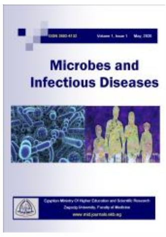
Microbes and Infectious Diseases 2025; 6(4): 6732-6742



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Phenotypic and molecular characterization of aerobic bacteria from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria

Paul Habila Mamman[1 *], Bala Ningi Umar[1], Abubakar Nafi'u Kakudi[1], Akeem Olayiwola Ahmed[2], Kennedy Chah[3], and Haruna Makanjuola Kazeem[1]

1- Department of Veterinary Microbiology, Ahmadu Bello University, Zaria, Nigeria

2- Department of Veterinary Microbiology, University of Ilorin, Nigeria

3- Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsuka

ARTICLE INFO

Article history:
Received 13 September 2024
Received in revised form 12 January 2025
Accepted 14 January 2025

Keywords:
Aerobic bacteria
Gemella palaticanis
Schaalia canis
16S rRNA sequencing

ABSTRACT

Background: Infertility is a major constraint to successful dog breeding in many developing countries including Nigeria. The aim of this study was to establish the aerobic bacterial microflora of the vagina of both apparently healthy and clinically infertile Nigerian bitches in a bit to inferring the likely bacterial causes of infertility in the dogs examined. **Methods**: Bacteria were culturally isolated and characterized using conventional biochemical tests and sequencing of their 16S rRNA; then antimicrobial susceptibility testing was carried out using commonly available antimicrobial agents that were also implicated in the treatment of dogs in the area. **Results**: A total of 169 bacteria were isolated; of which 103 (60.9 %) were from apparently healthy bitches and 66 (39.1%) from bitches with reproductive anomalies. These include *Staphylococcus saprophyticus* (27.2 %), *Streptococcus* species (14.8 %), and *Corynebacterium* species (13.6 %). Also, *Gemella palaticanis* (1.8%), and *Schaalia canis* (1.3%) which are new to the study area were isolated for the first time in Nigeria. Nearly all the infertile bitches (90.1%) had mixed infections which might be responsible for their illness. **Conclusion**: Gentamicin was the most effective drug *in vitro*. *Micrococcus* species and *Escherichia coli* exhibited the highest multiple drug resistance. The authors recommend thorough screening of dogs for pathogenic bacteria before breeding.

Introduction

Reproduction in dogs is a complex biological process that is governed by various external and internal parameters having different influencing factors. Similarly, canine reproductive disorders are usually multi-factorial in origin, involving numerous predisposing factors [1-3]. Failure to detect and correct these anomaly-causing factors might lead to irreversible syndromes of the reproductive tract in dogs [4-8]. Infertility is one of the common canine reproductive disorders which is often associated with one or a combination of anatomical, hormonal, nutritional, genetic, management and/or infectious causes, which are broadly categorized into infectious and non-infectious causes [2,9-12].

Bacteria are regarded as the leading cause of infectious canine infertility [10,12-15]. however,

---

DOI: 10.21608/MID.2025.320466.2212

* *Corresponding author:* Paul Habila Mamman
E-mail address: *embeemaa@gmail.com*

in spite of the vast advances in the knowledge of these agents, the relationship between the presence of bacteria in the reproductive tract and infertility in bitches remains poorly understood [16]. Nonetheless, vaginal swab culture during an overt disease process has been clinically valuable for diagnosis and selection of appropriate therapy. Results can be combined with proper history, clinical examination, and vaginal cytology, to adequately deduce clinical significance.

Some clinicians advocate the employment of systemic antibiotic therapy in bitches when a heavy growth of bacteria is identified at the anterior vagina [17]. Moreover, in Nigeria, dog breeders often treat bitches with antibiotics at the time of mating to guard against genital infections, and this indiscriminate use may result in the development of partial or complete antimicrobial resistance. Hence, antibiogram studies on bacterial isolates assume great importance, and such studies are essential prerequisites for rational use of antimicrobial agents. Therefore, to forestall transmission during breeding, the present study assessed, characterized, and established the antimicrobial profile of vaginal aerobic bacteria from both apparently healthy and infertile bitches in Zaria, Nigeria.

The Null hypothesis to be tested was: ‘‘There are no aerobic bacteria present in the vagina of bitches capable of causing infertility in dogs in Zaria, Nigeria”

Materials and Methods

Sampled Dogs

The study was carried out on both clinical and non-clinical (presented for vaccination) cases of bitches presented to the Small Animal Clinic of the Veterinary Teaching Hospital, Ahmadu Bello University (VTH-ABU), Zaria; as well as bitches at their homes within Zaria metropolis i.e. dog owners who gave consent for their bitches to be sampled. In each case, the consent of clients (dog owners) was graciously sought and obtained with elaborate explanations on what the study was all about. Medical history of each bitch was taken. Bitches were termed healthy or infertile based on the history of previous mating and outcomes. They were considered infertile when presented with a history of having been mated three or more times during previous estrous cycles without viable conceptions I.e. mated with a healthy male dog that had sired pups before. Based on these inclusion criteria, available 70 healthy, and 30 infertile bitches were evaluated. The Committee on Animal Use and Care, resident in of Ahmadu Bello University, Zaria gave an approval for the research to be conducted.

Sample Collection

A total of 100 samples were obtained from the anterior vagina of the bitches using sterile disposable swabs. Each bitch was restrained in a standing position on the examination table and the tail deflected laterally to expose the peri-vulvar area. The peri-vulvar area was initially cleaned with 5% povidone iodine antiseptic solution (WOSAN®). An eight-inch sterile swab was then carefully introduced, directing the swab initially dorso-cranially to avoid the clitoral fossa and urethral orifice and then longitudinally as previously described [18]. The tip of the swab was dipped into the anterior vagina then softly rolled around the vaginal wall. Thereafter, the swab was withdrawn gently into its labeled sterile container. The samples collected were packed in a cool box containing ice pack and transported to the Department of Veterinary Microbiology laboratory, ABU Zaria for analysis.

Bacterial Isolation and Phenotypic Identification

The samples collected were inoculated unto prepared plates of blood agar base (Oxoid, Hampshire, UK) enriched with 10% sheep blood for primary isolation. The inoculated plates were incubated aerobically at 37 ^{0}C for 24 hours, and the isolated colonies were Gram-stained for preliminary identification. Gram-negative organisms from the primary culture were sub-cultured on MacConkey agar (Oxoid, Hampshire, UK) for differentiation based on their lactose fermentation properties [19]. The resulting lactose and non-lactose fermenters were sub-cultured on eosin methylene blue agar (Oxoid, Hampshire, UK) and xylose lysine deoxycholate agar (Oxoid, Hampshire, UK) for probable identification of *Escherichia coli* and *Salmonella* species respectively. The isolates were then subjected to biochemical characterization, including sugars fermentation, fermentation of sugars, oxidase, triple sugar iron, urease, citrate, methyl red, indole, Voges–Proskauer, phenylalanine deaminase, catalase and motility tests as previously described [20].

Genetic Identification of Some Isolates

Five of the bacterial isolates (1ZN2019 - 5ZN2019) not clearly identified phenotypically were characterized by molecular means. DNA extraction was carried out in accordance with the manufacturer's instruction using ZR fungal/bacterial DNA miniPrep^{TM} as previously described [21]; and conventional polymerase chain reaction (PCR) assay targeting the 16S rRNA gene was done as per standard protocol described by Wagner *et al*. [22] using Ribose 1: 5'-GGACTACAGGGTATCTAAT-3', and Ribose 2: 5'-AGAGTTTGATCCTGG-3' primers. A partial genome sequencing of the amplified product was done at Inqaba biotec laboratory (South Africa). The obtained 16S rDNA sequences were edited and aligned using BioEdit, and MEGA X software, then compared with sequences available in the GenBank database of the National Center for Biotechnology Information (NCBI) using the algorithm BLASTn program [20,23]. A phylogenetic tree was constructed as unrooted Neighbour-Joining Tree built with Kimura 2 Parameter and Gama distribution (K 2-G) [24]. A Bootstrap replicate of 1000 was used to test the robustness of the tree. Seven hundred and eight (708) nucleotide regions were used for the construction of the phylogenetic tree. The main identified limitation encountered as far as the techniques used in this work are concerned, is that of a limited number of isolates that were selected for molecular analyses and also, instead of subjecting all the genotypically identified isolates to full genome sequencing, only a partial gene sequencing of the16S rRNA was carried out. This full genome sequencing would have been expedient especially for the new isolates being reported in this research.

Antimicrobial Susceptibility Testing

The pure bacterial isolates were subjected to an antimicrobial susceptibility test using the Kirby-Bauer disk diffusion method [25], then interpreted based on Clinical Laboratory Standards Institute Guidelines [26]. The drug impregnated disks (Oxoid, Hampshire, UK) included: tetracycline (30µg), gentamicin (10µg), streptomycin (10µg), amoxycilin/clavulanic acid (30µg), ampicillin (10µg), penicillin (10 units), oxacillin (1µg), cefoxitin (30µg), vancomycin (30µg), erythromycin (15µg), and ciprofloxacin (5µg).

Data Analyses

Descriptive statistics were applied to calculate frequencies, and percentages of bacterial occurrence rates using SPSS software version 26 (NY, USA). Statistical analysis was performed to determine significant differences in the occurrence rate of each microbial bacterial species within the tested groups using Fisher's exact test. P-values less than 0.05 were considered statistically significant.

Results

A total of 169 bacterial isolates were identified from the entire samples analyzed, out of which 103 were obtained from apparently healthy bitches while 66 were from infertile ones. *Staphylococcus saprophyticus* had the highest occurrence rate of 27.2%, followed by *Streptococcus* species (14.8%), and *Corynebacterium* species (13.6%). While the lowest occurrence rates were the molecularly identified *Schaalia canis* (1.3%) and *Gemella palaticanis* (1.8%) both of which were isolated solely from infertile bitches **(Table 1)**. Broadly, 15 different categories of bacteria were isolated with an overall occurrence rate of 11.3%. Samples from the healthy bitches yielded 9.8% isolation rate while 14.7% was recorded from the infertile bitches. Therefore, infertile bitches had significantly higher (P = 0.007) bacterial occurrence rate than the healthy bitches. Among the different bacteria isolated, only *G. palaticanis* and *Streptococcus* species (other than *Strept. hyovaginalis*) differ significantly (P < 0.05) between the infertile and healthy bitches (Table 1). Similarly, majority of the isolates were Gram-positive with only *Escherichia coli* and *Proteus vulgaris* being Gram-negative **(Tables 2 and 3)**.

Based on the sequence results of the16S rRNA gene, isolates 1ZN2019 with 762bp sequence size was identified as *Enterococcus dispar* (Accession No: MN585805); 2ZN2019 with 739bp sequence size as *Schaalia canis* (Accession No: MN585806); and 3ZN2019 with 761bp sequence size as *Streptococcus hyovaginalis* (Accession No: MN585807). Isolates 4ZN2019 which has 768bp sequence, and 5ZN2019 with 757bp sequence sizes were respectively identified as *Enterococcus faecium* (Accession No: MN585808) and *Gemella palaticanis* (Accession No: MN585809). The dendogram of the phylogenetic tree drawn from the five identified bacterial isolates and other related organisms earlier deposited from different parts of the world is presented as **Figure 1**.

The bacteria isolated in the present study exhibited varying degrees of sensitivity to different antimicrobials. Among all the 11 antimicrobials tested, gentamicin proved to be the most effective as 89.25% of all the isolates tested were sensitive to it. The highest rate of resistance was recorded among the penicillins (penicillin, oxacillin, amoxicillin/clavulanic acid, and ampicillin) which had about 73.00% average resistance to all the isolates tested **(Table 4)**. The detailed antimicrobial resistance pattern of the isolates is shown in Table 5. Several categories of the isolates particularly *E. coli*, *Streptococcus* species (other than *Strep. hyovaginalis*), and *Enterococcus* species (other than *E. faecium*) exhibited multidrug resistance (MDR), resisting at least three different classes of antimicrobials. Similarly, *Micrococcus* species showed an extended spectrum of drug resistance (XDR) as they were resistant to at least one agent in nearly all the classes of antimicrobials tested.

Table 1: Distribution of aerobic bacteria isolated from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria

S/No	Name of isolate	Healthy Bitches (n = 70)		Infertile Bitches (n = 30)		P-value
		Frequency	Percentage (%)	Frequency	Percentage (%)	
1	*Staphylococcus saprophyticus*	32	45.7	14	46.7	1.000
2	*Staphylococcus aureus*	10	14.3	6	20.0	0.554
3	*Staphylococcus schleiferi*	6	8.6	0	0.0	0.174
4	*Streptococcus hyovaginalis*	0	0.0	1	3.3	0.300
5	Other *Streptococcus species*	12	17.1	12	40.0	0.021*
6	*Corynebacterium species*	14	20.0	9	30.0	0.306
7	*Enterococcus faecium*	0	0.0	1	3.3	0.300
8	*Enterococcus dispar*	0	0.0	1	3.3	0.300
9	Other *Enterococcus species*	8	11.4	1	3.3	0.272
10	*Micrococcus species*	5	7.1	6	20.0	0.082
11	*Proteus vulgaris*	6	8.6	5	16.7	0.298
12	*Escherichia coli*	7	10.0	3	10.0	1.000
13	*Baccilus species*	3	4.3	2	6.7	0.635
14	*Gemella palaticanis*	0	0.0	3	10.0	0.025*
15	*Schaalia canis*	0	0.0	2	6.7	0.088
	Total	103		66		

P-values with an asterisk*are statistically significant (P<0.05).

Table 2: Morphological and Biochemical characteristics of some aerobic Gram-positive bacteria isolated from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria

S/No	**Colonial morphology**	**Microscopic features**	**Biochemical tests**	**Bacterium identified**
1	Small, raised, β-hemolytic colonies on blood agar	Gram-positive cocci in clusters	Catalase, coagulase, glucose, trehalose, maltose, mannitol, and sucrose positive; while negative for xylose, and arabinose	*Staphylococcus aureus*
2	Small, raised, β-hemolytic colonies on blood agar	Gram-positive cocci in clusters	Catalase, glucose, trehalose, mannitol, and sucrose positive; while negative for coagulase, maltose, xylose, and arabinose	*S. saprophyticus*
3	Small, raised, whitish β-hemolytic colonies	Gram-positive cocci in clusters	Catalase, glucose, and sucrose positive; while negative for coagulase, trehalose, mannitol, maltose, xylose, and arabinose	*S. schleiferi*
4	Glossy-dew drop-like, α, and β-hemolytic colonies	Gram-positive cocci in short and long chains	Positive for glucose; and negative for catalase	*Streptococcus spp* other than *S. hyovaginalis*
5	Glossy dew-drop-like, γ-hemolytic colonies	Gram-positive cocci in short chains and branches	Glucose, lactose, dextrose, raffinose, arabinose, and urease positive; while negative for catalase, coagulase, trehalose, mannitol, maltose, xylose, and sucrose	*Enterococcus* species other than *E. faecium and E. dispar*
6	Small, raised, β-hemolytic colonies with little yellowish pigmentation on blood agar	Gram-positive cocci with tetrad appearance	Positive for catalase, and glucose; but negative for coagulase	*Micrococcus* species
7	Large, flat, β-hemolytic colonies with serrated edges	Gram-positive rods in short chains	Catalase, glucose, lactose, and urease positive; while negative for sucrose	*Baccilus species*
8	Small, raised, moist, greyish, γ-hemolytic colonies	Gram-positive, pleomorphic rods with Chinese-letter appearance	Positive for catalase, glucose, and lactose; but negative for coagulase, sucrose, indole, and urease	*Corynebacterium* species
9	Pin-point, β-hemolytic colonies on blood agar	Gram-positive cocci mostly in pairs	***	*Gemella palaticanis*
10	Tiny granular-like, β-hemolytic colonies	Gram-positive coccobaccilli in chains	***	*Schaalia canis*

Note: Organisms with*** were identified via molecular characterization

Table 3: Biochemical characteristics of some aerobic Gram-negative bacteria isolated from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria

S/ No.	**Biochemical Tests Conducted**														**Name of Bacterium**
	CAT	OF	TSI	NIT	IND	URE	CIT	MR	VP	MOT	OXD	GLU	SUC	LAC	
1	+	F	K/A.H2S	+	+	+	-	+	-	+	-/+	+	+	-	*Proteus vulgaris*
2	+	F	A/A. G	+	+	-	-	+	-	+	-	+	-	+	*Escherichia coli*

Key: CAT=catalase, OF=oxidative/fermentative, TSI=triple sugar iron, NIT=nitrate reduction, IND=indole, URE=urease, CIT=citrate, MR=methyl red, VP=Voges Proskauer, MOT=motility, OXD=oxidase, GLU=glucose, SUC=sucrose, LAC=lactose, F=fermenter, A=acid, K=alkaline, G=gas, H2S= hydrogen sulphide, -=negative, and +=positive

Table 4: Percentage antimicrobial susceptibility profile of aerobic bacteria isolated from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria

Antibiotic (µg)	***Staphylococcus***			***Streptococcus***			***Enterococcus***			***Micrococcus***			***Corynebacterium***			***Schaalia***			***Gemella***			***Escherichia***		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
TE (30)	7	25	68	50	0	50	0	0	100	0	0	100	0	100	0	70	20	10	0	0	100	90	10	0
CN (10)	94	0	6	100	0	0	90	10	0	50	0	50	90	10	0	90	10	0	100	0	0	100	0	0
AMC (30)	5	0	95	20	20	60	0	0	100	0	0	100	0	10	90	0	10	90	80	20	0	0	0	100
FOX (30)	80	15	5	80	20	0	80	20	0	50	0	50	80	20	0	80	10	10	100	0	0	10	90	0
OX (1)	0	40	60	20	0	80	0	0	100	20	30	50	0	50	50	0	40	60	80	20	0	0	0	100
VA (30)	40	30	30	80	20	0	0	10	90	0	100	0	0	100	0	0	30	70	60	20	20	0	0	100
AMP (10)	0	20	80	0	40	60	0	20	80	0	0	100	0	10	90	0	20	80	0	40	60	0	0	100
P (10)	0	10	90	20	40	40	0	20	80	0	10	90	0	20	80	20	70	10	20	20	60	0	0	100
E (15)	50	10	40	40	20	40	80	20	0	0	50	50	50	30	20	80	10	10	80	20	0	0	0	100
S (10)	40	30	30	40	40	20	80	20	0	0	0	100	80	20	0	70	20	10	20	60	20	20	80	0
CIP (5)	30	60	10	40	40	20	0	100	0	0	0	100	50	30	20	100	0	0	40	40	20	50	50	0

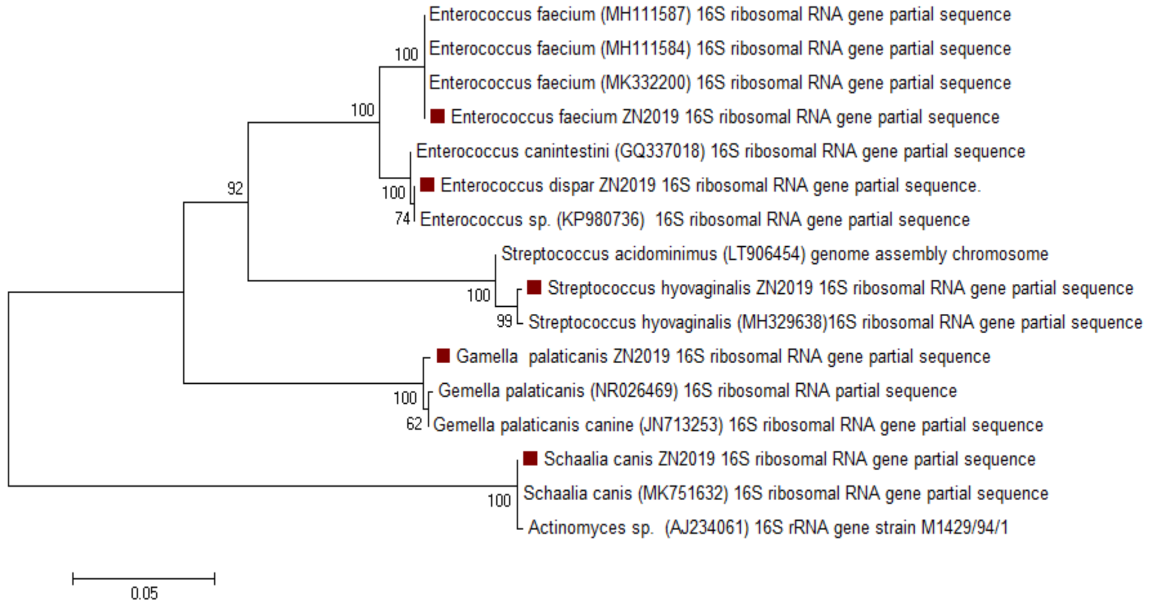
Note: *Staphylococcus = (S. aureus & S. saprophyticus), Streptococcus = (Strep. hyovaginalis* & other species), *Enterococcus = (E. faecium, E. dispar*, & other species), *Micrococcus = (Micrococcus* species), *Corynebacterium (Corynebacterium* species), *Schaalia = (S. canis), Gemella = (G. palaticanis*), *Escherichia = (E. coli*), TE =Tetracycline, CN=Gentamicin, AMC=Amoxicillin/Clavulanic acid, FOX=Cefoxitin, OX=Oxacillin, VA=Vancomycin, AMP=Ampicillin, P=Penicillin, E=Erythromycin, S=Streptomycin, CIP=Ciprofloxacin. Using the CLSI, 2016: R=Resistant, I=Intermediate, and S=Sensitive

Table 5: Antimicrobial resistance patterns of some aerobic bacteria isolated from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria

S/No	Name of Isolate	Resistance Pattern	Number of the Classes of Antimicrobials Resisted
1	*Staphylococcus saprophyticus*	TE-AMC-OX-AMP-P	2
2	*Staphylococcus aureus*	AMC-AMP-P-OX	1
4	*Streptococcus hyovaginalis*	AMC-OX-AMP-P	1
5	Other *Streptococcus* species	TE-AMC-OX-AMP-P-E	3
6	*Corynebacterium* species	AMC-OX-AMP-P	1
7	*Enterococcus faecium*	AMC-OX-AMP-P	1
8	*Enterococcus dispar*	TE-AMC-OX-VA-AMP-P-S	4
9	Other *Enterococcus* species	TE-AMC-OX-VA-AMP-P	3
10	*Micrococcus species*	TE-CN-AMC-FOX-OX-AMP-P-E-S-CIP	6
11	*Escherichia coli*	AMC-OX-VA-AMP-P-E	3
12	*Gemella palaticanis*	TE-AMP-P	2
13	*Schaalia canis*	AMC-OX-VA-AMP	2

TE =Tetracycline, AMC=Amoxicillin/Clavulanic acid, OX=Oxacillin, AMP=Ampicillin, P=Penicillin, E=Erythromycin, VA=Vancomycin, S=Streptomycin, CN=Gentamicin, FOX=Cefoxitin, and CIP=Ciprofloxacin

Figure 1: Phylogenetic tree with bootstrap values showing the relationships between some selected bacteria in the gene bank (each having its accession number in parenthesis) and the five aerobic bacterial isolates (each having a red square box) from the vagina of infertile bitches in Zaria, Nigeria.



Discussion

This study assessed the presence and antibiogram of aerobic bacterial populations in the vagina of apparently healthy and infertile bitches. The findings showed some level of variation in the number and type of bacteria isolated between the two different groups. The incidence of common pathogens, particularly, *Staphylococcus saprophyticus*, *S. aureus*, *E. coli*, *Streptococcus* species, *Bacillus* species, *Enterococcus*, and *Corynebacterium* species was similar between the healthy and infertile bitches. This outcome correlates well with the results of other parallel studies which documented *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Corynebacterium*, *Proteus* and *Escherichia* as the most common genera colonizing the vagina of dogs [6,14,27-29]. Interestingly, the present study established a significant difference in the overall bacterial occurrence rates between healthy and infertile bitches; where the higher isolation rate was from the infertile bitches implying that the presence of these bacterial species could be responsible for the infertility recorded in the affected bitches in agreement with other studies [14,30,31]. This, however contradicts the findings of Hutchins *et al.* [30] and Golińska *et al.* [16] who documented that the total number of bacteria in the vagina of healthy and non-healthy dogs at different stages of estrus cycle was almost the same when conducting similar studies. The exact reason for this variation is not clear, but one possible explanation is the relatively large sample size (100) of the current study as against the 44 and 39 respectively utilized by the two previous studies. This implies that, a probable significant difference in the bacterial population would have been identified if much higher sample sizes were employed in those preceding studies.

The restricted vaginal presence of *Staphylococcus schleiferi* within healthy bitches could have a competitive protective role against potentially more dangerous pathogens like *Gemella palaticanis* and *Schaalia canis* which were never isolated from the healthy bitches in the present study. This statement is in line with the findings of Feldman and Nelson [32] who documented that normal vaginal microbiota has been shown to protect the genito-urinary tract against pathogenic bacteria through nutrient and epithelial cell receptors competition. Conversely, the relatively higher incidence of *Proteus vulgaris* observed in infertile bitches concurs with the findings of Golińska *et al.* [16] who reported a significantly higher prevalence of Gram-negative rods (other than *E. coli*) in the vagina of dogs with genital tract infections. Likewise, the significantly higher occurrence rate of *Streptococcus* species in infertile bitches might be responsible for infertility in those dogs. This is because β-haemolytic *Streptococci*, as well as *Proteus, E. coli* and *Enterococcus* species were previously associated with infertility in bitches [31,33,34]. Similarly, the significantly higher occurrence of *G. palaticanis* in the anterior vagina of infertile bitches could be associated with the development of genital tract infection that might eventually result in infertility, however, a focused case-control study would be required to ascertain this hypothesis.

Bacteria of the Genera *Pasteurella, Pseudomonas, Klebsiella,* and *Enterobacter* which were previously reported to reside within the dog's reproductive tract [29,33] were not identified in the present study. This variation could be due to the canine intrinsic and genetic variability, indiscriminate use of antibiotics in the study area, and/or inherent limitations of culture-based systems when compared with molecular identification methods as earlier highlighted by Lyman *et al*. [33]. Another likely cause of this disparity in the type of vaginal microflora is the possibility of sample contamination from the vestibule or vulva, especially in the case of John *et al*. [34] who used vaginal discharges rather than guided aseptic swab collection from the anterior vagina.

Varying grades of antimicrobial sensitivity were observed among bacterial isolates from both healthy and infertile bitches, with cefoxitin and gentamycin being the most effective drugs as similarly observed by John *et al*. [34]. Likewise, several isolates displayed resistance to at least two classes of antibiotics termed multidrug-resistant phenotypes. In this study, the β-lactam antibiotics namely: ampicillin, penicillin, oxacillin, and amoxicillin/clavulanic acid which are known to be effective against many Gram-positive pathogens were surprisingly found to be resistant (with average resistance of 81.25%, 68.75%, 62.50% and 79.38% respectively). Gram-negative bacteria, (such as *E. coli*) have been established to develop resistance to multiple antibiotics mostly due to their inherent resistance to macrolide antibiotics (such as erythromycin), efflux system, as well as decreased drug uptake mediated by chromosomal mutations or via acquisition of resistance genes [35,36]. However, the non-susceptibility of *E. coli* to multiple antimicrobials observed in the current study is alarming. Consequently, the extremely drug-resistant *Micrococcus* species might have resulted from the acquisition of multiple resistance determinants over a long period sequel to the known indiscriminate prescription, acquisition and usage of antimicrobials in the country [37,38]. Therefore, the multidrug resistance nature of *Streptococcus* species (other than *Strep. hyovaginalis*), *Enterococcus* species (other than *E. faecium* and *E. dispar*), as well as the extensive antimicrobial resistance of *Micrococcus* species recorded in the present study highlight the need for an improved fight against antimicrobial resistance in Nigeria. The advice on an urgent need for curbing antimicrobial resistance becomes even more expedient with the realization of the disturbing resistance patterns recorded in this study that showed over 70% of the isolates exhibiting multiple drug resistance which is a phenomenon that prolongs treaments even causing deaths when infection set in and or produces non-response to treatment when trying to prevent infertility by pre-mating administration of drugs as advised in this study. Multiple antimicrobial resistant bacteria such as are being reported in this study are an urgent threat to public health especially with limited antibiotic therapy. These setbacks are common consequences of multiple antimicrobial resistance in bacterial infections as have been established in other studies [39,40].

Conclusion

The present study has established the most common bacteria colonizing the anterior vagina of bitches in Zaria, Nigeria. The occurrence of aerobic bacteria was significantly higher in infertile bitches compared to the healthy ones suggesting the possibility of some identified pathogens being responsible for the infertility experienced. Furthermore, varying degrees of sensitivity to antimicrobials and the development of multiple drug resistance were identified among the isolates highlighting the need for rational and judicious selection of drugs when managing infertility in dogs. Dog breeders need to screen their bitches and studs before breeding especially for the presence of possible multi-drug resistant bacteria, which can pose a threat to pregnancy. This should be preceded by thorough clinical and vagina cytological examinations. Since bacterial screening including susceptibility testing would take a bit of time which the breeder may not have the luxury of when heat sets in, it is hereby advised that while the screening results are being awaited, the best drugs from this study could be used to carry out a general bacterial treatment pending the outcome of laboratory results. Further research that would explore the possibility of whole genome sequencing of all or at least the new isolates identified, and experimental infection of bitches with the new isolates are hereby recommended.

Acknowledgment

The authors wish to thank Dr. K. H. Ahmad of Veterinary Microbiology, ABU Zaria for his help in reading and correcting the manuscript.

Funding statement

None

Conflict of interests

The authors declare no conflict of interest.

Data availability

All data generated or analyzed during this study are included in this puplished article.

Authors' contribution

All authors made significant contributions to the work presented, including study design, data collection, analysis, and interpretation. They also contributed to the article's writing, revising, or critical evaluation, gave final approval for the version to be published.

References

1- De Paula Antunes JMA, de Carvalho Freire DE, de Medeiros Oliveira IVP, Moura GHF, de Castro Demoner L, Ferreira HIP. Infectious Causes of Abortion, Stillbirth and Neonatal Death in Bitches. In: Abdelhay Essayed KH (Ed). Can Med R T Adv ResI. 2016; pp. 55-73.

2- Hollinshead FK, Hanlon DW. Factors affecting the reproductive performance of bitches: A prospective cohort study involving 1203 inseminations with fresh and frozen semen. Therio. 2017; 101: 62–72.

3- Long ST, Hien NTT, Hang PT, Hoai NT, Bach PX. Canine Dystocia: The Risk Factors and Treatment Methods in Dogs of Hanoi, Vietnam. World Vet J. 2022; 12 (3): 290-295. doi: https://dx.doi.org/10.54203/scil.2022.wvj36.

4- Gavrilovic BB, Andersson K, Forsberg C. Reproductive patterns in the domestic dog: A retrospective study of the Drever breed. Therio. 2008; 70(5): 783-794.

5- Runcan EE, da Silva MAC. Whelping and dystocia: maximizing success of medical management. Top Companion Anim Med. 2018; 33(1): 12-16.

6- Bhuyan M, Baruti M, Deka R, Deka A, Phungsubapu H, Pachoni S et al. Canine Reproductive Disorders at Veterinary Clinical Complex, College of Veterinary Science, Guwahati, India: A Retrospective Study from 2016 to 2021. Ind J Anim Res. 2022; Online: doi: 10.18805/IJAR.B-4851.

7- Xavier RGC, Santana CH, de Castro YG, de Souza TGV, do Amarante TGV, Santos RL et al. Canine Pyometra: A short review of Current Advances. Anim. 2023; 13(21): 3310. doi: 10.3390/ani13213310.

8- Dejneka GJ, Ochota M, Bielas W, Niżański W. Dystocia after unwanted mating as one of the risk factors in non-spayed bitches - a retrospective study. Anim. 2020; 10(9): 1697. doi: https://www.doi.org/10.3390/ani10091697.

9- Fontbonne A. Infertility in bitches and queens: recent advances. Revista Brasila Reprod Anim. 2011; 35(2): 202-209.

10- Zubair M, Ubaid-Ur-Rehman S, Sajid SM. Causes of infertility in bitches. Adv Anim Vet Sci. 2014; 2(10): 565-573.

11- Anonymous. High Street Epping Veterinary Clinic: Factors Leading to Infertility in Bitches. 2021; Retrieved on October 28, 2021 from https://www.highstreeteppingvetclinic.com/factors-leading-to-infertility-in-bitch.pml.

12- Nazer AT, Bibin B, Jayakumar C, Unnikrishnan P, Devi SS. Occurrence of canine transmissible venereal tumour. J Vet Anim Sci. 2023; ISSN (Print): 0971-0701, (Online): 54(2): 2582-0605. doi: https://doi.org/10.51966/jvas.2023.54.2.382-387.

13- Xavier RGC, Santana CH, da Silva PHS, Aburjaile FF, Pereira FL, Figueiredo HCP et al. Transmission of Escherichia coli Causing Pyometra between Two Female Dogs. Microbiol. 2022; 10(12): 2465. doi: 10.3390/microorganisms10122465.

14- Jagodka D, Kaczorek-Łukowska E, Graczyk R, Socha P. Vaginal aerobic bacteria of healthy bitches and those with fertility problems. Polish J of vet Sci. 2023; 26(4), 733–739.

15- Leps AS, Klein B, Schneider M, Meyer C, Soba A, Simon C et al. The Canine Vaginal Flora: A Large-Cohort Retrospective Study. Vet Sci, 2024; 11(2): 55

16- Golińska E, Sowińska N, Tomusiak-Plebanek A, Szydło M, Witka N, Lenarczyk J et al. The vaginal microflora changes in various stages of the estrous cycle of healthy female dogs and the ones with genital tract infections. BMC Vet Res. 2021; 17(1): 1-8.

17- Johnston SD, Kustritz MVR, Olson PNS. Canine and Feline Theriogenology. Saunders, Philadelphia, USA. 2001; p. 592.

18- Olson PS. Steptococcus canis: An isolate from the canine uterus. Vet Med Small Anim Clin. 1975; 70: 933–934.

19- Cottral GE. Manual of Standardised Methods for Veterinary Microbiology. National Research Council. Subc Stand Met Vet Microbiol. 1978; Cornell University Press, Ithaca, New York, Pp. 731.

20- Cowan ST, Steel KJ. Manual for the Identification of Medical Bacteria. 2nd edition. Cambridge University press, United Kingdom. 2002; pp. 217.

21- Ahmed OA, Mamman PH, Raji MA, Kwanashie CN, Raufu IO, Aremu A. Distribution of Virulence Genes in Salmonella Serovars isolated from poultry farms in Kwara State, Nigeria. Ceylon J Sci. 2017; 46: 69–76. doi: https://doi.org/10.4038/cjs.v46i4.7469.

22- Wagner J, Short K, Catto-Smith AG, Cameron DJ, Bishop RF, Kirkwood CD. Identification and characterisation of Pseudomonas 16S ribosomal DNA from ileal biopsies of children with Crohn's disease. 2008. 3(10), e3578.

23- Thomas J, Thanigaivel S, Vijayakumar S, Acharya K, Shinge D, Seelan TSJ et al. Pathogenecity of Pseudomonas aeruginosa in Oreochromis mossambicus and treatment using lime oil nano emulsion. Colloids and Surfaces B: Biointerfaces 2014 116, 372-377.

24- Kimura MA. Simple Method for Estimating Evolutionary Rates of Base Substitutions Through Comparative Studies of Nucleotide Sequences. J Mol Evol. 1980; 16: 111-120.

25- Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966; 45: 493–496.

26- Clinical Laboratory Standards Institute (CLSI) M100S27. Performance Standards for Antimicrobial Susceptibility Testing. Clinical Laboratory Standards Institute 2016; M100S27.2016

27- Findik M, Maral N, Keskin O, Kalender H, Erdeger J, Aslan J. The relationship between the stages of sexual cycle, the pregnancy and postpartum periods and vaginal flora in kangal breed bitches. Turk J V Anim Sci. 2003; 27: 761-765.

28- Groppetti D, Pecile A, Barbero C, Martino PA. Vaginal bacterial flora and cytology in proestrous bitches: Role on fertility. Therio. 2012; 77(8): 1549-1556.

29- Antonov A. Normal and Pathological Vaginal Microflora in the Bitch - A Review. J Adv

Microbiol; 2019; 15: 1-5. doi: 10.9734/JAMB/2019/v15i130074.

30- Hutchins RG, Vaden SL, Jacob ME, Harris TL, Bowles KD, Wood MW et al. Vaginal microbiota of spayed dogs with or without recurrent urinary tract infections. J Vet Intern Med; 2014; 28(2): 300–304.

31- Markus TP, Orakpoghenor O, Mamman PH, Nafi'u L. Polymicrobial Vaginal Infection and Chronic Infertility in a 3-year-old Bitch: Isolation of Escherichia coli, Morganella morganii, and Staphylococcus spp. SVU-International J Vet Sci, (accepted) 2024.

32- Feldman EC, Nelson RW. Canine and feline endocrinology and reproduction. 3rd Ed. Saunders, Philadelphia, USA. 2003; Pp. 1044-1104.

33- Lyman CC, Holyoak GR, Meinkoth K, Wieneke X, Chillemi KA, Desilvao U. Canine endometrial and vaginal microbiomes reveal distinct and complex ecosystems. PLoS ONE. 2019; 14(1): e0210157. doi.org/10.1371/journal.pone.0210157.

34- John A, Nair DR, Sreejith JR, Deepthi L, Praseeda R, Ajitkumar G. Vaginal Microflora and its Antibiogram in Infertile Bitches. Online Vet J. 2011; 6(1):e79.

35- Lartey PA, Nellans HN, Tanaka SK. New developments in macrolides: structures and antibacterial and prokinetic activities. Adv Pharmacol. 1994; (San Diego, Calif.), Elsevier Academic Press, Inc. pp. 307-343.

36- Rossolini GM, Arena F, Giani T. Mechanisms of Antibacterial Resistance. In: Jonathan Cohen, William Powderly, Steven Opal (Eds.). Infect Dis. 2017; (4thEdition), Elsevier Ltd. 2, Pp. e1181-1196.

37- Federal Ministries of Agriculture, Environment, and Health; (FMAEH). Antimicrobial Use and Resistance in Nigeria: Situation Analysis and Recommendations. 2017; pp. 1-105.

38- Ogwuche A, Ekiri AB, Endacott I, Maikai BV, Idoga ES, Alafiatayo et al. Antibiotic use, practices of veterinarians and para-veterinarians and the implications for antibiotic stewardship in Nigeria. JSAVA. 2021; 92: 1-14.

39- Catalano A, Iacopetta D, Ceramella J, Scumaci D, Giuzio F, Saturnino C, et al. Multidrug resistance (MDR): a widespread phenomenon in pharmacological therapies. Molecules, 2022; 27:616. 10.3390/molecules27030616 - DOI - PMC - PubMed

40- Korczak L, Majewski P, Rombel K, Iwaniuk D, Sacha P, Modzelewski et al. In vitro evaluation of tigecycline synergy testing with nine antimicrobial agents against Enterobacter cloacae clinical strains. Front Microbiol. 2024; 18;15:1490032. doi: 10.3389/fmicb.2024.1490032. PMID: 39493842; PMCID: PMC11527652

Mamman PH. Umar BN, Kakudi AK, Ahmed AO, Chah K, Kazeem HK. Phenotypic and molecular characterization of aerobic bacteria from the vagina of apparently healthy and infertile bitches in Zaria, Nigeria. Microbes Infect Dis 2025; 6(4): 6732-6742.