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MOLECULAR CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY OF BACTERIAL ISOLATES FROM PIGS IN ENUGU STATE

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Abstract

Antibiotics which now represent a major class of antimicrobial agents and has been very useful in treating diseases caused by microorganisms. Resistance to this antibiotic by microbes has become a threat to global health. Hence, the need to identify them and their successive resistance patterns. This study has been undertaken to isolate and identify isolates in piggery in Enugu. Eighteen samples were collected from the feaces and anus of the pigs from each local government. Both E. coli and Staphylococcus aureus demonstrated high susceptibility to Levofloxacin (83%) and Azithromycin (83%). Gentamycin also showed considerable effectiveness, with a susceptibility rate of 55%. Bacteria isolates exhibited notable resistance to certain antibiotics. Augmentin posed the highest resistance, with both organisms showing an 83% resistance rate, followed by Amoxicillin at 72%. Cefotaxime and Sparfloxacin demonstrated similar resistance rates of 67%. In the molecular dimension of this research, the extracted DNA from all eighteen isolates exhibited a notably high molecular weight. Subsequently, the amplification of this DNA was carried out using the 16SrRNA gene of the bacteria, resulting in fragments of approximately 1500 base pairs. The molecular characterization was further advanced by sequencing two representative samples. Upon sequencing, these samples demonstrated robust strain identification, particularly when compared with the NCBI accession number database. Isolate AA1displayed a significant 87.8% pairwise identity with a strain of Escherichia coli, affirming the accurate genetic association of this isolate with the specified bacterial species. Similarly, isolate NE2 exhibited a noteworthy 86.50% pairwise identity with a strain of Staphylococcus aureus, confirming its genetic alignment with this particular bacterial strain. This molecular analysis not only validates the bacterial species identification but also provides insights into the genetic relatedness of the isolates to known strains. The use of the 16SrRNA gene amplification and sequencing, coupled with pairwise identity comparisons, enhances the precision of the molecular characterization, contributing valuable data to the overall understanding of the genetic composition of the bacterial isolates under investigation.

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I. INTRODUCTION

The demand for animal protein has increased in recent decade and this has caused the increase in the consumption of antibiotics by livestock notably pigs. To be used on livestock, an antimicrobial agent must exhibit selective toxicity; it must exhibit greater toxicity to the infecting pathogens than to the host organism. Antibiotics must be selectively harmful since they are produced by single bacteria and have varied degrees of toxicity against others. Bacteria specie such as *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hemolyticus, Clostridium perfringes,* (White *et al.,* 2019), *E. coli, Pseudomonas aureginosa, Klebsiella aerogenes, Proteus vulgaris, Corynobacterium xerosis* have been isolated from piggery (Adewale, 2018). These bacteria cause disease and they heavily affect the health of pigs, especially, respiratory system and digestive system disease which are reported to be associated with intensive pig production. For example, *Escherichia coli* and *Salmonella enteria* can lead to diarrhea and other gastrointestinal disease (Kaper *et al*., 2004).

Even more alarming is that some of these species, particularly bacteria, have developed resistance to the majority of the antibiotics originally employed to kill them. Although antibiotic resistance (ABR) occurs naturally, it has been exacerbated by inappropriate and excessive use, poor therapy adherence, and over-use of antibiotics in food-producing animals, the inability to administer antibiotics by weight of pigs, and poor hygiene and sanitation (Marinho *et al.,* 2016).

The use of antibiotics on animal has been a concern especially when the drug classes are the same or related to the pharmaceutical used in the control of human infection. Philip *et al*., (2004) reported that the use of antibiotics in food animal selects for bacteria resistant to antibiotics used in human and these might spread via the food to human and cause human infection.

Antibiotic-resistant bacteria can develop and move between food-producing animals and humans by direct exposure or through the food chain and the environment, irrespective of geographical or ecological borders (Marinho *et al.,* 2016).

The emergence of antibiotic resistance is a major concern around the world. And so, it is necessary to investigate the effect of these antibiotics on pigs which serve as a high source of protein to Enugu indigenes.

II PROBLEM STATEMENT

Antibiotics which now represent a major class of antimicrobial agents and has been very useful in treating diseases caused by microorganisms. Resistance to these antibiotics by microbes has become a threat to global health. Hence, the need to identify them and their successive resistance patterns. For instance, in 2021 there was serious case of swine endemic experienced by pig Farmers which led to heavy loss as a lot of pig farmers had to look for alternative businesses. Swine flu is a concern has proved to be resistant to antibiotic and even china has battled in time past. Pigs are more in danger from June - November; hence Farmers are more meticulous during these periods.

III LITERATURE REVIEW

TABLE: I SUMMARY of LITERATURE REVIEW

S/no	Name of Authors	Year of Publication	Title of Paper	Work Done	Research Gap
1.	Eze, J. C., Ezeh, C., and Okeke, N. E.	2017	Antibiotic resistance profiles and molecular characterization	This research focused on analyzing antibiotic resistance profiles and molecular characteristics of	Provided further insight into the prevalence of antibiotic resistance

			of bacteria isolated from pigs in Ebonyi State.	bacteria from pigs. It utilized PCR and other molecular techniques to identify bacterial species and ascertain their resistance patterns against various antibiotics.	among bacterial isolates from pigs in Ebonyi State.
2.	Sharma, R., Kumar, P., & Sharma, D.	2019	Antibiotic Resistance Patterns and Molecular Characterization of Bacterial Isolates from Pigs in India: A Review.	This review article comprehensively analyzes the antibiotic resistance patterns and molecular characterization of bacterial isolates from pigs in India. The authors delve into various aspects, including the prevalence of antibiotic resistance, the genetic mechanisms underlying resistance, and the implications for both animal and human health. They explore the spectrum of bacterial pathogens affecting pigs, such as Escherichia coli, Salmonella spp., Staphylococcus aureus, and others, shedding light on their resistance profiles and genetic determinants. The review synthesizes findings from multiple studies conducted across different regions of India, highlighting the diversity and complexity of antibiotic resistance in pig farming. It discusses the factors contributing to the emergence and spread of resistance, including the misuse and overuse of antibiotics in livestock production, inadequate surveillance, and limited enforcement of regulations. Moreover, the review elucidates the role of mobile genetic elements, such as plasmids and integrons, in disseminating resistance	Despite the wealth of information presented in the review, several research gaps remain to be addressed. One notable gap is the limited understanding of the socio-economic factors influencing antibiotic use in pig farming and their implications for resistance selection. Additionally, there is a need for more longitudinal studies to elucidate the temporal trends in resistance patterns and the effectiveness of control measures over time. Furthermore, while the review provides insights into the genetic basis of resistance, further research is needed to unravel the intricate interplay between genetic determinants, bacterial ecology, and host factors shaping resistance dynamics in pig populations. Addressing these

				genes among bacterial populations.	gaps is essential for developing evidence-based interventions to combat antibiotic resistance in the pig farming sector and safeguard public health.
3.	Obeta, M. C., Odugu, M., and Anyanwu, M. U.	2021	Genetic diversity and antibiotic susceptibility patterns of bacteria isolated from pigs in Enugu State, Nigeria.	This study investigated the genetic diversity and antibiotic susceptibility patterns of bacteria isolated from pigs in Enugu State. It employed molecular techniques such as PCR and PFGE to identify bacterial species and assess their genetic diversity, alongside antibiotic susceptibility testing.	Explored the genetic diversity of bacterial isolates from pigs and their antibiotic susceptibility patterns, providing insights into the evolution and spread of antibiotic resistance in the region.
4.	, I., Kabic, J., Kekic, D., Jovicevic, M., Milenkovic, M., Mitic Culafic, D., Trudic, A., Ranin, L., and Opavski, N.	2022	Antimicrobial Susceptibility Testing: A Comprehensive Review of Currently Used Methods.	This article discusses common antimicrobial susceptibility testing (AST) methods, their advantages, and disadvantages. It emphasizes the need for innovative, rapid, and accurate diagnostic tools for AST in the context of increasing antimicrobial resistance.	Contributed to the understanding of the molecular diversity and antibiotic susceptibility of bacterial pathogens in pigs, filling gaps in knowledge about antibiotic resistance in the region.
5.	Mulemba Tillika Samutela, Bruno Stephen July Phiri, Edgar Simulundu, Geoffrey Kwenda, Ladslav Moonga, Eugene C. Bwalya, Walter Muleya, Therese Nyirahabimana, Kaunda Yamba, Henson Kainga,	2022	Antimicrobial Susceptibility Profiles and Molecular Characterization of *Staphylococcus aureus* from Pigs and Workers at Farms and Abattoirs in Zambia	The study investigated the prevalence, phenotypic and genotypic characteristics of *Staphylococcus aureus* from pigs and workers at farms and abattoirs in Lusaka Province, Zambia. They collected nasal swabs from pigs and humans, isolated *S. aureus*, determined antimicrobial susceptibility patterns, and evaluated genetic diversity	The presence of plasmid-mediated resistance genes and immune evasion cluster genes among the isolates is of great public health concern. Continuous surveillance of *S. aureus* using a "One Health" approach is warranted to monitor infections and the spread of

	Simegnew Adugna Kallu, Innocent Mwape, Andrew Frey, Matthew Bates, Hideaki Higashi, and Bernard Mudenda Hang'ombe			using spa typing.	antimicrobial resistance.
6.	Wang, C., He, T., Shen, J., Zhang, P., Guo, D., Xiao, Q., & Zhang, B.	2018	Molecular characterization and antimicrobial susceptibility of Salmonella isolated from pigs with diarrhea in China.	This study aimed to investigate the molecular characteristics and antibiotic susceptibility of Salmonella strains isolated from pigs with diarrhea in China. The researchers conducted molecular typing using pulsed-field gel electrophoresis (PFGE) and assessed antimicrobial susceptibility using the disk diffusion method.	Although the study provides valuable insights into Salmonella strains in pigs, further research could explore the transmission dynamics and factors contributing to antimicrobial resistance in these strains.
7.	Alali, W. Q., Thakur, S., Berghaus, R. D., Martin, M. P., & Gebreyes, W. A.	2010	Prevalence and distribution of Salmonella in organic and conventional broiler poultry farms.	This study investigated the prevalence and distribution of Salmonella in organic and conventional broiler poultry farms in the United States. The researchers analyzed Salmonella isolates using molecular techniques and assessed their antibiotic resistance profiles.	While the study sheds light on Salmonella prevalence in poultry farms, there is a need for further investigation into the specific antibiotic resistance patterns and molecular characterization of Salmonella isolates from pigs, as this study focused on poultry.
8.	Szmolka, A., Nagy, B.	2013	Multidrug resistant commensal Escherichia coli in animals and its impact for public health.	This review summarizes the prevalence, antibiotic resistance profiles, and molecular characteristics of multidrug-resistant Escherichia coli (MDR E. coli) in various animal species, including pigs. It	While the review provides a comprehensive overview of MDR E. coli in animals, including pigs, more research is needed to understand the

				discusses the potential public health implications of MDR E. coli transmission from animals to humans.	specific antibiotic resistance patterns and molecular mechanisms of MDR E. coli isolates from pigs worldwide.
9.	Tang, K. L., Caffrey, N. P., Nóbrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W, & Polachek, A. J.	2017	Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis.	This systematic review and meta-analysis aimed to assess the association between restricting antibiotic use in food-producing animals and antibiotic resistance in both animals and humans. The study synthesized evidence from various global studies and analyzed the impact of antibiotic restrictions on antibiotic resistance patterns.	While the review provides insights into the association between antibiotic use in food-producing animals and antibiotic resistance, further research specifically focusing on bacterial isolates from pigs could provide more targeted insights into the molecular characterization and antibiotic resistance patterns of these isolates.
10.	Iroha, I. R., Oji, A. E., and Ugwu, M. C.	2019	Prevalence and molecular characterization of antibiotic-resistant bacteria in pigs from Enugu State, Nigeria.	This research aimed to determine the prevalence of antibiotic-resistant bacteria in pigs and characterize them molecularly. It utilized PCR and sequencing techniques to identify bacterial species and examine their antibiotic resistance profiles.	Provided updated information on the prevalence and molecular features of antibiotic-resistant bacteria in pigs, contributing to the understanding of antibiotic resistance dynamics in the region.

IV METHODOLOGY

Kirby- Bauer disc diffusion method was used to determine the sensitivity of the bacteria to antibiotics. The following antibiotics were subjected to susceptibility test:

LEV – Levofloxacin, CF – Cefotaxim, SP – Sparfloxacin, CPX – Ciprofloxacin, AM – Amoxacillin, AU – Augmentin, CN – Gentamycin, PEF – Pefloxacin, OFX – Tarivid, AZ – Azithromycin, CH – Cephalothin, SXT – Sulfamethoxazole, S – Streptomycin.

V. MATERIALS AND METHOD

Study Area

The study was conducted at three different local governments in Enugu state, which are Awgu, Nsukka and Orji-river.

Study Population

Two piggery farms were visited in each local government which is Awgu, Nsukka and Orji-river.

Ethical Clearance

The Enugu State University Teaching Hospital granted ethical clearance.

Methodology for Antibiotic Susceptibility Test

Kirby- Bauer disc diffusion method was used to determine the sensitivity of the bacteria to antibiotics. The following antibiotics were subjected to susceptibility test:

LEV – Levofloxacin, CF – Cefotaxim, SP – Sparfloxacin, CPX – Ciprofloxacin, AM – Amoxacillin, AU – Augmentin, CN – Gentamycin, PEF – Pefloxacin, OFX – Tarivid, AZ – Azithromycin, CH – Cephalothin, SXT – Sulfamethoxazole, S – Streptomycin.

Sample Collection

Eighteen samples were collected from the feaces and anus of the pigs from each local government. Awgu local government had six samples, Nsukka local government had 6 samples and Oji-River had six samples. Each sample was collected using sterile swab stick and sterile container. The samples were labeled according to the time it was collected; they were immediately taken to Godfrey Okoye University microbiological lab for examination.

Preparation of Media

Mac Conkey agar, Nutrient agar, Nutrient broth, Peptone water, Citrate agar and Urease agar were all prepared using manufacturers guidance.

Isolation of Bacteria

Isolation of bacteria refers to the process of obtaining pure cultures of bacterial cells from a mixed population or sample. It's an essential step in microbiological research, clinical diagnostics, and various biotechnological applications. The goal of bacterial isolation is to separate individual bacterial cells or colonies so that they can be studied individually or characterized for specific purposes.

Serial Dilution

In the process of assessing microbial load, a comprehensive serial dilution technique was employed to systematically reduce the concentration of microorganisms in a series of steps. The following steps were undertaken to achieve accurate and reliable dilutions:

Eighteen samples containing feaces from pigs were transferred into a sterile container and labeled according to where they were gotten from. Serial dilution was carried out using 5-fold dilutions. The original samples were transferred into a sterile test tube containing 3ml of distilled water and mixed thoroughly, and then 5 extra test tubes were arranged in a test tube rack which was filled with 9ml of distilled water. One mil was extracted from the original sample using a sterile pipette and transported into the first test tube containing 9ml of distilled water, thorough mixing of the contents ensured an even distribution of microorganisms. Transfers of known volumes (1ml) from each preceding dilution to subsequent tubes containing diluents were carried out systematically; mixing was performed thoroughly after each dilution step.

The third test tubes from each dilution were plated onto nutrient agar plates using an appropriate volume, pour plate methods were implemented based on the expected microbial load.

The agar plates were placed in an incubator at 37^{o}C for 24 hours in order for the microorganisms to facilitate growth. After the incubation period, colonies were meticulously counted on plates with a countable number, typically ranging between 30 and 300 colonies, ensuring accuracy.

The microbial load in the original sample was calculated using the colony counts from plates, considering the dilution factors. The formula employed was: Microbial Load = Number of Colonies multiplied by Dilution Factor divided by Volume Plated.

After the microbial load has been calculated the 18 feaces samples were cultured using both nutrient agar and MacConkey agar and were incubated for 24 hours in an incubator under aseptic measures. A stock solution was then made after the cultivated plates had been incubated for 24 hours at 37^{o}C and checked for bacterial growth. Biochemical assays of catalase test, oxidase test, indole test, methyl red test, Urease test and Gram staining were conducted.

Colony Morphology

The incubated plates were characterized by identifying the media macroscopically via texture, color, shape.

Gram Staining

The preparation of bacterial smears for microscopic examination involves a series of meticulous steps. Initially, the organisms were distributed thinly and evenly on a slide, after which they were allowed to air dry. Subsequently, the slide undergoes heat fixation by passing through a flame three times, securing the bacteria firmly onto the slide. To facilitate staining, a few drops of crystal violet stain were applied to the smear, and a waiting period of one minute ensued. Following this, the excess crystal violet stain was gently rinsed off using distilled water. The next step involved the application of Gram's iodine solution to the smear, allowing it to sit for another minute. Notably, Gram's iodine functions as a mordant in this context, forming a complex with crystal violet. This interaction serves to enhance the staining process, contributing to the clarity and precision of the microscopic examination of the bacterial smear. The systematic execution of these steps is crucial in ensuring accurate and reliable results in the Gram staining procedure. The Gram iodine was rinsed off using distilled water. Acetone-alcohol was gently added to decolorize the smear and distilled water was quickly used to rinse off the acetone-alcohol to stop the decolorization process. A few drops of safranin, serving as a counterstain, were delicately applied to the bacterial smear. The smear was allowed to sit with safranin for one minute, facilitating the staining of bacterial cells that might not have retained the initial crystal violet. Following the safranin application, the excess stain was gently rinsed off using distilled water. Subsequently, the stained bacterial smear was left to air dry, preparing it for observation under a microscope. This step is critical for the comprehensive examination of the bacterial cells, as the safranin counterstain imparts color to cells that did not retain the crystal violet during the initial Gram staining process.

Catalase Test

A pure culture of bacteria was streaked into a prepared agar medium which was salmonella shigella and incubated at 37^{o}C for 18-24 hours. After incubation, the pure colony of the organism was transferred into a sterile test tube containing a small volume of sterile saline solutions and mixed thoroughly to create a bacterial suspension. A small amount of bacterial suspension was transferred onto a clean slide. Few drops of hydrogen peroxide were added directly to the bacteria suspension on the slide. Bubbles were observed and it indicated a positive catalase reaction.

Citrate Test

Citrate agar was prepared according to manufacturer’s guide and 5ml was poured into a test tube and was allowed to solidify. Bacterial colonies were added into the test tube using wire loop and incubated at 37^{o}c for 72 hours. After incubation, some of the test tube colours at the top were blue which indicated a positive reaction while some remain the same having no colour change which indicated a negative reaction.

Indole Test

A pure culture of bacteria was streaked into a prepared agar medium which is *Salmonella shigella* and incubated at 37^{o}c for 18-24 hours. After incubation, a small loopful of the culture was transferred into a sterile test tube

containing peptone water. The broth will be mixed thoroughly and incubated at 37°c for 18-24 hours. After incubation, a few drops of Kovacs reagent were added to the test tube and gently mixed by swirling. No colour was observed in some test tubes which indicated a negative indole reaction while some test tubes had red colour at the top which indicates a positive reaction.

Oxidase Test

Stripe of Whatman's filter paper was soaked in a freshly prepared in 1% solution of the oxidase reagent. The colony was picked up using a wire loop under aseptic measures and was smeared over the moist area. The result was observed for 3 minutes and it was a negative reaction.

Urease Test

A pure culture of bacteria was streaked into a prepared agar medium which is salmonella shigella and incubated at 37°C for 24 hours. After incubation, a well isolated colony of bacteria was selected using a sterilized loop or needle and inoculated onto a urease agar slant tube ensuring that the inoculums is evenly spread or mixed. It was incubated at 37°C for 24 hours. There was no colour change in some test tubes which indicated a negative reaction while there was colour change pink in some test tubes which indicated a positive reaction.

Methyl Red Test

Peptone water was prepared according to manufacturer's guide and 5ml was poured into a sterile test tube. Bacterial colonies were added into the test tube using wire loop under aseptic measures and incubated at 37°C for 48 hours. Then 5 drops of methyl red reagent were added to the test tube and observed for colour change. There was red colour at the top and it indicated a positive reaction

DNA Extraction Using ZR Fungal/Bacterial DNA Miniprep (Manufactured by Zymo Research)

Two milliliters of bacterial cell broth were added to a ZR Bashing TM Lysis Tube, and 750 microliters of Lysis Solution were added to the tube. The tube was secured in a bead fitted with a 2 ml tube holder assembly and processed at maximum speed for more than 5 minutes. The ZR Bashing Bead TM Lysis Tube was centrifuged in a microcentrifuge at more than 10,000 x g for 1 minute. Up to 400 microliters of supernatant were transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 x g for 1 minute. One thousand two hundred microliters of Fungal/Bacterial DNA Binding Buffer were added to the filtrate in the Collection Tube from the previous step. After being moved to a Zymo-SpinTMIIC Column in a Collection Tube, eight hundred microliters of the Step 5 mixture were centrifuged at 10,000 x g for one minute. After discarding the flow-through from the Collection Tube, Step 6 was carried out once more. After adding 200 microliters of DNA Pre-Wash Buffer to the Zymo-Spin TM IIC Column in a fresh Collection Tube, centrifugation was carried out for one minute at 10,000 x g. The Zymo-SpinTMIIC Column was filled with 500 microliters of Fungal/Bacterial DNA Wash Buffer, and centrifugation was carried out at 10,000 x g for one minute. After moving the Zymo-SpinTMIIC Column to a sterile 1.5 ml microcentrifuge tube, 100 microliters (35 ul at the very least) of DNA Elution Buffer were added straight to the matrix of the column. To elute the DNA, centrifugation was done for 30 seconds at 10,000 x g.

Electrophoresis for DNA and PCR

For DNA electrophoresis, 1 gram of agarose was measured, while 2 grams were used for PCR. The agarose powder was mixed with 100 mL of 1xTAE in a microwavable flask. After microwaving for 1-3 minutes until complete dissolution (being cautious not to over boil to prevent buffer evaporation), the agarose solution cooled to around 50 °C, taking approximately 5 minutes.

Next, 10µL of EZ vision DNA stain was added to the agarose solution, facilitating DNA visualization under UV light. The agarose mixture was poured into a gel tray with the well comb in place. The newly poured gel was either placed at 4 °C for 10-15 minutes or left at room temperature for 20-30 minutes until it solidified.

Loading Samples and Running an Agarose Gel

For loading samples and running the agarose gel, loading buffer was added to each DNA sample or PCR product. Once solidified, the Agarose gel was placed in the gel box, filled with 1xTAE until the gel was covered. A molecular weight ladder was carefully loaded into the first lane, followed by the samples in the additional wells. The gel was run at 80-150 V for about 1-1.5 hours. After turning off the power, disconnecting

the electrodes, and carefully removing the gel from the gel box, DNA fragments or PCR products were visualized under a UV transilluminator.

16SrRNA Gene Amplification of the Bacterial Isolate

The PCR mix is made up of 12.5µL of Taq2X Master Mix from New England Biolabs (M0270); 1µL each of 10µM forward (27F: AGAGTTTGATCMTGGCTCAG) and reverse (1525R: AAGGAGGTGWTCCARCCGCA) primer; 2µL of DNA template and then made up with 8.5µL Nuclease free water.

Gene

36 cycles of denaturation at 94˚C for 30 seconds, annealing at 56˚C for 30 seconds, and elongation at 72˚C for 45 seconds were performed after the initial denaturation at 94˚C for 5 minutes. Followed by a final elongation phase at 72˚C for 7 minutes and hold temperature at 10 ˚C indefinitely.

Sequencing

Using the BigDye Terminator v3.1 cycle sequencing kit, the amplified fragments were sequenced using an Applied Biosystems Genetic Analyser 3130xl sequencer, following the manufacturer's instructions. For all genomic analyses, MEGA X and Bio-Edit software were utilized.

Table II: Shows the breakdown of Isolates collected from each location

S/no	Location	Number of E- *Coli* Isolates	Number of Staphylococcus Aeurus	Total Number of Isolates Per Location
1.	Awgu LGC	4	2	6
2.	Nnsuka LGC	3	6	9
3.	Oji-River LGC	1	2	3
	Total Number of Isolates	**8**	**10**	**18**

E – Coli:

Awgu LGC has the highest count with four (4)

Nnsuka LGC follows with three (3) occurrences.

Oji-River LGC has the lowest count with only one (1) occurrence.

Staphylococcus:

Nnsuka has the highest count with six (6).

Awgu and Oji-River LGC’s tie with two (2) occurrences each

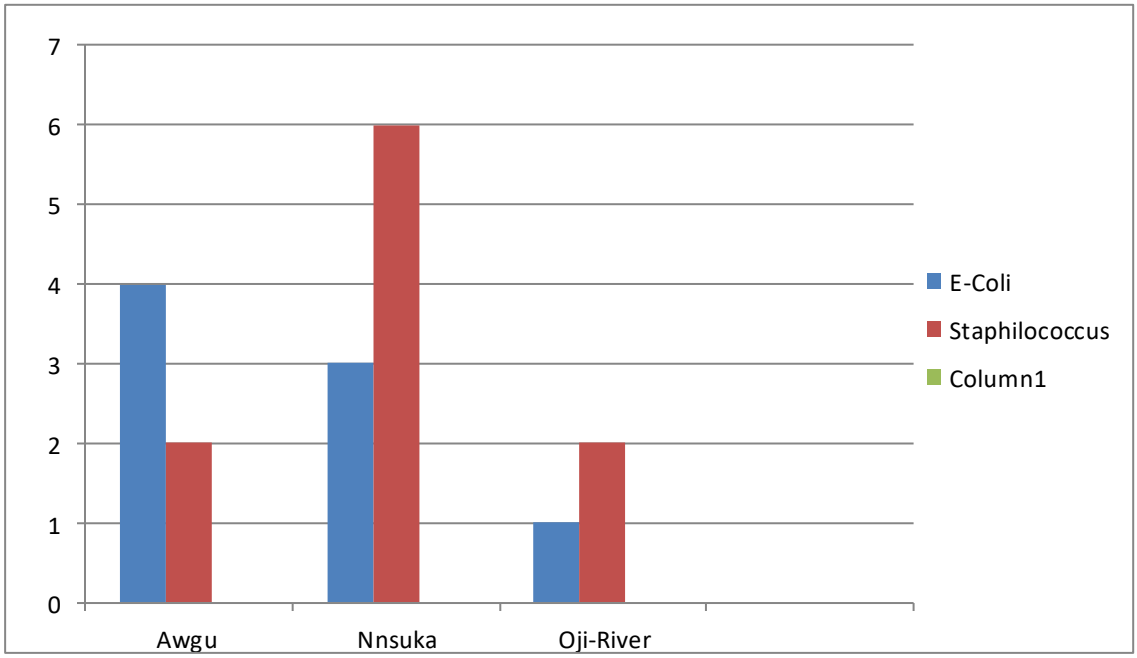


Fig. 1. Shows a Bar Chart Representing the Isolates from the 3 Locations

VI. RESULTS

Eighteen samples were collected from the feaces and anus of the pigs from each local government. Awgu local government had six samples, Nsukka local government had 6 samples and Orji-River had six samples. Eight (8) isolates were E. coli while the remaining ten isolates were Staphylococcus aeurus. E coli was labelled as AA1, AA2, OC3, NF2, NF3, NE1, OD2 and AB3 while Staphylococcus aeurus were labelled as AA3, OC1. OC2, NF1, NE2, NE3, OD1, OD3, AB1 and AB2.

I limited the number of isolates for analysis to two (2) so as to streamline my research by allowing for deeper investigation into each individual sample, reducing resources and time demands, ensuring statistical power and facilitating clearer interpretation of results.

Table III: Shows morphological characteristics of the bacterial isolates found. Samples AA1, AA2, OC3, NF2, NF3, NE1, OD2 and AB3 were identified as E. coli because of their circular, smooth, pink, rod shape features and they were all gram negative. While AA3, OC1. OC2, NF1, NE2, NE3, OD1, OD3, AB1 and AB2 were identified as Staphylococcus aeurus because of their irregular, smooth, yellow colour, grapelike features and they were all gram positive.

Table III: Morphological Characterization of Bacterial Isolates

Bacterial Isolates	Shape	Texture	Colour	Microscopy	Characterization	Probable organism
AA1	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*
AA2	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*
AA3	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
OC1	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
OC2	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
OC3	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*
NF1	Irregular	smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
NF2	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*
NF3	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*
NE1	Circular	Smooth	Pink	Rod shape	Gram-negative	*E.coli*
NE2	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
NE3	Irregular	Smooth	Yellow	Grapelike Shape	Gram-positive	*Staphylococcus aureus*
OD1	Irregular	Smooth	Yellow	Grapelike Shape	Gram-positive	*Staphylococcus aureus*
OD2	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*
OD3	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
AB1	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
AB2	Irregular	Smooth	Yellow	Grapelike shape	Gram-positive	*Staphylococcus aureus*
AB3	Circular	Smooth	Pink	Rod shape	Gram-negative	*E. coli*

Table IV: Shows Biochemical characteristics of the bacterial isolates found. All the isolates were all positive in catalase, methyl red test and all were also negative in oxidase test. Samples AA1, AA2, OC3, NF2, NF3, NE1, OD2 and AB3 were all negative in citrate and Urease test but positive in indole test while sample AA3, OC1. OC2, NF1, NE2, NE3, OD1, OD3, AB1 and AB2 were all positive in citrate and Urease test but were negative in indole test.

Table IV: Biochemical Characteristics of Isolates

Bacterial Isolates	Catalase Test	Indole Test	Oxidase Test	Methyl red Test	Urease Test	Citrate Test	Probable Organism
AA1	+	+	-	+	-	-	*E. coli*
AA2	+	+	-	+	-	-	*E. coli*
AA3	+	-	-	+	+	+	*Staphylococcus aureus*
OC1	+	-	-	+	+	+	*Staphylococcus aureus*
OC2	+	-	-	+	+	+	*Staphylococcus aureus*
OC3	+	+	-	+	-	-	*E. coli*
NF1	+	-	-	+	+	+	*Staphylococcus aureus*
NF2	+	+	-	+	-	-	*E. coli*
NF3	+	+	-	+	-	-	*E. coli*
NE1	+	+	-	+	-	-	*E. coli*
NE2	+	-	-	+	+	+	*Staphylococcus aureus*
NE3	+	-	-	+	+	+	*Staphylococcus aureus*
OD1	+	-	-	+	+	+	*Staphylococcus aureus*
OD2	+	+	-	+	-	-	*E. coli*
OD3	+	-	-	+	+	+	*Staphylococcus aureus*
AB1	+	-	-	+	+	+	*Staphylococcus aureus*
AB2	+	-	-	+	+	+	*Staphylococcus aureus*
AB3	+	+	-	+	-	-	*E. coli*

Table V: Shows Antibiotic susceptibility of bacterial isolates. The measurement of zone of inhibition was in millimeter and they were compared to those of Clinical Laboratory Standards Institute Chart and was reported as susceptible (S), intermediate (I) and resistant (R). Both organisms were highly Susceptible to Levofloxacin (83%) and Azithromycin (83%), followed by Gentamycin (55%) while both isolates had the highest resistance to Augmentin (83%), followed by Amoxicillin (72%), Cefotaxime and Sparfloxacin had (67%) each. The calculation of the Multiple Antibiotic Resistance Index (MARI) was undertaken, isolate AA1 had the highest multiple resistance index (1.0), followed by OD3 having 0.9 and NE1, NE3 having 0.7. The lowest multiple resistance index was in NE2 having 0.1 respectively.

Table V: Antibiotic Susceptibility of bacterial isolates

Bacterial isolates	LEV	CF	SP	CPX	AM	AU	CN	PEF	OFX	AZ	MAR Index
AA1	S	S	S	S	S	S	S	S	S	S	1
AA2	R	R	S	S	S	R	S	S	S	S	0.4
AA3	R	R	R	S	R	R	S	R	R	R	0.2
OC1	S	R	R	R	R	R	R	R	S	S	0.3
OC2	S	R	R	S	R	R	S	R	S	S	0.5
OC3	S	R	R	R	R	R	R	S	S	S	0.4
NF1	S	R	R	R	R	R	R	R	R	S	0.2
NF2	S	R	R	R	R	R	R	R	R	S	0.2
NF3	S	R	R	R	R	R	S	R	S	S	0.4
NE1	S	S	I	S	I	I	S	S	S	S	0.7
NE2	S	R	R	R	R	R	R	R	R	R	0.1
NE3	S	S	S	S	S	R	S	R	S	R	0.7
OD1	R	S	S	S	R	R	S	S	S	S	0.6
OD2	S	R	R	R	R	R	R	R	R	S	0.2
OD3	S	S	S	S	I	S	S	S	S	S	0.9
AB1	S	R	R	R	R	R	R	R	R	S	0.2
AB2	S	S	R	R	R	R	S	S	S	S	0.6
AB3	S	R	R	I	R	R	R	R	S	S	0.3

Key

R – Resistant, S – Susceptible, I – Intermediate, LEV – Levofloxacin, CF – Cefotaxim, SP – Sparfloxacin, CPX – Ciprofloxacin, AM – Amoxacillin, AU – Augmentin, CN – Gentamycin, PEF – Pefloxacin, OFX – Tarivid, AZ – Azithromycin, CH – Cephalothin, SXT – Sulfamethoxazole, S – Streptomycin, MAR- Multiple antibiotic resistance.

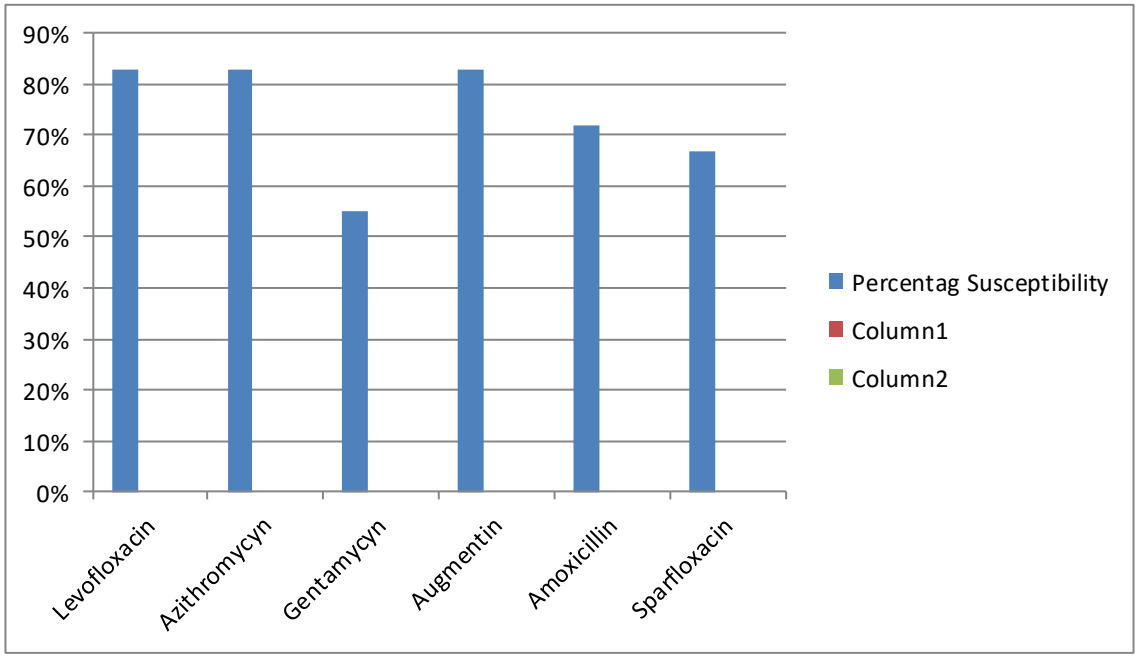


Fig 2. Shows the Representation of Percentage Susceptibility to Different Antibiotics among Isolates

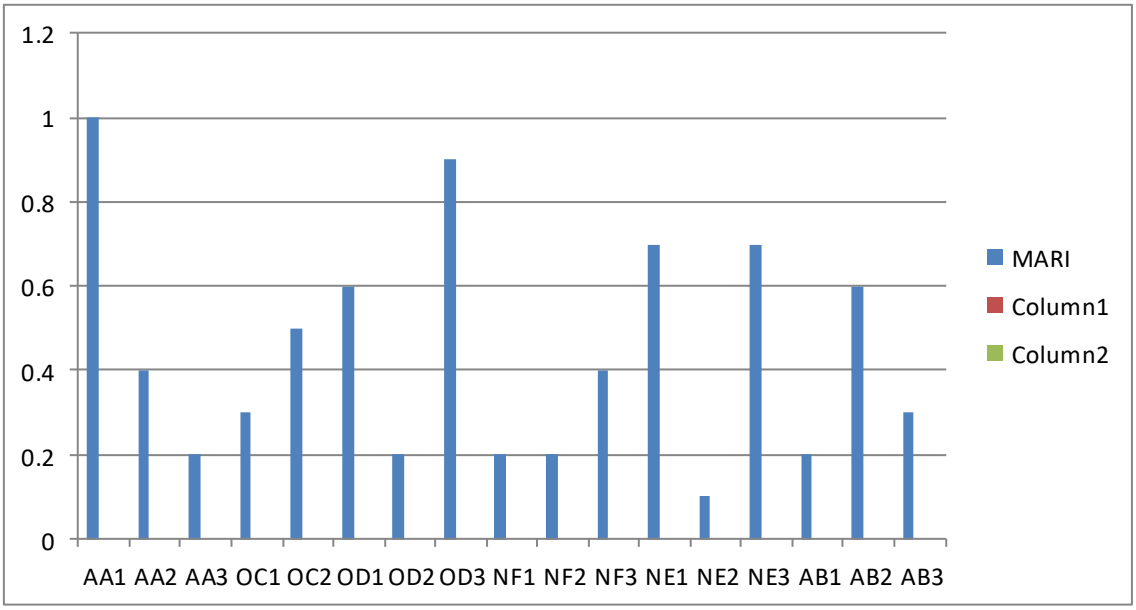
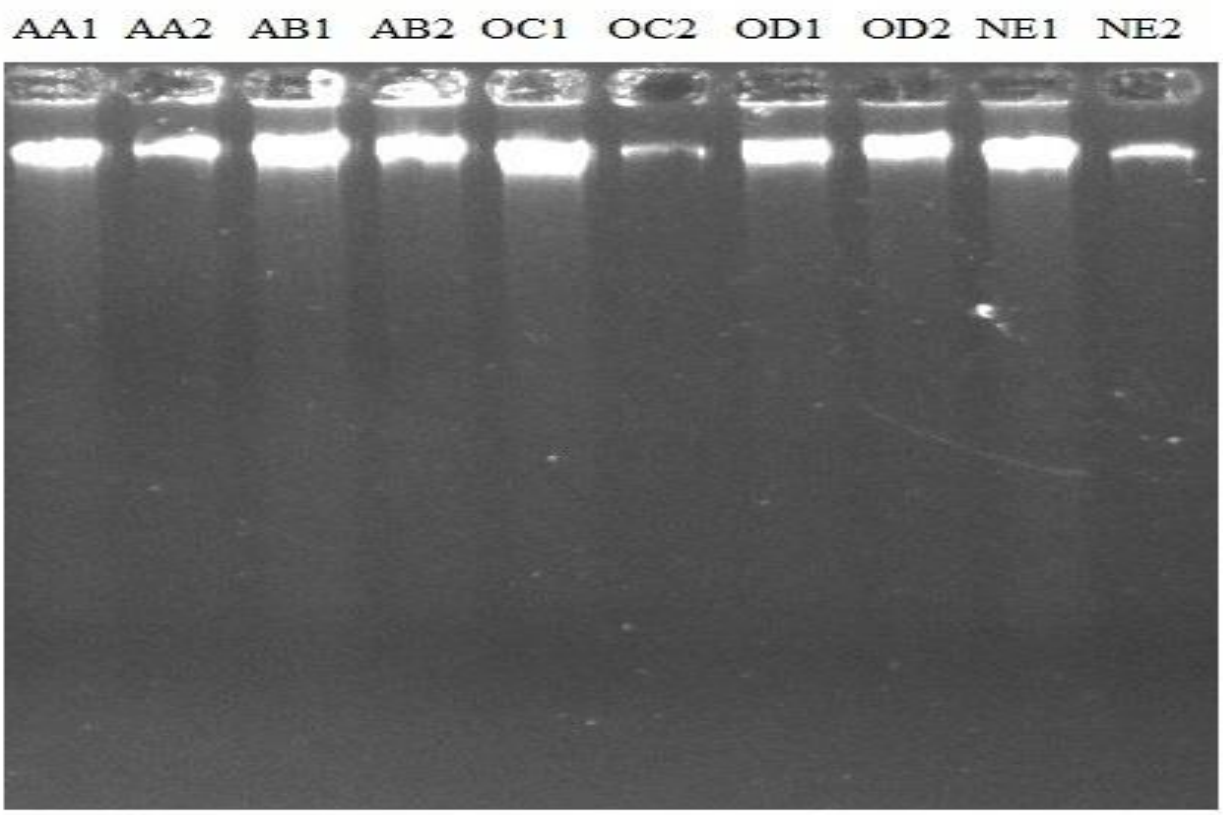
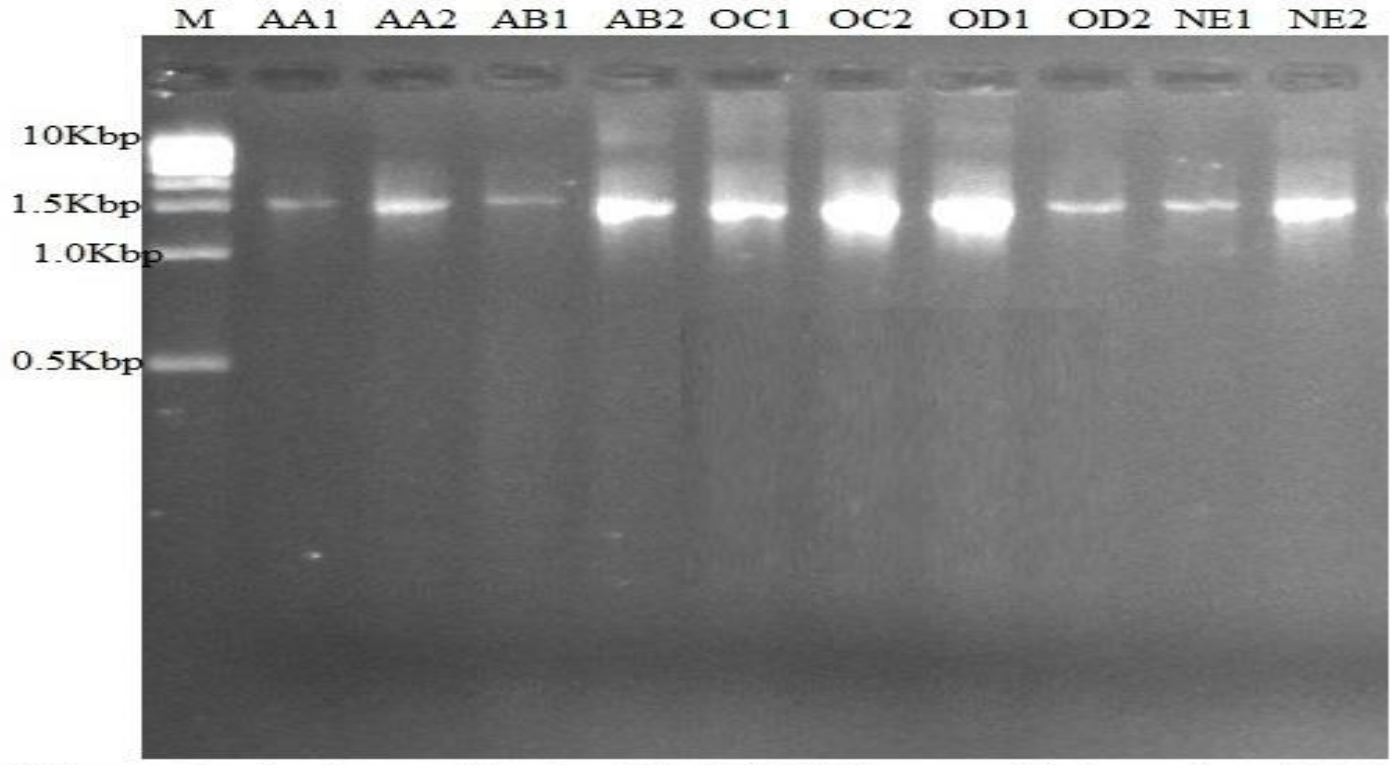


Fig. 3. Shows the Multiple Antibiotic Resistance Index (MARI)



Gel image showing high Molecular weight DNA extracted from the isolates

Fig. 4. Shows the Gel Image High Molecular Weight DNA Extracted from Isolates



Gel image showing the amplification of the 16SrRNA gene of the bacteria at 1500bp

Fig 5**.** Shows Gel image Amplification of the 16SrRNA Gene of the bacteria at 1500bp

Fig. 6. Rooted Neighbor-Joining phylogram depicting the evolutionary relationships (phylogeny) among 16S rRNA gene sequences of selected bacterial species alongside closely related Genbank relatives. Pisolithus sp. served as the outgroup

The numbers preceding the organism's names are the accession numbers of the closest Genbank relatives. The Neighbor-Joining (NJ) method stands as a widely favored algorithm in constructing Phylogenetic trees from genetic distance data. Renowned for its computational efficiency and relatively strong performance in delineating Phylogenetic relationships, NJ is a distance-based approach frequently relied upon in scientific research.

VII SUMMARY OF FINDINGS

A total of eighteen samples were systematically collected from both the feces and anus of pigs residing in each of the local governments under study. The distribution of samples among the local governments was as follows: Awgu local government contributed six samples, Nsukka local government provided an additional six samples, and Nsukka itself yielded six samples for analysis. Upon analyzing these samples, it was observed that out of the total isolates, eight were identified as belonging to the bacterial species E. coli. These specific *E. coli* isolates were designated with distinct labels: AA1, AA2, OC3, NF2, NF3, NE1, OD2, and AB3. In contrast, the remaining ten isolates were identified as *Staphylococcus aureus*. The *Staphylococcus aureus* isolates were assigned the labels AA3, OC1, OC2, NF1, NE2, NE3, OD1, OD3, AB1, and AB2. The findings are detailed in Table 4.1, which outlines the morphological characteristics of the bacterial isolates. Samples AA1, AA2, OC3, NF2, NF3, NE1, OD2, and AB3, identified as E. coli, exhibited specific morphological features, including circular shapes, smooth textures, a pink coloration, rod-like structures, and a gram-negative classification. Conversely, samples AA3, OC1, OC2, NF1, NE2, NE3, OD1, OD3, AB1, and AB2, identified as *Staphylococcus aureus*, displayed different morphological characteristics, such as irregular shapes, smooth

textures, a yellow coloration, grapelike structures, and a gram-positive classification. This comprehensive analysis provides a detailed account of the sample collection process, the distribution of isolates across local governments, and the specific characteristics used to differentiate between *E. coli* and *Staphylococcus aureus*. The use of distinct labels for each isolate facilitates clear identification and tracking throughout the study. Table 4.2 illustrates the biochemical characteristics of the bacterial isolates under examination. Notably, all isolates exhibited positive results in catalase and methyl red tests, while uniformly displaying negative outcomes in the oxidase test. This consistent pattern indicates a shared biochemical profile among the isolates.

Further differentiations were observed in specific biochemical tests. Samples AA1, AA2, OC3, NF2, NF3, NE1, OD2, and AB3 exhibited negativity in citrate and urease tests but tested positive in the indole test. Conversely, samples AA3, OC1, OC2, NF1, NE2, NE3, OD1, OD3, AB1, and AB2 demonstrated positivity in both citrate and urease tests but yielded negative results in the indole test. This detailed biochemical analysis provides insights into the varied metabolic characteristics of the bacterial isolates. The positive results in catalase and methyl red tests suggest common enzymatic activities, while differences in citrate, urease, and indole tests reveal distinctive biochemical profiles among the isolates. These findings contribute to a comprehensive understanding of the diverse nature of the bacterial populations under investigation. Table 4.3 outlines the antibiotic susceptibility of the bacterial isolates, with the zone of inhibition measurements reported in millimeters and compared to the Clinical Laboratory Standards Institute Chart. Results were categorized as susceptible (S), intermediate (I), and resistant (R). Notably, both E. coli and Staphylococcus aureus demonstrated high susceptibility to Levofloxacin (83%) and Azithromycin (83%). Gentamycin also showed considerable effectiveness, with a susceptibility rate of 55%. Conversely, the isolates exhibited notable resistance to certain antibiotics. Augmentin posed the highest resistance, with both organisms showing an 83% resistance rate, followed by Amoxicillin at 72%. Cefotaxime and Sparfloxacin demonstrated similar resistance rates of 67%. To further assess antibiotic resistance patterns, the Multiple Antibiotic Resistance Index (MARI) was calculated. Isolate AA1 displayed the highest multiple resistance index at 1.0, followed by OD3 with 0.9, and NE1 and NE3 both having a multiple resistance index of 0.7. Notably, NE2 exhibited the lowest multiple resistance index at 0.1. These findings shed light on the antibiotic susceptibility and resistance profiles of the bacterial isolates, providing crucial insights into their potential response to various antibiotic treatments. The MARI calculations offer additional information on the prevalence of multiple antibiotic resistance within specific isolates, contributing to a comprehensive understanding of the antibiotic resistance landscape in the studied bacterial populations. The molecular aspect in this research showed high molecular weight DNA extracted from all eighteen isolates and were also amplified using 16SrRNA gene of the bacteria at 1500bp. The two samples that were sequenced showed high strain identification when compared with NCBI accession number. Isolate AA1 had 87.8% pairwise identity with *Escherichia coli* strain while isolate NE2 had 86.50% pairwise identity with *staphylococcus aureus* strain.

The study encompassed multiple local governments within Enugu State, including Awgu, Nsukka, and others. Data analysis revealed variations in the prevalence rates of E. coli and Staphylococcus aureus among the sampled populations across these local governments. Further analysis indicated that various factors contributed to these differences in prevalence and distribution. These factors include environmental conditions, agricultural practices, waste management systems, and livestock management techniques prevalent within each local government. The research underscores the importance of understanding regional variations in microbial prevalence for effective public health measures and livestock management strategies tailored to each locality within Enugu State.

The potential sources of contamination leading to bacterial isolates in pig feces and anus samples in Enugu State resulting in E. coli and Staphylococcus aureus can be attributed to the following factors:

i. **Feed and Water Quality:** The Study reveals that Contamination originates from the feed and water given to the pigs. Poor quality feed or water contaminated with bacteria leads to bacterial presence in feces and anus samples.

ii. **Hygiene and Sanitation Practices:** The research revealed that inadequate hygiene and sanitation practices in pig farms contribute significantly to bacterial contamination. Lack of proper cleaning of pig pens, equipment, and surrounding areas leads to the proliferation of bacteria.

iii. **Manure Management:** The thesis explains that improper management of pig manure can result in environmental contamination. If manure is not adequately treated or disposed of, it can contaminate the surrounding soil and water sources, which in turn can lead to bacterial contamination of pigs and their feces.

VII. Contribution to Knowledge

This thesis on molecular characterization and antibiotic susceptibility of bacterial isolates from pigs in Enugu State advances our knowledge of antibiotic resistance in livestock, sheds light on the genetic basis of resistance, supports a One Health approach to combating antibiotic resistance, informs surveillance and control efforts, and provides region-specific insights into the epidemiology of antibiotic resistance.

IX. Conclusion

In conclusion, the research on molecular characterization and antibiotic susceptibility of bacterial isolates from pigs in Enugu State provides valuable insights into the prevalence, genetic basis, and regional epidemiology of antibiotic resistance in livestock. By employing molecular techniques to analyze bacterial isolates, the study enhances our understanding of the mechanisms underlying antibiotic resistance and highlights the importance of prudent antibiotic use in animal husbandry. The findings contribute to a One Health approach to addressing antibiotic resistance by recognizing the interconnectedness of human, animal, and environmental health. Furthermore, the research informs surveillance and control strategies aimed at mitigating the spread of antibiotic-resistant bacteria in pig populations, ultimately promoting the health and welfare of both animals and humans in Enugu State and beyond.

XI. Recommendation

Based on the findings of the thesis "Molecular Characterization and Antibiotic Susceptibility of Bacterial Isolates from Pigs in Enugu State," the following recommendations can be made:

1. **Promote Antibiotic Stewardship in Livestock Farming:** Encourage responsible use of antibiotics in pig farming by implementing guidelines and regulations that promote judicious use of antibiotics. This includes educating farmers on proper antibiotic administration, dosage, and withdrawal periods.
2. **Enhances Biosecurity Measures:** Improve biosecurity practices on pig farms to prevent the introduction and spread of antibiotic-resistant bacteria. This may involve implementing stricter hygiene protocols, controlling access to farm facilities, and monitoring animal movements.
3. **Implementation of Surveillance Programmes:** Establish surveillance programs to monitor the prevalence and spread of antibiotic-resistant bacteria in pig populations. Regular monitoring can help identify emerging resistance patterns, track transmission dynamics, and inform targeted intervention strategies.
4. **Diversification of Treatment Options:** Establish surveillance programs to monitor the prevalence and spread of antibiotic-resistant bacteria in pig populations. Regular monitoring can help identify emerging resistance patterns, track transmission dynamics, and inform targeted intervention resistance.

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