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First report of Carbapenemase producing gram negative bacteria in Republic of Benin: *Pseudomonas aeruginosa* Vim-2 New ST

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Abstract

The spread of carbapenem resistance is increasingly a concern and a serious health challenge for Africa. The present study aims to detect resistance to carbapenem in Benin. 157 Gram negative bacilli were isolated in the period of July to September 2015 in southern Benin. After identification with Maldi Tof Mass Spectrometer, sensitivity to carbapenems (Ertapenem and Imipenem) was determined by antibiotic disk diffusion on MH-2 Agar. Resistance to Imipenem were confirmed by E-test and Carba NP test. The presence of OXA-48 was investigated in all strains exhibiting resistance to one of the carbapenems tested and the search for KPC, NDM, IMP and VIM in strains resistant to Imipenem by real-time and gel-based PCR. The OPRD gene was also investigated by PCR in *Pseudomonas aeruginosa* strains. The genes found by PCR were sequenced and MLST was performed.17 of the 157 strains had a phenotype of Ertapenem resistance against only 5 strains resistant to Imipenem. Resistance to carbapenems was observed in 8 *Escherichia coli* isolates, 5 *Enterobacter cloacea*, 2 *Enterobacter asburiae* and one *Klebsiella pneumoniae* isolate. 3 strains of *Pseudomonas aeruginosa* were isolated and showed resistance to imipenem and Ertapenem. The search for resistance genes showed that only *Pseudomonas* strains carried the VIM-2 gene. MLST analysis of the *Pseudomonas sequences* showed an unidentified clone. This demonstrates the need for urgent surveillance and control of resistance to antibiotics in Benin.

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Introduction

The problem of antibiotics resistance remains a topical and worrying issue in developing countries. The situation is more worrying because new resistance patterns that have never been identified in developing countries are increasingly emerging (Ouedraogo, 2016). This is the case of the resistance to carbapenems in full expansion in African countries (Manenzhe *et al.*, 2015).

The strong resistance of Enterobacteriaceae by the production of ESBL led to the clinical prescription of carbapenems favoring the emergence of carbapenemases which inactivate these antibiotics, the latest molecules of the therapeutic arsenal to fight Enterobacteriaceae (Nordmann & Poirel, 2014). Carbapenemases belong to the class B metallo-beta-Lactamase (MBL) family, which is characterized by their ability to hydrolyze penicillin's, cephalosporins and carbapenems and by a low ability to hydrolyze Aztreonam (Franco *et al.*, 2010). Several families of these enzymes have already been described. IMP-1 is the first carbapenemase and the first MBL to be detected in 1991 in Japan (Watanabe *et al.*, 1991), followed by VIM-1 discovered later in 1997 at Verona, Italy (Lauretti *et al.*, 1999). They are the two most widely distributed carbapenemases (Meletis, 2016). There is also in few cases KPC, SPM GIM, SIM, AIM, DIM, FIM and POM carbapenemases, and the last NDM enzyme detected in India in 2008 and which has since undergone a global spread (Khan *et al.*, 2017; Yong *et al.*, 2009).

VIM1 and ViM2 are the two most widely used variants of the VIM gene and isolated for the first time in strains of *Pseudomonas aeruginosa* in Verona (Italy) and Marseille (France) (Lauretti *et al.*, 1999; Poirel *et al.*, 2000). Since then, there has been a spread of strains of *Pseudomonas aeruginosa* VIM throughout the world. In Africa, several studies have reported the presence of *Pseudomonas aeruginosa* VIM (Pitout *et al.*, 2008; Samuelsen *et al.*, 2009; Jacobson *et al.*, 2012; Jeannot *et al.*, 2013; Touati *et al.*, 2013; Bayssari *et al.*, 2014; Manyahi *et al.*, 2014; Zafer *et al.*, 2015). However, no study in Benin Republic has focused on the search for production of Carbapenemase by Gram Negative bacilli in general and *Pseudomonas aeruginosa* strains in particular. This study is therefore a first report of *Pseudomonas aeruginosa* VIM-2 in Benin.

Material and methods

Identification and sensitivity to antibiotics

157 strains of Gram Negatif Bacillus were isolated from three hospitals in Benin (Bethesda Hospital, Zonal Hospital of Menontin and Regional University Hospital of Ouémé-Plateau) in from July to September 2015. The strains were isolated from Urine and cervico-vaginal secretions. An identification of the strains was carried out by Maldi Tof SM (Seng *et al.*, 2013). Sensitivity to antibiotics was determined by disk diffusion on MH agar (CA-SFM, 2017). The antibiotic discs used are Amoxicillin (AX 25), Fosfomycin (FF50), Ciprofloxacin (CIP5), Amoxicillin + Clavulanic Acid (AMC30), Ertapenem (ERT10), Trime thoprim + Sulphamet hoxazol (SXT25), Imipinem IMP10), Amilkacin (AK30), Gentamycin (CN15), Ceftriazone (CRO30), Cefotaxime (CTX30), Ticarcillin + Sulphamethoxazol (TIM85), Cefoxitin (FOX 30), Rifampicin (RA 30), Aztreonam (ATM 30). After incubation for 24 h, the zones of inhibition were measured and compared with the diameters recommended by (CA-SFM, 2013).

E-test and Carba Test

Imipenem resistance was tested using imipenem E-test Imipenem on MH agar and Carab-NP test (Bakour *et al.*, 2015).

Search for resistance genes

The search for resistance genes was carried out by real time PCR and conventional PCR. In strains with resistance to imipenem or Ertapenem, the Oxa-48 gene was sought. The NDM, IMP, KPC, VIM genes were searched in Imipenem resistant strains. The oprD gene was investigated in *Pseudomonas aeruginosa* strains (Touati *et al.*, 2013). The reaction mix was composed of Quantitec 10µl, Primer F 1µl, Primers R 1µl, DNase free water 2µl, probe 1µl and 5µl of DNA for qPCR and Quantitec 12.5µl, Primers F 0.5µl, Primers R 0.5µl, DNase free water 6.5µl and 5µl DNA for conventional PCR. The primers used as well as the positive controls are recorded in Table 1.

Sequencing

PCR products were purified and a Big Dye PCR was carried out with the same primers. For each sample, the primers were used differently in two reactions. The reaction medium for the Big Dye PCR was Buffer Big Dye 3µl, Big Dye 2µl, Primers 1µl, DNase free water 10µL. The product of the big Dye PCR was then filtered on sephadex and subjected to sequencing by the Sanger method. Obtained sequences were aligned according to the two primers R and F and blasted in NCBI and Arg-Annot databases. The Mega8 software was used for the construction of the phylogenetic tree.

MLST

The MLST of *Pseudomonas aeruginosa* strains carrying the vim-2 gene was carried out by sequencing the seven housekeeping genes acs A, aro E, gua A, mut L, nuo D, pps A and trp E. The sequences obtained for the seven housekeeping genes were processed on the *Pseudomonas aeruginosa* MLST website (http://pubmlst.org/peruginosa/) to determine the number of alleles and sequence types (ST) (Cholley *et al.*, 2014).

Results

Of the 157 strains of Gram-negative bacilli isolated in our study, 17 showed resistance to Ertapenem and 5 demonstrated intermediate resistance to imipenem. *Escherichia coli* showed the highest resistance to Ertapenem (Table 1).

The strains of *Pseudomonas aeruginosa* were resistant to imipenem and Ertapenem. All carbapenem resistance resulted from urine samples.

Table 1. Primers and probes used for the PCR reactions.

Genes (T +)	Type of PCR	Primer or probe	Oligonucleotide sequences	References
OprD (P. aeruginosa O1)	*PCR-Std*	*OprD-F*	*GGAACCTCAACTATCGCCAAG*	*AY539833*
		OprD-R	*GTTGCCTGTCGGTCGATTAC*	
VIM (P. aeruginosa VIM2)	*qPCR*	*ALLVIM_RT_F*	*CACAGYGGCMCTTCTCGCGGAGA*	HG530068.1
		ALLVIM_RT_R	*GCGTACGTYGCCACYCCAGCC*	
		ALLVIM_RT_Probe	*6FAM-AGTCTCCACGCACTTTCATGACGACCGCGTCGGCG-TAMRA*	
	PCR-Std	*ALLVIM_STD_F*	*ATTGGTCTATTTGACCGCGTC*	
		ALLVIM_STD_R	*TGCTACTCAACGACTGCGCG*	
OXA-48 (E. coli CMUL64)	*qPCR*	*OXA48_RT_F*	*TCTTAAACGGGCGAACCAAG*	AY236073
		OXA48_RT_R	*GCGTCTGTCCATCCCACTTA*	
		OXA48_RT_Probe	*6-FAM-AGCTTGATCGCCCTCGATTTGG-TAMRA*	
	PCR-Std	*OXA48_STD_F*	*TTGGTGGCATCGATTATCGG*	
		OXA48_STD_R	*GAGCACTTCTTTTGTGATGGC*	
KPC (K.pneumoniae ST512)	*qPCR*	*KPC_RT_F*	*GATACCACGTTCCGTCTGGA*	
		KPC_RT_R	*GGTCGTGTTTCCCTTTAGCC*	
		KPC_RT_Probe	*CGCGCGCCGTGACGGAAAGC*	
NDM (Kpnasey)	*qPCR*	*NDM-1_RT_F*	*GCGCAACACAGCCTGACTTT*	
		NDM-1_RT_R	*CAGCCACCAAAAGCGATGTC*	
		NDM-1_RT_Probe	*CAACCGCGCCCAACTTTGGC*	
IMP (P.aeruginosa IMP-1)	*PCR-Std*	*Bla(Gr3)Imp(295)F*	*GACAGYACRGSDGGAATAGAGTGGCT'*	
		Bla(Gr3)Imp(606)R	*CATTAATATTTDGCGGACTYTGGCCAAGC*	
OprD (P. aeruginosa O1)	*PCR-Std*	*OprD-F*	*GGAACCTCAACTATCGCCAAG*	*AY539833*
		OprD-R	*GTTGCCTGTCGGTCGATTAC*	
VIM (P. aeruginosa VIM2)	*qPCR*	*ALLVIM_RT_F*	*CACAGYGGCMCTTCTCGCGGAGA*	HG530068.1
		ALLVIM_RT_R	*GCGTACGTYGCCACYCCAGCC*	
		ALLVIM_RT_Probe	*6FAM-AGTCTCCACGCACTTTCATGACGACCGCGTCGGCG-TAMRA*	
	PCR-Std	*ALLVIM_STD_F*	*ATTGGTCTATTTGACCGCGTC*	
		ALLVIM_STD_R	*TGCTACTCAACGACTGCGCG*	
OXA-48 (E. coli CMUL64)	*qPCR*	*OXA48_RT_F*	*TCTTAAACGGGCGAACCAAG*	AY236073
		OXA48_RT_R	*GCGTCTGTCCATCCCACTTA*	
		OXA48_RT_Probe	*6-FAM-AGCTTGATCGCCCTCGATTTGG-TAMRA*	
	PCR-Std	*OXA48_STD_F*	*TTGGTGGCATCGATTATCGG*	
		OXA48_STD_R	*GAGCACTTCTTTTGTGATGGC*	
KPC	*qPCR*	*KPC_RT_F*	*GATACCACGTTCCGTCTGGA*	

Genes (T +)	Type of PCR	Primer or probe	Oligonucleotide sequences	References
(K.pneumoniae ST512)		*KPC_RT_R*	*GGTCGTGTTTCCCTTTAGCC*	
		KPC_RT_Probe	*CGCGCGCCGTGACGGAAAGC*	
NDM (Kpnasey)	*qPCR*	*NDM-1_RT_F*	*GCGCAACACAGCCTGACTTT*	
		NDM-1_RT_R	*CAGCCACCAAAAGCGATGTC*	
		NDM-1_RT_Probe	*CAACCGCGCCCAACTTTGGC*	
IMP (P.aeruginosa IMP-1)	*PCR-Std*	*Bla(Gr3)Imp(295)F*	*GACAGYACRGSDGGAATAGAGTGGCT'*	
		Bla(Gr3)Imp(606)R	*CATTAATATTTTDGCGGACTYTGGCCAAGC*	

Table 2. Phenotypic features of the 19 imipenem and/or Ertapenem resistant in clinical isolates.

N°	Identification	Source	Ertapenem	Imipenem
1	*Escherichia coli*	Urines	R	S
2	*Enterobacter asburiae*	Urines	R	S
10	*Enterobacter asburiae*	Urines	R	S
19	*Escherichia coli*	Urines	R	S
52	*Escherichia coli*	Urines	R	S
59	*Enterobacter cloacea*	Urines	S	I
60	*Escherichia coli*	Urines	R	S
90	*Escherichia coli*	Urines	R	S
93	*Escherichia coli*	Urines	R	S
107	*Escherichia coli*	Urines	R	S
112	*Enterobacter cloacea*	Urines	S	R
119	*Enterobacter cloacea*	Urines	R	S
148	*Escherichia coli*	Urines	R	S
153	*Pseudomonas aeruginosa*	Urines	R	R
168	*Pseudomonas aeruginosa*	Urines	R	R
173	*Pseudomonas aeruginosa*	Urines	R	R
246	*Klebsiella pneumoniae*	Urines	R	S
302	*Enterobacter cloacea*	Urines	R	S
303	*Enterobacter cloacea*	Urines	R	S

R: Resistant; S: Sensible; I: Intermediate.

Table 3. Result of E-test and CarbaNP test of imipenem resistant in clinical isolates

N°	Identification	Source	E-test	CarbaNP test
52	*Escherichia coli*	Urines	-	-
112	*Enterobacter cloacea*	Urines	-	-
153	*Pseudomonas aeruginosa*	Urines	+	+
168	*Pseudomonas aeruginosa*	Urines	+	+
173	*Pseudomonas aeruginosa*	Urines	+	+

Table 4. Genotypic features of the 19 imipenem and/or Ertapenem resistant in clinical isolates.

N°	Identification	Phe	OXA-48	IMP	KPC	NDM	VIM	oprD
1	*Escherichia coli*	*ERT*	-					
2	*Enterobacter asburiae*	*ERT*	-					
10	*Enterobacter asburiae*	*ERT*	-					
19	*Escherichia coli*	*ERT*	-					
52	*Escherichia coli*	*ERT-IMP*	-	-	-	-	-	
59	*Enterobacter cloacea*	*ERT*	-					
60	*Escherichia coli*	*ERT*	-					
90	*Escherichia coli*	*ERT*	-					
93	*Escherichia coli*	*ERT*	-					
107	*Escherichia coli*	*ERT*	-					
112	*Enterobacter cloacea*	*ERT-IMP*	-	-	-	-	-	
119	*Enterobacter cloacea*	*ERT*	-					
148	*Escherichia coli*	*ERT*	-					
153	*Pseudomonas aeruginosa*	*ERT-IMP*	-	-	-	-	+	+
168	*Pseudomonas aeruginosa*	*ERT-IMP*	-	-	-	-	+	+
173	*Pseudomonas aeruginosa*	*ERT-IMP*	-	-	-	-	+	+
246	*Klebsiella pneumoniae*	*ERT*	-					
302	*Enterobacter cloacea*	*ERT*	-					
303	*Enterobacter cloacea*	*ERT*	-					

ERT : Ertapenem; IMP: Imipenem.

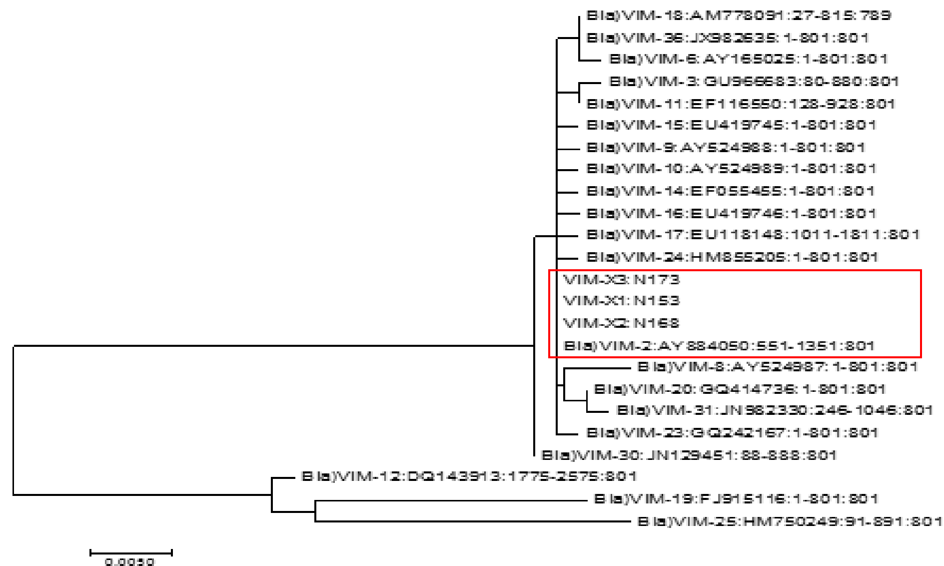

Fig. 1. Phylogenetic tree of three *Pseudomonas aeruginosa* Vim-2 isolates.

Discussion

The occurrence and rapid worldwide spread of carbapememase-producing bacteria (CPB), particularly carbamemia-producing Enterobacteriaceae (CPE), is a serious public health problem worldwide (Cantón *et al.*, 2012; Manenzhe *et al.*, 2015). In this study, 19 of the 154 strains had resistance to 1 or both carbapenems, 3.2% to Imipenem and 11% to Ertapenem. A similar prevalence (3.6%) was observed in hospital strains in Benin (Anago *et al.*, 2015). Cases of resistance to Imipenem were reported in foods in Benin by Ahouandjinou *et al.*, 2016; Mousse *et al.*, 2016 13,6%) 13.6% and 4%, respectively. None of these studies, however, researched the genes of resistance to Carbapenem. In addition, Ertapenem is often not tested for antibiotic sensitivity.

This raises the problem of harmonization and updating of antibiotic panels for the detection of antibiotic susceptibility, which is a handicap for the surveillance of antibiotic resistance in Africa. Only the three strains of *Pseudomonas aeruginosa* were positive for E-test and Carba NP test. Other authors (Bakour *et al.*, 2015; Girlich *et al.*, 2013) also reported the effectiveness of these tests for resistance to imipenem. None of the Ertapenem resistant strains was carriers of the OXA-48 gene.

The sequence analysis for resistance genes revealed the presence of *Pseudomonas aeruginosa* Vim-2 after blast in GenBank NCBI and Arg Annot database. We also notice a alignment of the three sequence of *Pseudomonas aeruginosa* isolates with the Vim-2 sequence of the given Arg Annot database. This gene is identified for the first time in Benin.

A number of studies have reported the presence of Pseudomonas Vim-2 in Africa. These include, Algeria: n = 14 (Touati *et al.*, 2013); Lebanon: n = 18 (Bayssari *et al.*, 2014); Egypt: n = 28 (Zafer *et al.*, 2015), Tanzania: n = 8 (Manyahi *et al.*, 2014); Kenya n = 57 (Pitout *et al.*, 2008b); Côte d'Ivoire: n = 6 (Jeannot *et al.*, 2013); Ghana: n = 1 (Samuelsen *et al.*, 2009); South Africa: n = 11 (Jacobson *et al.*, 2012). A new sequence type was identified during MLST analyses.

This new sequence type is very similar to the isolated ST in Ghana (Samuelsen *et al.*, 2009). The only difference lies in the allele of the trpE gene, which is 31 for our strains of *Pseudomonas aeruginosa* vim-2 and 41 for the strains of *Pseudomonas aeruginosa* vim-2 ST 233.

The emergence of a new multidrug-resistance bacterium clone should draw attention to the control and monitoring of antibiotic resistance.

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