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Antibiotic Resistance among Bacteria isolated from Pigs in Selected Farms in Ekiti State, Nigeria

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Abstract

Emergence of antibiotic resistance among bacteria isolated from animals has been recognized to pose serious threat to health when these bacteria are disseminated into food chain. This study determined the incidence of antibiotic resistant bacteria isolated from pigs at different pig locations in Ekiti State, Nigeria. Sixty-three (63) faecal samples were collected from all selected poultry locations in Ekiti State and cultured in the microbiology laboratory at the Federal University Oye Ekiti. Distinct colonies of bacterial isolates were picked per plate and subcultured to obtain pure cultures. All bacteria were identified using biochemical tests and were subjected to antibiotic susceptibility tests using the following antibiotics: ertapenem (10µg), meropenem (10µg), ceftazidime (30µg), ceftriaxone (30µg), gentamicin (10µg), ampicillin (10µg), tetracycline (30µg), norfloxacin (10µg) and pefloxacin (5µg). All the bacteria were tested for biofilms and haemolysis. 57/63 (90.5%) bacterial isolates were recovered from 63 samples. The bacteria recovered from the faecal samples included Escherichia coli, Klebsiella pneumonia, Proteus mirabilis and Yersinia enterocolitica. Generally, the bacteria showed the highest rate of reduced susceptibility to ampicillin 44 (77.2%) and least resistance to norfloxacin 9 (15.8%). All the bacterial isolates were biofilm producers while 12 of the total isolates were haemolytic (21.1%). The observed high resistance rates of the bacterial isolates suggested the need for swift special intervention on antibiotic usages in livestock production as this will help to reduce the incidence of antibiotic resistant bacteria from animals and hence, reducing the havoc that might arise when these bacteria enter food chain.

Keywords: antibiotics, antibiotic resistance, biofilms, Pigs.

INTRODUCTION

Pork is a popular source of animal protein in Nigeria, ranking immediately after beef, chicken and fish (Adeshinwa *et al.,* 2003; Monger *et al.,* 2021). In an effort to cater for the growing demand for pork, pig farming has become a major economic phenomenon in Nigeria. Small

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and medium scale pig farms are common business entities that supply pork to supplement the protein dietary needs for human consumption (Ogunniyi and Omoteso, 2011).

In order to keep animals in good health and increase productivity, antibiotics have been used in different forms, either as food additives, drink supplement or intramuscular injections (Scoppetta *et al.,* 2017; Liu *et al.,* 2022). In the case of pigs for pork production, antibiotic injections, especially streptomycin, are usually given to treat or control infections (Ahmed *et al.,* 2010). However, the use of these antibiotics raises serious concerns due to the emergence of antibiotic resistant bacteria. Not only is the emergence of antibiotic resistant bacteria in human clinical setting considered a serious threat, use of antibiotics in animals and subsequent emergence of antibiotic resistant bacteria in animals also contributes significantly to the current burden of antibiotic resistance (Osterberg *et al.,* 2016).

In view of the concerns raised about the serious threat posed by antibiotic resistant bacteria that emerge from animals, it has been recognized that periodic surveillance of antibiotic resistant bacteria from animals is crucial, not only to understand the extent of the problem, but also to prevent the emergence of germs resistant to antibiotics and their subsequent spread throughout the food chain (Founou *et al.,* 2016). Furthermore, there is little knowledge regarding the incidence of resistance to antibiotics considered as last resort in veterinary clinical settings in Ekiti State, Nigeria. On this note, this study aimed to determine the prevalence of antibiotic resistant Gram negative bacteria from pigs at different pig farms in different towns in Ekiti-State, Nigeria.

MATERIALS AND METHODS

Study area

This study was carried out at three different towns in Ekiti State: Ado Ekiti, Oye Ekiti and Ayegbaju Ekiti. Ado Ekiti is the biggest of the three towns and also the state capital with characteristics of urban population. The other towns are not as big in size and population but also have students and locals that make up a bulk of the population. The common factor in these locations is the consumption of pork and its related products to satisfy some protein and nutritional needs of the population and there are commercial pig farms and outlets that supply pork to the population in varying quantities. One pig far was selected per town and the appropriate permissions were sought from the farmers before samples were collected at the different locations.

Collection of samples

A total of 63 fresh faecal samples were collected from pigs at pig farms at the different pig farms in Ekiti State. The pigs were between 3 months and 3 years old. The owners of the pig farms were consulted and permission was granted before samples were taken. A total number of 63 fresh faecal samples were collected from the pigs at the different pig the farms. All samples were transported immediately to the microbiology laboratory, Federal University Oye Ekiti within two hours of collection for analyses. Twelve (12) samples were collected at Ado Ekiti, while 28 and 17 samples were collected at Ayegbaju and Oye Ekiti respectively.

Laboratory procedures

Isolation of selected bacteria

All faecal samples were brought to the laboratory within 2 hours after collection. Faecal swabs were initially pre-enriched by inoculating into tryptone soy broth (TSB) and were incubated at

37^{0}C for 2-3 hours. Each sample broth containing bacteria was streaked onto freshly prepared MacConkey agar plates and incubated at 37^{0}C for 24 hours. After incubation, distinct red colonies were picked and inoculated onto sterile nutrient agar (NA) plates and incubated at 37^{0}C for 18 hours for pure culture. One distinct colony was picked from the pure culture plate and transferred onto sterile NA slants. The slants were kept in refrigerator at 4^{0}C for future use. All bacteria were subjected to Gram's staining and biochemical tests for identification. The biochemical tests carried out were indole, methyl red test, Vogues Proskauer test, citrate utilization tests and sugar fermentation tests.

Antibiotic Susceptibility Test

All bacterial isolates were subjected to antibiotic susceptibility testing using the disk diffusion method, with results interpreted in accordance with Clinical Laboratory Science Institute recommendations (CLSI, 2020). Some colonies were put into sterile normal saline that had been adjusted to meet the 0.5 McFarland turbidity criterion after the bacteria were cultured overnight on sterile Mueller-Hinton agar plates. Ertapenem (10µg), meropenem (10µg), ceftazidime (30µg), ceftriaxone (30µg), gentamicin (10µg), ampicillin (10µg), tetracycline (30µg), norfloxacin (10µg), and pefloxacin (5µg) were the antibiotic disks that were employed for the susceptibility resting. Sterile Mueller-Hinton agar plates were inoculated using a standardized innocula, and antibiotic disks were positioned as needed. All plates were incubated at 37^{0}C for twenty-four hours. Using the conventional interpretation chart, plates were examined for zones of inhibition and categorized as resistant and susceptible. The resistance to two or more antibiotics from separate classes of antibiotics was designated as multiple drug resistance. For every antibiotic susceptibility test, the *E. coli* ATCC 25922 was employed as a control.

Detection of other parameters

The Congo red agar method was employed to identify biofilm-forming bacteria among all the isolated specimens, following the previously reported protocol. On agar plates supplemented with Congo red, the bacteria were cultivated and incubated for 24 hours at 37°C. The development of black crystalline colonies was interpreted as evidence that the bacterial isolates had formed biofilm. Intermediate biofilm producers were represented by colonies that were gray, whereas non-biofilm producers were interpreted as those that had red colonies. By cultivating the bacteria on Mueller Hinton agar plates supplemented with 5% sheep blood and incubating them for 24 hours at 35°C, the haemolytic activity of each and every bacteria was found. Following incubation, the typical alpha and beta haemolytic patterns were examined on each plate.

Results

A total of fifty- seven bacterial isolates were isolated from the sixty-three different samples collected. All isolates were identified biochemically as *E. coli* 14(24.14%), *Klebsiella pneumonia* 30(52.6%), *Proteus mirabilis* 11(19.3%) and *Yersinia enterocolitica,* 2(3.5%) (Table 1). It was also observed that the highest number of bacterial isolates was detected at Ayegbaju while the least number of separate bacterial isolates were recovered from the pig farm at Ado Ekiti (Table 2). *K. pneumonia* appeared the most common organism with 30 isolates across the different locations (Table 2). The overall results that emerged from the antibiotic susceptibility tests showed that resistance to ampicillin was highest 44 (77.2%), followed by resistance to ertapenem and meropenem (Table 3). All the bacteria also showed the least resistance to norfloxacin. The distribution of the organisms according to resistance by individual organisms is shown in Table 3. Majority of the bacteria from the different locations produced biofilms (Table 4) while 12 (21.0%) bacterial isolates showed haemolysis (Table 5). The

distribution of the resistant bacteria according to their respective locations are shown in Figures 1-3.

Table 1: Frequency of occurrence of the selected isolated bacteria

S/N	Bacterium	Frequency of occurrence (%)
1	*Escherichia coli*	14 (24.14)
2	*Klebsiella pneumoniae*	30 (52.6)
3	*Proteus mirabilis*	11 (19.3)
4	*Yersinia enterocolitica*	2 (3.5)
Total		57

Table 2: Frequency of bacterial isolation based on location

Bacterium	Ayegbaju	Ado	Oye	Total (%)
E.coli	3 (10.7)	5 (41.7)	6 (35.3)	14 (24.6)
K. pnemoniae	18 (64.3)	4(33.3)	8 (47.1)	30 (52.6)
P. mirabilis	7 (25)	1 (8.3)	3 (17.6)	11 (19.3)
Y. enterocolitica	0 (0)	2 (16.7)	0 (0)	2 (3.5)
Total	28	12	17	57

Table 3: Antibiotic Resistance pattern for isolated bacteria

Antibiotic	*Escherichia coli*	*Klebsiella. pneumoniae*	*Proteus mirabilis.*	*Yersinia enterocolitica*
AMP	10 (71.4%)	23(76.7%)	9 (81.8%)	2 (100%)
AMC	4 (28.6%)	12(40%)	4 (36.4%)	2 (100%)
CRO	8(57.1%)	10(33.3%)	5 (45.5%)	2 (100%)
CAZ	6 (42.9%)	7(23.3%)	3 (27.3%)	2 (100%)
ETP	7 (50%)	22(73.3%)	6 (54.5%)	2 (100%)
MEM	6 (42.9%)	19(63.3%)	5 (45.5%)	2 (100%)
CN	5 (35.7%)	6(20%)	3 (27.3%)	1 (50%)
TE	6 (42.9%)	20(66.7%)	8 (72.7%)	2 (100%)
PEF	9 (64.3%)	11(36.7%)	6 (54.5%)	2 (100%)
NOR	4(28.6%)	3(10%)	1 (9.1%)	1 (50%)

Total number of bacteria isolates = 57 (90.5%), AMP - Ampicillin, AMC - Amoxilin, CRO - Ceftriaxone, CAZ - Certazidime, ETP - Ertapenem, CN - Gentamycin, TE - Tetracycline, PEF =-Pefloxaxin, NOR -Norfloxacin.

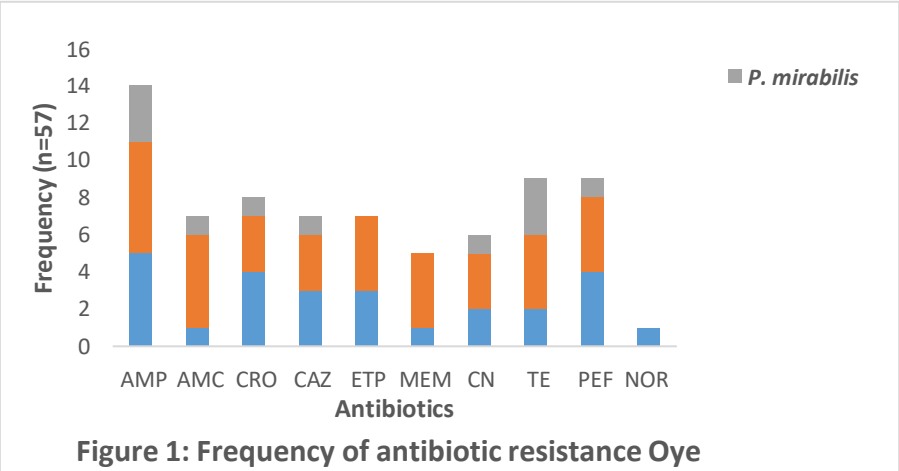

Figure 1: Frequency of antibiotic resistance Oye

***Keys**: AMP - Ampicillin, AMC - Amoxilin, CRO - Ceftriaxone, CAZ - Certazidime, ETP - Ertapenem, CN - Gentamycin, TE - Tetracycline, PEF - Pefloxacin, NOR -Norfloxacin.*

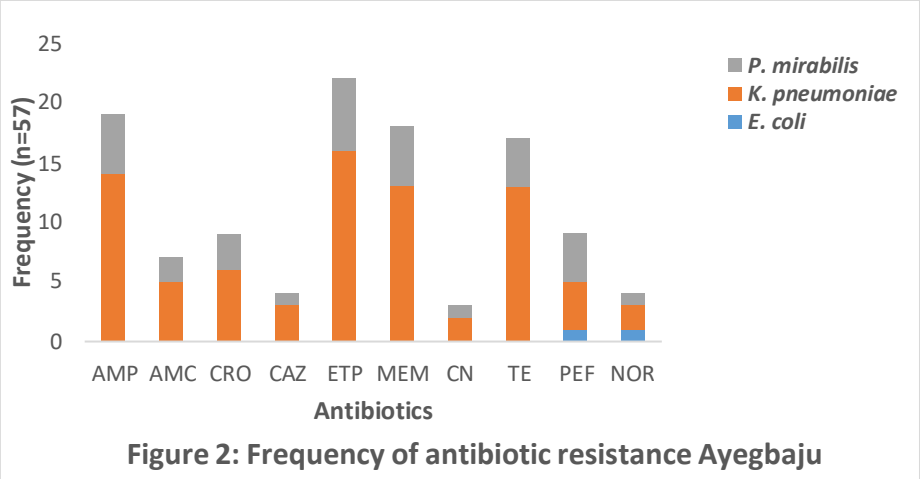

Figure 2: Frequency of antibiotic resistance Ayegbaju

***Keys**: AMP - Ampicillin, AMC - Amoxilin, CRO - Ceftriaxone, CAZ - Certazidime, ETP - Ertapenem, CN - Gentamycin, TE - Tetracycline, PEF - Pefloxacin, NOR -Norfloxacin.*

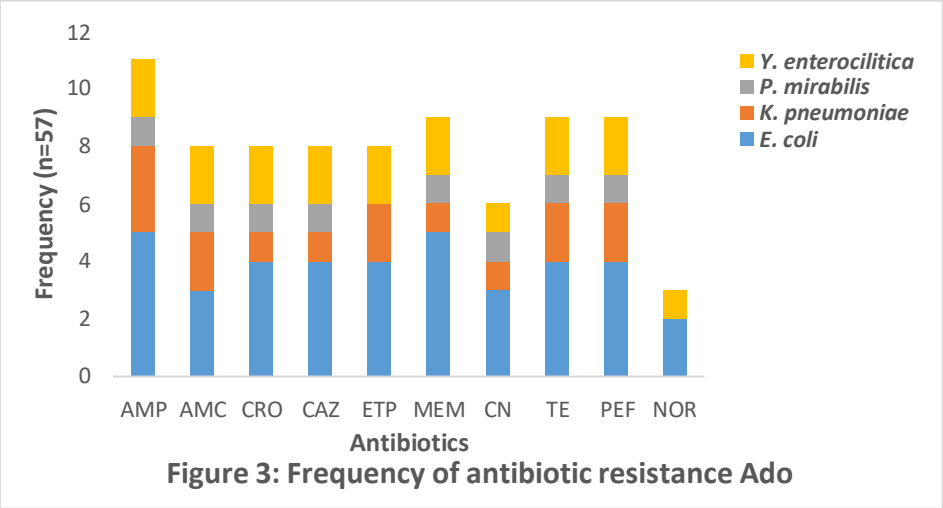

Figure 3: Frequency of antibiotic resistance Ado

***Keys**: AMP - Ampicillin, AMC - Amoxilin, CRO - Ceftriaxone, CAZ - Certazidime, ETP - Ertapenem, CN - Gentamycin, TE - Tetracycline, PEF - Pefloxacin, NOR -Norfloxacin.*

Table 4: Biofilm production among organisms

	Ayegbaju	Ado	Oye	Total
E. coli	3	5	6	14
K. Pneumoniae	18	4	8	30
P. mirabilis	7	1	3	11
Y. enterocolitica	0	2	0	2
Total	28	12	17	57

Table 5: Hemolysis patterns among bacteria from pigs

	E. coli	*K. pneumonia*	*P. mirabilis*	*Y. enterocolitica*	Total
Alpha-hemolysis	-	2	2	-	4
Beta-hemolysis	2	5	1	-	8
Non-hemolysis	12	23	8	2	45
Total	14	30	11	2	57

DISCUSSION

This study was carried out to evaluate the prevalence of antibiotic resistant bacteria isolated from pigs in three selected piggeries in Ekiti-State, Nigeria. The places included Ayegbaju-Ekiti, Ado- Ekiti and Oye-Ekiti. A total of 57 (90.5%) bacteria were isolated from a total of 63

faecal samples collected across the three piggery locations within the stipulated study period. The use of antibiotics in animals, including pigs has been implicated in the emergence of antibiotic resistant bacteria and this contributes significantly to the current burden of antibiotic resistance in humans, animals and the ecosystem (Osterberg *et al.,* 2016; Mitchaothai, and Srikijkasemwat, 2022). Antimicrobial resistance in fecal *E. coli* from different pig production systems has been a recurring phenomenon in several veterinary settings. This menace therefore necessitates the need to ascertain the antibiotic resistance profiles of bacteria isolates from pig.

In the present study, *K. pneumonia* was observed to be the most frequently isolated bacteria with a frequency rate of 30 (52.6%), followed by *E. coli* 14 (24.14%). This is in contrast to previous studies that have reported *E. coli* as the most dominant bacterium (Urumova, 2016; Kallau *et al.,*2018). It has been observed that different bacteria isolated from different veterinary settings could reflect the peculiarity of the environmental settings where the animals are reared (Xu *et al.,* 2023). This bacterium is one of the most commonly isolated from animals' settings, most especially pigs. The increased frequency of *K. pneumonia* could be attributed to the increased rate of intensive farming that is used foe rearing pigs (Founou *et al.,* 2018, Leangapichart *et al.,* 2021). It was also observed that *K. pneumonia* was the predominant bacterium at Ayegbaju-Ekiti and Oye-Ekiti with a frequency rate of 18 (64.3%) and 8 (47.1%) respectively. However, *E. coli* was the most frequently isolated organisms at Ado-Ekiti with a frequency rate of 5 (41.7%). *P. mirabilis* was the second most isolated bacteria at Ayegbaju-Ekiti 7 (25%) while *Y. enterolitica* was only isolated from samples collected at Ado-Ekiti with only two strains of the bacterium (2 (16.7%) (Table 3). The differences in the type of bacteria observed, compared with previous findings reflected the differences in the predominant antibiotic resistant bacteria across different geographical locations (Mittal *et al.,* 2018; Liu *et al.,* 2022). Generally, previous studies also reflect the fact that *P. mirabilis* and *Y. enterocolitica* are not frequent organisms commonly isolated in food animals although the presence of antibiotic resistance among these two organism is significant (Qu *et al.,* 2022).

A closer observation of the overall antibiotic susceptibility shows that resistance to ampicllin was highest 44 (77.2%), followed by resistance to ertapenem 37 (64.9%), tetracycline 36 (63.2%) and meropenem 32 (56.1%). The organisms showed the highest susceptibility to gentamicin 41 (71.9%) and norfloxacin 36 (63.2%). Different organisms showed different proportions of antibiotic resistance to the different antibiotics, relative to the total number of each organism isolated and tested for antibiotic resistance. The high level of resistance shown by the organisms to ampicillin in the present study is in agreement with previous studies that have shown that organisms isolated from pig faeces were found to be highly resistant to ampicillin, tetracycline, sulphonamides and spectinomycin (Chander *et al*. 2007). In addition, ampicillin has been onfirmed as one of the most commonly abused antibiotics in human and veterinary settings, contributing to the high level of resistance against the antibiotic (Monger *et al.,* 2021). Furthermore, Amador *et al*. (2019) and Kim *et al*. (2005) have also documented a high level of antibiotic resistance of bacteria from pig farms to tetracycline. However, resistance to carbapenems appears to be relatively high, as opposed to previous studies that have confirmed increased susceptibility to carbapenems among bacteria from pigs (Gao *et al.,* 2015, Amador *et al.,* 2019). The resistance to the carbapenem antibiotics observed in this present study is a serious threat to public health. This is because of the clinical significance of this set of antibiotics as drugs of last resort in clinical medicine. This development obviously limits the use of carbapenems in humans and efforts must be made to restrict their use in animals (Bonardi and Pitino, 2019, Mollenkopf *et al.,* 2019). In addition, the results of the susceptibilities of the bacteria to gentamicin and norfloxin in the present study appear to be

relatively high as compared with previous studies (Pandey *et al*. 2016). There is a deluge of scientific evidence that the use of gentamicin, and more importantly, fluoroquinolones in pigs and other food animals is on the increase and risks could emerge in the fluoroquinolone and gentamicin resistant bacteria to farm workers (Kenyon, 2021). Studies have also shown that resistance to fluoroquinolones among bacteria from animals is increasing (Kenyon *et al.,* 2021). The successful treatment of enteric and blood infections in core clinical settings depends heavily on use of fluoroquinolones and emergence of resistance to them may compromise their clinical use (Bhatt, *et al.,* 2022). The high rate of occurrence of resistance to some of the antibiotics tested in this study could be due to the indiscriminate use of some of the antibiotics in pig farms, in view of established facts that antibiotic use usually predates antibiotic resistance. (De Jong *et al.,* 2014; Moawad *et al.,* 2017). These findings have implications because the presence or emergence of antibiotic resistant bacteria from pigs and livestock imperils human health through the possible transfer of such antibiotic resistant bacteria from pigs to humans through a contaminated food chain (Marshall *et al.,* 2011*)*.

In the present study, all the bacterial isolates 57 (100%) produced biofilms. Bacteria that form biofilms are better able to agglomerate on surfaces and produce bacterial illnesses that are persistent and recurring (Ajayi *et al.,* 2019). The introduction of these biofilm-producing bacteria into food chain could be detrimental because such bacteria during infections are very difficult to treat and require higher doses of antibiotics to eliminate such bacteria during treatment (Flores-Mireles *et al.,* 2015). Also in this study, twelve bacterial isolates equivalent to 21.1% of the total bacterial isolates were confirmed to be haemolytic. This indicates the potential pathogenicity of the bacterial isolates from the three pig farms. The low frequency of haemolytic bacteria observed in this study is comparable to the findings of Pandey *et al*. (2016), who observed that approximately 35.2% of antibiotic resistant bacteria produced haemolysins. It should be noted that low rate of haemolysin among the bacteria recovered from the pigs could indicate that the organisms may not be pathogenic. However, the high frequency of resistance to the antibiotics constitutes a serious threat to individual, public and ecosystem health.

CONCLUSION

The bacterial isolates in this study were observed to exhibit high resistance to majority of the antibiotics tested. The observed high resistance to antibiotics in the carbapenem family is a serious cause for concern. The production of biofilm by the bacterial isolates in this study further buttresses the need for special intervention on antibiotic usages in livestock production as these biofilms will make the bacteria resistant to antibiotics. This intervention will help to reduce the incidence of antibiotic resistant bacteria from animals. Also, the appropriate body enforcing antibiotic usage in livestock production should be more proactive and continuous surveillance on the incidence of resistant bacteria from animals and their resistance profiles should be promoted. It is strongly recommended that more studies should be carried out with emphasis on the molecular mechanisms of resistance to clinically relevant antibiotics among bacteria isolated from pigs and other food producing animals.

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