Antibiotic Resistance Patterns of *Escherichia coli* Detected in Pig Faecal Samples from Anambra State

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: Pig farmers use antibiotics for therapeutic, metaphylaxis, prophylaxis and growth promotion in their farm animals. This work was aimed to determine the antibiogram of *Escherichia coli* isolated from pig faecal samples from pig farms in the three senatorial zones of Anambra State.
Materials and Methods: A total of 80 faecal samples were collected from forty pig farms in the three senatorial zones (Anambra North, Anambra Central and Anambra South) of Anambra State from February, 2018-January, 2019 and analyzed microbiologically using MacConkey agar and Eosin Methylene Blue (EMB) Agar and appropriate culture conditions. Recovered isolates were further characterized based on their morphological and biochemical features. Antimicrobial susceptibility of the isolates was tested to 10 conventional antibiotics using Kirby Bauer Disc Diffusion method.
Results: A total of 140 *Escherichia coli* isolates were recovered and characterized. Percentage occurrences were 36.4, 30.1 and 33.5 in Anambra North, Anambra Central and Anambra South

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respectively. Antimicrobial susceptibility testing of the bacterial isolates revealed that resistance of the isolates ranged from 7% resistance to Imipenem to 67% resistance to Streptomycin. A one way analysis of variance showed no significant difference ($p > 0.05$) in resistance to imipenem, amoxicillin/clavulanic, ceftazidime, tetracycline, gentamicin, ceftriaxone, ciprofloxacin, trimethoprim/sulfamethoxazol and significant difference ($p < 0.05$) in resistance to streptomycin and cefotaxime in the three senatorial zones studied. 51.4% of the isolates were multidrug resistant.

Conclusion and Recommendation: Misuse and overuse of antibiotics in piggery have resulted in antibiotic resistance in isolates from pig faecal samples. Policies regarding prudent use of antibiotics in pig farms should be enforced to reduce the spread of antibiotic resistance.

1. INTRODUCTION

Pig (*Sus scrofa*) is one of the sources of animal protein in Nigeria. Porcine production among other species has a high potential to contribute to high economic gains [1]. Pig production has been identified as a solution to protein inadequacy due to certain attributes which pigs possess that are not in other domestic livestock. First, the pigs have high fecundity, high feed conversion efficiency, early maturity, short generation interval and relatively small space requirement. They excel above other red meat animals such as cattle, sheep and goat in converting feed to flesh and their annual growth rate (3.8%) is higher than that of the human population (2.30 – 2.80%) [2].

Pig farmers use antibiotics for treatment, metaphylaxis, prophylaxis and growth promotion in their farm animals [3,4]. Antibiotics have been used routinely in farm animal production since the 1950s to treat, control and prevent disease and to increase productivity. Based on the estimated continued increase in global demand for animal products, global antimicrobial consumption of livestock is expected to increase by two-thirds over the next decade. Within the livestock sector, antimicrobial usage is estimated to be highest in pigs, compared with poultry and cattle [5].The use of antibiotics in pigs is complex and associated with the interrelating domains of animal health, animal welfare and economics [6].

The use of antibiotics in animal husbandry is one of the major drivers for the emergence of resistant bacteria and dissemination of resistance genes. The long-term and extensive use of antibiotics in food animals is not only a regional or national phenomenon, but part of a global problem [7].

The profitability of pig farming has recently attracted the interest of many investors in

Anambra leading to a proliferation of pig farms in Anambra State. There is need study antimicrobial resistance in indicator microorganisms such as *E. coli*, in these farms as a means of monitoring prudent antibiotic use in the farms.

2. MATERIALS AND METHODS

2.1 Description of Study Area

The study area is Anambra State of Nigeria. It covers an area approximately 4416 km2, and lies at Latitude of 6°20'N and Longitude 7°00'E [8]. Forty farms were randomly selected from three senatorial zones of Anambra State (Anambra South, Anambra North and Anambra Central) and included in the study.

2.2 Sample Collection

A total of 80 pig faecal samples were collected randomly from 40 pig farms using sterile sample bottles from. The samples were labeled and transferred to Nnamdi Azikiwe University Microbiology laboratory for processing within 24 h.

2.3 Sample Processing

1g of the mashed faecal sample was added into a test tube containing 9ml of 0.9% (w/v) saline, 20% (v/v) glycerol, allowed to stand for 5min and then stored at 4^0C until required. This served as stock culture. Samples not treated after 48h of collection were discarded.

2.4 Isolation *Escherichia coli* from Faecal Samples

The stock sample was diluted tenfold in phosphate buffered saline (PBS), pH 7.2 and a 0.1ml of 10^{-5} dilution was plated onto sterile Mac

Conkey agar (TM Media India) using spread plate technique and incubated at 37^{0}C for 24h under aerobic condition. Candidate lactose fermenting colonies were subcultured on sterile Eosin Methylene Blue (EMB) Agar and Nutrient agar media and subjected to biochemical tests.

2.5 Biochemical Characterization of the Bacterial Isolates

The bacterial isolates were characterized based on their morphological and biochemical features.

2.5.1 Gram reaction

A thin smear of the isolate on a clean slide was air dried and heat-fixed. The smear was stained using Gram's reagents and the slide dried and viewed under the oil immersion microscope (X100). Purple colour indicates Gram positive bacteria while red colour indicates Gram negative bacteria [9].

2.5.2 Motility test

A loopful of a 24h broth culture of the isolate was placed on a clean, dry cover slip. Vaseline was smeared on the edge of concavity of a concave shaped slide, which was then placed gently but firmly over the cover slip. The slide was inverted and observed for motility under high power objective lens (x40) [9].

2.5.3 Indole test

The isolate was inoculated into different test tubes containing peptone water and incubated at 37^{0}C for 48h. Few drops of Kovac's reagent (Amyl or isoamyl alcohol) were added. A red colour in the alcohol layer indicates a positive result [9].

2.5.4 Methyl red test

About seven drops of methyl red solution were added to 5m1 of a 5-day old culture of the isolate inoculated in glucose-phosphate broth. Red colour change indicates a positive test while yellow colour indicates a negative test [9].

2.5.5 Voges-proskauer test

A mixture of 3ml of 5% alpha-naphthol dissolved in absolute alcohol and lml of 40% potassium hydroxide were added to 5m1 of a 5- day old culture of the isolate in glucose-phosphate medium. Bright pink or red colour indicates a

positive test whereas a yellow colour indicates negative [9].

2.5.6 Citrate test

The isolate was inoculated into Simmon's citrate agar slant and incubated for 24-96h at 37°C. A colour change from green to deep blue indicates a positive result [9].

2.5.7 Catalase test

A few drops of 3% hydrogen peroxide were added to a thick emulsion of the isolates on a clean slide and observed for effervescence. Effervescence indicates a positive result [9].

2.5.8 Coagulase test

An emulsion of a 24h old culture of the isolate was made on a clean grease-free slide and a loopful of plasma added to it. Clumping within 10sec indicates a positive result [9].

2.5.9 Oxidative - fermentative test

Two test tubes containing 10ml each of the oxidative — fermentative basal medium and 10% glucose were inoculated with 24h culture of the isolate by stabbing. Bromothymol blue was added as an indicator. One of the test tubes was added lml of sterile paraffin oil (fermentative) while the other (oxidative) was without paraffin. Both tubes were incubated at 37°C for 14 days. A change in colour from green to yellow in both tubes indicates the isolate is both oxidative and fermentative. Colour change in tube with paraffin only indicates fermentative isolate while a change in colour in tube without paraffin indicates an oxidative isolate [9].

2.5.10 Sugar fermentation test

Sugars such as glucose, sucrose, lactose and maltose were added in peptone water in 1% (w/v). Two drops of Bromothymol blue were added as an indicator. Inverted Durham tube was inserted into each of the tubes with the tubes containing the broth. The sugar solutions were sterilized by autoclaving at 115^{0}C for 15 min. 200µl of a 24h broth culture of the bacterial isolates was inoculated in each tube and then incubated at 37^{0}C for 24h. Colour change from green to yellow indicates a positive result and presence of bubbles in the inverted Durham tubes indicate gas production [9].

2.6 Antimicrobial Sensitivity Testing

The antimicrobial sensitivity test was carried out following the methods described by Clinical and Laboratory Standards Institute. The antibiotics discs (Oxoid Hampshire, UK) were amoxicillin/clavulanic acid (20μg/10 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), streptomycin (10 μg), gentamicin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), trimethoprim/ sulfamethoxazole, (1.25/23.75 μg), chloramphenicol (30μg) and imipenem (10 μg). Susceptibility of the enteric bacterial isolate was tested using the standard Kirby-Bauer disc diffusion method. Multidrug resistant (MDR) strains were identified as those resistant to at least three different classes of antimicrobials [10,11].

Percentage resistance (PR) was calculated thus;

$$PS\ (\%) = \frac{No.of\ Resistant\ organisms\ to\ an\ antibiotic \times 100}{Total\ No.of\ organisms\ tested}\ (1)$$

2.7 Statistical Analysis

The data collected were analyzed for significant differences using one-way Analysis of Variance (ANOVA) by Student-Newman-Keul (SNK) test at 95% confidence level. IBM SPSS statistics version 20 was used for the correlation and ANOVA.

3. RESULTS

3.1 Isolation of Bacterial Organisms from Pig Faecal Samples

A total of 140 *Escherichia coli* isolates were recovered from the 80 faecal samples collected from the forty farms visited. Table 1 shows the percentage occurrence of *E. coli* in the senatorial zones. Anambra North had the highest percentage occurrence of *E. coli* (36.4) while Anambra Central had the least (30.1).

3.2 Antibiogram of the *Escherichia coli* Isolates

The 140 *Escherichia coli* isolates recovered were subjected to antimicrobial susceptibility testing and the antibiogram of the bacterial isolates according to senatorial zones are presented in Table 2. Imipenem was the least resisted antibiotics in all the senatorial zones studied with only 7% of all the isolates resistant to imipenem (Table 2, Plate 1A) while Streptomycin (67%) was the most resisted antibiotics in the senatorial zones. A one way analysis of variance showed no significant difference (*p* >0.05) in resistance to imipenem, amoxicillin/clavulanic, ceftazidime, tetracycline, gentamicin, ceftriaxone, ciprofloxacin, trimethoprim/sulfamethoxazol respectively and significant difference (*p* < 0.05) in resistance to streptomycin and cefotaxime respectively in the three senatorial zones studied. 51.4% of the isolates were multidrug resistant (Plate 1B).

4. DISCUSSION

Antibiotics are used in both humans and animals and have contributed immensely to better human and livestock health. As a result, animal health, welfare and productivity have been improved in the livestock sector, and ultimately food safety, food security and nutrition and economic growth have shown positive development. However, the achievements in modern medicine and in the livestock sector due to the discovery and development of antibiotics are threatened by the global emergence and spread of antimicrobial resistance [12]. Antibiotic use in livestock is increasing in different parts of the world [5], and it has been shown that antimicrobial resistance can be transmitted from animals to humans through consumption of animal products [13]. The aim of this work was to determine antibiotic resistance pattern of *E. coli* isolated from pig faecal samples in the three senatorial zones of Anambra State. Pig industry is rapidly growing in Anambra State as in other part of the country and knowledge and skills related to biosafety management in pig production are still low among pig farmers. This

Table 1. Percentage occurrence of *E. coli* isolates according to senatorial zones

Senatrial Zones	Number of Isolates	Percentage
Anambra North	51	36.4
Anambra Central	42	30.1
Anambra South	47	33.5
Total	140	100

Table 2. Antimicrobial resistance patterns of isolates according to senatorial zones

		IPM (10µg)	CXT (30µg)	AMC (30µg)	CAZ (30µg)	Te (30ug)	CN (10µg)	CEF (30µg)	CPX (5µg)	S (10µg)	SXT (1.25/23.75 µg)
Senatrial Zones											
AN	*E. coli (n= 51)*	4	13	40	11	32	11	11	19	31	28
AC	*E. coli (n= 42)*	4	7	31	16	32	13	9	12	29	28
AS	*E. coli (n= 47)*	2	14	18	9	14	9	14	12	34	23
	Total (%)	10(7)	34 (24)	89(63)	36(25)	78(55)	33(23)	34(24)	43(30)	94(67)	79(56)

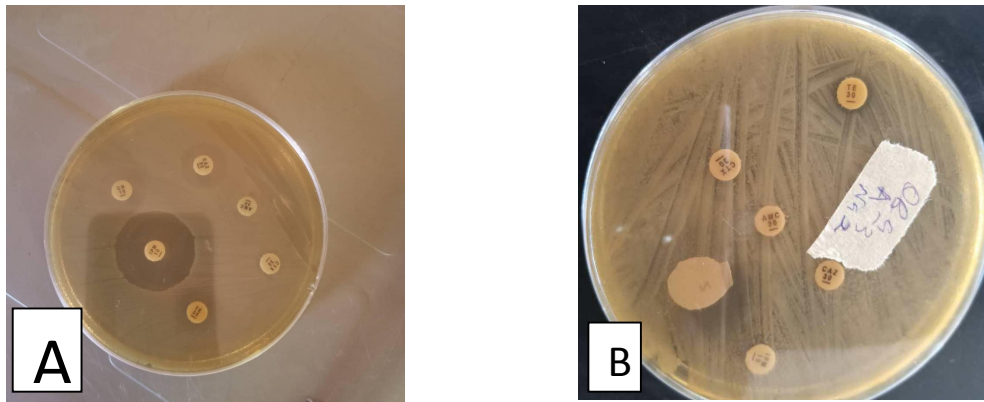*Key: AN: Anambra North, AC: Anambra Central, AS: Anambra South*

Plate 1. A: Organisms Showing Marked Sensitivity to Imipenem and Resistance to Other Antibiotics. B: Organism showing resistance to all the antibiotics tested in the plate

may be the cause of high numbers of *E. coli* isolates obtained from the pig samples which were 36.4%, 30.1% and 33.5% in Anambra North, Anambra Central and Anambra South respectively Table 1. This high prevalence of *E. coli* obtained in pig faecal samples from these farms is a concern as these farms can be the principal source of contamination of pork if the pig carcass is poorly processed at slaughter. Other researchers have isolated *E. coli* from pig faecal samples and pig farm environment [14,15]. Pig faecal samples are used mainly in the studied location in crop production as manure. Animal manure is an important source of bacteria, which can cause serious illness both in animals and humans and *E. coli* is one of the major manure-borne zoonotic pathogen. The application of manure leads to the spread of these bacteria in the environment [16,17,18]. *E. coli* bacterial organisms are involved in various disease conditions in human and livestock population and in the hosting and exchange of resistance determinant [13,19].

The *E. coli* isolates showed varying susceptibility and resistance to the antibiotics tested [Table 2]. The susceptibility of the isolated bacterial organisms to conventional antibiotics revealed that the organisms had least percentage resistance (7%) to Imipenem and highest percentage resistance (67%) to Streptomycin. Although resistance to antibiotics is a natural phenomenon, their overuse and misuse in humans and livestock have significantly increased the antibiotic resistance levels [20,21]. The resistance of the isolates to antibiotics could be attributed to indiscriminate use of these antibiotics in piggery [22].

The result of this work agrees with the work of [13], who observed resistance to tetracycline, trimethoprim/sulfamethoxazole, chloramphenicol and gentamicin by Enterobacteria in their study. While we observed highest resistance of the bacterial organisms to streptomycin, [23], observed the highest resistance to tetracycline, chloramphenicol, ampicillin, and sulfamethoxazole-trimethoprim. A surveillance study in China that investigated the antibiotic resistance trends in *E. coli* originating from food animals during 2008–2015 [24], reported a high resistance rate to tetracycline, sulfamethoxazole, and ampicillin. The report of this survey further supports the fact that ampicillin and tetracycline have a long history of use in animals [23,24]. [25], stated that factors behind the emergence and spread of resistant bacteria are complex. They may be due to coselection, whereby using one antibiotic selects for resistance to other antibiotics.

Interestingly, imipenem (a carbapenem) was the least resisted antibiotics in our study (Plate 1A) which is similar to the report of [23] which studied *E. coli* from an intensive pig production system in South Africa and observed low resistance to imipenem. Notwithstanding the low percentage resistance to carbapenems, the emergence of carbapenems resistance is a serious health challenge as the WHO classifies them as critically important antibiotics and carbapenems are the last-resort antibiotics for treating a wide range of bacterial infections caused by multidrug-resistant Gram-negative bacteria [26].

Organisms expressing *in vitro* resistance to three or more antimicrobial classes are referred to as multidrug-resistant organisms [27]. A total of

51.4% of all our isolates exhibited multidrug resistance to the antibiotics tested. Extensive use of antimicrobial agents, particularly β-lactams and tetracyclines, in pig production may be responsible for the emergence of multidrug-resistant (MDR) enteric bacteria as suggested by [26].

5. CONCLUSION

There is abundance of *E. coli* bacterial organisms in faecal samples collected from piggery in the three senatorial zones in Anambra State. These isolate showed appreciable antimicrobial resistance to 10 conventional antibiotics. Antimicrobial stewardship should be monitored in pig farms to reduce the spread of antibiotic resistance.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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