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Original article

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

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ABSTRACT

Background: Epidemic of carbapenemase-producing bacteria has become worldwide. Thus, during the last decade, the prevalence of carbapenem-resistant *Enterobacteriaceae* and non fermentative Gram-negative bacteria in human patients have increased. Carbapenemase-producing bacteria are usually multidrug resistant. Therefore, early recognition of carbapenemase producers is critical to prevent their spread. **Objectives**: The aim of this study was to contribute establishing the prevalence of isolates producing metallo-β-lactamase isolated from patients admitted to Schiphra Hospital of Ouagadougou. **Material and methods:** Susceptibility of bacteria to antimicrobial agents was evaluated by disc diffusion method using imipenem as screening antibiotic. The combination of imipenem-EDTA was used after detecting the resistance to imipenem. **Results:** A total of 52 isolates resisting one of the third generation cephalosporins were collected. Five isolates showed an intermediary resistance to imipenem (9.61%). Two isolates were resistant to imipenem-EDTA (3.85%). The test of imipenem-EDTA was done to confirm the production of metallo-β-lactamase. The hydrolysis of bacterial extract by meropenem was confirmed by production with a kinetic activity at spectrophotometer V_0=4.77x10^{-5}µM/min for *Pseudomonas aeruginosa* and V_0=1.183x10^{-4} µM/min for *Stenotrophomonas maltophilia*. **Conclusion:** This study showed that bacterial resistances by production of metallo-β-lactamases are a reality in Burkina Faso.

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 common a basic structure called beta-lactam ring. The diversity of structures molecular these antibiotics allow them to

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be classified into several subgroups with mains 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 (peniillinase), 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 Schiphra 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 VITEK2system (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 V0 = $\Delta A\lambda/\Delta t$ where $\Delta A\lambda/\Delta t$ represents the slope at origin of curve variation Absorbance at wavelength λ 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% (cefotaxim) 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 ‘‘cefotaximases’’ type reported by **Ghiglione et al.** [16]. Antibiogramm 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 (carbapenemase enzymes) are increasingly found in infections [19]. Among these bacteria, *Pseudomonas* and *Acinetobacter*, responsible of 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 aeruginosa* 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]. Those 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 hydrolyse carbapenem establishes the enzyme as metallo-β-lactamase. This enzyme is able to hydrolyse 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 $V0 = 4.77*10^{-4}$ $UAb.min^{-1}$ **(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.

Isolates	Pathological products				
	Stool	Urine	Pus	Vaginal swab	Total (%)
Escherichia coli	21	16	03	03	43 (82.69)
Proteus mirabilis	00	01	00	00	01 (1.92)
Klebsiella pneumoniae	00	02	02	01	05 (9.62)
Pseudomonas aeruginosa	00	00	01	00	01 (1.92)
Stenotrophomonas maltophilia	00	02	00	00	02 (3.85)
Total	21	21	06	04	52 (100)

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

Antimicrobial	MIC	Interpretation
Piperacillin/Tazobactam	16	I
Ceftazidim	4	S
Imipenem	≥ 16	R
Amikacin	≥ 64	R
Gentamicin	≤ 1	S
Tobramycin	≥ 16	R
Ciprofloxacin	1	I
Ofloxacin	1	S

Legend: R: Resistance, I: Intermediary, S: Susceptible, MIC: minimum inhibitory concentrations**.**

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

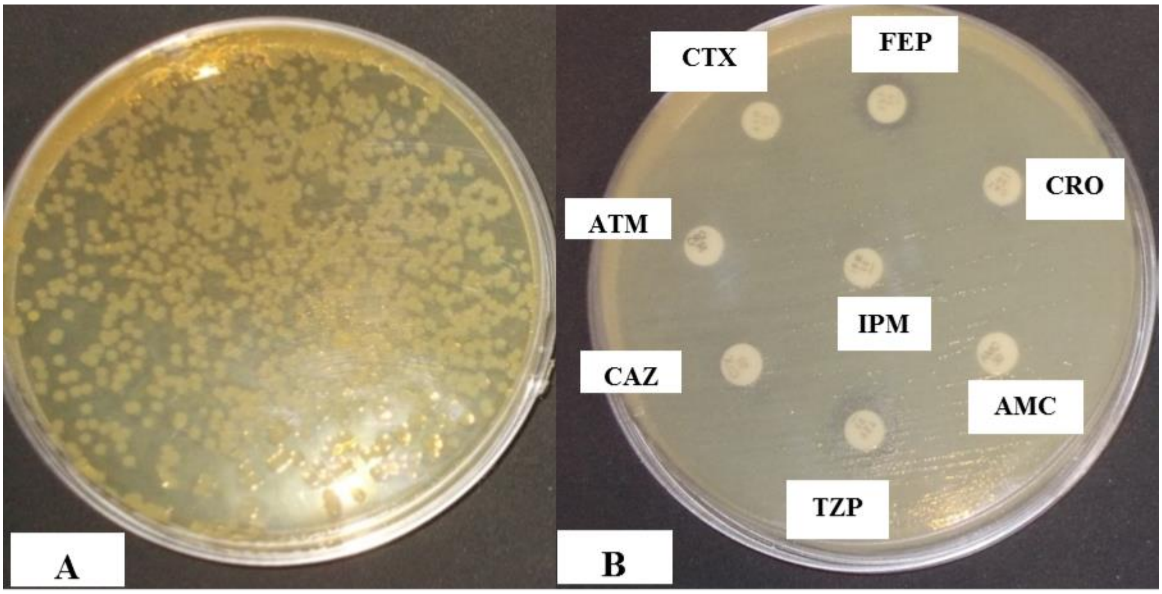


Figure 2. Detection of Metallo-beta-lactamase producer by *Pseudomonas aeruginosa.*

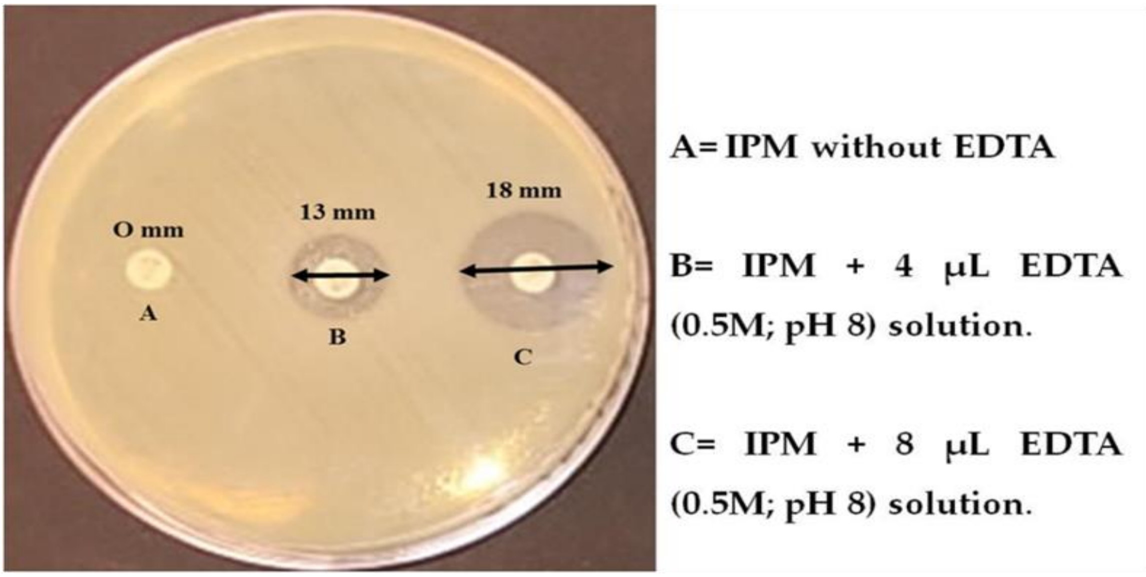
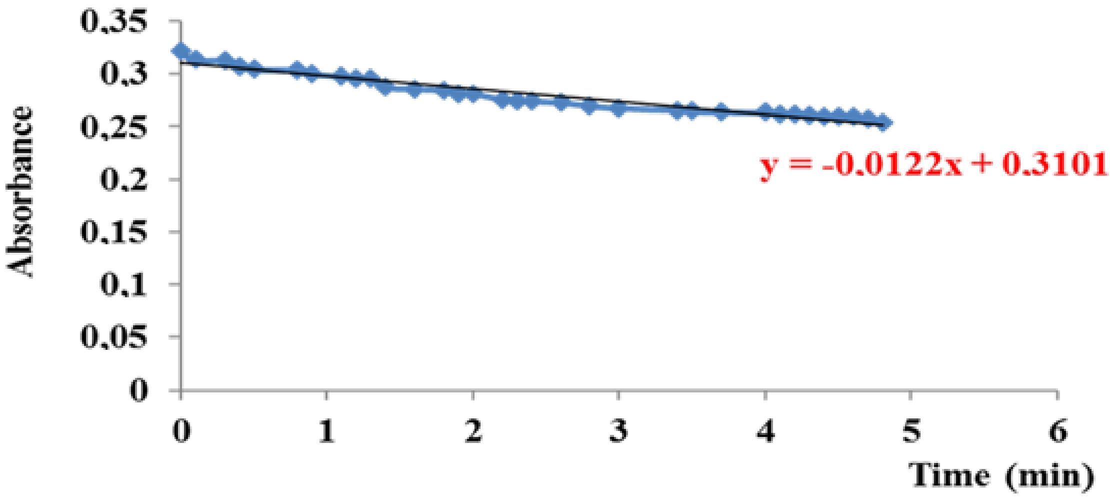


Figure 3. Monitoring of the kinetics hydrolysis of meropenem by *Pseudomonas aeruginosa.*



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-latamase. 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.

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Supplementary file: Bacterial tested by Vitek 2

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