IJPSR (2015), Vol. 6, Issue 8 **(Review Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 14 December, 2014; received in revised form, 21 February, 2015; accepted, 18 April, 2015; published 01 August, 2015

ENZYMATIC INACTIVATION OF PENICILLINS: AN EMERGING THREAT TO GLOBAL PUBLIC HEALTH

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Keywords:

Penicillins Resistance, β-lactamases, Gram-positive bacteria, Gram-negative bacteria, Public Health

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ABSTRACT: Penicillin antibiotics have been the main-stay armamentarium in the fight against bacterial diseases due to their availability and ease of use. Penicillin resistance has been attributed to modification of penicillin-binding proteins, production of β-lactamases, overexpression of efflux pumps and reduced permeability. In the past 10 years, research was centred on Gram-positive bacteria, particularly Methicillin-Resistant *Staphylococcus aureus* (MRSA). The key determinant of resistance in MRSA strains is the penicillin-binding protein (PBP2a). Currently, the production of β-lactamases by Gram-negative bacteria has garnered a degree of attention that seems to be rising rapidly. Gram-negative bacteria pose the greatest risk to public health because of the global emergence and spread of metallo-β-lactamases, including Imipenemase (IMP-types), Verona integrin-encoded-metallo-β–lactamases (VIM-types) and New Delhi metallo-β-lactamases (NDM-types). Not only is the increase in resistance of Gram-negative bacteria to beta-lactams faster than in Gram-positive bacteria, but also there are no current and developmental antibiotics active against metallo-β-lactamase-producing Gram-negative bacteria. This review critically examines the current issues in beta-lactamases responsible for penicillins resistance in relation to the current clinically important bacteria pathogens.

INTRODUCTION: Penicillin antibiotics have been major agents in the treatment of most bacterial infections since their introduction in 1941. They mainly act by inhibiting the transpeptidases (penicillin binding proteins-PBPs) which are responsible for the cross-linkage of the peptidoglycan layer of the bacterial cell wall [1]. Bacterial resistance to penicillins is mainly as a result of the production of beta-lactamases or by modification and/ or production of defective penicillin-binding proteins.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(8).3151-60

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(8).3151-60

The modification or inhibition of PBPs could then lead to reduced cell wall permeability and increase in efflux pumps activity. The modification of target site is the most frequent resistance mechanism in Gram-positive cocci such as MRSA, penicillin-resistant *Streptococcus pneumoniae*, and penicillin-resistant *Neisseria gonorrhoeae*. Resistance in MRSA is as a result of the production a low affinity PBP2a (**Fig. 1**) whereas alteration in PBP2b is the major cause of penicillin resistance in *Streptococcus pneumoniae* [1].

In contrast, penicillin resistance in Gram-negative bacteria is the production of intrinsic or horizontally acquired beta-lactamases, which destroys the amide bond of the beta-lactam ring of penicillins rendering them ineffective [2]. This poses serious threat to public health and a concerted

global action is required to contain this emerging antibiotics resistance [29, 30, 31].

In the past 10 years, research has been focused on Gram-positive bacteria, particularly MRSA which are resistant to penicillins as a result of the presence of PBP2a [1]. Currently, production of β-lactamases by Gram-negative bacteria has garnered a degree of attention that seems to be rising rapidly. However, the most significant contributory factor to penicillins resistance in bacteria is "the enzymatic inactivation." This review critically examines the current clinically relevant issues on penicillins resistance in order to evaluate the degree of risks posed by both Gram-positive and Gram-negative bacteria to global public health.

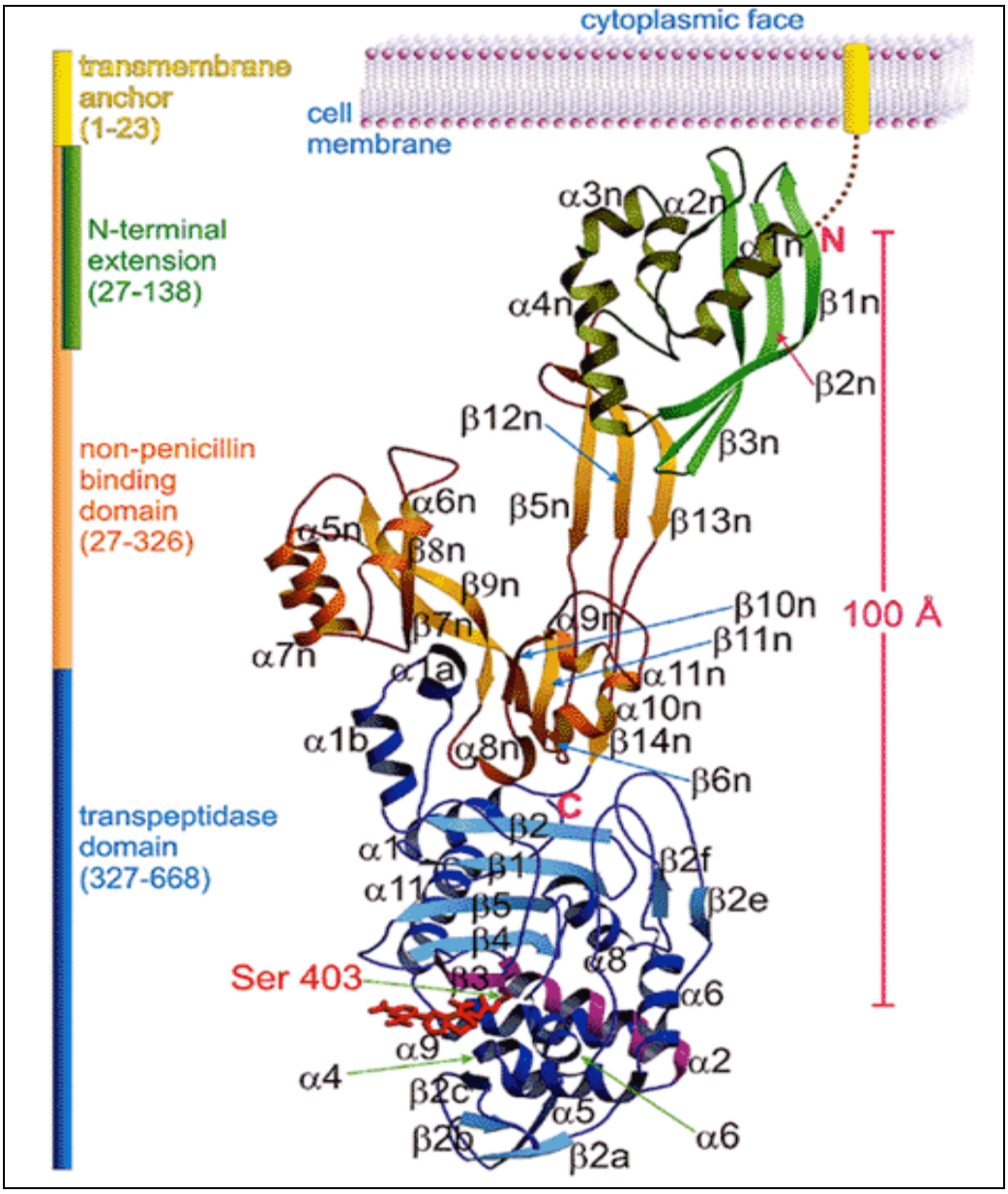

FIG.1: STRUCTURE OF SAUPBP2a*

Classification of β-lactamases:

Generally, β-lactamases are a group of enzymes that hydrolyses β-lactam antibiotics by opening of the β-lactam ring and turning the antibiotic inactive [3]. There are two predominantly β-lactamases classification schemes:

i. Ambler classification system: this classification is based on the sequence of amino acids and tend to group β-lactamases into class A, B, C and D enzymes.These groups are further sub-divided into metal-dependent (Zn2 +- requiring class B) and

metal-independent (serine utilising classes A, C and D), and

ii. **Bush-Jacoby-Medeiros system:** this is an updated classification system and is also known as the functional classification scheme. The classification is based on the substrates and inhibitors of the beta-lactamase enzymes such as clavulanic acid, sulbactam, and Tazobactam [4]. Here, numbers are assigned to the groupings.

Table 1 compares the structural classifications schemes (Ambler classification system) with the functional classification scheme (Bush et. al classification). It can be seen that there are still some important issues particularly with the functional classification scheme despite the updates; the hydrolysis of a particular β-lactam class and inactivation properties of the inhibitors are still retained. Also, it is obvious that the functional classification scheme overlaps with structural classification despite its diverse new sub-groups. For instance, the functional classification group 2 contains molecular classes A and D which represent large groups of β-lactamases.

However, structural β-lactamase classification scheme provides a much comprehensive and encompassing format for classifying new and emerging enzymes. This is because enzymes continue to evolve but through use of new molecular techniques such as Polymerase Chain Reaction (PCR) and other methods of genetic analysis, they can be discovered and subsequently classified. Of particular concern here are the classes of enzymes that readily confer resistance to penicillins and other structurally related antibiotics in the Ambler classification and pathogens of clinical relevance.

TABLE 1: CLASSIFICATION SCHEMES FOR BACTERIAL β-LACTAMASES, ADAPTED FROM BUSH AND JACOBY [4].

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

[a] CA, clavulanic acid; TZB, tazobactam.
[b] NI, not included.

Resistance mechanisms to penicillins in selected bacteria:

(i). *Haemophilus influenza*: *Haemophilus influenzae* is one of the major bacteria causing opportunistic infections in children, including tonsillitis, sinusitis, pneumonia, epiglottitis, meningitis and sepsis. The introduction of Hib conjugate vaccine in developed countries has

reduced the morbidity and mortality linked to *H. influenzae*. In developing countries where the Hib vaccine is not readily available, *H. influenzae* is still a frequent cause of morbidity and mortality, especially in children less than 5 years of age [2]. In addition, the wide spread use of pneumococcal conjugate vaccine in children has led to a sudden increase in pneumonia caused by non-typeable *H. influenzae*.

Therefore, penicillin antibiotic medication became the last resort for the treatment of these infections. This sudden increase in resistance is thought to be caused by the production of β-lactamases, a major resistance mechanism in H. influenzae. The β-lactamase producing rate varies worldwide. The β-lactamase-producing strains of H. influenzae have been shown to be 42% in USA, 35.8% in Kenya, 52.4% in Korea, 55% in Taiwan, 48.5% in Thailand and 35.8% in China [2]. *H. influenzae* resistance to ampicillin has been linked to the β-lactamase-producing genes, bla_{TEM-1} and bla_{ROB-1} [2]. Thus, the resistance of this opportunistic bacterium to penicillins is enhanced leading to increase in morbidity and mortality associated with its infection.

(ii) *Escherichia coli:*

E. coli is a Gram-negative rod and has been associated with bacteraemia, urinary tract infections, peritonitis, neonatal meningitis, skin and soft tissue infections and food-borne infections. There was an increase in resistance of *E. coli* to aminopenicillins in Europe in 2011 [5]. Its resistance to penicillins is usually mediated by plasmid coded β-lactamases, mainly of the TEM type and to a lesser extent, the SHV type [6]. It is also resistant to carbapenems through the mediation of metallo- β-lactamases or serine-carbapenemases and this could lead to resistance to most or all available beta-lactam antibiotics. Extended spectrum beta-lactamases (ESBLs) have also been identified in *E. coli* [6]. The production of Verona integrin-encoded-4 (VIM-4) metallo- β –lactamase and CTX-M-15 extended-spectrum β –lactamase was identified in an *E. coli* isolate from a urine sample in Russia and their presence were found to be mediated by plasmids [7]. About 90% of ESBL-producing *E. coli* isolates from urine of 28 patients from Innsbruck, Austria and 34 patients from Bolzano, Italy contained CTX-M group 1 enzymes [6]. Similarly, isolates of the Enterobacteriaceae group producing TEM, SHV, OXA, CTX-M have been reported in Nigeria [32, 33]. Kluytmans *et al.* [8] also reported the isolation of ESBL-producing *E. coli* from retail meat in Netherlands. The presence of extended-spectrum β–lactamases from broiler chickens and turkey have been reported in United Kingdom [9] and in Czech Republic, respectively [10].

These are potential risks factors for the transmission of resistant ESBL-producing bacteria and antibiotic-resistance genes to humans through the food chain. It could also serve as a source for the outbreak of multidrug resistant pathogens in the human population since most of the resistance genes are plasmid encoded. Besides these, CTM-M and SHV extended-spectrum β–lactamases have been identified in *E. coli* isolates from dogs and cats [11] and CMY-2 and CTX-M-15 extended-spectrum β –lactamases have been found in *E. coli* from wild birds (seagull and pelican faeces) in USA[12]. Extended-spectrum-β-lactamase-producing and plasmid-mediated AmpC β –lactamase-producing E. coli from dogs have also been reported in South Korea 13 whereas multi-drug resistant E. coli producing extended-spectrum β-lactamases have been isolated from the faeces of wild animals in central Europe [14]. ces of wild animals in central Europe [14]. The colonisation of companion animals, wild animals and birds by resistant *E. coli* can become important reservoirs and vectors for human infection and the possibility of transferring resistance by conjugation to human strains is unavoidable.

More recently, the discovery of New Delhi Metallo- β-lactmase-1 (NDM-I) in *E. coli* and *Klebsiella pneumoniae* worldwide (**Fig. 2**), especially in United Kingdom, India, Pakistan [15, 34], Australia [16], Canada [21], and South Africa [28, 35] is a major concern. The NDM-1 is mediated by plasmids carrying bla_{NDM-1} gene and these plasmids harbours "numerous resistance genes associated with other carbapenemase genes (Oxacillinase-48), plasmid-mediated cephalosporinase genes, ESBLs genes, aminoglycoside resistance genes (16S RNA methylase), macrolide resistance (esterase), rifampin (rifampin-modifying enzymes) and

sulfamethoxazole resistance as a source of multidrug resistance and pan-drug resistance" [17].

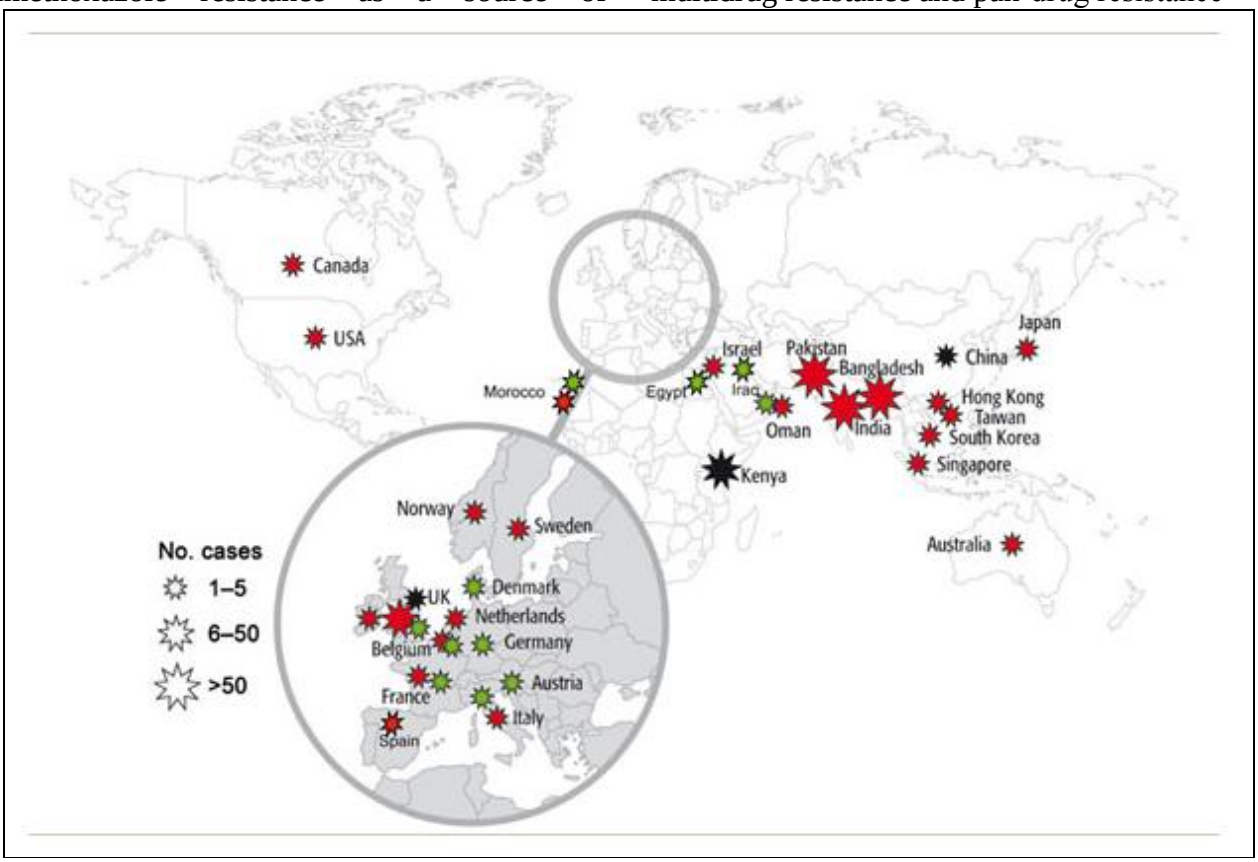

FIG.2: GEOGRAPHIC DISTRIBUTION OF NEW DELHI METALLO-B-LACTAMASE-1 PRODUCERS, JULY 15, 2011. STAR SIZE INDICATES NUMBER OF CASES REPORTED. RED STARS INDICATE INFECTIONS TRACED BACK TO INDIA, PAKISTAN, OR BANGLADESH, GREEN STARS INDICATE INFECTIONS TRACED BACK TO THE BALKAN STATES OR THE MIDDLE EAST, AND BLACK STARS INDICATE CONTAMINATIONS OF UNKNOWN ORIGIN SOURCE: Nordmann *et al.* [17].

(iii) *Klebsiella pneumonia:*

The metallo-β-lactamases and extended-spectrum β-lactamases are also present in *K. pneumoniae*. These isolates are common in neonatal intensive care units and are major causes of hospital-acquired infections in this group [18]. Nosocomial outbreaks in a neonatal intensive care unit attributed to extended-spectrum-β-lactamase-producing *K. pneumoniae* have been reported [19]. In cultures of clinical samples from Neonatal care unit, the hands of healthcare workers and the environment, Lin et al. [18] reported that 2.6% of the neonates had infections while 4.5% had colonisation with extended-spectrum-β-lactamase-producing *K. pneumoniae*. In addition, 44.9% of the environmental samples yielded extended-spectrum-β-lactamase-producing *K. pneumoniae*. The relatively immature immune system of these neonates and the absence of passive antibodies through the placenta and breast feeding tend to increase the risk of neonatal colonisation by multi-drug resistant *K. pneumoniae*.

DISCUSSION: Even though there was an increased attention on resistant Gram-positive organisms such as MRSA, Penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococcus* species in the past 10 years, [15] there is now a continuous decline of MRSA resistance, especially in UK [5]. This decrease in infections by resistant Gram-positive organisms in the UK is attributed to the implementation of different programmes, including hand washing by hospital staff as well as enhanced surveillance of MRSA in hospitals. The *mecA* gene acquired by MRSA is responsible for the expression of PBP2a which has low affinity to beta-lactam antibiotics and penicillins. Penicillins PBP1a, PBP2x and PBP2b are also known to be responsible for penicillin resistance in *Streptococcus pneumoniae* [1].

The antibiotic, ceftaroline has recently been approved in the US for the treatment of acute bacterial skin and skin-structure infections, including community-acquired bacterial infections whilst in Europe, it's been approved for the treatment of complicated and soft tissue infections, and community-acquired pneumonia [1]. Ceftaroline has high efficacy in the treatment of infections caused by MRSA and penicillin-resistant Streptococcus pneumoniae as result of its strong affinity for PBP2a, mostly found in MRSA and PBP1a, PBP1b, PBP1x, PBP2a/b and PBP3 proteins responsible for antibiotics resistance in MRSA and penicillin-resistant Streptococcus pneumoniae [1]. For antibiotics resistance in MRSA and penicillin-resistant *Streptococcus pneumoniae* [1]. Consequently, the global threat initially posed by MRSA and penicillin-resistant *Streptococcus pneumoniae* is becoming a thing of the past due to development of this antibiotic.

The spread of multidrug-resistant Gram negative bacteria, especially the *Enterobacteriaceae* are a major threat to public health [15]. This is because of the global spread of resistance genes among Gram-negative bacteria through horizontal gene transfer mainly mediated by plasmids, transposons and integrons [16]. The emergence of metallo-β-lactamase-producing *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*, and their isolation from life-threatening infections, has increased globally.

Metallo-β-lactamases are mainly of two types; Imipenemase (IMP) and Verona integrin-encoded metallo-β-lactamases (VIM). The enzymes of the IMP-type metallo-β-lactamase were first described in a strain of *Serratia marcescens* from Japan and they were able to hydrolyse all β-lactams, except monobactams [17]. The IMP-type metallo-β-lactamases have been identified in *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae* and reported worldwide [5]. The VIM-type was described in an isolate of *Pseudomonas aeruginosa* in Italy, also hydrolyses all β-lactams, except monobactams [20]. Several VIM-type enzymes; VIM-1, VIM-2, VIM-3, VIM-4, VIM-5, VIM-6 and VIM-7 have been discovered in *P. aeruginosa, K. pneumoniae,* and *E. coli* isolates worldwide (**Fig.3**), with high incidence in Europe and Far East [7, 20].

In a recent study in Thailand, Piyakul *et al.* [20] reported the prevalence of 17.3% of *P. aeruginosa*-producing metallo-β-lactamases with both IMP-14 and VIM-2 enzymes. They further reported that all the IMP-14 strains were identical or closely related suggesting clonal dissemination.

The major challenge in the antibiotics armamentarium is the emergence of New Delhi Metallo-β-lactamases (NDM) in Enterobacteriaceae. NDM-1 was first detected in a strain of *Klebsiella pneumoniae* isolated in 2008 in a patient returning to Sweden from India, where NDM-1 is widespread in Enterobacteriaceae [7]. Kumarasamy *et al.* [15] identified 44 isolates with NDM-1 in Chennai, 26 in Haryana, 37 in the UK, and 73 in other sites in India and Pakistan even though NDM-1 was mostly found among *Escherichia coli* (36) and *Klebsiella pneumoniae* (111).

The 37 isolates with NDM-1 in UK were identified as *K. pneumoniae* (21), *E. coli* (7), Enterobacter spp. (5), *Citrobacter freundii* (2), *Morganella morganii* (1) and Providencia spp. (1). These isolates were from urine, blood, burn or wound swab, sputum, central line tip and throat swab. It is disturbing as most of the Indian isolates from Chennai and Haryana were from community-acquired infections, suggesting that $bla_{\text{NDM-1}}$ is widespread in the environment. This is corroborated by the isolation of NDM-1 β-lactamase-producing bacteria from 2 of 50 (4%) water and 51 of 171 (30%) sewage seepage samples in India indicating that the environment can be a potential source for dissemination [36].

Since India act as a medical tourism centre for Europeans, Americans and Africans, $bla_{\text{NDM-1}}$ will likely spread worldwide. This is true because Multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli* isolates harboring New Delhi metallo-β-lactamase (NDM-1) have been isolated from a patient who had returned to Canada from India [21]. In the same vein, Poirel *et al.* [26] reported seven carbapenem-resistant NDM-1-positive *Klebsiella pneumoniae* isolates recovered from

patients hospitalised between 2007 and 2009 in different wards at a referral and tertiary care center in Nairobi, Kenya. In Australia, an *E. coli* isolate from the urine produced NDM-1 metallo-β-lactamase [16]. In addition, this *E. coli* isolate expressed the extended-spectrum-β-lactamase CTX-M-15, together with two 16S rRNA methylases, namely, ArmA and RmtB, conferring a high level of resistance to aminoglycosides. Similarly, *K. pneumoniae*-producing NDM-1 which was also carrying genes for CTX-M 15, TEM-1 and SHV-11 has been reported in Malaysia [27] while *K. pneumoniae*-producing NDM-1 was detected in South Africa [28].

The dissemination of this novel carbapenemase gene is considered a serious threat since the reservoir of NDM-1 producers is at least in part related to the Indian subcontinent, which is inhabited by the second-largest population in the world and where NDM-1 producers are reported also in community-acquired infections. These NDM-1 variants have been shown to possess high hydrolytic activity to carbapenems and various cephalosporins [25]. Currently, seven variants of NDM (NDM-1 to -7) have been detected in various countries [37].

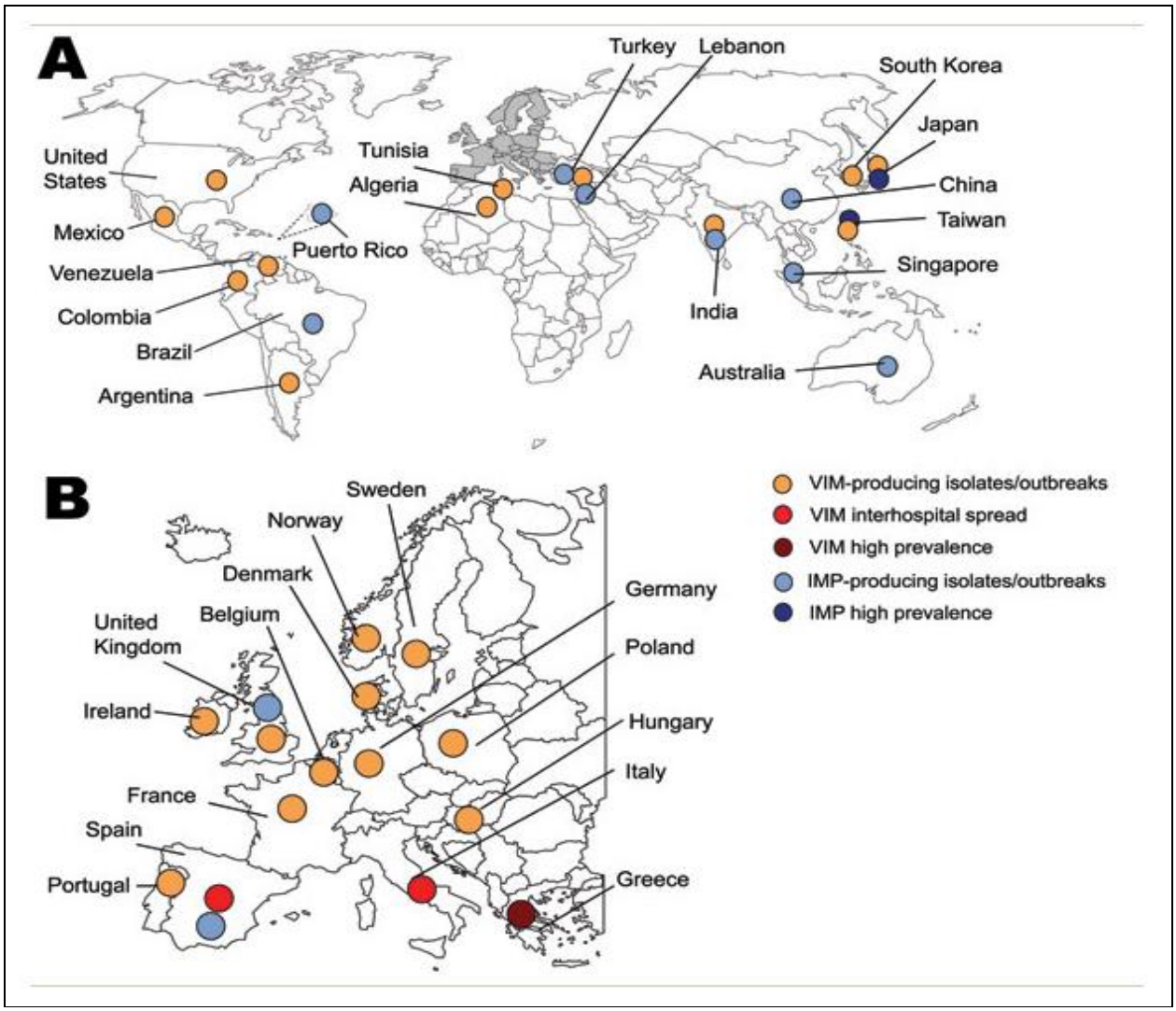

FIG. 3: WORLDWIDE (A) AND EUROPEAN (B) GEOGRAPHIC DISTRIBUTION OF VERONA INTEGRON–ENCODED METALLO-B-LACTAMASE (VIM) AND IMP ENTEROBACTERIAL PRODUCERS SOURCE: Nordmann *et al.* [17].

Despite the widespread discovery of NDM-1, NDM-2-producing *A. baumannii* isolates have been reported from Egypt and Israel [22, 23]. NDM-2 differs from NDM-1 by a single amino acid substitution (Pro28Ala) located in the leader peptide of the enzyme [23]. In contrast to the spread of NDM-1 which is associated with travel to the Indian subcontinent, the five (5) *A. baumannii* isolates

producing NDM-2 enzymes reported in Israel were not associated with international travel. Consequently, there is a possibility for the NDM-2 to spread globally since Israel is a country where many Christians from different parts of the world sojourn for pilgrimage. In addition, NDM-4 and NDM-5 have been reported from *Escherichia coli* isolates from United Kingdom, India and Japan [24, 25, 30]. Another variant, NDM-6 with a point mutation at position 698 (C to T) was identified in an *E. coli* isolate in New Zealand [38] whereas NDM-7 have been reported in Germany and this differ from NDM-1 as a result of mutations at positions 388 (G to A) and 460 (A to C) corresponding to amino acid substitutions Asp130Asn and Met154Leu, respectively [39].

The continuous spread of metallo-β-lactamases across Enterobacteriaceae and *Pseudomonas aeruginosa* is worrisome because there is no current drug for the management of infections caused by these super bugs, although colistin, fosfomycin and tigecycline has shown promising activity [40, 41, 42.]

CONCLUSION: Bacteria from clinical and non-clinical environments are becoming increasingly resistant to conventional antibiotics, including penicillins. Resistance to penicillins is usually due to modification of target site, inactivation of the beta-lactam ring, and/or presence of efflux pumps. In the past 10 years, concern was centred on Gram-positive bacteria, particularly meticillin-resistant *Staphylococcus aureus, Streptococcus pneumoniae* and vancomycin-resistant *Enterococcus* spp which are resistant as a result of modified target. Now, however, clinical microbiologists increasingly agree that multidrug resistant Gram-negative bacteria pose the greatest risk to public health as a result of the emergence of metallo-β-lactamases, including IMP-types, VIM-types and NDM-types. Not only is the increase in resistance of Gram-negative bacteria faster than in Gram-positive bacteria, but also there are no current and developmental antibiotics active against metallo-β-lactamase-producing Gram-negative bacteria.

ACKNOWLEDGEMENTS: The authors sincerely give thanks to the entire academic staff members of Department of Medical Laboratory Sciences, College of Health Sciences, Ebonyi State University, Abakaliki for help and useful comments on the manuscript. We also thank Dr. Prosper Kanyong (Ulster University, Northern Ireland, UK) for finding time to proof-read the manuscript and also making useful comments.

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How to cite this article:
Eze, UA, Eze NM and Mustapha A: Enzymatic Inactivation of Penicillins: An Emerging Threat to Global Public Health. Int J Pharm Sci Res 2015; 6(8): 3151-60.doi: 10.13040/IJPSR.0975-8232.6(8).3151-60.