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Pseudomonas species from cattle dung producing extended spectrum and metallo beta-lactamases

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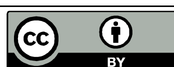

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ABSTRACT: Indiscriminate use of antibiotics in livestock contributes to emergence of antimicrobial resistance in pathogens co-habiting the gastro-intestinal tract of animals. This study was to determine the Extended Spectrum Beta-Lactamase (ESBL) and Metallo-Beta-Lactamase (MBL) production in *Pseudomonas* species from cattle fecal samples. Cattle dungs were collected from the University of Ibadan Cattle Ranch and the *Pseudomonas* species isolated using *Pseudomonas* Base Agar with *Pseudomonas* CN Selective Supplement were identified using standard tests. Phenotypic detection of ESBL and MBL was by double disk synergy test and Ethylene Di-amine Tetra Acetic Acid Combined Disk Test respectively. Antibiotics susceptibility tests was done using the disc diffusion technique against ten antibiotics. A total of 144 *Pseudomonas* species were isolated and identified as *P. aeruginosa* (71.5%), *P. fluorescens* (19.4%) and *P. stutzeri* (9.1%) and 19 (37.1%) produced ESBL including *P. aeruginosa* (15), *P. fluorescens* (2) and *P. stutzeri* (2) while, one (6.7%) ESBL *P. aeruginosa* produced MBL. All the ESBL producers were resistant to cefotaxime and trimethoprim; resistance of *P. aeruginosa* to ciprofloxacin was 93.3% and to ceftazidime was 80.0%, while it was 13.3% (colistin) and 6.7% (imipenem). The ESBL producing *P. fluorescens* were resistant to ceftazidime, ciprofloxacin and trimethoprim, likewise, the ESBL producing *P. stutzeri* showed resistance to gentamicin, ciprofloxacin and trimethoprim. The production of ESBL and MBL observed among the *Pseudomonas* species in this study with high level of resistance to some antibiotics portend public health risk, hence a need for caution in the use of antibiotics in animal husbandry.

Keywords: *Pseudomonas* species; Cattle dung; ESBL; MBL; Antibiotic resistance.

1. INTRODUCTION

In other to improve animal health and productivity especially in intensive reared species in agricultural industry, the use of antimicrobial agents is relied upon [1, 2]. The indiscriminate use of antimicrobial agents in livestock management either as food supplements or growth promoters has led to the emergence of Extended Spectrum Beta-lactamases (ESBLs) and Metallo-Beta-Lactamases (MBLs) producing bacteria including opportunistic pathogens such as *Pseudomonas* species of the gastrointestinal tract owing to mutations, selective pressure and the widespread of multi-drug resistance genes amongst bacteria [3]. The genus *Pseudomonas* is one of the most important members of the family Pseudomonadaceae which are Gram-negative bacilli, aerobic gamma-Proteobacteria with straight or sometimes marginally bent rod shape and one

or more polar flagella [4]. The use of antibiotics in the livestock production chain is usually seen as important in continuing a consistent supply of healthy and substantial animals, leading to greater profitability and efficiency [5]. Interestingly, many of the antimicrobial agents such as tetracycline, penicillin and sulphonamides that are important in human health are also used in animal food production [6]. It has also been observed that the application of mass medication known as metaphylaxis and the use of broad-spectrum antibiotics in animal husbandry especially for nontherapeutic use is linked to resistance in people who live on and near farms and even the general population via the food chain [7].

Extended Spectrum Beta-lactamases (ESBLs) are plasmid mediated beta-lactamases that mediate resistance to Extended Spectrum Cephalosporins (ESCs) including ceftriaxone, ceftazidime and cefotaxime and the monobactam such as aztreonam but have no effect on cephamycins and carbapenems. The ESBLs hydrolyzes the oxyimino-cephalosporins by cleaving structural beta-lactam ring but are inhibited by beta lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam [8]. Extended Spectrum Beta-lactamases have been increasingly reported to be produced by the members of family Enterobacteriaceae and by *Pseudomonas* species [9]. Metallo-Beta-Lactamases (MBLs) is enzymes capable of hydrolysing bicyclic beta-lactam antibiotics such as penicillins, cephalosporins and carbapenems with the exception of the monobactams. Metallo-Beta-Lactamases producing *Pseudomonas* species was first reported in Japan in 1991 and since then, there had been substantial increase in *Pseudomonas* producing MBLs worldwide [10]. The association of MBLs genes to mobile genetic elements has facilitated the dissemination of these enzymes among prevalent pathogens thereby, increasing the spread and outbreak of community and hospital acquired infection [10, 11]. This study was designed to determine the occurrence of ESBLs and MBLs production in *Pseudomonas* species isolated from cattle dung collected from the cattle ranch of the University of Ibadan, Nigeria and determine their antibiotic susceptibility pattern.

2. MATERIALS AND METHOD

2.1. Study site and sample collection

The study site was the University of Ibadan Cattle Ranch located in Abadina end of the University. A total of thirty (30) cattle dung samples were collected from the Ranch. The sample bottles were labelled appropriately, placed in ice packs and immediately transported to the Microbiology laboratory of the University of Ibadan for processing.

2.2. Isolation and identification of the *Pseudomonas* species

Pseudomonas species were isolated using the method of Kathiravan et al. [12]. *Pseudomonas* base agar (CM0559, Oxoid) supplemented with Pseudomonas C-N supplement (SR102, Oxoid), a selective media, prepared according to the manufacturers' instruction was used for the isolation of the *Pseudomonas* spp. One ml of the serial diluents (10^{-1}) of the samples was dispensed into appropriately labelled Petri dishes. Aseptically, *Pseudomonas* base agar cooled to about 45°C was dispensed into the aliquots of the samples in the Petri dishes and swirled gently, allowed to solidified and incubated at 37°C for 24-48 hours [12]. The isolates were characterized using standard morphological and biochemical tests including Gram staining, catalase, motility, oxidase, growth at 4°C, growth at 42°C and sugar fermentation tests including glucose, lactose, maltose, mannitol, sucrose, nitrate reduction, citrate [13].

2.3. Screening for potential Extended Spectrum Beta-Lactamases (ESBL) producing *Pseudomonas* species

All the isolates were subjected to antibiotics susceptibility testing using Kirby-Bauer disk diffusion test. The antibiotics discs used were ceftazidime (30 µg), cefotaxime (30 µg) and cefepime (30 µg) purchased from Oxoid, UK. Pure distinct colonies of 18-24 hours old culture were inoculated into sterile test tubes containing normal saline and its turbidity was adjusted to 0.5 McFarland standards. Mueller Hinton agar plates were prepared according to manufacturer's instructions and the standardized bacterial suspension was evenly inoculated on the surface of the agar plate by swabbing the entire surface of the agar. The antibiotics were placed on the culture plates with the aid of a sterile forceps and incubated at 37°C for 18-24 hours. The diameters of the zones of inhibitions were measured, recorded in mm and interpreted using Clinical Laboratory Standard Institute (CLSI) and those with reduced susceptibility were selected as potential ESBL-producers [14].

2.4. Phenotypic detection of Extended Spectrum Beta-Lactamases (ESBL) producing *Pseudomonas* species using Double Disk Synergy Test (DDST)

All the isolates that showed reduced susceptibility to ceftazidime (30 µg), cefotaxime (30 µg) and cefepime (30 µg) were selected for ESBL detection using double disk synergy test. This was done using disks of ceftazidime (30 µg), cefotaxime (30 µg) and cefepime (30 µg); which were placed adjacent to augmentin (amoxicillin-clavulanate 20 µg/10 µg) disk at the distance of 20 mm from it (centre to centre). The standardized bacterial test suspension was inoculated on Mueller Hinton agar plates by uniformly swabbing the entire surface of the agar plates and the inoculated plates were incubated for 18-24 hours at 37°C. Isolates producing ESBL were those with a clear cut indentation towards the amoxicillin-clavulanate disc [15].

2.5. Screening for potential Metallo-Beta-Lactamase (MBL) producing *Pseudomonas* species

Phenotypic screening of the isolates for MBL production was carried out by subjecting the isolates that produced ESBL to imipinem (30 µg) purchased from Oxoid (UK), using Kirby-Bauer disk diffusion test. Pure distinct colonies of 18-24 hours old culture of the isolates were inoculated into sterile test tubes containing normal saline and turbidity adjusted to 0.5 McFarland standards. The standardized bacterial suspension was evenly inoculated on the entire surface of Mueller Hinton agar plates by swabbing. The antibiotics were placed on the culture plates with the aid of a sterile forceps and incubated at 37°C for 18-24 hours. Isolates that showed inhibition zone diameter (IZD) of $\leq$ 23 mm were considered and suspected to produce MBL enzyme and these isolates were further tested using a phenotypic confirmation test [14].

2.6. Phenotypic detection of Metallo-Beta-lactamase (MBL) producing *Pseudomonas* species

The metallo-beta-lactamase production of the isolates was determined phenotypically using Ethylene Di-amine Tetra Acetic Acid (EDTA) Combined Disk Test (EDTA-CDT). One disk of imipenem (10 µg) and one with imipenem (10 µg) in combination with 0.5 M EDTA were placed at a distance of 20 mm, centre to centre, on Mueller Hinton agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity and incubated at 37°C for 18-24 hours. The MBL producers were those with zone of inhibition with a difference of 7 mm and above around imipenem disk containing EDTA compared to imipenem disk without EDTA [16].

2.7. Antibiotics susceptibility tests of the ESBL producing *Pseudomonas* species

Antibiotics susceptibility test of the ESBL-producing *Pseudomonas* species were carried out using the standard disk diffusion method recommended by Clinical Laboratory Standard Institute against ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), amoxicillin-clavulanate (20 µg/10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), Imipenem (10 µg), colistin (10 µg), trimethoprim (5 µg) and aztreonam (30 µg). The susceptibility test was carried out using pure colonies of 18-24 hours old culture adjusted to 0.5 McFarland Standards. The culture suspension was inoculated unto the surface of Mueller Hinton agar plates using sterile swab sticks. The antibiotics discs were placed on the inoculated plates with the aid of a sterile forceps and incubated at 37°C for 18-24 hours. The zones of inhibition were measured and interpreted according to Clinical Laboratory Standard Institute [14].

3. RESULTS

The average mean value of the total heterotrophic bacteria count obtained from the cattle dung was 2.3×10^{-6} cfu/g, with the highest mean value of 2.7×10^{-6} cfu/g from paddock 4 and the least (1.8×10^{-6}cfu/g) from paddock 1 (Table 1). A total of 144 *Pseudomonas* species were isolated including *P. aeruginosa* (71.5%), *P. fluorescens* (19.4%) and *P. stutzeri* (9.1%) (Table 2). Of the 144 *Pseudomonas* species, 19 (37.1%) were positive for ESBL production, comprising 15 (14.6%) *P. aeruginosa,* 2 (7.1%) *P. fluorescens* and 2 (15.4%) *P. stutzeri* (Table 2). In addition, only 1 (5.3%) *Pseudomonas aeruginosa* that produced ESBL also produced MBL (Table 2).

Table 1. Total Heterotrophic Bacteria Count (THBC) of isolates from the cattle dung.

	Sum of THBC ($\times10^{-4}$CFU/g)	Mean ± SD
Paddock 1	544	181.3 ± 17.0
Paddock 2	734	244.7 ± 31.1
Paddock 3	790	263.3 ± 19.4
Paddock 4	819	273.0 ± 17.7
Paddock 5	576	192.0 ± 13.1
Paddock 6	723	241.0 ± 24.0
Paddock 7	796	265.3 ± 13.0
Paddock 8	703	234.2 ± 20.5
Paddock 9	759	253.0 ± 8.2
Paddock 10	550	183.3 ± 15.0
Total	6994	233.1 ± 16.1

Table 2. Occurrence of Extended Spectrum Beta-Lactamases (ESBLs) and Metallo Beta-Lactamases (MBL) producing *Pseudomonas* species from cattle dung.

Isolates	No. of tested isolates	n (%) of positive ESBL	n (%) of positive MBL
P. aeruginosa	103	15 (14.6)	1 (6.7)
P. fluorescens	28	2 (7.1)	0 (0)
P. stutzeri	13	2 (15.4)	0 (0)
Total	144	19 (13.2)	1 (5.3)

The patterns of the antimicrobial resistance of the ESBL isolates showed that all the 19 (100%) isolates were resistant to trimethoprim and cefotaxime. Of the 15 *P. aeruginosa* that produced ESBL, 12 (80.0%)

showed resistance to ceftazidime, 9 (60.0%) to gentamicin while, 1 (5.3%) and 2 (13.3%) were resistant to imipenem and colistin respectively. However, none of these isolates showed resistance to cefepime and aztreonam. Furthermore, the two *P. fluorescens* that produced ESBL also showed resistance to ciprofloxacin and ceftazidime. Similarly, the two *P. stutzeri* ESBL producers showed resistance to gentamicin and ciprofloxacin, but the two *P. fluorescens* and *P. stutzeri* were fully susceptible to cefepime, aztreonam, imipenem and colistin (Table 3).

In addition, all the ESBL producers showed resistance to at least four different classes of antibiotics. Five (26.5%) of the isolates showed resistance to a combination of four antibiotics (CAZ-CTX-CIP-SXT) including four *P. aeruginosa* and one *P. fluorescens* while two isolates including one each of the *P. aeruginosa* and one *P. fluorescens* showed resistance to a combination of six (AMC-CAZ-CTX-CIP-GEN-SXT) antibiotics and one *P. aeruginosa* also showed resistance to eight (AMC-CAZ-CTX-CIP-GEN-CST-IPM-SXT) antibiotics (Table 4).

Table 3. Antibiotics resistant pattern of the ESBL producing *Pseudomonas* species isolated from the cattle dung.

Antibiotics	***P. aeruginosa*** **n=15 (%)**	***P. fluorescens*** **n=2 (%)**	***P. stutzeri*** **n=2 (%)**
Amoxicillin-clavulanate	6 (40)	1 (50)	0 (0)
Ceftazidime	12 (80)	2 (100)	1 (50)
Cefotaxime	15 (100)	2 (100)	2 (100)
Cefepime	0 (0)	0 (0)	0 (0)
Aztreonam	0 (0)	0 (0)	0 (0)
Imipenem	1 (6.7)	0 (0)	0 (0)
Gentamicin	9 (60)	1 (50)	2 (100)
Colistin	2 (13.3)	0 (0)	0 (0)
Ciprofloxacin	14 (93.3)	2 (100)	2 (100)
Trimethoprim	15 (100)	2 (100)	2 (100)

Table 4. Antibiotypes of the Extended Spectrum Beta-Lactamases (ESBLs) producing *Pseudomonas* species from the cattle dung.

Antibiotypes	***Pseudomonas aeruginosa*** **n=15**	***Pseudomonas fluorescens*** **n=2**	***Pseudomonas stutzeri*** **n=2**	**Total n=19**
CAZ-CTX-CIP-SXT	4 (26.7%)	1 (50%)	-	5 (26.5%)
CAZ-CTX-GEN-SXT	2 (13.3%)	-	-	2 (10.5%)
AMC-CTX-CIP-SXT	1 (6.7%)	-	-	1 (5.3%)
CTX-CIP-GEN-SXT	0 (0%)	-	1 (50%)	1 (5.3%)
CAZ-CTX-CIP-GEN-SXT	3 (20%)	-	-	3 (15.8%)
AMC-CAZ-CTX-CIP-SXT	2 (13.3%)	-	-	2 (10.5%)
CAZ-CTX-CIP-GEN-SXT	0 (0%)	-	1 (50%)	1 (5.3%)
AMC-CAZ-CTX-CIP-GEN-SXT	1 (6.7%)	1 (50%)	-	2 (10.5%)
AMC-CAZ-CTX-CIP-GEN-CST-SXT	1 (6.7%)	-	-	1 (5.3%)
AMC-CAZ-CTX-CIP-GEN-CST-IPM-SXT	1 (6.7%)	-	-	1 (5.3%)

Footnote: CAZ: Ceftazidime; CTX: Cefotaxime; FEP: Cefepime; AMC: Amoxicillin-clavulanate; CIP: Ciprofloxacin; CST: Colistin; GEN: Gentamicin; SXT: Trimethoprim; IMP: Imipenem.

4. DISCUSSION

The average mean value of the total heterotrophic bacterial count (THBC) (2.3×10^6 cfu/g) observed in this study is similar to the average mean value of 2.71×10^6 cfu/g reported from a previous study on cattle's faecal sample from an abattoir in Gombe State, northern part of Nigeria [17]. The observed highest mean value (2.7×10^6 cfu/g) in this study is not in agreement with 8.65×10^7 cfu/g from cow dung in Cross River, a city in the southern part of Nigeria [18]. The disparity might be due to differences in the plating techniques, while pour plate technique was employ in this study, spread plate technique was used in the latter study. Similarly, the least mean value (1.8×10^6 cfu/g) from this study is lower compared to 2.29×10^8 cfu/ml reported from another study on cow dung in India [19]. The difference might be due to the geographical locations. The high THBC mean value obtained from the cattle dung in each Paddock revealed the presence of high microbial load and a similar report attributed this to rich microbial diversity of animal guts [20].

The prevalence *P. aeruginosa* (71.5%) in this study is not in agreement with the previously reported 30.0% obtained in a similar study in Ebonyi, Southeastern Nigeria [21]. *Pseudomonas* species had been predominantly reported to be found on plant and water bodies and the animals from the present study were allowed to freely graze on open field pasture (grasses) surrounding the ranch with their water source from a nearby river [22]. In addition, the prevalence (9.1%) of *P. stutzeri* obtained from the present study was a bit higher than the 2.1% recently reported from a study carried out on fishes in Uganda [23]. The reason for the differences might be the studied samples and the fact that the source of drinking water for the animals in the present study is a nearby river, and aquatic environment had been reported to be potential reservoirs for *Pseudomonas* species [24, 25].

Furthermore, the 37.1% ESBL producing *Pseudomonas* species in this study is comparably similar to the 38.9% from a recent study on *Pseudomonas* species isolated from selected rivers in Ibadan [26]. This finding is also similar to 37.8% ESBL production from a study in Bangladesh on human clinical samples [27]. However, a much lower occurrence (15.0%) was reported in a study carried out on *Pseudomonas* species from mixed human samples in Enugu, Nigeria [28]. The reason for this disparity could be due the number of isolates studied. While 144 isolates were used in this study, only 20 isolates were studied in the latter research. More so, animals from which samples were collected from the present study were pre-exposed to treatment with various antibiotics including the beta-lactams classes and previous report had attributed high prevalence of ESBL producing *Pseudomonas* species in animal husbandry to the wide misuse and abuse of antibiotics [29]. In addition, the prevalence (6.7%) of ESBL and MBL co-production among the *P. aeruginosa* is comparably similar to the 5.1% previously reported from another study carried out on *P. aeruginosa* isolated from human clinical samples in India [30]. However, this observation is slightly higher than the 2.8% and 3.3% reported from studies on *P. aeruginosa* on clinical samples in France and Abeokuta, Nigeria respectively [31, 32]; but lower compared to the 18.6% previously reported in a similar study in Abakaliki, Nigeria [21]. The coexistence of ESBLs and MBLs enzymes in a single *P. aeruginosa* poses a public health risk to mankind owing to the fact that these genes are reported to be plasmid encoded and could be transferred from one organisms to another within the guts, thus conferring resistance to antimicrobial agents such as aminoglycosides, macrolides, carbapenems and sulphamethoxazole [33, 34].

Furthermore, the total resistance of the ESBL-producing *Pseudomonas* species observed in this study to trimethoprim is in agreement with the report of studies on commensal *Pseudomonas* species from wastewater and freshwater Milieus in the Eastern Cape Province, South Africa and human clinical samples in Iran [25, 34]. However, this observation is not in agreement with the 38.0% resistance reported on similar

isolates from camel in Egypt [35]. The discrepancy might be due to the differences in sampling source. In addition, the observed total resistance of ESBL-producing *P. fluorescens* and *P. stutzeri* and high (93.3%) resistance of *P. aeruginosa* to ciprofloxacin in this study contradict the report of other studies on abattoir wastewater in Ibadan, Nigeria and camels samples in Egypt from which none of the isolates and 33.3% *Pseudomonas aeruginosa* showed resistance to ciprofloxacin respectively [36, 37]. The reason for the high level of resistance in the present study may be due to the indiscriminate use of these classes of antibiotics in livestock management that might have led to selective pressure and development of resistance. It has also been revealed in previous report that the use of antibiotics as supplement in commercial feeds and as growth enhancers might have initiated resistance [38]. In addition, the mechanisms for resistance employ by *P. aeruginosa* to quinolones include: decreased in the amount of quinolones entering the cell because of the defect in the function of the porin channels and various efflux systems in the bacterial membrane [39].

The 6.7% resistance to imipenem among the ESBL-producing *P. aeruginosa* in this study is in agreement with the 6.0% previously reported on *P. aeruginosa* isolated from human blood, wound, sputum, cerebral spinal fluid, stool, ear and eye swab in Tehran [40]. However, that none of the ESBL-producing *P. fluorescens* and *P. stutzeri* showed resistance to imipenem is not in agreement with 6.7% that was obtained in another study carried out on ESBL producing *Pseudomonas* species isolated from human wounds, pus, urine aural sputum, throat umbilicus and conjunctiva samples [27]. The low resistance to imipenem may be because it is not readily available for the treatment of animal infections. Because the carbapenems such as imipenem are considered as last resort for treatment, there is a need for continued surveillance and judicious use of these antibiotics especially in livestock management [21]. Moreover, the 13.3% resistance to colistin by ESBL-producing *P. aeruginosa* is a public health challenge because colistin is regarded as one of the drug of choice and last drug of resort for the treatment of infections caused by multidrug resistance *P. aeruginosa*. The implication of this is that infections caused by these organisms may be difficult to treat. Furthermore, the observation that none of the ESBL-producing *P. fluorescens* and *P. stutzeri* showed resistance to cefepime, aztreonam and colistin is in contrast with the report of Chen et al. [41] in a study where the ESBL producing *P. aeruginosa* isolated from clinical specimens in Chinese Teaching Hospital showed a higher resistance (52.4%) to both cefepime and aztreonam. The reason for the differences may be due to samples studied.

The observation from this study that showed the ESBL-producing *P. aeruginosa* exhibiting multiple drug resistance to a combination of seven (7) different classes of antibiotics (AMC-CAZ-CTX-CIP-GEN-CST-IPM-SXT) is similar and comparable to a study carried on *P. aeruginosa* isolated from wastewater generated from an abattoir in Ibadan, Nigeria which showed resistance to a combination of 8 different classes of antibiotics (AMP-TET-CHL-CRO-OFX-CLX-STR-SXT) but higher than the one reported in another study on commensal *Pseudomonas* species from wastewater and fresh water milieus in South Africa which showed resistance to the combination of four (4) different classes of antibiotics (PG-OX-CD-RP) [25, 36]. The observed resistance in the strains of *Pseudomonas* species obtained from cattle is alarming as these animals could serve as potential reservoirs of these resistant strains and could be deposited into the environment in the form feces/urine often used as manure on crop produce. These bacterial strains could be transmitted directly and indirectly to human because cattle meat are frequently consumed in Nigeria as part of diet in a roasted or cooked form and thus, posing serious health threat when such meat product harboring these pathogens are not properly cooked [22].

The resistance patterns of ESBL-producing *Pseudomonas* species against the various antibiotics tested in the present study showed that all the isolates obtained from cattle dung were multidrug resistant as they showed resistance to a combination of three or more different classes of antibiotics. The ability of

Pseudomonas species to acquire and harbour various resistance determinants allows only limited classes of antibiotics for effective treatment of infection caused by it.

5. CONCLUSION

The ESBL and MBL producing *Pseudomonas* species in this study showed high level of resistance to some commonly available antibiotics especially the beta-lactams. The emergence and spread of these bacteria in cattle might be as a result of the indiscriminate use of these antibiotics and intrinsic resistance properties which could portend public health risk to mankind and the environment. Hence, the use of antibiotics in animal husbandry should be regulated.

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