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Intestinal flora of selected Agricultural animals and their antibiotic resistance and plasmid profiles.

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Abstract
Intestinal samples from cattle, goat and chickens were collected following laid down procedures to identify their genomic antibiotic resistance status. The bacteria were isolated using Nutrient agar, MacConkey agar,Eosin Methylene Blue agar (EMB), Blood, agar, Muller Hinton (MH). The isolates were subjected to colonial, morphological and biochemical characterization after which they were subjected to pathogenicity, antibiotic resistance tests and the molecular identification of their resistance genes.
The bacteria species isolated wereProteus spp, E. coli, Klebsiella spp. Proteus sppshowedbetaheamolysiswhile, E. coli showed alphaheamolysis and Klebsiellasppshowed gamma heamolysis.Antibiotic susceptibility test showed all isolates to be antibiotic resistant with Multiple antibiotic resistance index (MARi) of 1. Plasmid profiling showed all isolates to have plasmids with bandwidth of 10,000 bp.
Proteus spp, andKlebsiellaoxytoca,were positive for beta lactamase genes (bla SHV). Only Proteus sppwas positive for resistance to aminoglycosides (aac(3)-IV).Proteus spp and Escherichia coli, were found to have the resistance genes for quinolones (qnrA gene) while all the isolates possess genes against the sulphonmides(sul1 gene) used.The result of this work establishes the antibiotic resistance status of bacteria isolated from aquatic habitat and intestine of agricultural animals
***Key words; Antibiotic**, resistance, plasmids, integrons, primers, multiple antibiotic resistance index*

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I. Introduction.

Antimicrobials are important therapeutic agents that have found use against clinical infectionsdecades ago but whose efficacy is seriously compromised as a result of widespread antibiotic resistance (Marshall and Levy, 2011). Antibiotic resistant bacteriaassociated with the animals pose a great health risk because they may be pathogenic to humans, easily transmitted to humans through food chains, and may be widely disseminated in the environment through animal wastes (Manyi-Loh, *et al*., 2018)..

The use of antibiotic in agriculture vary across regions and countries in the developing world and antibiotics that have been banned developed countries are still being used in most developing countries (Moyane*et al*., 2013). Antibiotics are being extensivelyused in livestock farming, in the treatment of diseases, at sub-therapeutic levels in concentrated animal feed for growth promotion, improved feed conversion efficiency and for the prevention of diseases (You and Silbergeld, 2014). Coincidentally, the types, and mode of actions of the antibiotics employed in agriculture and veterinary practice are closely related or similar to those prescribed for humans (Islam *et al*., 2016).

Several authors (Davies & Davies, 2010; Sharma*et al*., 2016) have also reported that antibiotic resistance of environmental bacteria can be intrinsic, acquired via spontaneous mutations (de novo) or horizontal gene transfer (HGT).

The aim and objective of this work is to determine the antibiotic resistance profiles of bacteria isolated from animal intestines using specific primers and their plasmid profile.

II. Materials And Method

2.1 Study Area

Owo is a Local Government Area in Ondo State, Nigeria. The local government has a population of 222,262, based on 2006 population census. Owo is situated in South-Western Nigeria, at the southern edge of the Yoruba Hills, and at the intersection of roads from Akure, Kabba, Benin City, and Siluko. Owo is situated halfway between the towns of Ile Ife and Benin City.

2.2 Preparation of samples

Three different animal intestines and three water bodies were sampled within Owo metropolis. The animal intestines were purchase from abbatoirs and transported in clean sterile bags into the laboratory.

Secondly, a total of 6 intestinal samples from 3 animals (cattle 2; Goats 2; Chickens 2) were collected from commercial farms and abattoir located in Owo, Ondo State. All sample were transported into the laboratory. Thereafter, internal part of the intestines were swabbed using sterile swab sticks and the sticks kept until use.

2.3 Culture media

The culture media were prepared following the manufacture direction. The culture media used for isolation, stocking and for biochemical characterization of the bacteria isolates were eosin methylene blue agar (EMB), Nutrient agar, MacConkey agar, Blood, agar, Mannitol salt agar (MSA) Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar, Muller Hinton (MH) and Nutrient broth.

2.4 Isolation Procedure (Preparation of Stock Culture) for animal intestines.

A test tube containing sterile peptone water was prepared and placed on a test tube rack. Already inoculated swab sticks were dipped into the prepared peptone water and were swirled gently to dislodge the organisms on it. The inoculated peptone water was allowed to stand for 4 hours and then standardized to0.5McFarland's constant ($1.5X10^{-8}$) by comparing with $BaSO_4$ in the spectrophotometer at 500nm (Souza *et al*., 2020).

2.3.1. Standardization of Inocula

2.3.1.1. Preparation of BaSO4 (McFarland's constant).

A.0.5 McFarland's constant was prepared by mixing 0.05ml of 1.1175% of Barium chloride ($BaCl_2.2H_2O$) with 9.95ml of 1% sulphuric acid (H_2SO_4). This stock as prepared in 2.4. above was placed in the spectrophotometer and was compared with the McFarland's constant at 500nm. Peptone water was gradually added to the stock culture until a constant was attained that tallied with the $BaSO_4$ solution.

2.4.1.Isolation of Organisms

The stock standardized culture was used to carry out serial dilution and the inoculum was taken from the test tube containing 10^{-6} dilution. The inoculation was done by the pour plate method. The plates were then incubated at 37^{O}Cfor 24 hours after which the plates were observed for growth. Plate count was carried out using theJ-3colony counter, China.Inocula containing isolates from animal intestine were cultured on Nutrient agar, MacConkey agar, Salmonella –Shigella agar.

2.4.2. Preparation of Pure Culture

After observing the plates distinct colonies from the previously incubated plates were lifted using a sterile wire loop and used to streak a freshly prepared but gelled agar plate. The freshly streaked plates were allowed to incubate at 37^{O}C for 24 hours after which plates were observed for pure culture. Subsequently, pure culture of each isolates was subjected to colonial, morphological and biochemical analysis for characterization.

2.5 Gram staining.

Pure isolates were subjected to Gram staining to determine their Gram status following the laid down procedure.

2.6 Colonial and morphological characterization of isolates

Colonial identification of isolates was based on criteria like morphology, growth parameter such as size, color, texture, elevation, appearance and shape were observed for identification of colonies.

2.7 Biochemical characterization

The following biochemical tests were carried out to identify isolates following Standard methods. Sugar fermentation, Citrate utilization Test, Oxidase Test, Indole Test, Urease Test, Methyl red Test &VogesProskauer Test as described by Fawoleand Oso (1988).

2.8 Pathogenicity Test

In-vitro pathogenicity test of isolates was done by streaking pure culture of the isolated bacteria on blood agar plate using the modified method described by Buxton, (2005). The plates were incubated at 30±2ºC for 7days. The pathogenicity test was confirmed by observing α, β and ϒ heamolytic zones of the growing bacteria on the plate.

2.9 Antibiotic Susceptibility Testing

The antibiotic susceptibility patterns of the isolates were determined using Kirby-Bauer (1966) method on Mueller-Hilton agar according to CLSI guidelines for antimicrobial susceptibility testing (CLSI, 2017). The following standard antibiotics were used were Chloramphenicol (C) 30µg, Nalixidic acid (NAL) 25µg, Ciprofloxacin (CIP) 5µg, Ampicillin (AMP) 30µg, Gentamycin (CN) 10µg, Vancomycin (VAN) 10µg Penicillin (PEN) 25µg.

2.10 Multiple Antibiotic Resistance Index

The multiple antibiotic resistance index for the resistant bacteria isolates, was determined according to the procedure described by Krumperman (1983). This is essentially to determine the degree of bacterial resistance to antibiotics. These indices were determined by dividing the numbers of antibiotics to which the organism were resistant to (a) by the numbers of antibiotics tested. (b) Resistance to three or more antibiotics is takenas multiple antibiotics resistance and MAR greater than 0.2 is considered a health risk.

2.11 Plasmid profiling of isolates;

Plasmids were isolated using the QIAGEN Plasmid Purification mini kit.

2.11.1. Gel Integrity

The integrity of the extracted plasmid was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 10µl of each PCR product and loaded into the wells after the 100bp-10kbp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the plasmid was estimated by comparison with the mobility of the molecular weight ladder that was run alongside experimental samples in the gel. Extracted plasmid was then used as the template for PCR amplification.

2.13. Molecular identification

Primer sequences were as earlier documented by (Barghouthi 2011). Reaction cocktail used for all PCR per primer set included (Reagent Volume µl) - 5X PCR SYBR green buffer (2.5), MgCl2 (0.75), 10pM DNTP (0.25), 10pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06) and made up to 10.5 with sterile distilled water to which 2 µl template was added. Buffer control was also added to eliminate any probability of false amplification. PCR was carried out in a Gene Amp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair.

III. Result and discussion

3.1. Total microbial count of samples

The total count of the samples are as shown in table 1. The total plate count for each sample is indicated. Intestinal samples of animals were plated on Nutrient agar, MacConkey agar and Salmonella-Shigella agar (SSA) and sample collected from cattleson thenutrient agar plate showedthe highest count of 6.0 x 10^{-6}cfu/ml, followed by the sample from goat with a count of 2.0x10^{-6}cfu/ml and then the sample from chicken with 1.0x10^{-6} cfu/ml. The coliform counts for the samples were 3.0 x 10^{-6} cfu/ml,2.6 x10^{-6}cfu/ml and 2.0 x10^{-6}cfu/ml for cattle, goat and chicken respectively. On Salmonella- Shigella agar, the sample from cattle and chicken had the highest count of 5.0 x10^{-6}cfu/ml each, while that of goat was 3.5 x 10^{-6}cfu/ml. This result indicates that there are more bacterial count in cattles than goat and chicken.

3.2. Colonial and morphological presentation of isolates.

Based on the colonial, morphological and biochemical characterizations, the organisms isolated from the animal intestines were*Proteus spp, Escherichia coli and Klebsiellaspp*In the study of normal microbiome of chicken, Pan and Yu (2014) reported a diverse group of bacteria which include Gram-positive rods, Gram-negative rods and Gram-positive cocci which agrees with the result of this study.Galler*et al*., (2021) also discovered in their work strains of *E. coli* in swine and broilers.

Table 1. The bacterial countof different samples from animal intestines and water bodies.

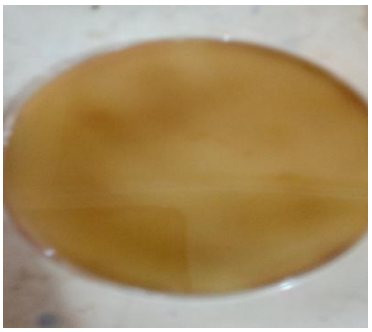
	Animal intestines		
Media/ samples (Cfu/ml)(x10^{-6})	Cattle	Goat	Chicken
Nutrient Agar MacConkey agar Salmonella–Shigella agar	6.0 x 10^{-6} 3.0 x 10^{-6} 5.0 x10^{-6}	2.0 x10^{-6} 2.6 x10^{-6} 3.5 x 10^{-6}	1.0 x10^{-6} 2.0 x10^{-6} 5.0 x10-^{6}

Table 2. Morphological and biochemical characteristics of isolates

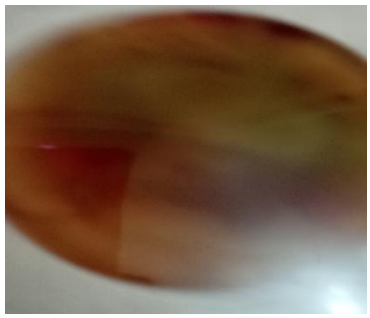
Isolate	morphology	Gram	Sugar fermentation						oxidase	catalase	Vogues Proskeur	indole	Methyl red	Citrate	Suspected org.
			Sucrose	fructose	lactose	glucose	galactos	maltose							
1	Rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	*Proteus spp*
2	Rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	*E. coli*
3	Rod	-ve		+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	*Klebsiellaspp*

3.2. **Pathogenicity properties of isolates**.

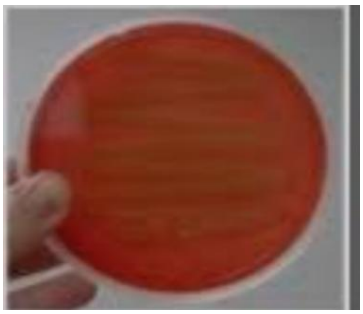
The pathogenicity test carried out on the isolates showed that they were all pathogenic with different degrees of heamolysis. *Proteu*s spp had beta (complete) hemolysis by completely lysing the blood cells presenting clear zones around the colonies after incubation. *Escherichia coli*showedaalphaheamolysis with evidence of green patches around the colonies signifying partial heamolysis while *Klebsiellaoxytoca* presented gamma heamolysis by not showing lysing of blood. (Fig 1.3).Hemolysins, considered an important virulence factor, are compounds produced by a variety of bacterial species. These compounds are responsible for membrane damage, cell lysis and destruction of neighboring cells and tissues in order to provide nutrients, mainly iron, for the toxin-producing bacteria (Bullen *et al.,* 2005).Mogrovejo-Arias*et al*., (2020) pointed out that bacteria can exhibit three different types of hemolytic activity (Buxton 2005): Beta (β) hemolysis, when the toxin causes the complete lysis of the red blood cells. Often referred to as true lysis, it manifests as a clear, transparent area in the blood agar cultures; alpha (α) hemolysis, on the other hand manifests when lysis does not occur but the hemoglobin of the red blood cells is reduced to methemoglobin and a brown/green colored area is observed in blood agar cultures; gamma (γ) hemolysis, or non-hemolysis occurs when no damage to the cells is caused and no change in the agar plate is observed.Indeed many of the coliformsincluding strains of *E. coli* comprise entero-pathogenic serogroups and have been found to be hemolytic and verocytotoxigenic (Pelczar*et al*.,1986; Welch *et al.,* 2000). All isolates are pathogens of human, and animals. . *Proteus* is known to cause gastroenteritis and urinary tract infections in humans (Hamilton, *et al*., 2018), Pathogenic *E. coli* are known to cause diarrheal infections such as gastroenteritis (Lim *et al.*, 2010).



betaheamolysis of Proteus spFig 1



alphaheamolysis of *Escherichia coli* Fig 2



Gamma heamolysis of *Klebsiellaoxytoca* Fig 3.

Fig 4. Control (Blood agar)

3.3. Antibiotic sensitivity of isolates

All isolates were resistant to the standard antibiotics used. (Table 3).The result of the antibiotic susceptibility tests carried out on the isolate echoes the danger of using antibiotics in animal husbandry. The result of this work agrees with the reports of several authors who recoded widespread resistance in agricultural animals (Thakur *et al.,* 2003;Bughe*et al*., 2016;Mishra *et al*, 2010;Nakamura *et al.,* 2012; Shirin and Micheal, 2020;Girlich*et al*., 2020). Emergence and spread of multi drug resistantin *P. mirabilis* isolates including those producing Extended spectrum beta lactamase (ESBLs) AMPC cephalosporins and carbapenems have been severally reported (Girlich*et al*., (2020).Abdelwahab*et al*, (2022) in their work recorded a high percentage resistance of *E. coli* isolated from livestock in the Emirates to several classes of antibiotics.*E. coli*is also documented to be a culprit in the horizontal transfer of resistant gene to other bacteria strains (Li *et al*., 2019).

3.4. Multiple Antibiotic Resistance Index (MARi)

Multiple antibiotics resistance index is used to determine the risk level of antibiotic resistant organisms. The result of this work showed that all isolates had MARi of 1 (Table 4). Multiple antibiotic resistance (MAR) analysis was introduced to discern bacteria from sources using antimicrobialagents regularly approved for human therapy.MAR is most frequently associated with presence of plasmids which harbours single of multiple resistance genes (Ruppe*et al*., 2015). It is recorded that MARi above 0.2. is an evidence of high risk source of contamination (Osundiya*et al.,* 2013).

Table 3. Antibiotic resistance profile of isolates

Antibiotics	*Proteus spp*	*Escherichia coli*	*Klebsiellaoxytoca*
Ciprofloxacin	R	R	R
Sparfloxacin	R	R	R
Augmentin	R	R	R
Ampicillin	R	R	R
Pefloxacin	R	R	R
Streptomycin	R	R	R
Gentamycin	R	R	R
Chloramphenicol	R	R	R
Septrin	R	R	R
Ciprofloxacin	R	R	R

High prevalence of multidrug resistance indicates a serious need for broad based antimicrobialresistance surveillance and the resultant planning of intervention to reduce multidrug resistance in pathogens (Olayinka*et al.,* 2004), The MARi index of all isolates shows they are high risk resistant bacteria both to humans during consumption causing zoonotic infections or to other animals in cross contamination. Baquero*et al*., (2008) posited that any MARi greater than 0.2 constitute public health risk.

Plasmid profile of isolates and gene expression of resistance using antibiotic primers.

Plates 1a presents the plasmid profiles of isolates from intestines of animals**.**The results show that all the isolates have plasmidmediated resistance genes with bandwidth of 10,000 base pairs and above.The large plasmid observed in the isolates is an indication of resistance to multiple antibiotics. This type of plasmids belongs to the class 1 integron because it has resistance to a variety of antibiotics.

Table 4. Multiple Antibiotic Resistance Index (MARi)

organisms	MAR (a/b)	indices
Proteus spp	10/10	1
Escherichia coli	10/10	1
Klebsiellaoxytoca	10/10	1

Class 1 integrons are central players in the problems of antibiotic resistance because they can capture and express diverse resistance genes and are often embedded in promiscuous plasmid and transposons (Gillings*et al*., 2008). Gillings also reported the presence of class 1 integron plasmid from Betaproteobacteria isolated from freshwater.Generally, conjugative plasmids are distributors to horizontal gene transfer and carry a wide variety of accessory genetic elements (Frost *et al*., 2005). The role of plasmid in the dissemination of antibiotic resistance is becoming worrisome for human and animal health. Several authors have documented the presence of plasmids in *E. coli* (Babu*et al*., (2009), *Proteus*spp ((Michea*et al*., 2008), *spp*(Reig*et al*., 1997).Although it appears that plasmids occur naturally in some strains of *E. coli*, but incidence of plasmid is probably higher in countries where antibiotics are readily available and used indiscriminately like Nigeria (Akanbi*et al*., 2004).

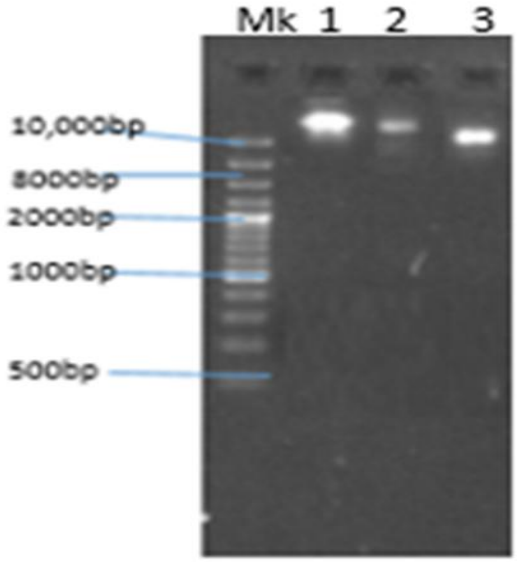


Plate 1

Plate 1. Agarose gel electrophoresis showing the profiling of the plasmid extracted from Proteus spp(1) Escherichia coli (2) and Klebsiellaoxytoca (3).

Plate 2 presents the molecular fingerprints of the isolates showing positive result to the primers used in detecting the resistance genes. Plate 2 shows that *Proteus* spp and *Klebsiellaoxytoca*were positive for beta lactamase genes (bla SHV). Plates 3 showed positive for resistance to aminoglycosides (aac(3)-IV). Only *Proteus* sppwas positive for resistance to aminoglycosides. Plates 4 showed positive result for the presence of resistance gene to quinolones (qnrA gene). *Proteus spp* and *Escherichia coli* were observed to have the resistance gene. In the same manner, Plate 5shows the resistance pattern of the isolates to the sulphonamides (sul1 gene). All the isolates possess genes against the sulphonamides.

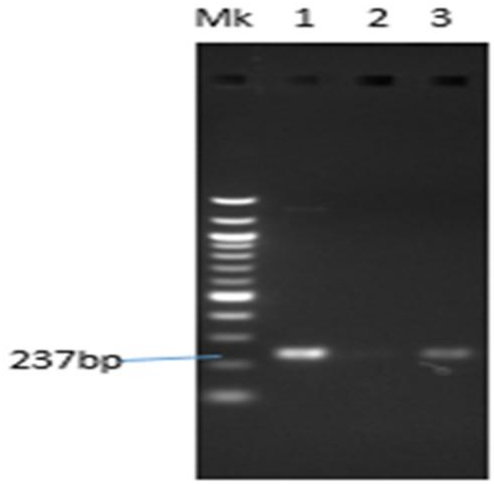


Plate 2

Plate 2; Agarose gel electrophoresis of the PCR bla SHV gene fragment amplified from selected bacteria isolates. Band size approximately 237bp indicates positive amplification. Gel image indicates a positive amplification in samples 1 indicating the presence of ESBL- Bla SHV gene.

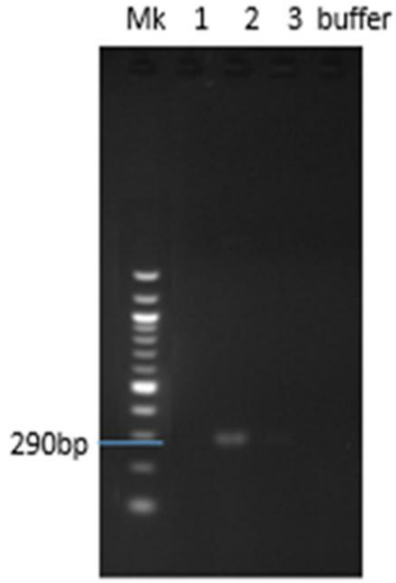


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Plate 3

Plate 3. Agarose gel electrophoresis of the PCR aac(3)-IV gene fragment amplified from selected bacteria isolates. Band size approximately 290bp indicates positive amplification.

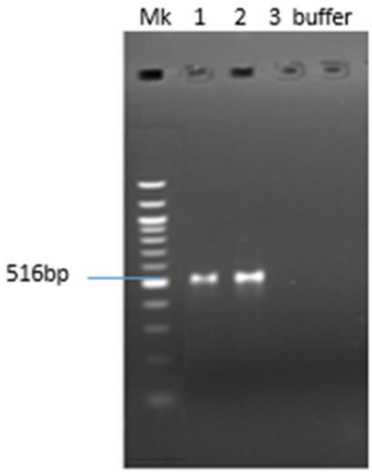

Plate 4.

Plate 4. Agarose gel electrophoresis of the PCR qnrA gene fragment amplified from selected bacteria isolates. Band size approximately 516bp indicates positive amplification. Gel image indicates a positive amplification of Proteus spp and Escherichia coli indicating the presence of qnrA gene

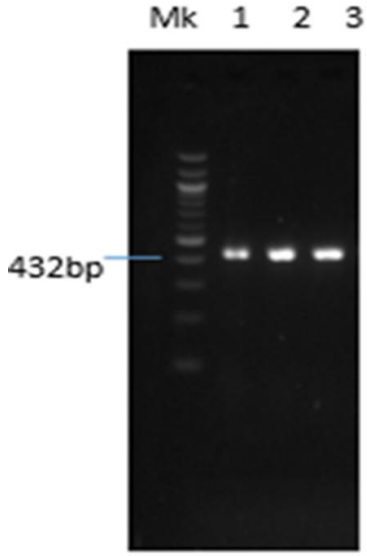

Plate 5

Plate 5 Agarose gel electrophoresis of the PCR sul1 gene fragment amplified from selected bacteria isolates. Band size approximately 432bp indicates positive amplification. Gel image indicates a positive amplification in all samples indicating the presence of sul gene.

The resistance of enterobactericeae rods to aminoglycosides has been reported by several authors (Fernandez- Martinez, *et al*., 2015). Odjana*et al*., (2018) reported that this resistance development reduces therapeutic options for infections caused by multi drug resistant organisms because of the changing epidemiology of extended spectrum beta lactamase and resistance to aminoglycosides.Resistance of enterobacteriacea to fluoroquinolones and cephalosporin have been documented by several authors. Fluoroquinolone resistance occur primarily through mutation in the DNA gyrase (A and B) and topoisomerase 4 (Nakato*et al*., 2019). Quinolone resistant *E. coli* in animals have increased in number after quinolone introduction in a number of studies as carried out by Webber *and*Piddock (2001). They observed that the resistance mechanism in their isolates were the same as those in resistant strains found in humans.Proteus has been reported to show resistance to sulphonamides, ß lactams, tetracycline, cephalosporin and quinolones (Algammal*et al*., 2021). *E. coli* have been reported to show resistance to sulphonamides. Perreten and Boerlin (2003) documented that Sul 3 gene was detected in one third of the sulphonamide resistant pathogenic *E. coli* isolated from pigs in Switzerland. Algammal*et al*., (2021) reported the isolation of *P. mirabilis* carrying the resistance gene Sul1 to sulphonomide.

Klebsiellaoxytoca has also been found to carry multi antibiotic resistance genes (Moradigarav *etal.,* 2017). Adekanmbi*and*Adeleke (2020), did not detect sulphonamide carrying plasmid in Klebsiellaspp they worked with. Similarly, Moradigaravand*et al.,* (2017) did not record the presence of resistance gene to sulphonamide in *K. oxytoca.* This could be because *K. oxytoca* has not been well studied and therefore, there is a need for a holistic studying of this organism which is well implicated in nosocomial infections.

Generally speaking, *Proteus*spp are commensal enterobacteriaceae of the human digestive tract and the same time involved urinary tract infections. They however have developed antibiotic resistance to several classes of antibiotics. This trait has turned *Proteu*s spp to dreadful pan drug resistant bacteria resulting in difficult to treat infections. *Proteus*spp can acquire resistance to ampicillin and other antibiotics through plasmid mediated Beta lactamases. In the last decade, there have been numerous reports on the production of extended spectrum Beta lactamases by Proteus spp (Philippon*et al*., 1989). This is aptly observed in our result where *Proteus* tested positive to Bla SHV primer showing the presence of Beta lactamase.

Girlich*et al*., (2020) posited that quinolones and aminoglycosides are usually active against *P. mirabilis* strain. This work however disputed this fact and the results in this study shows distinctly the presence of resistant strain to quinolones and aminoglycosides in the *Proteus* spp isolated.

As an opportunistic pathogen, *K. oxytoca* is known to have environmental sources and therefore the environment may serve as a potential reservoir for multidrug resistant *K. oxytoca*. The resistance factors of most antibiotic resistant pathogens are encoded in transferable plasmids that encode resistance genes and the acquisition of these genes by commensal of feacal isolates leads in turn to multidrug resistant pathogens. Evidence that antibiotics used in food animals can result in antibiotics resistant infection in humans abound for several diseases (Landers *et al*., 2012).

Conclusively, conjugative plasmids are distributors to horizontal gene transfer and carry a wide variety of accessory genetic elements. The role of plasmid in the dissemination of antibiotic resistance is becoming worrisome for human and animal health. Plasmids are very important vehicles for the communication of genetic information between bacteria. The exchange of plasmids transmits pathogenically and environmentally relevant trait. The fact that most of the antibiotics used in this study are of major therapeutic importance in human medicine, this is a public health concern. This study has been able to identify some common intestinal bacteria and their level of resistance to some common classes of antibiotics. And has also established the fact that microorganisms can acquired plasmid mediated resistance to antimicrobials irrespective of the ecosystem and this is a cause for concern as most ecosystem are interconnected and interrelated. Human and animal activities also contribute to the transfer of organisms into different habitat which is an undeniable factor in the transfer of antimicrobial resistance to previously non- resistant strains. It is evident that there is a need to devote resources to studying antibiotic resistance in different ecosystems. It is no gain saying that the use of antibiotics in in farm animals is one of the major contributions to the development of antibiotic resistant organisms involved in life threatening human infections.it to the host bacteria promoting their rapid evolution and adaptation to various environment.

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