45.6</td>
<td>20</td>
<td>76.9</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>46</td>
<td>40.4</td>
<td>19</td>
<td>73.1</td>
</tr>
<tr>
<td>Cefoperazone/Sulbactam</td>
<td>31</td>
<td>27.2</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>37</td>
<td>32.5</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>Cefepime</td>
<td>18</td>
<td>15.8</td>
<td>15</td>
<td>57.7</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>27</td>
<td>23.7</td>
<td>17</td>
<td>65.4</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>23</td>
<td>20.2</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>24</td>
<td>21.1</td>
<td>16</td>
<td>61.5</td>
</tr>
<tr>
<td>Amikacin</td>
<td>3</td>
<td>2.6</td>
<td>3</td>
<td>11.5</td>
</tr>
<tr>
<td>Trimethoprim/Sulfa</td>
<td>30</td>
<td>26.3</td>
<td>18</td>
<td>69.2</td>
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<tr>
<td>Aztreonam</td>
<td>48</td>
<td>42.1</td>
<td>22</td>
<td>84.6</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>3</td>
<td>2.6</td>
<td>3</td>
<td>11.5</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Figure 3.** ERIC typing electrophorogram. Left to Right: Number 1 to 25. 1: type1 (1 isolate); 2: type2 (1 isolate); 5-7: type3 (9 isolates); 8: type4 (2 isolate); 9 – 10: type5 (2 isolates); 11-12: type6 (2 isolates); 13-14: type7 (8 isolates); 15-16: type8 (2 isolates); 17-20: type9 (26 isolates); 21-22: type10 (2 isolates); 23-25: type11 (8 isolates); 3: DNA Marker (4 kb, up to down: 4 kb – 0.1 kb); 4: Negative sample.

infection in general surgery (6, 23.1%). These findings indicated that cloning spread played an important role in the increased infections of MDR E. cloacae. These strains showed high resistance to cephalosporin and patients all had a failure of cefotaxime or ceftazidime treatment in clinical treatment according to the data offered by medical records. The most effective therapy for these patients was still meropenem or imipenem.

In conclusion, there was scattered transmission among E. cloacae with enzymes in our hospital according to the results of ERIC typing, even across more than three years. And there was a widespread of beta-lactamases and class I integron in E. cloacae except for carbapenemase, but the resistance determinants ESBLs, AmpC β-lactamases, class I integron had rapidly becoming responsible for carbapenem reduced susceptibility or resistance.

**REFERENCES**


APPENDIX

Aac(6')-Ib (482bp)

ISEcp1 (300-500bp)

Class 1 integron (160bp)

Class 1 variation regions