Metallo-B-Lactamase Production by *Escherichia Coli* and *Klebsiella Species* Isolated from Hospital and Community Subjects in Lagos, Nigeria.

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Abstract: Metallo β-lactamases (MBL) producing *Enterobacteriaceae* are of clinical concern globally. β-Lactam antibiotic is the treatment option for serious bacterial infections. Carbapenems is active against Extended-Spectrum β-lactamase (ESBL) producing *Enterobacteriaceae*, particularly, *Escherichia coli* and *Klebsiella pneumonia; Cephalosporinases* and carbapenemases producers as well. This study was designed to evaluate Metallo β-Lactamase producing *E. coli* and *Klebsiella spp* amongst hospitalized and community subjects in Lagos Nigeria, between March and July 2008. Sixty bacteria from hospital and community were analyzed. Antimicrobial susceptibility and Metallo- β-Lactamase-Production were determined using Disk Diffusion method, Double Disk Synergy Test and Combined Disk Test respectively. Carbapenems had the highest (100%) activity against the bacteria tested. Two strains of *Kleb. spp* susceptible to imipenem were found to be MBL producers. Ceftazidime had 52% resistance in both organisms. Among the 17 strains of *E. coli* from hospital patients, 7 were resistant to ceftazidime and were found to be MBL producers. Out of the 24 *Kleb. spp* (hospital) tested, 8 were resistant to ceftazidime, and 4/8 were subsequently found to be MBL producers. One *E. coli* and two *Kleb. spp* were resistant to ceftazidime; and only one strain of *Kleb. spp* was found to be MBL producer from the community. Metallo-β-Lactamase in *E. coli and Kleb Spp.* is a threat within the hospital and community studied from the public health view point. Early detection of MBL-producers routinely in clinical laboratories is a tool for its containment and is hereby advocated.

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1. Introduction

The increase in the rates of antibiotic resistance is becoming a major cause for concern in isolates of the *Enterobacteriaceae* family (Dongeun Yong *et al.*, 2009). Beta-lactams have been the drugs of choice for the treatment of serious bacterial infections. The most active is the carbapenems, which are employed in the treatment of infections caused by Extended- Spectrum β-lactamase (ESBL) producing *Enterobacteriaceae*, particularly *Escherichia coli* and *Klebsiella pneumonia* (Peterson, 2006). However, clinical utility of carbapenems is under a serious threat with the emergence of acquired carbapenemases, mainly; class B Metallo-β-lactamases (MBLs) (Hemalatha *et al*., 2005).

These enzymes (MBLs) have been reported in many geographical locations to often confer high-level resistance to all β-lactams except aztreonam (Clare *et al.*, 2006). Five variants of MBLs have been described (IMP and VIM [most dominant] SPM, GIM, and SIM), and their prevalence are increasing rapidly (Yan *et al.*, 2004).

Acquired MBL genes are located on the integron structures that reside on mobile genetic elements such as plasmids or transposons (Walsh *et al.*, 2005), thus, enabling widespread dissemination. They are characterized by the ability to hydrolyze carbapenems and are inhibited by EDTA chelators of Zn2+ (Queenan and Bush, 2007).

Eschericher coli and *Kleb. spp* have been reported as opportunistic, worrisome nosocomia and community-associated pathogens (Todar, 2007; Umeh and Berkowitz, 2009). Records have shown that they are the leading causes of pneumonia, bacteremia, thrombophlebitis, Urinary tract infections (UTI) They are also associated with cholecystitis, diarrhea, upper respiratory tract infections, wound infections, osteomyelitis, and meningitis (Melzer and Petersen, 2007; Nordmann *et al*., 2009). βeta-lactam antibiotics have been the drug of choice in treating these health conditions. However, multiple antibacterial resistance in recent decades among *E. coli* and *Kleb spp* as extended-spectrum β-lactamase producers have become common, very difficult to treat and may result to death (Paterson and Bonomo, 2005). In 2009, strains with gene called 'New Delhi' metallo-beta-lactamase, was described in India and Pakistan which is resistant even to intravenous carbapenem preparations (Umeh and Berkowitz, 2009; Kumarasamy *et al.*, 2010).

The phenotypic appearance of MBL-carrying organisms varies depending on the bacteria host (Scoulica, *et al.*, 2004; Peleg, *et al.*, 2005). Several reports have shown that MBL-carrying organisms may be susceptible to carbapenems invitro (using standard methods), but is actually resistant invivo (Clare *et al.*, 2006). Peleg *et al.* (2005) reported that over 30% MBL-carrying isolates which were predominately *Enterobacteriaceae*, were found to be susceptible to imipenem having MIC ≥ 4 µg/ml. Yan and colleagues (2001) also reported an outbreak of *Klebsiella pneumoniae* isolates carrying *bla*IMP-8 and 88% (35/40) were susceptible to carbapenems, however, the clinical use of carbapenems for in handling these cases did not reflect reported susceptibility profile.

Considering, that ESBLs and MBL-producing *E. coli* and *Kleb.* spp. are on the increase and pose a serious clinical challenges, this study was designed to evaluate the antibiotic resistance pattern, the prevalence of MBL-producing *E. coli* and *Kleb spp* and the laboratory primary detection method, using patients from tertiary hospital and individuals from some communities in Lagos, Nigeria.

2. Materials and Methods

A total of 251 (118 from hospital patients and 133 healthy community individuals) various human specimens were processed, between March and July, 2008. All bacteria isolates were characterized using standard methods.

Antibiotic susceptibility testing

Susceptibility tests were determined on Mueller-Hinton agar (CM337-Oxoid, UK) by standard disk diffusion procedures of Bauer *et al.*, (1966) which conforms to the recommended standard of the Clinical and Laboratory Standard Institute (CLSI, 2000). The following standard commercial disks were used: Gentamycin (10µg), Nalidixic acid (30µg), Nitrofurantoin (200µg), Cotrimoxazole (25µg), Amoxycillin (25µg), Tetracycline (25µg), Augmentin (30µg), Ofloxacin (5µg), (Ranbaxy) Ceftazidime (30µg), and Imipenem (10µg), (Oxoid). Standard ESBL positive *K. pneumoniae* ATCC 700603 and E. *coli* ATCC 25922 were used as control strains. The control strains were run simultaneously with the test organisms. Results were interpreted according to CLSI interpretative criteria (2002) and Manual of Antimicrobial Susceptibility Testing guidelines (Coyle, 2005; Cheesbrough, 2006).

Detection of Metallo–Beta-Lactamase (MBL) Producing Strains

Organisms resistant to ceftazidime and susceptible to imipenem were tested for MBL production by using a method according to Lee *et al.*, (2003) and Clare et *al.* (2006) with modification.

Combined Disc Diffusion Test (CDDT)

Two 30µg ceftazidime discs (one impregnated with 10µl of 750µg EDTA) were placed on the Mueller Hinton (MH) agar medium inoculated with test organism standardized with 0.5 McFarland standards. After 24 hours of incubation at 35°C, the zones of inhibition around ceftazidime and ceftazidime +EDTA discs were compared. Zone of inhibition of ceftazidime +EDTA discs compared with ceftazidime alone greater than 4 mm, was considered as MBL producing (Clare et *al.*, 2006). The same test was done with imipenem discs and imipenem impregnated with EDTA.

Double Disc Synergy Test (DDST)

Ceftazidime disc (30µg) was placed on MH agar medium inoculated with test organism and 10 mm apart, a blank filter study disc impregnated with 10µl of 750µg EDTA solution was added, another ceftazidime disc was placed on the far side of the medium. The zone of inhibition around ceftazidime disc that expanded towards EDTA disc, compared to ceftazidime disc placed on the far side of the medium, was indicative of a positive result. The same test was conducted with imipenem disc with10µl of 750µg EDTA solution as well.

3. Result and Discussion

Out of 118(hospital) and 133(community) specimens analyzed for *E.coli* and *Kleb spp,* 41(hospital) and 19(community) specimens showed positive bacterial growth. Out of these, 28(47%) were *E. coli.* 17 and 11 were from hospital and community respectively. A total of 32 (53%) isolates were *Kleb. spp*. (24 from Hospital and 8 from Community specimens). From this result one can deduce that the prevalence of *Kleb. spp* from hospital was higher than that of *E. coli* and that of *E. coli* in community higher than *Kleb. spp.*

The drug resistance pattern of *E. coli and Kleb. spp* isolates were shown in table 1 : . Resistance was least in ceftazidime antibiotic, 8 (41%) showed a form of resistance: 7 from hospital and only 1 isolate from the community. Eight (33%) *Kleb. spp. (*hospital*) showed* resistance to ceftazidime compared with *2*(25%) from the community. It is heartwarming to observe that all the sixty bacterial isolates (hospital and community) were susceptible (100%) *to* imipenem, a third generation β-lactam antibacterial agent.

The result of this study showed relatively low resistance to ceftazidime, oflaxacin, gentamycin, nitrofurantoin and nalidixic acid by *E. coli* and *Kleb. spp* in the community, when compared with the pattern from the hospital. The indication may imply that these drugs have not been grossly misused in the vicinity studied. Therefore, they may still be useful for treating uncomplicated cases where the organisms are indicated as aetiologic agents of diseases in hospital OPDs. Hospital acquired infections by these agents may require a different approach since they have shown invitro resistance patterns that may require alternative drugs or in combination with another drugs.

Table 1: Resistance profile of *E. coli* and *Kleb. Spp*.

Antibacterial agent	Resistance pattern *E. coli*		Resistance pattern *Kleb. spp*	
	Hospital N=17 No (%)	Community N=11 No (%)	Hospital N=24 No (%)	Community N= 8 No (%)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)
Ceftazidime	7 (41)	1 (9)	8 (33)	2 (25)
Ofloxacin	9 (53)	2 (18)	12 (50)	1 (13)
Nitrofurantoin	9 (53)	3 (27)	10 (42)	3 (38)
Nalidixic acid	9 (53)	3 (27)	16 (67)	4 (50)
Augumentin	10 (59)	8 (73)	18 (75)	6 (75)
Gentamycin	12 (71)	2 (18)	17 (71)	2 (25)
Amoxicillin	15 (88)	10 (91)	21 (88)	7 (88)
Cotrimoxazol	15 (88)	7 (64)	20 (83)	5 (63)
Tetracycline	16 (94)	7 (64)	21 (88)	7 (88)

Sensitivity pattern for Metallo-β-lactamase production, By DDST and CDDT methods.

Hospital isolates

CODE	CAZ(mm)	Imp(mm)	CazEDTA(mm)	ImpEDTA(mm)
E. coli	11	28	17	29
E. coli	16	26	23	27
E. coli	14	26	23	28
E. coli	0	28	22	30
E. coli	0	24	20	26
E. coli	16	25	21	28
E. coli	11	26	20	27
Kleb. Spp	16	23	18	25
Kleb. Spp	9	26	24	27
Kleb. Spp	10	23	16	25
Kleb. Spp	15	24	17	27
Kleb. Spp	17	25	20	40
Kleb. Spp	14	24	17	25
Kleb. Spp	0	28	19	30
Kleb. Spp	0	26	18	28
Kleb. Spp	16	21	19	24

Community isolates

CODE	CAZ(mm)	Imip (mm)	CaEDTA (mm)	ImEDTA (mm)
E. coli	16	24	19	25
Kleb. Spp	0	38	24	40
Kleb. Spp	12	32	14	36

Key: Caz – Ceftazidime, Imp – Imipenem, CazEDTA – Ceftazidme + EDTA, ImpEDTA –Imipenem + EDTA.

The combined disc diffusion and double disc synergy test methods showed a clear result for primary detection of MBL and non-MBL production. Of the 60 bacterial strains tested, 14(23%) (Hospital =12) and (community =2) were confirmed to be MBL producers. In hospital isolates, 7(50%) *E coli* and 5(36%) Kleb. spp are MBL producers; with significant cazEDTA increase in zone diameter. In the community, only 2 *Kleb spp* are MBL producers: one with cazEDTA and one with impEDTA significant increase in zone diameter. However no *E. coli* was identified as MBL producer amongst community isolates. A breakpoint of >4 mm, was highly effective at discriminatory between MBL and non-MBL producers (Clare Franklin *et al*., 2006). Interestingly, 12 MBL producing isolates had increased zone diameter of >6 mm.

The forgone is a phenotypic MBL method that is highly sensitive and specific at detecting both carbapenem-susceptible (98.33%) and carbapenem-resistant MBL-carrying isolates among two gram-negative genera from clinical and community specimens. The method is simple to perform and the materials cheap and easily accessible, therefore, it is highly applicable for routine clinical diagnosis. The selection for phenotypic MBL detection is more challenging with the emergence of carbapenem-susceptible MBL-carrying organisms; therefore, screening only carbapenem-resistant organisms, as is mostly performed by clinical laboratories is suboptimal. On the other hand, selecting all isolates creates unnecessary work with a lower yield; practical on-the-bench experience laboratory professional becomes imperative.

Yan *et al.*, (2004) evaluated three methods, DDST, combined-disk test and E-test for this procedure. He reported that, E-test is not applicable to carbapenem-susceptible MBL-carrying organism (MIC $\leq$ 4µg/ml).

In the current study, all MBL-carrying isolates tested, including the carbapenem-susceptible isolates, were resistant to ceftazidime. The DDST and CDDT performed concurrently on a different agar plate. For the combined-disk test, we recommend a lower cutoff (>4 mm) (Clare Franklin *et al*., 2006), than ≥7 mm (Yan *et al.*, 2004; Pitout *et al*., 2005) for the increase in zone diameter with Imip-EDTA as opposed to Imip alone. This value provided a good discriminatory power for detecting MBL-producing isolates (14 MBL-producing isolates). Interestingly, 12 of the reported MBL-producers had increased zone. This results showed that both methods are sensitive and specific in detecting MBL producer, this is in agreement with Clare *et al*. (2006), who reported on DDST and a combined disk test MBL detection methods in which they correctly detected 51 out of 52 PCR-confirmed MBL-negative isolates. Their report showed, using PCR that the sensitivity and specificity of the phenotypic MBL detection method was 100% and 98%, respectively. As described above, no single method demonstrated absolute positive results for MBL detection, and therefore, we recommend the combination of both techniques.

There are reports of MBL production among gram negative organisms from various countries like India, Pakistan (Miriagou *et al*. 2010), Brazil (Gales *et al*., 2003) and Korea (Lee *et al*., 2002). This enzyme (MBL) was first reported as a zinc dependent enzyme in *Bacillus cereus* in mid 1960s (Sabath and Abraham. 1966). A few decades later imipenem hydrolyzing metallo-enzymes was described in *Aeromonas hydrophila* (Shannon *et al.*, 1986) and *Bacteroides fragilis* (Cuchural *et al*., 1986). From this study, MBL production among isolates of *E.coli* and *Kleb.spp* is a foreseable problem.

Our study has demonstrated a high level of resistance in *E.coli* and *Kleb.spp* to most of antibiotics tested within the environment studied. The outcome of this study may be associated with excessive use of broad-spectrum antibacterial agents. Thus, early detection of the occurrence of this enzyme using this simple methodology replicated in this study will smack off a control strategy. The awareness of the existence of MBL initializes indication for the need for proper use of antibiotics to stem selective pressure and spread of MDR bacterial strains within these hospital and communities. Continued surveillance of MBL enzymes within these settings is a novel tool to provide the necessary information for care handlers and policy progenitors on the nature and spread of this type of resistance.

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