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Occurrence of Metallo-Beta-Lactamase-Producing *Enterobacteriaceae* from a Local Poultry Farm in Abakaliki, Nigeria

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ABSTRACT:

The production of beta-lactamases especially the expanded/extended beta-lactamases such as the metallo beta-lactamase (MBL) enzymes by Gram negative bacteria (GNB) are amongst the top arsenal of pathogenic bacteria used to make the therapeutic effect of some available drugs less efficacious. This study evaluated the phenotypic production of MBLs from 50 cloacal swabs of poultry birds in a poultry farm in Abakaliki metropolis, Ebonyi State, Nigeria. The samples were bacteriologically analyzed using eosin methylene blue (EMB) agar and MacConkey agar; and MBL production was phenotypically detected using the Kirby-Bauer disk diffusion method (for antibiogram) and the inhibition based assay technique (for MBL production). Out of the 50 cloacal swab samples analyzed in this study, 39 isolates were *Escherichia coli* while 33 isolates were positive for *Klebsiella* species. All the *E. coli* and *Klebsiella* species showed high resistance to most of the tested antibiotics especially to imipenem, meropenem, ciprofloxacin, sulphamethoxazole trimethoprim, cefoxitin, ertapenem and gentamicin. Nine (9) isolates of *E. coli* (23.1 %) were phenotypically detected as MBL producers while 6 isolates of *Klebsiella* species (18.2 %) were confirmed as MBL producing strains. This present day study accentuates the growing resistance mechanism in the community; and thus calls for concerted effort to detect and prevent the dissemination of antibiotic resistant microbes in the community. Further molecular characterization is required to classify the genetic elements responsible for the dissemination of MBL-producing microbes in this environment.

1. Introduction

Antibiotic resistance (especially those mediated by metallo beta-lactamase enzymes and AmpC enzymes) is a global health problem that is posing a menace to our ability to effectively treat and manage infections caused by organisms producing these enzymes (Ejikeugwu *et al*., 2014). And the continued development and dissemination of drug resistant microbes in the community (following undue antibiotic usage as growth promoting agents in the rearing and development of food-producing animals) has contributed to this global dilemma of antimicrobial resistance. Metallo – β – lactamases (MBLs) are carbapenem hydrolyzing β – lactamases which belong to Molecular Class B of Ambler β – lactamase classification, and which have the ability to

hydrolyze and confer resistance to carbapenems (imipenem, meropenem, ertapenem) and other β – lactam antibiotics but not aztreonam and which are yet inhibited by chelating agents like ethylenediamine tetra-acetic acid (EDTA) (Pitout *et al*., 2007; Walsh *et al*., 2002; Aibinu *et al*., 2007, Franklin et al., 2006). The carbapenems are very potent antimicrobial agents used for the treatment of serious Gram – negative infections including those mediated by Extended-spectrum β-lactamases (ESBLs) (Walsh *et al*., 2002). They are mostly used by hospitals worldwide under restricted conditions to treat and manage severe Gram – negative infections because of their broad – spectrum activity (Franco *et al*., 2010). The MBLs are known to confer variable range of high resistance to all β – lactam antibiotics except the monobactams and their presence in clinically important organisms like *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella* species have put the use of the carbapenems as the last treatment option for treatment of serious Gram – negative bacterial infections under threat (Ejikeugwu *et al*., 2014; Walsh *et al*., 2002; Varaiya *et al*., 2008). MBLs belong to a group of β – lactamases which requires divalent cations of zinc as cofactors for their enzyme activity, and they share four main characteristics including: activity against carbapenem antibiotics, no clear hydrolysis of monobactams, inhibition by chelating agents (e.g. EDTA), and requirement of zinc ions for enzyme activity (Walsh *et al*., 2002; Varaiya *et al*., 2008; Franco *et al*., 2010). The emergence of bacteria producing these enzymes together with their ability to transmit their resistant genes to other susceptible bacteria through plasmids, transposons, integrons and other means of genetic transfer poses a serious problem especially in settings where they still remain undetected (Bush and Jacoby, 2010; Jacoby and Munoz-Price, 2005). These β – lactamases are now found worldwide, and they are increasingly being observed as important causes of multidrug resistance in Gram – negative bacteria all over the world (Nigeria inclusive). The menace posed by multidrug resistant bacteria necessitates the need to detect their presence in community samples and/or isolates owing to the fact that antibiotic resistance is an increasing problem in our health sector worldwide (Ejikeugwu *et al*., 2014; Akinduti *et al*., 2012; Chakraborty *et al*., 2010). This study presumptively evaluated the occurrence of metallo-beta-lactamase (MBL) positive *Enterobacteriaceae* from a poultry farm in Abakaliki metropolis, Ebonyi State, Nigeria.

2. Materials and methods

2.1 Sample collection and preparation:

Fifty (50) cloacal swab samples were aseptically collected from the cloacae of poultry birds in a local poultry farm in Abakaliki metropolis, Ebonyi State, Nigeria from the period of January-April, 2016. The samples were collected from poultry birds that received antibiotics two months before the period of sample collection. Each of the collected samples was collected by rotating the swab sticks thrice around the cloacae region of the poultry birds and they were returned to their containers and labeled individually. The samples were processed in the Department of Applied Microbiology, Ebonyi State University, Abakaliki, Nigeria; and each of the samples was inoculated on double strength nutrient broth (Oxoid, UK) and incubated at 30^{o}C for 18-24 hrs.

2.2 Isolation and identification of bacteria:

A loopful of each of the turbid samples (as previously inoculated on double strength nutrient broth) was aseptically inoculated on eosin methylene blue (EMB) agar and MacConkey (MAC) agar for the isolation of *Klebsiella* species and *Escherichia coli* respectively. Each of the culture plates were incubated at 30^{o}C for 18-24 hrs. *Klebsiella* species produces mucoid colonies without a metallic sheen on EMB agar while *E. coli* produces pinkish colonies on MAC agar due to lactose fermentation. Suspect colonies of *Klebsiella* species and *E. coli* were aseptically subculture onto freshly prepared MAC and EMB agar plates for the isolation of pure cultures; and these were incubated at 30^{o}C for 18-24 hrs. These colonies were purified on nutrient agar (Oxoid, UK) plates and identified using standard microbiological identification techniques as described previously (Cheesbrough, 2000).

2.3 Antibiogram:

Antimicrobial susceptibility testing was carried out using the Kirby-Bauer disk diffusion method on Mueller-Hinton (MH) agar plates as per the guidelines of the Clinical Laboratory Standard Institute, CLSI (CLSI 2005; Ejikeugwu *et al*., 2016). Overnight cultures of the test bacteria (adjusted to 0.5 McFarland turbidity standards) were aseptically swabbed on MH agar plates. The plates were allowed on the bench for 10 mins before single antibiotic disks including ofloxacin (30 µg), ciprofloxacin (5 µg), nalidixic acid (5 µg),ceftazidime (30 µg), ertapenem (10 µg), imipenem (10 µg), gentamicin (10 µg), cefoxitin (30 µg), meropenem (10 µg), and sulphamethoxazole-trimethoprim (25 µg) [Oxoid, UK] were aseptically placed on each of the plates using laboratory forceps. The antibiogram plates were incubated at 30^{o}C for 18-24 hrs; and the results of the susceptibility studies was reported as per the guidelines of the CLSI (CLSI, 2005).

2.4 Screening for the production of metallo-beta-lactamase (MBL) enzymes:

Organisms that harbour genes for resistance to the carbapenems including imipenem, meropenem and ertapenem are usually resistant to the antimicrobial actions of these drugs. Thus, it is proper to use either of

the carbapenems (imipenem or meropenem) for screening bacterial pathogens for the production of MBLs. In this study, the test bacteria were screened for MBL production by determining their susceptibility to imipenem (10 µg) and meropenem (10 µg) (Ejikeugwu *et al*., 2014). According to the CLSI breakpoints, MBL production should be suspected for those organisms with reduced susceptibility to imipenem (IPM) and meropenem (MEM). Strains with reduced susceptibility to IPM and MEM (both inhibition zone < 22 mm) are suspected to produce MBL phenotypically (Aibinu *et al*., 2007; Ejikeugwu *et al*., 2016).

2.5 Inhibition-based assay for detection of MBL:

The production of MBL enzymes in the test bacteria was phenotypically carried out using the inhibition-based assay (Aibinu *et al*., 2007; Saderi *et al*., 2008; Ejikeugwu *et al*., 2014). Test isolates found to be resistant to imipenem or meropenem (as described by the CLSI breakpoints for MBL screening) was evaluated phenotypically for MBL production. The test bacteria (adjusted to 0.5 McFarland turbidity standards) were aseptically swabbed on MH agar plates. Imipenem (10 µg) and meropenem (10 µg) disks impregnated with EDTA (1 µl) was aseptically placed on the MH agar plates. Supplementary imipenem (10 µg) and meropenem disks without EDTA was also placed alongside the carbapenem disks encumbered with the chelating agent (EDTA). The chelating agent (EDTA) was tested on the test bacteria prior to the inhibition based assay to ensure they had no inhibitory effect on the test organisms. All plates were incubated at 30^{o}C for 24 hrs, and the zones of inhibition recorded as per the CLSI criteria. Inhibition zones with a difference of ≥ 7 mm between the zones of inhibition of any of the carbapenem disks with and without the chelating agent (EDTA) infer metallo-β-lactamase production phenotypically (Aibinu *et al*., 2007; Ejikeugwu *et al*., 2016).

3. Results

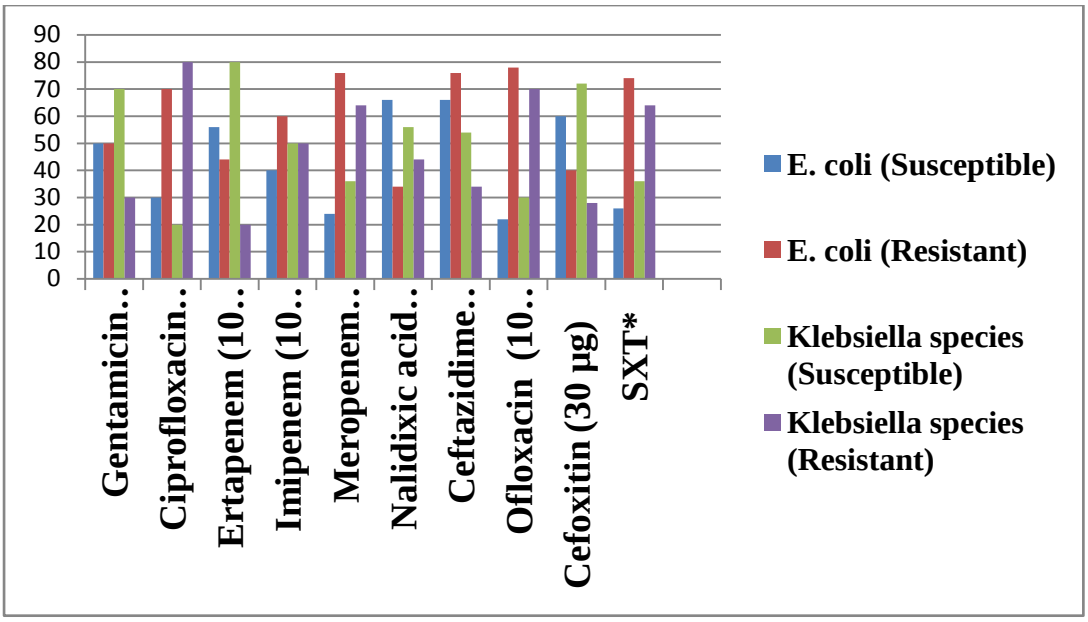
A total of fifty (50) cloacal swab samples from the cloacae of poultry birds in a local poultry farm in Abakaliki metropolis were bacteriologically and microscopically analyzed in this study for the isolation of *Escherichia coli* and *Klebsiella* species. The result of the isolation of the *Enterobacteriaceae* is shown in Table 1. *E. coli* was the most frequent enteric organism isolated (78 %), followed by *Klebsiella* species (Table 1). A total of 39 *E. coli* isolates and 33 *Klebsiella* species isolates were recovered from the cloacal swab samples. The antimicrobial susceptibility profile of the test bacterial isolates is shown in Figure 1. More than 50 % of the isolated *E. coli* isolates were resistant to gentamicin (10 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), ceftazidime (30 µg), ofloxacin (10 µg) and sulphamethoxazole-trimethoprim (25 µg). However, some of the *E. coli* isolates showed some level of susceptibility to cefoxitin (60 %), a cephamycin; and also to nalidixic acid (66 %), and ciprofloxacin (30 %). *Klebsiella* species isolates were resistant to ciprofloxacin (80 %), meropenem (64 %), ofloxacin (70 %) and sulphamethoxazole-trimethoprim (64 %) (Figure 1). The percentage susceptibility of the *Klebsiella* species to gentamicin, ertapenem, imipenem, nalidixic acid, ceftazidime, and cefoxitin were 70 %, 80 %, 50 %, 56 %, 54 % and 72 % respectively (Figure 1).

The result of the screening of the *Enterobacteriaceae* isolates for the presence of metallo beta-lactamase (MBL) and the confirmation of the production of MBL enzymes by phenotypic confirmation test as per the CLSI criteria is as shown in Table 2. Out of the 39 isolates of *E. coli* screened for the production of metallo beta-lactamase (MBL) enzyme, only 29 isolates of *E. coli* were found to be resistant to either of the carbapenems, imipenem or meropenem. For *Klebsiella* species, only 21 isolates were suspected to produce MBL enzymes. The result of the phenotypic confirmation of MBL production in the enteric organisms showed that 9 isolates of *E. coli* (23.1 %) produced MBL enzyme phenotypically while only 6 isolates of *Klebsiella* species (representing 18.2 % of the total isolated *Klebsiella* species) produced MBL enzyme phenotypically (Table 2).

Table 1: Frequency of *Enterobacteriaceae* isolated from cloacal swab samples

Organism	Source	No of isolates (%)
Escherichia coli	Cloacae swabs	39 (78)
Klebsiella species	Cloacae swabs	33 (66)

Figure 1: Antibiogram of *Enterobacteriaceae*



Key: SXT = Sulphamethoxazole-trimethoprim

Table 2: Distribution of MBL-producing *Enterobacteriaceae*

Bacteria	No of isolates screened	No of isolates suspected to produce MBL	MBL negative n (%)	MBL positive n (%)
Escherichia coli	39	29	30 (77.0)	9 (23.1)
Klebsiella species	33	21	27 (81.8)	6 (18.2)

4. Discussion

Escherichia coli and *Klebsiella* species are amongst the most important Gram negative bacteria that cause several health problems clinically as a result of their high resistance to some available antibiotics. These organisms also account for a good percentage of many hospital visits across the globe; and most strains of *Escherichia* and *Klebsiella* are multidrug resistant in nature because they produce enzymes (such as metallo beta-lactamase) with exceptional ability to hydrolyze and render inefficacious some broad-spectrum antibiotics like the carbapenems (Ejiekugwu *et al*., 2014; Walsh *et al*., 2005). The production of metallo beta-lactamases (MBLs) in Gram negative bacteria especially those in the *Enterobacteriaceae* family including *Escherichia coli* and *Klebsiella* species is responsible for the multidrug resistant nature of these organisms. Metallo beta-lactamases (MBLs) are bacterial enzymes that are majorly plasmid-borne, and which mediate bacterial resistance to the carbapenems such as imipenem and meropenem – which are clinically used to treat multidrug resistance infections including those caused by extended spectrum beta-lactamase (ESBL)-producing bacteria (Ejikeugwu *et al*., 2014; Walsh *et al*., 2005). This present day study presumptively evaluated the occurrence of MBL-producing Gram negative bacteria (particularly *E. coli* and *Klebsiella* species) isolated from the cloacal swab samples of poultry birds in Abakaliki, Nigeria. The isolated *E. coli* and *Klebsiella* species isolates showed varying levels of resistance to the tested antibiotics used in this study. Notably, the *E. coli* isolates were multiply resistant to more than one class of antibiotics. The *E. coli* isolates showed resistance to gentamicin (50 %), ciprofloxacin (70 %), nalidixic acid (34 %), ceftazidime (76 %), ofloxacin (78 %), cefoxitin (40 %) and

sulphamethoxazole-trimethoprim (74 %). The resistance rates of *E. coli* isolates in this study is similar to the work of Eze *et al*., 2013 and Nwankwo *et al*., 2014 who reported higher resistance rates of *E. coli* from poultry origin to Gram negative antibiotics. The *E. coli* isolates showed varying rates of resistance to the carbapenems including imipenem (60 %), ertapenem (44 %) and meropenem (76 %). These results of *E. coli* resistance to the carbapenems agreed to results obtained by Chakraborty *et al*., 2010 and Franklin *et al*., 2006 who reported similar prevalence of MBL-producing bacteria in their respective studies. Our results of carbapenem resistance amongst the *E. coli* isolates were also close to the results obtained by Akinduti *et al*., 2012. The *Klebsiella* species isolated from the cloacal swab samples were resistant to gentamicin (30 %), ciprofloxacin (80 %), nalidixic acid (44 %), ceftazidime (34 %), ofloxacin (70 %), cefoxitin (28 %) and sulphamethoxazole-trimethoprim (64 %). Our study also shows that the isolated *Klebsiella* species were resistant to the carbapenems including imipenem (50 %), ertapenem (20 %) and meropenem (64 %). These results are in line with the work of Akinduti *et al*., 2012 – who also reported higher resistance rate of *Klebsiella* species to the carbapenems (particularly imipenem). The resistance rate of the isolated *Klebsiella* species to imipenem in our study (50 %) is lower than that reported by Akinduti *et al*., 2012. Bacterial resistance to the carbapenems especially imipenem and meropenem is indicative of the production of metallo beta-lactamase (MBL) enzymes by the organism as was previously reported (Ejikeugwu *et al*., 2014; Pitout *et al*., 2007). All the *Klebsiella* species isolates and *E. coli* isolates were phenotypically tested for the production of metallo-beta-lactamase (MBL) enzyme using the inhibition based assay – in which EDTA was incorporated as a chelating agent – since bacterial organisms producing MBL enzymes require zinc ions at the active site of the enzyme (i.e. MBL) for enzyme activity (Walsh *et al*., 2005). It was found in our study that 23.1 % of the *E. coli* isolates were positive for MBL enzyme while 18.2 % of the *Klebsiella* species were also positive for MBL enzyme production phenotypically. This result is similar to the work of Chakraborty *et al*., 2010 and Franklin *et al*., 2006 – who reported similar prevalence of metallo-beta-lactamase-producing Gram negative bacteria from clinical isolates. The prevalence of MBL-producing *Enterobacteriaceae* in Nigeria especially from environmental isolates is not known comprehensively due to so many factors. Limited surveillance studies on MBL-producing bacteria in both the community and hospital environments coupled with the difficulty experienced in screening bacterial isolates for MBL production may be responsible for the paucity of data on metallo-beta-lactamase-producing bacteria in this region. Present study emphasizes the growing trend of multidrug resistant Gram negative bacteria especially those in the *Enterobacteriaceae* family community samples. Bacterial organisms producing MBL enzymes pose grave threat to antimicrobial therapy – since these organisms are multidrug resistant in nature; and thus can remain viable even in the face of potent antimicrobial onslaught including the antimicrobial activity of the carbapenems (e.g. imipenem and meropenem). Strict antibiotic policy and alternative measures for rearing and production of food-producing animals (that does not include the use of antibiotics) is required now than ever to protect and sustain the efficacy of available antibiotics.

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