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Occurrence and phenotypic characterization of ESBL-producing *Escherichia coli* from piggery farms wastewater in South Western Nigeria

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Abstract: This study examined the occurrence of extended spectrum beta lactamase activity in *Escherichia coli* isolated from wastewaters of selected pig farms. Bacterial isolation was done by standard method and identification was achieved following recommended biochemical tests. Isolates obtained were tested for susceptibility to antibiotics using the Kirby Bauer disk diffusion method while phenotypic characterization to detect ESBL activity was done using the combination disc method. A total of 131 *E. coli* isolates were gotten from the study farms with the highest prevalence from the farm settlement. Antibiotic resistance (AR) among *E. coli* ranged between 0% and 89.6% to colistin and tetracycline respectively. Resistance levels were observed to be highest for ampicillin (82.1%) and tetracycline (89.6%) across sampled farms. ESBL activity among isolates ranged between 10.6% and 59.6% and all ESBL positive isolates were multiple antibiotic resistant (MAR) to at least three unrelated antibiotic classes. Predominant MAR phenotypes included ampcilin-tetracycline-cefpodoxime-sulfamethoxazole/trimethoprim-erythromycin-nalidixic acid-chloramphenicol and ampicillin-tetracycline-cefpodoxime-sulfamethoxazole/trimethoprim-erythromycin-ceftriaxone-nalidixic acid-chloramphenicol-gentamycin; found in isolates from the three farms with the highest occurrence in the farm settlement. Our study showed that piggery wastewater could be a significant reservoir of multiple antibiotic resistant bacteria with ESBL activity.

Keywords: Antibiotic Resistance. ESBL. Wastewater. Piggery

Introduction

Upsurge in antimicrobial resistance is an increasing crisis in clinical as with veterinary medicine worldwide. With the expansion in animal farming practises comes the attendant use of antibiotics for the treatment of infectious diseases and general maintenance of animals. The huge application of antibiotics in animal husbandry has been the focus of recent research due to its contribution to the development and spread of antibiotic resistance (Li, 2017; Tang *et al*., 2017). Though, much of the problems due to antibiotic resistance has been credited to its use in human medicine in the past, studies have however come to substantiate the role played by livestock management, in addition to use in human medicine as key drivers in the increasingly emergence of resistance in a range of microorganisms (Ventola, 2015).

As with other countries of the world, there has been a marked increase in livestock farming especially pig farming in Nigeria. While many rears in large scales in secluded farm settlements, many others, perhaps due to low financially exerting requirements of rearing pigs keep these animals intensively in-home backyards placing them in close proximity to immediate human environments (Manyi-Loh *et al*., 2018).

The peculiarity of animal farming allows to a great length, the misuse of antimicrobials as they are widely administered for purpose of prophylaxis, as well as for growth enhancement in addition to treatment of infectious diseases (Jacopo, *et al.,* 2020). Pig rearing particularly has been associated with application of more antimicrobials than most of other animal husbandry practises especially the prophylactic administration of antibiotics (Dawangpa *et al*., 2022). Varied types of antibiotics are used in pig farming and studies have reported the use of clinically relevant drugs with the beta lactam being a prominent member (Zhang *et al*., 2015). Large application of antibiotics in pig farming has been correlated to development of antibiotic pressures in resulting wastewater which support the acquisition of antibiotic resistant bacteria and AR genes (Manyi-Loh *et al*., 2018). The production of ESBL remain one of the leading means by which bacteria may express this fitness. ESBLs are a group of hydrolytic enzymes able to cause loss of potency of Beta lactam drug thereby resulting in therapeutic failure (Rawat and Nair, 2010). Furthermore, ESBL-producing organisms have the capacity to extend resistance to even unrelated classes of antibiotics. The uninhibited access to antibiotics by farmers, near zero monitoring to enforce hygiene standard that characterise our farming operations in Nigeria clearly raises a red flag. More so, its most worrisome that quite a number of pig farms spring up on daily basis and wastewater generated from are simply discharged unto the environment with no prior treatment.

Numerous pathways of risk can arise from this action as resistant bacteria can move to contaminate groundwater and surface water via runoff can lead to further build-up of reservoirs of antibiotic resistance and antibiotic residue. The objective of this study is to investigate the prevalence and level of antibiotic resistance among *Escherichia coli* isolated from three selected farms located on Lagos and Ogun State, Southwestern Nigeria.

Materials and Methods

Description of study site and collection of samples

Three commercial pig farms were selected for this study. One of these is a pig farm settlement in Lagos state prominently bothering with Ogun state. The others are situated in Awa and Ijebu Igbo towns of Ogun State (Fig 1). They are designated Farm A, B, C, respectively. The farm in Lagos was the largest of the studied farms. It provided a space for different breeders to keep their pig pens separately but within a common space. Farm B is moderately large, intensively farmed piggery while Farm C is managed as a home sited pig farm. A verbal one on one interaction was conducted on the pig managers who majorly appeared semi-literates. Information sought revealed the use of antibiotics majorly in the tetracycline and penicillin class. They admitted to engaging veterinary doctors only when self-medication appeared to have failed. Dosing did not follow a particular pattern. A farmer in one of the sites admitted stepping up recommended dose when the action of the drug appears slow. Free accessibility to antimicrobials was evident as farmers were able to purchase drugs and antibiotics off the counter of pet shops and only one of the farm managers could relate with the consequences that may arise with arbitrary use of antimicrobials.

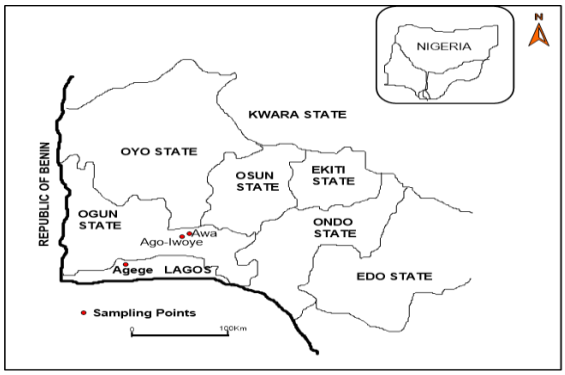

Fig 1. Map showing the location of study pig farms

Sample collection

Samples of wastewaters were collected from three independent points on each farm into sterile sample bottles weekly for a period of three months spanning June-July 2019. Samples were transported in ice packs to the Microbiology Laboratory, Department of Microbiology, Olabisi Onabanjo University Ago-Iwoye, Ogun state where they were analyzed within eight hours of collection.

Isolation of *Escherichia coli*

Escherichia coli were isolated by aseptically inoculating two microliters (2ml) of wastewater samples from each pig farm's wastewater samples into Luria Bertani broth (Oxoid, Basingtoke, UK) as a pre-enrichment medium for the growth of bacteria. The broth was incubated at 37ºC for 18-24 hours. After incubation, the above-described setup was inoculated (streaked) on a selective medium, Eosin Methylene Blue (EMB) Agar to isolate *Escherichia coli*. The inoculated agar plates were incubated at 37ºC for 18-24 hours. The plates were observed for growth and morphological distinct colonies of bacteria were selected and repeatedly sub-cultured to obtain pure cultures. Isolates were stored on nutrient agar slants and kept at 4ºC, to serve as working cultures for further analysis. Identification of bacteria was aided by carrying out biochemical testing indole, sugar fermentation tests, lysine decarboxylase test, urease, citrate utilization, methyl red, coagulase and motility tests (Edward and Ewing, 1972).

Antimicrobial susceptibility testing

Isolates were screened for susceptibility to antibiotics using the disc diffusion method and following the protocol recommended by the Clinical laboratory standard institute (Kirby-Bauer, 1996). CLSI (2018) guideline. Antibiotics used consisted of a panel of 10 antibiotics; ampicillin, tetracycline, cefpodoxime, sulfamethoxazole/trimethoprim, gentamycin, chloramphenicol, colistin, erythromycin, ceftriaxone, and nalidixic acid. The inoculums were prepared following the growth method and the turbidity of resulting suspension was adjusted to 0.5 MacFarland standard. The suspension was introduced uniformly onto the surface of already prepared Mueller Hinton agar plate with the aid of sterile swab stick, followed by the discs and the set-up was incubated for 18-24 hours at 35±2ºC. The diameter of zones of inhibition were measured and interpreted using the clinical and laboratory standard institute (CLSI, 2018) criteria. Multidrug-resistance (MDR) was defined as isolates resistant to at least three (3) different classes of the antibiotics tested

Phenotypic Detection of ESBL

Phenotypic detection of ESBL producing isolates was done using the combination disc test method. Isolates that were resistant to cefpodoxime were selected and screened on ready prepared plates of brilliance ESBL agar (Oxoid, Basingstoke UK) by inoculating suspension of single well isolated colonies of test isolates on said agar. The plates were incubated aerobically for 24hr at 37C and thereafter assessed following manufacturers directive. Isolates were then subjected to the combination disk test using cefpodoxime and cefpodoxime/clavulanic disc set (Oxoid, Basingstoke UK).

Results and Discussion

A total of one hundred and thirty-one *E. coli* isolates were recovered from collected wastewater samples. *E. coli* was detected in all the sampled wastewaters. The highest prevalence was at the Lagos state piggery farm A with eighty-nine (44.3%) isolates. Sixty-five and 47 isolates were recovered from Farms B and C, which corresponds to 32.3% and 23.3% of the total isolated *E. coli* respectively (Table 1).

Table 2 is showing the result of antibiotic sensitivity testing. Generally, isolates from the three farms displayed varying levels of resistance to antibiotics, the highest being from Farm A and closely followed by isolates from Farm B (Table 2). Highest AR was against tetracycline 89.6% among Farm A isolates; correspondingly, there was zero resistance displayed towards colistin (100% susceptibility) across the three sampled farms while moderate to high levels of resistances were displayed towards cefpodoxime (54.2%), sulfamethoxazole/trimethoprim (52.2%), erythromycin (55.2%), ceftriaxone (28.9%) and chloramphenicol (25.4%) (Table 2).

Table 1. Distribution of isolates in the three sampled towns under study

Sampling town	Number of recovered isolates (%)
Agege, Lagos	89 (44.28%)
Awa	65 (32.34%)
Ago-Iwoye	47 (23.38%)
Total	201

Table 2. Antibiotic resistance patterns displayed by *E. coli* isolates from pig farm wastewater

Antibiotics	Number (%) of antibiotic resistant isolates			
	Farm A n= 89	Farm B n= 65	Farm C n= 47	Total no of AR *E. coli* n=201
Ampicillin	77 (86.5)	54 (83.1)	34 (72.3)	165 (82.1)
Tetracycline	81 (91.0)	57 (87.7)	42 (89.3)	186 (89.6)
Cefpodoxime	55 (61.7)	33 (50.7)	17 (36.2)	105 (52.2)
Sulfamethoxazole/ Trimethoprim	45 (50.6)	27 (41.5)	37 (78.7)	109 (54.2)
Ceftriaxone	31 (34.8)	18 (27.7)	9 (19.1)	58 (28.9)
Erythromycin	51 (57.3)	37 (56.9)	23 (48.9)	111 (55.2)
Nalidixic acid	20 (22.4)	11 (16.9)	4 (8.5)	35 (17.4)
Chloramphenicol	37 (41.5)	10 (15.4)	4 (8.5)	51 (25.4)
Gentamicin	24 (26.9)	5 (7.6)	10 (21.3)	39 (19.4)
Colistin	0 (0)	0 0)	0 (0)	0 (0)

Results in this study clearly accentuate the place of piggery wastewater as reservoirs of antibiotic resistant bacteria. Specifically, wastewater from pig farms in this study originated from daily pen floor cleaning, cooling/bath troughs and drinking water troughs; all of which were most times, inevitably smeared with faecal matter. We observed pens in this study were structured to contain feeding area and dipping troughs within same confined space. Resulting wastewater contained leftover feed (sometimes medicated with antibiotics), remnant of disinfection material, faecal and urine of pigs which may contain antibiotics in different stages of metabolism, all of which provide a supporting pressure for the propagation and transfer of resistance genes. The discharge of this unto soil without any form of treatment which was observed in all sampled sites in this study could be a major route for dispensing antibiotic resistant bacteria into shared environment soil as soil, surface water and groundwater. The observed levels of resistance in this study were consistent to those reported in recent related studies conducted on *E coli* from pig farm environs (Liu *et al*., 2016; Nuangmek *et al*., 2018; Dawangpa *et al*., 2022).

Though the immediate scope of this study did not include the examination of adjoining environments, other studies have reported the contribution of wastewater from animal husbandry to the contamination of surrounding environment (Adelowo *et al*., 2018; He *et al*., 2020).

Escherichia coli in this study displayed particularly the highest level of resistances to ampicillin and tetracycline where n=165 and n=186 which corresponds to 82.1% and 89.6% respectively. The high resistances recorded against these antibiotics may be a reflection of the large use of tetracycline and beta lactams in farms under study as these two antibiotics were mentioned as the most applied antimicrobials on all three farms. This was closely followed by resistance to erythromycin and sulfamethoxazole/trimethoprim with n=109 and n=105 respectively. The continual usage of antibiotics creates selection pressure in the wastewater which enhance the selection of resistant genes. Studies from other climes have also reported the antibiotics in the tetracycline and penicillin class as most applied in pig farming and the resultant fall out in resistance (Nuangmek *et al*., 2018; Dawangpa *et al*., 2022).

Table 3A. Phenotypic antibiotic resistance profiles of *Escherichia coli* from farm A

Resistance profiles	No of isolates
Te.	7
Amp. Te.	4
Amp. Te. Cpd. Sxt. Cro. E.	5
Amp. Te. Cpd. Sxt. Cro. Nal	3
Amp. Te. E. C. CN	9
Amp. Te. Cpd. Sxt. Cro. Nal. E. C. GN	6
TE. Amp. Cpd. Sxt. E.Nal	7
Amp. Te. Cpd. E. Cro	3
Amp.	3
Amp. Te. Cpd. Sxt. Cro. E. Nal. C. GN	4
Amp. Te. Cpd. Sxt. Cro. E	4
Amp. Te. Cpd. E. Cro. C.	8
Amp. Te. Cpd. Sxt. E. C	10
Te. Sxt	6
Amp. Cpd. GN	5
Amp. Te. E	7

Amp = ampicillin; **Te** = tetracycline; **Cpd** = cefpodoxime; S**xt** = sulfamethoxazole/ trimethoprim; **Cro** = ceftriaxone; **E** = erythromycin; **nal** = nalidixic acid; **C** = chloramphenicol; **GN** = gentamicin

Table 3B. Phenotypic antibiotic resistance profiles of *Escherichia coli* from farm B

Resistance profiles	No of isolates
Te.	10
Amp	2
Amp. Te.	4
Amp. Te. Cpd. Sxt. E. Nal. C.	7
Amp. Te. E	3
Amp. Te. Cpd. Sxt. Cro. E.	5
Amp. Te. Cpd. E	7
Amp. Te. Cpd. E. Cro.	4
Amp. Sxt	4
Amp. Te. Cpd. Sxt. Cro. E. Nal. C. GN	3
Amp. Te. Cpd. Sxt. E. Cro.	5
Amp. Te. Cpd. GN	2
Te. Sxt	3
Amp. Te. Sxt. E	3
Amp. Cpd. Sxt. E. S.	1

Amp = ampicillin; **Te** = tetracycline; **Cpd** = cefpodoxime; S**xt** = sulfamethoxazole/ trimethoprim; **Cro** = ceftriaxone; **E** = erythromycin; **nal** = nalidixic acid; **C** = chloramphenicol; **GN** = gentamicin

Table3C. Phenotypic antibiotic resistance profiles of *Escherichia coli* from farm C

Resistance profiles	No of isolates
Te.	7
Amp. Te.	6
Amp. Te. Cpd. Sxt. Cro. E.	5
Amp. Te. Cpd. Sxt.	3
Amp. Te. E. C.	3
Amp. Te. Cpd. E	6
TE. Sxt. E.	2
Amp.	1
Amp. Te. Cpd. Sxt. E. Cro. Nal. C. GN.	4
Amp. Te. Cpd. Sxt. E. GN	2
Te. Sxt	4
Amp. Cpd. C.	1

Amp = ampicillin; **Te** = tetracycline; **Cpd** = cefpodoxime; S**xt** = sulfamethoxazole/ trimethoprim; **Cro** = ceftriaxone; **E** = erythromycin; **nal** = nalidixic acid; **C** = chloramphenicol; **GN** = gentamicin

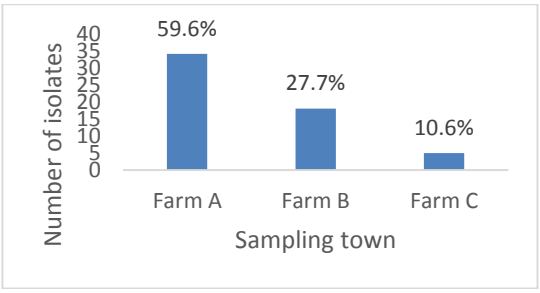

Fig 2. Positive ESBL-producing isolates from each sampled farm.

Fig 2. is showing result of phenotypic detection of ESBL among isolated organisms. A total of 57 out of 105 cefpodoxime resistant isolates screened were found to be phenotypically positive for the production of ESBL. Going by geographic areas, prevalence of ESBL-producing *E. coli* bacteria was highest among isolates from Lagos farm n=34

(59%). In contrast, a much lower percentage of the isolates from Ago town n=5 (10.1%) were found to express the ESBL activity. The higher prevalence in the Farm A could be a result of variations in animal production practice of the studied farms. Farm C is a piggery settlement with pig pens in close proximity and kept in high density with lesser attention paid to hygiene in comparison to the other study farms. The hygiene situation may aid the propagation and spread of resistance determinants as ESBL. In consonance with present study, poor hygiene practise and subsequent prophylactic use of antibiotics to prevent disease breakouts has been reported to aid the spread of resistance in pig farming. Dawangpa *et al*., (2022) revealed very high level of antibiotic resistance; about 100% to all test antibiotics, among isolates from drinking trough.

All the ESBL positive isolates were multiple drug resistant which reflected in the resistant phenotypic patterns displayed. Generally, 16 phenotypic patterns of resistance were displayed at Farm A, and 15 and 13 patterns were displayed by isolates from farm B and farm C respectively (Tables 3A, B, and C). Result of multidrug resistance and prevalence is similar to that reported in the work of Nuagunek *et al*., 2018). Dawangpa *et al*. (2022) in a related study, reported high proportion of antibiotic resistance genes from pig farm effluents, the highest of the resistance genes being ESBL genes. In their study, all of the sampled piggery wastewaters were found to be reservoirs of resistant genes, suggesting the transferability of resistance genes may likely occur from pig wastewater to immediate and remote environment of pig farms. This calls for better attention and monitoring of antibiotic use in piggery to avert risk to public health.

Conclusion

Wastewater samples from the three pig production farms in this study reflected the presence of antibiotic resistant bacteria with diverse MDR phenotypes. Our study also revealed that these wastewaters are reservoirs of bacteria with ESBL activity. The pig farm settlement was highest in their possession of ESBL activity, followed by the moderately large farm. In contrast, the home sited farm had the least number of MDR bacteria, but was not any less significant in risk, though with a smaller number of ESBL bacteria. ESBL characterization was not exhaustive in this study, further work especially molecular characterization of resistant genes will provide more insight to the current investigation.

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