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Extended spectrum and metallo-beta-lactamase *Pseudomonas* species from poultry and piggery waste

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ABSTRACT: Beta-lactamase producing bacteria have become a public health burden due to antibiotics usage in livestock production. This study was carried out to detect extended spectrum beta-lactamase (ESBL) and metallo-beta-lactamase (MBL) producing *Pseudomonas* spp. from poultry droppings and piggery dung in Ibadan. Poultry droppings and piggery dung were collected from the University of Ibadan livestock farms while isolation of *Pseudomonas* spp. was done using *Pseudomonas* base agar supplemented with *Pseudomonas* C-N supplement and were conventionally characterized. Detection of ESBL and MBL producing isolates were by double disc synergy test and imipenem-EDTA combined disc test respectively. Antimicrobial susceptibility test was by disc diffusion method against trimethoprim (5 µg), amoxicillin/ clavulanate (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), impenem (10 µg), gentamicin (10 µg) and ciprofloxacin (10 µg). A total of 108 *Pseudomonas* spp. were isolated comprising 53.7% from poultry droppings and 46.3% from piggery dung. The isolates include *P. aeruginosa* (63.0%), *P. putida* (24.0%) and *P. stutzeri* (13.0%). While the ESBL producers were *P. aeruginosa* (10.2%) and *P. stutzeri* (1.9%), none of the isolates produced MBL. However, 63.6% the ESBL producers showed resistance to trimethoprim while 61.5% were multidrug resistant. The high prevalence of antibiotics resistance and multidrug resistant strains observed among the *Pseudomonas* spp. infer that poultry droppings and piggery dung can serves as a reservoir for growth and dissemination of clinically significant antibiotics resistance among bacterial species.

Keywords: Livestock; Antibiotics resistance; ESBL; MBL; *Pseudomonas* species.

1. INTRODUCTION

The use of antibiotics in livestock production has aggravated the risk factor associated with the development and distribution of antibiotics resistance from animal husbandry [1]. Antibiotic resistance is a worldwide problem and new forms of antibiotic resistance emerges each year which has cross international boundaries and spread between continents with ease. The World Health Organisation (WHO) described antibiotic-resistant bacteria as “nightmare bacteria” or “superbugs” that pose catastrophic threat to the public health [2]. According to the United State report, it was estimated that at least 2,049,442 illnesses were caused by antibiotics resistant infections resulting into about 23,000 deaths [3]. The use of antibiotics in agricultural

settings has been reported to exert selective pressure which favours the survival of resistant strains of bacteria over susceptible ones, resulting to increase in resistant bacteria within the microbial communities [4].

Antibiotics has been reported to be widely and indiscriminately used in Nigeria as additives to feeds and water, to treat diseases and promote animal growth in livestock production [5, 6]. This practice has led to increase in the emergence of antibiotics resistant in livestock production such as poultry and piggery. More so, majority of the animal farms in Nigeria have no waste treatment facility, hence, all the waste generated in the farm are either dumped in a farmland or in a remote area such as in or close to water bodies [7]. Wastes generated from the livestock are also used as feed supplement to generate maggot for feeding fishes in aquaculture. This could constitute threat to humans inhabiting the vicinities where these animal wastes are dumped because of the possibilities of being exposed to antibiotics resistant bacteria in the waste.

Pseudomonas species are emerging opportunistic pathogen of clinical importance that survives in harsh conditions and are also reported to be naturally resistant to penicillin and most related beta-lactam antibiotics. *Pseudomonas aeruginosa* is one of the bacteria most frequently responsible for nosocomial and community acquired infections. Development of resistance to β-lactams in clinical strains of *P. aeruginosa* has been reported to be associated with the production of acquired β-lactamases, constitutive overproduction of the cephalosporinase, AmpC or non-enzymatic mechanisms such as drug efflux or outer membrane impermeability [8, 9].

Extended spectrum beta-lactamase and metallo-beta-lactamase production have been reported as some of the mechanisms that leads to the increase in resistance of *Pseudomonas* spp. to beta-lactam drugs [10]. A lot of studies have investigated the occurrence of ESBL and MBL producing Enterobacteriaceae in livestock waste but there is dearth of information of ESBL and MBL producing *Pseudomonas* spp. in livestock wastes. Hence, the present study is aimed at determining the occurrence of ESBL and MBL producing *Pseudomonas* spp. isolated from poultry droppings and piggery dung in Ibadan, Nigeria.

2. MATERIALS AND METHODS

2.1. Sampling site and sample collection

Poultry droppings and piggery dung were collected from the University of Ibadan poultry farm (latitude 7.4401, longitude 3.900) and piggery farm (latitude 7.4401, longitude 3.8996) between the months of March and April 2018. The samples were labeled appropriately, preserved in ice packs and transported to Environmental Laboratory, Department of Microbiology University of Ibadan for immediate analyses.

2.2. Isolation and identification of the *Pseudomonas* species

Isolation of *Pseudomonas* species was done using the standard pour plate technique on *Pseudomonas* base agar (CM0559, Oxoid, Basingstoke, UK) supplemented with pseudomonas C-N supplement (SR102, Oxoid, Basingstoke, UK) a selective medium for the isolation of the *Pseudomonas* species. The inoculated plates were incubated at 37^{o}C for 48 hours. Distinct bacteria growth with either bluish or yellowish green pigment, irregular margin, smooth or crenate or rugose surface, semi translucent or raised colony were selected as probable *Pseudomonas* spp. and sub cultured by repeated streaking on nutrient agar to obtain the pure culture. Characterization of these isolates was done based on their distinct pigment production and conventional biochemical tests [11].

2.3. Screening for potential extended spectrum beta-lactamase producing *Pseudomonas* species

Potential ESBL-producing *Pseudomonas* spp. screening was done using ceftazidime (30 µg) and

cefotaxime (30 µg). Isolates showing reduced susceptibility to at least one of these drugs with zone of inhibition for ceftazidime ≤ 22 mm and cefotaxime ≤ 27 mm were considered as potential ESBL producing strains [12].

2.4. Phenotypic detection of extended spectrum beta-lactamase producing *Pseudomonas* species

Pseudomonas species that were suspected to be potential ESBL producers were confirmed for ESBL production using the double disk synergy test [13]. Sterile cotton swab was used to inoculate the standardized inoculum (corresponding to 0.5 McFarland standard) on Mueller Hinton agar plate. Two disks of 3rd generation cephalosporins (ceftazidime 30 µg, cefotaxime 30 µg) and fourth generation cephalosporin (cefepime 30 µg) disks were placed at 20 mm distance center to center from amoxicillin/clavulanate (30 µg) disk preceding incubation for 18-24 hours at 35°C. Augmentation of the zone of inhibition of any one of the three cephalosporin antibiotics disks towards amoxicillin/clavulanate indicated the presence of extended-spectrum beta-lactamases.

2.5. Antibiotics susceptibility test of the extended spectrum beta-lactamase producing *Pseudomonas* isolates

Antibiotic susceptibility test of the ESBL producing *Pseudomonas* species was carried out against nine antimicrobial agents using the standard Kirby-Bauer disc diffusion method [12]. The antibiotics used include the following classes of antibiotics: beta-lactam inhibitor (amoxicillin/clavulanate 30 µg), broad spectrum cephalosporin from the 3rd and 4th generation (ceftazidime 30 µg, cefotaxime 30 µg, and cefepime 30 µg), monobactam (aztreonam 30 µg), carbapenem (imipenem 10 µg), aminoglycosides (gentamicin 10 µg), fluoroquinolone (ciprofloxacin 5 µg), and folate pathway inhibitor (trimethoprim 5 µg).The antibiotic disks were placed on Mueller Hinton agar plates inoculated with the standardized inoculum and incubated at 35^{o}C for 18-24 hours. The zones of inhibition were measured in millimeter and interpreted based on CLSI guidelines [12]. Isolates showing resistance to at least three different classes of antibiotics were considered as multidrug resistant strains [14].

2.6. Phenotypic detection of metallo β-lactamase (MβL) activity in ESBL-producing *Pseudomonas* species

Imipenem resistant ESBL-producing *Pseudomonas* isolates were tested for MBL production using imipenem and ethylenediaminetetraacetate (EDTA) combined disk test. Two imipenem disks were placed at a distance of 5 cm from each other on Mueller Hinton agar plate inoculated with the standardized inoculum and 10 µl of 0.5 M EDTA solution were added to one of the imipenem disk. Any of the isolates that showed increase of zone of inhibition ≥7 mm between imipenem+EDTA disk in comparison with the imipenem disk alone after 18-24 hours incubation were considered MBL producers [15].

3. RESULTS

A total of 108 *Pseudomonas* spp. were isolated comprising 58 (53.7%) from poultry droppings and 50 (46.3%) form piggery dung. Identification of the isolates showed that 63.0% of the *Pseudomonas* species were *P. aeruginosa*, 24.0% were *P. putida* while 13.0% were *P. stutzeri* (Table 1). The ESBL producers were *P. aeruginosa* (10.2%) and *P. stutzeri* (1.9%), while none of the isolates produced MBL (Table 2). The result of the antibiotics susceptibility test showed that 71.4% and 50.0% of the ESBL producing *P. aeruginosa* isolated from poultry droppings and piggery dung were resistant to trimethoprim respectively. Furthermore,

28.6% of the ESBL producing *P. aeruginosa* from the poultry droppings showed resistance to the following antibiotics: amoxicillin/clavulanate, ceftazidime, imipenem and ciprofloxacin. It was also observed that 42.9% of the ESBL producing *P. aeruginosa* were resistant to gentamicin and cefotaxime respectively, while none of the isolates exhibited resistance to cefepime and aztreonam.

Table 1. Prevalence of the *Pseudomonas* species isolated from the poultry droppings and piggery dung.

Samples	Isolates n (%)			Total n (%)
	P. aeruginosa	***P. stutzeri***	***P. putida***	
Poultry droppings	32 (47.1)	7 (50.0)	19 (73.1)	58 (53.7)
Piggery dung	36 (52.9)	7 (50.0)	7 (26.9)	50 (46.3)
Total	68 (63.0)	14 (13.0)	26 (24.0)	108 (100)

Table 2. Extended Spectrum Beta-lactamase producing *Pseudomonas* spp. from the poultry droppings and piggery dung.

Samples	No. of isolates screened	ESBL producing *Pseudomonas* species n (%)			Total n (%)
		P. aeruginosa	***P. stutzeri***	***P. putida***	
Poultry droppings	58	7 (12.1)	1 (1.7)	0 (0)	8 (13.8)
Piggery dung	50	4 (8.0)	1 (2.0)	0 (0)	5 (10.0)
Total	108	11 (10.2)	2 (1.9)	0 (0)	13 (12.0)

Table 3. Antibiotics resistance pattern of the ESBL-producing *Pseudomonas* spp.

Antibiotics	ESBL positive isolates from each samples, n (%)					
	Pseudomonas aeruginosa			***Pseudomonas stutzeri***		
	Poultry droppings (n=7)	Piggery dung (n=4)	Total (n=11)	Poultry droppings (n=1)	Piggery dung (n=1)	Total (n=2)
TMP	5 (71.4)	2 (50.0)	7 (63.6)	0 (0.0)	1 (100)	1 (50.0)
AMC	2 (28.6)	3 (75.0)	5 (45.5)	0 (0.0)	1 (100)	1 (50.0)
CTX	3 (42.9)	1 (25.0)	4 (36.4)	0 (0.0)	1 (100)	1 (50.0)
CAZ	2 (28.6)	3 (75.0)	5 (45.5)	0 (0.0)	1 (100)	1 (50.0)
FEP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	1 (50.0)
ATM	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
IPM	2 (28.6)	0 (0.0)	2 (18.2)	0 (0.0)	0 (0.0)	0 (0.0)
GEN	3 (42.9)	1 (25.0)	4 (36.4)	1 (100)	0 (0.0)	1 (50.0)
CIP	2 (28.6)	0 (0.0)	2 (18.2)	1 (100)	0 (0.0)	1 (50.0)

Key: TMP = Trimethoprim (5 µg), AMC = Amoxicillin/Clavulanate (30 µg), CTX = Cefotaxime (30 µg), CAZ = Ceftazidime (30 µg), FEP = Cefepime (30 µg), ATM = Aztreonam (30 µg), IMP = Imipenem (10 µg), GEN = Gentamicin (10 µg), CIP = Ciprofloxacin (5 µg).

In addition, 75.0% of the ESBL producing *P. aeruginosa* from the piggery dung were resistant to amoxicillin/clavulanate and ceftazidime; 25.0% to cefotaxime and gentamicin while none of these isolates showed resistance to cefepime, imipenem and gentamicin. Furthermore, the two *P. stutzeri* isolated from the poultry dropping that produced ESBL also showed resistance to trimethoprim, amoxicillin/clavulanate,

cefotaxime, ceftazidime and cefepime. More so, one of the two (50.0%) *P. stutzeri* that was isolated from piggery dung showed resistance to trimethoprim, amoxicillin/clavulanate, cefotaxime, ceftazidime, cefepime, gentamicin and ciprofloxacin (Table 3). The Antibiotypes of the ESBL-producing *Pseudomonas* species showed that eight (61.5%) of the isolates that produced ESBL and showed resistance to antibiotics were multidrug resistance. In addition, two *P. aeruginosa* were resistant to three different antibiotics that include TMP-AMC-CTX and one *P. stutzeri* also showed resistance to five different antibiotics including TMP-AMC-CTX-CAZ-FEP (Table 4).

Table 4. Antibiotypes of the ESBL-producing *Pseudomonas* species.

Antibiotypes	Classes of Antibiotics	*P. aeruginosa*	*P. stutzeri*	Total
CAZ	1	2	0	2 (15.4)
IMP	1	1	0	1 (7.7)
AMC-CAZ	2	1	0	1(7.7)
GEN-CIP	2	0	1	1(7.7)
TMP-AMC-CTX	3	2	0	2(15.4)
TMP-AMC-CAZ	3	1	0	1(7.7)
TMP-CAZ-GEN	3	1	0	1(7.7)
TMP-CTX-GEN	3	1	0	1(7.7)
TMP-AMC-CTX-GEN	4	1	0	1(7.7)
TMP-IMP-GEN-CIP	4	1	0	1(7.7)
TMP-AMC-CTX-CAZ-FEP	5	0	1	1(7.7)

Key: TMP = Trimethoprim (5 µg), AMC = Amoxicillin/Clavulanate (30 µg), CTX = Cefotaxime (30 µg), CAZ = Ceftazidime (30 µg), FEP = Cefepime (30 µg), ATM = Aztreonam (30 µg), IMP = Imipenem (10 µg), GEN = Gentamicin (10 µg), CIP = Ciprofloxacin (5 µg).

4. DISCUSSION

In the present study, the production of ESBL producing *Pseudomonas* species isolated from poultry waste and piggery was determined. The observation from this study that showed the occurrence of *P. aeruginosa* being the highest (63.0%) followed by *P. putida* (24.0%) and *P. stutzeri* (13.0%) is in agreement with the occurrence of similar isolates from a study on clinical samples in Bangladesh in which the occurrence of *P. aeruginosa* was higher than other species [16]. However, this is not in agreement with a report from a study carried out on water samples in Danube where the most prevalent isolate was *P. putida* and *P. aeruginosa* was the least [17]. The disparity may be attributed to different studied samples. Furthermore, the findings from this present study that showed 12.1% and 8.0% of the *P. aeruginosa* isolated from the poultry droppings and piggery waste being ESBL producers contradicts the report from another study on cattle fecal sample in Benin city, Nigeria where none of the *P. aeruginosa* was reported to have produced ESBL [18]. While the present study was on poultry droppings and piggery waste which could have been responsible for the observed difference, the numbers of isolates (10) considered in the latter study compared to the 108 isolates in this study might be responsible for the inability to detect ESBL producers. In addition, the 10.2% ESBL producing *P. aeruginosa* observed in this study is lower compared to the 27.5% ESBL producing *P. aeruginosa* reported in a previous study on clinical isolates in Egypt [19]. This noticeable

difference is not strange because a higher resistance to antibiotics including ESBL production is expected from clinical isolates compared to isolates from environmental samples.

The observation from this study that none of the ESBL producing *Pseudomonas* spp. produced MBL is in agreement with the report of a study on human urine, pus, body fluids, sputum and blood where none of the ESBL producing isolates also produced MBL [20]. The high resistance of *P. stutzeri* (100%) and *P. aeruginosa* (71.4%) from piggery dung and poultry droppings to trimethoprim in this study is of great concern. Although, trimethoprim has not been reported to be widely used in animal husbandry especially in Nigeria, sulphonamides has been reported to be widely used [21]; hence, resistance to trimethoprim might have co-evolved with resistance to sulphonamides since they have the same mechanism of action (inhibition of foliate synthase pathway). In addition, the intrinsic resistance possessed by *Pseudomonas* spp. against trimethoprim and the fact that the antibiotic is bacteriostatic might also be responsible for the higher resistance observed by *P. aeruginosa* and *P. stutzeri* to trimethoprim in this study.

In this present study, the observed high resistance of *P. stutzeri* and *P. aeruginosa* from piggery dung to amoxicillin/clavulanate which were 100.0% and 75.0% is similar to the report of a study on hospital drains in South Africa [22]. However, a lower resistance of *P. aeruginosa* (28.6%) and *P. stutzeri* (0%) among the ESBL producers from the poultry droppings to amoxicillin/clavulanate was obtained compared to the 90.0% previously reported from a study also on poultry droppings in Nigeria [23]. The reason for the differences may be as a result of different studied isolates; the latter study was on *E. coli* while the present study was on *Pseudomonas* spp. Similarly, the 28.6% and 0% resistance of ESBL producing *P. aeruginosa* and *P. stutzeri* respectively from the poultry droppings in this study to cefotaxime and ceftazidime is far lower compared to the 100% resistance reported on similar isolates in a study on clinical isolates in Nigeria and the 100% resistance of *P. aeruginosa* from a study on camel meat in Egypt [24, 25]. One of the reasons for the disparity may be the method of bacterial isolation, while in the latter study, the third generation cephalosporin (ceftazidime and cefotaxime) was incorporated into media for isolation, *Pseudomonas* spp. were isolated without the incorporation of any antibiotics in the present study to prevent eliminating species that may be susceptible to these antibiotics but resistant to others. Therefore incorporating antibiotics in medium for isolation might have exerted selective pressure for the survival of resistance strains over susceptible ones such that those isolated strains showed higher resistance to the incorporated antibiotics than other antibiotics.

Furthermore, the observation from this study that none of the *P. aeruginosa* obtained from the studied samples showed resistance to cefepime is in agreement with the report of another study on poultry droppings and cow dung in South-west Nigeria [24]. However, this finding is in contrast to the report of another study carried out in India on various clinical samples with the resistance of 74.0% of the *Pseudomonas* isolates to cefepime [26]. This may be an indication that the usage of cefepime is more in human medicine than in veterinary medicine.

Also, that none of the *P. aeruginosa* and *P. stutzeri* in this study was observed to have exhibited any resistance to imipenem is similar to the previously reported resistant patterns of the isolates from poultry droppings and cow dung collected from different geographical location in South-western Nigeria [24]. However, this finding is not in agreement with other studies from which it was reported that 8.7% and 1.6% showed resistance to imipenem from a study on bovine meat in Abidjan and dairy farm samples in Nottingham [27, 28]. The low resistance of *Pseudomonas* species to carbapenems such as imipenem observed in the studied samples in this present study as well as other environmental samples in other studies may be because carbapenems are considered as antimicrobial agent of last resort and effective against the treatment of

infections caused by ESBL-producing bacteria. These antibiotics are able to resist the hydrolytic activity of beta-lactamase enzymes [29, 30]. Furthermore, resistance of *P. aeruginosa* isolated from poultry droppings observed in the present study to gentamicin (42.9%) and ciprofloxacin (26.8%), is comparably similar to the 39.0% resistance reported from a previous study carried out on clinical samples in India [31]. Meanwhile, resistance to these antibiotics by *P. aeruginosa* has been previously reported to be linked to the mutation in the *gyrA* gene encoding the A subunit of the target enzyme, DNA gyrase [32]. The observed multidrug resistance (61.5%) of the ESBL producing *Pseudomonas* spp. in the present study is high and is also comparably similar to the 75.8% reported from Egypt on a study on clinical samples [19]. Such multiple antibiotics resistant in *Pseudomonas* spp. has been attributed to combination of acquisition of resistance gene through genetic exchange and mutation, as well as physiological mechanism such as the possession of specific protein, poor membrane permeability, biofilm formation and efflux pumps [33-35].

5. CONCLUSION

The high prevalence of antibiotics resistance as well as multidrug resistance strains of *Pseudomonas* spp. in this study is an indication that both the poultry droppings and piggery dung can serves as a reservoir for the development and dissemination of clinically significant antibiotics resistant among bacterial pathogens. Therefore, cautious efforts should be made to limit the misuse of antibiotics in animal husbandry and proper discharge of livestock waste into the environment should be enforce in other reduce emergence of antibiotics resistant organisms that can lead to outbreak of infection by antibiotics resistant bacteria.

Authors Contributions: OIF designed the study and protocol, supervised the study, managed literature search, data acquisition and analysis, revised the manuscript. EOI managed literature search, data acquisition and analysis, wrote the first draft. Both authors read and approved the final manuscript.

Conflict of Interest: The authors declare no conflicts of interest.

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