Original article

Phenotypic detection of metallo-β-lactamase in imipenem-resistant \textit{Pseudomonas aeruginosa} and \textit{Stenotrophomonas maltophilia} at Schiphra Hospital of Ouagadougou in Burkina Faso

Boukaré Kaboré \textsuperscript{1}, Hama Cissé \textsuperscript{1}, Koudbi Jacob Zongo \textsuperscript{2}, Idrissa Sanou \textsuperscript{3,4}, Boukaré Zeba \textsuperscript{1}, Yves Traoré\textsuperscript{1}, Aly Savadogo \textsuperscript{*1}

\textsuperscript{1} Department of Biochemistry and Microbiology, Laboratory of Applied Biochemistry and Immunology, University Joseph KI-ZERBO, Burkina Faso, BP 7021 Ouagadougou 03.
\textsuperscript{2} Department of Biochemistry and Microbiology, Faculty of Applied Science and Technology, Burkina Faso, BP 176 Dédougou.
\textsuperscript{3} UFR Health Sciences, University Joseph KI-ZERBO, Burkina Faso, BP 7021 Ouagadougou 03.
\textsuperscript{4} Laboratory of Bacteriology and Virology at Tengadogo University Hospital, Ouagadougou, Burkina Faso, BP 104 Ouagadougou.

\textbf{Introduction}

Global spread of antimicrobial resistant bacteria has been gradually constant since of introduction the antimicrobial agents into clinical medicine more than 70 years ago. The dissemination of Gram-positive antibiotic-resistant cocci is a challenge, but also the increasing incidence of Gram-negative antibiotic-resistant bacilli represent an increasingly pressing problem [1]. Indeed, beta-lactam are the most popular antibacterial agents used for treating bacterial infections due to their bactericidal activity and low toxicity, except for patients suffering from allergies. This family of antibiotics is a large group sharing a basic structure called beta-lactam ring. The diversity of structures molecular these antibiotics allow them to...
be classified into several subgroups with main characteristics.

The most significant development among carbapenem-resistance mechanisms is the gradual proliferation of carbapenemases. These carbapenemases are a new category of enzymes hydrolysing carbapenems [2]. Among, the β-lactamases, carbapenemases possess the most extensive substrate profiles of all β-lactamases and are therefore clinically important. They are associated with resistance to other classes of antibiotics (namely aminoglycosides, fluoroquinolones, and co-trimoxazole). The chromosomal carbapenemases have been recognized as not causing important effects for a long time. But the recombinant forms of these enzymes (acquired or imported) are a major threat and a crucial issue for infection control [3].

These carbapenemases have been identified for the molecular groups of β-lactamases corresponding to Ambler class A (penicillinase), class B (metallo-enzymes), class C (cephalosporinase or AmpI C) and class D (oxacillinase). Carbapenemases hydrolyze a group composed carbapenems, penicillins and cephalosporins at variable levels dependent of type enzyme [4,5]. In Burkina Faso, few studies are available on carbapenemases, hence the interest of this study [6]. The objective of this study was to screen the clinical Gram-negative bacteria having a metallo-β-lactamase gene for the resistance to imipenem.

Materials and Methods

Samples and screening the isolates

Samples of this study consisted of pathological specimens from patients at Schipra Hospital, Ouagadougou (Burkina Faso). These specimens were blood, urine, pus, vaginal swab and stools. Gram negative bacilli were isolated according to O’Hara [7]. Screening evaluate the resistance of these isolates to third generation cephalosporin (β-lactam).

Identification of isolates

All isolates were identified by API 20E according to the recommendation of manufacturer. Only isolates resistant to imipenem and sensitive to combination imipenem-EDTA were identified using VITEK2 system (Bio Merieux).

Antibiotic susceptibility and determination of minimal concentration by Vitek2

Antibiotic susceptibility was tested according to Andrews [8] and interpreted by the recommendation of CA-SFM [9]. Antibiotic disks used to test Gram negative bacilli for the monobactam (aztreonam, 30 µg), third generation cephalosporin (cefotaxim, 30 µg; ceftriaxone, 30 µg; ceftazidim, 30 µg), fourth generation cephalosporins (cefepim, 30 µg), and carbapenem (imipenem, 10 µg). Strains of interest were studied by Vitek for the determination of the minimum inhibitory concentration (MIC).

Combined disk diffusion method

The presence of metallo-β-lactamase was determined by combination disk diffusion method [8,10]. This method consists of placing two 10 µg imipenem disks on a Petri dish previously inoculated with the strain to be tested. Thus, 10µL of 0.5M EDTA was added to one the imipenem disk. Petri dishes were incubated during 24h at 37°C. The inhibition zones of imipenem and imipenem-EDTA were measured and compared. A difference of inhibition zones more 7mm between inhibition of imipenem and inhibition imipenem-EDTA confirmed that isolate produces the metallo-β-lactamase.

Extraction and kinetics activity of β-lactamase

Periplasmic enzyme extraction was carried according to the re-adapted methods of Shahid et al. [11], Deepak et al. [12] using young bacterial culture. After this treatment, the enzymatic solution was obtained by a final centrifugation at 8000 rpm for 20 minutes. For some bacteria, the enzyme production was induced by adding a 100 µg/mL ampicillin solution during cell culture. β-lactam activity of bacterial extracts obtained was evaluated with nitrocefin. The reaction medium composed of a phosphate buffer of pH 7 at 50 mM, enzyme (5 to 10 µL) and nitrocefin at a final concentration of 100 µM/mL. Mixing was carried out in a spectrophotometer tank. In order to determine their hydrolytic profile, the bacterial extracts with β-lactamase activity were tested with benzylpenicillin, cloxacillin, meropenem, imipenem at varying concentrations (25 µM, 50 µM, 75 µM and 100 µM) serving as a substrate in a reaction medium. Enzyme activities were followed at appropriate wavelengths using a UV/VIS dual beam spectrophotometer (Uvikon 923, XL), respectively at 482nm (nitrocefin), 235nm (benzylpenicillin), 260nm (cloxacillin) and 280 nm (meropenem, imipenem). These follow-ups were carried out at 30°C in a 50
mM phosphate buffer, pH7. Initial rate of hydrolysis of each compound (V0) expressed in absorbance units per minute was evaluated according to the relation $V_0 = \Delta A/\Delta t$ where $\Delta A/\Delta t$ represents the slope at origin of curve variation. Absorbance at wavelength $\lambda$ relative to time.

Results and Discussion
Isolates from pathological products
Table 1 shows the frequency of the bacterial species studied. The frequency of *Escherichia coli*, *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia* were 82.69%, 9.62% and 3.85% respectively. As for *Proteus mirabilis* and *Pseudomonas aeruginosa*, the frequency found was identical (1.92%). *Escherichia coli* was the major species in this study. In Burkina Faso, *Zongo et al.* [13] and *Serge et al.* [14] made the same observation but with frequencies less than that reported in this study. Their frequencies were 50% and 35% respectively for *Zongo et al.* [13] and *Serge et al.* [14]. This could be due to the size of sample. The values of the results obtained in this study are comparable to those reported by *Amine et al.* [15] in Morocco, this is explained by the diversity of species depending on the sites and regions of collection. *Escherichia coli* is increasingly resistant to antibiotics, including those of last resort (carbapenem).

Antibiotic susceptibility and minimal concentration according to Vitek2 system
Treatment of nosocomial infections is complicated by high rates of antibiotic resistance. Antibiotic susceptibility of bacterial species reported in this study was 86.54% (ceftazidim), 71.15% (ceftoxim) and 69.23% (amoxicillin/clavulanate). The bacterial species tested were multi drugs resistant. Antibiotic susceptibility of *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* by vitek 2 are recorded in an additional file (Supplementary file) because they are not strains of interest in this study.

Figure 1 shows the colonies of *Stenotrophomonas maltophilia* after 48 hours incubation on tryptic soy agar medium (TSA) supplemented with 100 μg/mL of ampicillin and their resistance of beta-lactams antibiotics. The high rate of resistance to ceftazidime may be linked to the presence of ESBL type “ceftazidimase” like CAZ-1 and recently CAZ-2 and as well as the enzyme “ceftoximases” type reported by *Ghiolone et al.* [16]. Antiogramm of *Stenotrophomonas maltophilia* realised by vitek 2 showed resistance for all antibiotics tested. One antibiotic was sensitive, trimethoprim /sulfamethoxazol, its MIC was ≤ 20 mg/L. It showed the multi-resistance of this pathogen. Several studies have shown the effectiveness of trimethoprim/sulfamethoxazol for *Stenotrophomonas maltophilia* [17,18]. Other antibiotics likes ciprofloxacin, chloramphenicol, tetracycline and levofloxacin were sensitive to strains tested [18].

Nowadays, bacteria harboring beta-lactamase enzymes capable of hydrolyzing carbapenems (carbapenemases enzymes) are increasingly found in infections [19]. Among these bacteria, *Pseudomonas* and *Acinetobacter*, responsible for nosocomial infections, are increasingly resistant to carbapenems. This resistance has resulted in an increase in the mortality rate in patients infected with strains of *Pseudomonas aeruginosa* producing metallo-β-lactamase, this is due to inadequate treatments empirically offered to patients [20].

Table 2 shows the values of MIC of *Pseudomonas aeruginosa* obtained by the vitek 2 system. The value of MIC obtained in this study for IMP is similar to that reported by *Qu et al.* [21] for the species *Pseudomonas aeruginosa* Hb21 producer of carbapenemase bla VIM-2.

Metallo-β-lactamase detection
Figure 2 shows the metal activity of *Pseudomonas aeruginosa*. In absence of EDTA, there is no inhibition (disc A). In presence of EDTA, the inhibition is function of EDTA load (disc B and C). *Pseudomonas aeruginosais* tested exhibits an inhibition diameter >4 mm around of imipenem-EDTA disc (disc B) compared to this imipenem disc alone (disc A). This same activity is observed with *Stenotrophomonas maltophilia*. EDTA is a chelating agent that prevents the expression of metallo-beta-lactamase activity. *Walsh* [19] reported that the using imipenem disk combined with EDTA confirm the detection of metallo-β-lactamase by clinical bacterial as *Pseudomonas* spp. and *Acinetobacter* spp. These authors concluded that the method using a disc with imipenem supplemented with 750 μg of EDTA allows the differentiation of producing metallo-β-lactamases by bacterial [19,20]. In a comparative study, *Qu et al.* [21] demonstrated that the combined disc method (IPM + EDTA), using 750 μg EDTA / disc for an inhibition diameter ≥ 6 mm, is best for the detection of metallo-β-lactamase in *Pseudomonas aeruginosa* species. The results obtained in this study are similar those reported by *Philippon and Arlet* [22].
authors had used a concentration of 750 μg of EDTA on an imipenem disc (i.e. 4 μL of a 0.5 M EDTA solution, pH 8) and the diameter obtained was significantly different from that of imipenem disc alone. The inhibition by EDTA and ability to hydrolyze carbapenem establishes the enzyme as metallo-β-lactamase. This enzyme is able to hydrolyze common beta-lactam antibiotics including benzyl penicillin, ampicillin, amoxicillin, cefalotin and cephaloridin, as well as powerful antibiotics such as cefotaxim, cefuroxime and imipenem [6]. The increasing and rapid spread of metallo-β-lactamase producing Gram-negative bacilli particularly Enterobacteriaceae constitutes a serious threat to public health worldwide.

**Kinetics activity of β-lactamase**

After analysis of the resistance profiles of these bacteria, bacterial enzymatic extracts were realized to follow the kinetic activity by UV/visible spectrophotometer double beam Uvikon 923 (Biotek kontron instruments). These enzymatic extracts showed hydrolysis activity of meropenem with an initial rate \( V_0 = 4.77 \times 10^{-4} \) UAb.min\(^{-1}\) (Figure 3). The monitoring of hydrolysis of the meropenem by the spectrophotometer revealed the nature of carbapenemase content in the extracts by inhibition of activity with 2mM EDTA and confirmed the resistance phenotype observed in figure (2). Figure 3 shows the hydrolysis of meropenem by the extract of *Pseudomonas aeruginosa*. Thus, the equation: \( y = -0.0122x + 0.3101 \) obtained from the plot allows to determine the rate of the reaction. These two resistance phenotypes, on Petri dish and kinetic analysis are similar and confirm the presence of metallo-beta-lactamase-type carbapenemase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*.

### Table 1. Frequency distribution of bacterial species.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Pathological products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stool</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>21</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>00</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>00</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>00</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>21</td>
</tr>
</tbody>
</table>

### Table 2. Antibiotic susceptibilities of *Pseudomonas aeruginosa* by Vitek 2 system.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Piperacillin/Tazobactam</em></td>
<td>16</td>
<td>I</td>
</tr>
<tr>
<td><em>Ceftazidim</em></td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td><em>Imipenem</em></td>
<td>≥ 16</td>
<td>R</td>
</tr>
<tr>
<td><em>Amikacin</em></td>
<td>≥ 64</td>
<td>R</td>
</tr>
<tr>
<td><em>Gentamicin</em></td>
<td>≤ 1</td>
<td>S</td>
</tr>
<tr>
<td><em>Tobramycin</em></td>
<td>≥ 16</td>
<td>R</td>
</tr>
<tr>
<td><em>Ciprofloxacin</em></td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td><em>Ofloxacin</em></td>
<td>1</td>
<td>S</td>
</tr>
</tbody>
</table>

Figure 1. (A) Colonies of *Stenotrophomonas maltophilia*; (B) Antibiogram of this species.

Figure 2. Detection of Metallo-beta-lactamase producer by *Pseudomonas aeruginosa*.

Figure 3. Monitoring of the kinetics hydrolysis of meropenem by *Pseudomonas aeruginosa*.
Conclusion

Carbapenem-resistant Gram-negative bacilli pose an exponentially increasing threat for public health worldwide. These bacteria possess diverse and versatile mechanisms of drug resistance, which makes control and early detection of infections caused by Gram negative bacilli difficult. Nowadays molecular detection methods are the most recommended, but it is indisputable that they remain inaccessible to low-income countries. Phenotypical procedures are common practices in diagnostic laboratories. The method imipenem-EDTA is an easy test to use in any case of decreased sensitivity to imipenem for the detection of metallo-beta-lactamase. Decreasing of sensitivity can be followed by spectrophotometry. Nevertheless, two isolates which produced metallo-beta-lactamase and resistant multidrug were determined. It is the Pseudomonas aeruginosa and Stenotrophomonas maltophilia. Strict application of hygiene rules and the rational use of antibiotics would limit the spread of these multidrug-resistant organisms.

Acknowledgements

The authors gratefully thank all personal of clinical laboratory of Hospital center Schipraof Ouagadougou and laboratory staff in the Ouagadougou center for sample collection.

Conflict of interest: The authors declare that they have no conflict of interest.

Funding: This study received no specific financial support.

Supplementary file: Bacterial tested by Vitek 2

References


