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Antimicrobial resistance profile of bacteria isolated from the vagina of nulliparous cattle

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Abstract

Breeding soundness evaluation and investigation of reproductive disorders require the profiling of resident microbial communities in the reproductive tract. This study was designed to investigate the antibiotic resistance profile of bacteria in the vagina of cattle. Deep vaginal swabs were collected from nulliparous cattle (n=82) at the lairage of the Akinyele Abattoir, Ibadan, Nigeria from September to November 2022. Bacterial isolation and characterization were done using standard techniques while the evaluation of the antimicrobial resistance pattern was conducted using the disc diffusion technique. Data were analyzed using descriptive statistics. Bacterial growth was obtained from 45 (55%) samples. The isolates were identified as *Pseudomonas aeruginosa* (34.1%), *Salmonella typhimurium* (29.3%), *Enterobacter aerogenes* (12.2%), *Citrobacter freundii* (7.3%), *Providencia alcalifaciens* (12.2%), and *Providencia stuartii* (4.9%). The bacterial isolates were completely resistant (100%) to penicillin, ceftriaxone, doxycycline, tetracycline, metronidazole, and furaltadone, highly resistant to amoxicillin (83.3%), moderately resistant to streptomycin (33.3%), and low resistance (16.7%) to gentamicin and enrofloxacin. The growing trend in antimicrobial resistance in microflora of food animals in Nigeria calls for stricter control of the use of antibiotics in livestock production.

Keywords: Antimicrobials, Vaginal Microflora, Nulliparous Cow, Resistance.

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Introduction

The economic importance of cattle and the need for a better understanding of resident microbial communities has led to the development of many studies to elucidate the relationship of the indigenous microbiota with the physiology of these animals. This microbiota can have a significant impact on the biology of the host. Most studies have focused on the skin, mouth, and gut microbiome, whereas relatively little is known about the vaginal environment. Despite the importance of the vaginal environment for further understanding the reproductive biology and diseases, there is limited research on its significance in cattle (Bicalho et al., 2017; Silvia et al., 2019), especially in the area of antimicrobial sensitivity.

Under normal conditions, the composition of the vaginal microbiota has a variable number of microorganisms and due to their invasive properties, may be in the uterus in a small number of healthy animals (Ramaswamy et al., 1991). Cows do not suffer continuous contamination from such agents; however, they colonize the genital tract if there is an opportunity.

In mammalian species, the genital tract is equipped with physical barriers, such as the vagina and cervix, as well as immune defenses, including mucous secretions, leukocyte infiltration, and the potential for inflammatory responses. These mechanisms are designed to protect against pathogen invasion (Wira et al., 2005). However, despite the protection, there is still a risk of microorganisms infiltrating the reproductive tract through the cervix (Rocha et al., 2004). The normal microorganisms of the vagina become pathogenic when animals exhibit a compromised immune system due to stress caused by various factors, demonstrating an opportunistic character at the source of infections (Borges and Paschoal, 2012; Amin et al., 2023). The vaginal environment is also protected by cellular and subcellular factors (Horne et al., 2008) as well as by a periodical change of temperature, humidity, pH, a nutritional substrate for bacteria, and inhibitory substances (Fernández et al., 2006) due to hormonal influences (Lewis, 2003). As a result, the normal vaginal environment is not conducive to excessive microbial proliferation. Therefore, the susceptibility patterns of bacteria derived from healthy animals are suggested as a good predictor of the resistance situation in the bacterial population as a whole (van den Bogaard and Stobberingh, 2000).

The microbiological flora of the lower female genital tract presents a dynamic and intricate example of microbial colonization, the regulation of which is not fully comprehended. For a disease to manifest, exogenous or endogenous bacteria possessing pathogenic prerequisites must attain a state of replicative dominance. Their ability to achieve this state is potentially associated with the inhibitory or synergistic interrelationships with other microbes (Horne et al., 2008). Furthermore, the microflora of the reproductive tract has been identified as a potential target for promoting both animal and human health through microbiota manipulation. Such manipulation is likely to be employed to achieve a balanced immune response, thereby increasing resistance to diseases and enhancing animal performance (Gomez et al., 2019). This repertoire of knowledge is vital for microbiologists and clinicians and serves as a starting point for better understanding dysbiosis or infections

caused by imbalances in the vaginal microflora, ultimately enabling the development of more appropriate treatments.

The majority of antimicrobials on a worldwide scale are consumed in the agricultural sector and this assumption has led to a 'mass action' hypothesis that selection in agriculture is a significant driving force for the evolution, persistence, and dissemination of antimicrobial resistance (AMR) traits worldwide (Heuer et al., 2006; Silbergeld et al., 2008). From a public health perspective there is potential for AMR pathogens and commensal organisms to disseminate to humans via direct contact with animals (Price et al., 2007) or via the food chain (Silbergeld et al., 2008).

This study was designed to investigate the bacteria population in the vagina region of the reproductive tract of nulliparous cattle presented for slaughter at the abattoir. Additionally, the antimicrobial resistance pattern was also investigated.

Materials and Methods

Sampling procedure

In a cross-sectional study, deep vaginal swab samples were purposively collected from 82 nulliparous White Fulani breed of cattle at the lairage of the Akinyele Central Abattoir, Ibadan Southwestern Nigeria from September to November 2022. The lairage of this central abattoir serves as a gathering point for cattle originating from smallholder farms from various ecological zones including rainforest, the derived Guinea savannah, Sahel, and sub-arid climatic zones of northern Nigeria, and border countries such as Niger, Chad, and Mali (Jeremiah and Banwo, 2019). Following clinical examination of 1,432 females, 82 nulliparous white-Fulani cows, apparently healthy and those free from the obvious reproductive disease were selected and sampled. The selective sampling was made based on the animal's history and examination of the genital tract.

Sampling procedures

The vulvar and perineal regions were cleaned and disinfected with distilled water followed by cotton wool soaked in 70% alcohol. Care was taken to avoid the moment of defecation and urination while sampling. The labia were held open, and deep vaginal swabs were collected from 82 apparently healthy nulliparous White Fulani breed of cows using 45-cm long sterile swabs (Equivet®, Kruuse) introduced into the vaginal tract via the opened vulva, without touching the vulva or the external urethral orifice until the cranial vagina, performing rotational movements to obtain material as described (Nugeyre et al., 2019; Quereda et al., 2020a). Samples were collected by gently swabbing the vaginal wall for 20 seconds. They were then placed into sterile tubes containing buffered peptone water as a transport medium. They were transported at 4°C to the Bacteriology Laboratory of the Department of Veterinary Medicine, University of Ibadan for analysis.

Bacteria isolation and identification

The samples were incubated at 37°C for 24 hours in the transport medium (buffered peptone) before serial dilution and inoculation on agar plates. The media were prepared according to the manufacturer's instructions, and the samples were cultured on Nutrient Agar at 37°C for 24 hours. Discrete colonies were aseptically sub-cultured on freshly prepared Nutrient Agar,

MacConkey agar, *Salmonella Shigella* agar and incubated at 37°C for 24 hours (Anifowose et al., 2023). Each distinct colony was further sub-cultured on fresh Nutrient Agar or MacConkey Agar (MAC) for evaluation of purity and colonial morphology. Bacterial colonies which show the typical characteristic and colour of lactose fermenter on MAC were sub-cultured on Blood Agar and Eosin Methylene Blue agar. Freshly recovered pure isolates were used for primary characterization. Colonies were identified based on their morphological characteristics such as size, elevation, outline, colour, and their effect on the medium and these were recorded. Biochemical identification of the isolates was performed using Gram's staining, motility, catalase, oxidase, Simon citrate, 6.5% NaCl Broth, Indole, Glucose (gas), Inositol, Mannitol, Hydrogen sulfide, and Urease, methyl red, Vogues Proskauer test, Triple sugar Iron agar slant culture and fermentation of sugars according to standard Taxonomic Schemes (Anifowose et al., 2023).

The results of the various biochemical tests were used to confirm the identity of the bacteria using the Bergeys Manual of Systemic Bacteriology (2012) and the Global Infectious Diseases and Epidemiology Network (GIDEON) Online microbiology database (2016). The biochemical results were entered on Gideon (a software for confirmation and differential diagnosis of infectious diseases) to confirm the isolated bacteria.

Antibiotic susceptibility tests

Antibiotic susceptibility testing was performed using the Kirby-Bauer method (Disc diffusion Technique) and interpreted using the Clinical and Laboratory Standards Institute catalog (CLSI, 2020). The susceptibility was tested using 10 antibiotics belonging to seven classes. The classes, antibiotics, and concentrations are: Penicillin; [procaine penicillin 10 µg (PP), amoxicillin 25 µg (AM)], Cephalosporin; [(third-generation) ceftriaxone 30 µg (CEF)], Tetracycline; [doxycycline 30 µg (D), tetracycline 30 µg (T)], Aminoglycoside; [streptomycin 10 µg (S), gentamicin 10 µg (G)], Fluoroquinolones; [Enrofloxacin 5 µg (E)], Nitroimidazole; [Metronidazole 5 µg (M)], Nitrofuram; [Furaltadone FUT (30 µg)] based on the Kirby-Bauer disk diffusion method (CLSI, 2020; Hartantyo et al., 2020).

The identified bacterial species, suspended in a sterile normal saline and mixed to an even turbidity density was adjusted to McFarland 0.5 by adding saline or more bacteria. Each bacterial isolate was then streaked on Muller-Hinton agar to form a smooth, homogenous lawn culture. The plate was prepared for each isolate and was properly identified using an indelible marker.

Antibiotic discs prepared according to the CLSI Mo2 disk diffusion guide were placed on the plate using sterile forceps and pressed gently to ensure proper contact with the media. Plates were incubated at 37°C for 18-24 hours. The inhibition zones of different antibiotics were measured in millimeters (mm) and the isolates were categorized into sensitive, intermediate, or resistant based on the guidelines in the Clinical and Laboratory Standards Institute (CLSI) catalogue for 2020. Data obtained were analyzed by descriptive statistics.

Results

Bacterial growth was obtained from 45 (55%) of the 82 samples.

The isolates were all Gram-negative and identified as *Pseudomonas aeruginosa* (34.1%), *Salmonella typhimurium* (29.3%), *Enterobacter aerogenes* (12.2%), *Citrobacter freundii* (7.3%), *Providencia alcalifaciens* (12.2%) and *Providencia stuartii* (4.9%) as shown in table 1.

Table 1: Bacterial growth from vaginal swabs of nulliparous white-Fulani Cattle.

S/No	Organism	Number of Isolates	Percentage	Gram staining
1	Salmonella typhimurium	24	29.3	Gram-Negative Rods
2	Pseudomonas aeruginosa	28	34.1	Gram-Negative Rods
3	Providencia stuartii	4	4.9	Gram-Negative Rods
4	Enterobacter aerogenes	10	12.2	Gram-Negative Rods
5	Citrobacter freundii	6	7.3	Gram-Negative Rods
6	Providencia alcalifaciens	10	12.2	Gram-Negative Rods
Total		82		

Table 2 shows the distribution of bacteria isolates from positive samples (n=45). The phenotypic and biochemical characteristics are presented in table 3, and sugar fermentation test results for the identified bacterial isolates are detailed in table 4. The bacterial isolates were resistant to gentamicin (16.7%), enrofloxacin (16.7%) and streptomycin (33.3%), highly resistant to amoxicillin (83.3%) while all were resistant to penicillin, ceftriaxone, doxycycline, tetracycline, metronidazole, and furaltadone as shown in table 5

Table 2: Distribution of bacterial isolates from positive samples.

S/No	Organism or combination of organisms	Number of Isolates	Percentage
1	Salmonella typhimurium	7	15.6
2	Salmonella typhimurium, Pseudomonas aeruginosa, Providencia alcalifaciens	8	17.8
3	Salmonella typhimurium, Pseudomonas aeruginosa, Enterobacter aerogenes	4	8.9
4	Salmonella typhimurium, Enterobacter aerogenes, Providencia alcalifaciens	2	4.4
5	Pseudomonas aeruginosa	13	28.9
6	Providencia stuartii	1	2.2
7	Enterobacter aerogenes	3	6.7
8	Citrobacter freundii	3	6.7
9	Salmonella typhimurium, Pseudomonas aeruginosa, Citrobacter freundii	2	4.4
10	Salmonella typhimurium, Providencia stuartii	1	2.2
11	Pseudomonas aeruginosa, Enterobacter aerogenes, Citrobacter freundii	1	2.2
Total		45	100

Discussion

The normal flora in the reproductive tract consists of a diverse community of microorganisms, mostly bacteria and, to a lesser extent, fungi (Ocando et al., 2010). This investigation provides information on the Gram-negative bacteria population in the vaginal environment of the nulliparous white Fulani breed of cattle selected from an aggregation point for cows from several locations within and outside Nigeria. Their antimicrobial-resistant pattern was also investigated to provide information on the characteristics of these reproductive tract associated microbes of apparently healthy females. Investigations on the microbial population in the reproductive tract remain an important component in breeding soundness

evaluation and investigation of reproductive problems in livestock.

Table 3: Phenotypic and biochemical tests for identification of bacteria isolates from vaginal swabs.

Biochemical Characteristics	Salmonella typhimurium	Pseudomonas aeruginosa	Providencia stuartii	Citrobacter freundii	Enterobacter aerogenes	Providencia alcalifaciens
Gram Staining	Negative rods	Negative rods	Negative rods	Negative rods	Negative rods	Negative rods
Catalase	Positive	Positive	Positive	Positive	Positive	Positive
Oxidase	Negative	Positive	Negative	Negative	Negative	Negative
Motility	Motile	Negative	Positive	Positive	Positive	Positive
Methyl-red	Positive	Negative	Positive	Positive	Negative	Positive
H_2S production	Positive	Negative	Negative	Positive	Negative	Negative
Indole production	Negative	Negative	Positive	Negative	Negative	Positive
Citrate	Negative	Positive	Positive	Negative	Positive	Negative
Voges-Proskauer	Negative	Negative	Negative	Negative	Positive	Negative
Urea Hydrolysis	Negative	Negative	Negative	Negative	Negative	Negative
Oxidative Fermentative	Fermentative	Negative	Fermentative	Facultative anaerobes	Facultative anaerobes	Fermentative
TSI Agar	alkaline slant, acidic butt	Alkaline, Alkaline	alkaline slant, acidic butt	alkaline slant, acidic butt, and gas production	alkaline slant, acidic butt, and presence of gas	alkaline slant, acidic butt
Gelatin Hydrolysis	Positive	Positive	Negative	-	-	Negative
Coagulase	Positive	Negative	-	-	-	-
Ornithine decarboxylase	Positive	Negative	-	-	-	-
Mannitol Salt Agar	Positive	Positive	-	-	-	-
MacConkey	Negative	LF			Mucoid and pinkish colonies	
Pigment		Greenish-blue				

EMB = Eosine methylene blue, **TSI =** Triple Sugar Iron Test

The organisms isolated from the cattle varied within the population. The variation observed in the bacterial population and bacterial load may be a reflection of variations in the environment, host and microbe factors including climate, management practices, hygiene, and the health status of the cows. The identified microbes include *Pseudomonas aeruginosa* (34.1%), *Salmonella typhimurium* (29.3%), *Enterobacter aerogenes* (12.2%), *Citrobacter freundii* (7.3%), *Providencia alcalifaciens* (12.2%) and *Providencia stuartii* (4.9%). These isolates with possible pathogenic, zoonotic potentials displayed varying degrees of antimicrobial resistance. Under normal conditions, the vaginal microbiota shows varied number and composition of microorganisms. These microorganisms generally do not cause continuous infection; however, they can colonize the genital tract when there is an opportunity.

Table 4: Sugar fermentation test on pure isolates from primary culture of bacterial organisms from vaginal swabs of Cattle.

ISOLATES	ORGANISM	GLUCOSE	LACTOSE	MALTOSE	MANNITOL	SUCROSE	ARABINOSE	XYLOSE
1	Salmonella typhimurium	+	-	+	+	-	*	+
2	Pseudomonas aeruginosa	-	-	-	*	-	-	*
3	Providencia stuartii	+	-	-	-	+	-	-
4	Enterobacter aerogenes	+	-	+	+	*	*	+
5	Citrobacter freundii	+	+	+	-	+	+	+
6	Providencia alcalifaciens	+	-	-	-	+	-	-

Negative = - , Positive = + **,** * Not available

After birthing, the vaginal microbiota can invade the uterus via the cervix. Most of the bacteria isolated from vaginal samples are Gram-negative bacteria which are also commensals in the gastrointestinal tract. Infertility and abortion of animals may be caused by various microorganisms whether or not they are part of the natural vaginal microbiota. These infections often occur when the environment is not ideal for optimal health conditions and can manifest at any stage of the estrous cycle, as reported by Kaltungo and Musa, (2013) and Szacawa et al., 2018). This underscores the critical role

of maintaining optimal conditions to minimize the risk of reproductive issues in cows.

Table 5: Antibiotic resistance pattern of bacterial isolated from nulliparous cow vagina.

		P	AM	CEF	D	T	S	G	E	M	FUT	Level of Resistance (%)
1	Salmonella typhimurium	R	R	R	R	R	S	S	S	R	R	70
2	Pseudomonas aeruginosa	R	R	R	R	R	S	S	S	R	R	70
3	Providencia stuartii	R	S	R	R	R	R	S	S	R	R	70
4	Enterobacter aerogenes	R	R	R	R	R	S	S	S	R	R	70
5	Citrobacter freundii	R	R	R	R	R	R	R	S	R	R	90
6	Providencia alcalifaciens	R	R	R	R	R	S	S	R	R	R	80
Spectrum of resistance (%)		100	83.3	100	100	100	33.3	16.7	16.7	100	100	
Range (Zone of Inhibition (mm)		R:2-6	S: Nil, R: 4-11	S:18-20 R:3-12	S: Nil R: 4-13	S: Nil, R: 10-14	S: Nil, R:4-12	S: 15-18 R:5-7	S: 16-20 R:8-10	S: 27-30 R: 5-13	S: Nil, R: 4-12	S: Nil

P:Penicillin, AM: Amoxicillin, CEF: Ceftriaxone, D: Doxycycline, T: Tetracycline, S: Streptomycin, G: Gentamicin, E: Enrofloxacin, M: Metronidazole, FUT: Furaltadone.

Changes in the vaginal environment may cause changes in the microbiota, such as vaginal pH changes during pregnancy or oestrus. Microbiota components can be opportunistic and play an important role in the development of infection in the upper reproductive tract, decreasing reproductive performance and therefore being responsible for significant economic losses, as reported by Barba et al., (2024). This highlights the importance of monitoring and managing vaginal health to sustain reproductive performance.

The current study indicates that bacterial isolates demonstrate total resistance (100%) to penicillin, ceftriaxone, doxycycline, tetracycline, metronidazole, and furaltadone. Additionally, they show high resistance to amoxicillin (83.3%), moderate resistance to streptomycin (33.3%), and low resistance (16.7%) to gentamicin and enrofloxacin. The high level of resistance to multiple antibiotics observed is evidence of a growing resistance of microbial population in livestock. This may be associated with routine, non-therapeutic use of antimicrobial agents in livestock practice in Nigeria or in the bordering countries from which these animals are sourced. Several studies announced the presence of resistance to antibiotic treatments that were used against different bacteria of the reproductive tract and nowadays new trend of applying different therapies rather than antibiotics was used to alleviate resistance of the bacteria in the reproductive tract (Amin et al., 2023; Mekibib et al., 2024).

The extensive system of management largely practiced in the country can expose the animals to a wide variety of microbes and environmental contaminants like antibiotics, biocides (Toghan et al., 2022), microplastics, Aflatoxins (Zakaria et al., 2019), and heavy metals (Zakaria et al., 2024), naturally present in shared environments. This can place selective pressure on bacteria and lead to increased resistance.

Pseudomonas aeruginosa has been associated with mastitis in cattle (Shah et al., 2021) and is also an opportunistic pathogen of zoonotic importance (El-Ghany, 2021). Higher levels could indicate an infection that needs treatment. Another frequently isolated and significantly important organism is Salmonella typhimurium which causes Salmonellosis in humans (Alley et al., 2002). This zoonotic pathogen has the potential to cause gastrointestinal illness in humans and plays a significant role in economic losses in

cattle production. It may occasionally be found in the vaginal tract of cows, with its presence potentially indicating fecal contamination during birth.

Citrobacter freundii was found to be resistant to the widest range of antimicrobials, being sensitive only to enrofloxacin. It has been found to be associated with subclinical mastitis (Lamari et al., 2021) and it is a known cause of diarrheal infection in humans.

Indiscriminate use of antibiotics in food animals such as cattle should be discouraged to reduce the risk of antimicrobial-resistant bacteria infections in human. Although cows possess a physical barrier that prevents colonization of the genital tract by opportunistic pathogens, they can still become susceptible to microorganisms passing through the cervix and reaching the uterus (Rocha et al., 2004). The presence of *Providencia stuartii*, normal commensal in the cow vaginal microflora, has been associated with metritis requiring antibiotic therapy in high numbers. *Enterobacter aerogenes* is a facultative anaerobe commonly found in the vaginal tract of healthy cows. Generally, it does not cause disease but can opportunistically infect if numbers increase substantially due to other predisposing factors.

Conclusion

The emergence of antimicrobial-resistant bacteria in farm animals has been associated with the wide use of antimicrobials by livestock farmers, both for therapeutic usage and non-therapeutic applications. The findings in this study reveals public health risks as the cattle may serve as a reservoir for antimicrobial-resistant bacteria. This calls for necessary advocacy for a more rational use of antimicrobials in livestock especially food animals given the fact that antimicrobial usage is the primary contributor to antimicrobial resistance.

The entire populace especially those that are immediately involved in the handling of food animals especially cattle, should be made aware of the public health risk posed by unnecessary and indiscriminate use of antibiotics in food animal production and how to, at best prevent such risks. The government should also implement stringent control on the use of antimicrobials in food animals in Nigeria. Future studies should provide molecular data on microorganisms isolated from the vaginal microflora of cattle to evaluate the nature of the resistance observed as some organisms have been observed to be intrinsically resistant to certain antimicrobial agents. Prudent antibiotic stewardship combined with good hygiene and biosecurity practices can help minimize further selection and spread of resistant strains on farms.

Conflict of interests

The authors declare no potential conflict of interest.

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