

## Special Issue:

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# Clinical Isolation and Genetic Established and Saprophytic Bacteria During Normal Estrus Cycle of Cow

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**Abstract** | There are many cases of vaginitis and metritis occurring in animals farms specially in cows In majority of these cases, the infection remained unnoticed caused by proliferation of saprophytic bacteria that present normally in mucosal membrane of vaginal. These infections predispose cow more susceptible to infection and bacterial proliferation during the stages of estrus periods (proestrus, estrus, diestrus and metestrus). Here, we investigated the presence of saprophytic bacteria in vaginal smears, and characterize them using PCR. All vaginal swabs collected from 43 sexually matured cows were subjected to Stuart transport media, before planted in blood agar media to identify the number of colonies counted. The bacteria was confirmed by Gram's staining and biochemical tests. The PCR products from the bacterial samples were genetically analyzed (Al-Mutar *et al.*, 2018). The result show that the most bacterial present in bovine vagina were *Streptococcus thermophilus* and *Streptococcus uberis* (92.65%) while the lowest percent of bacteria present in bovine vagina was *Corynebacterium frankenforstense* (78.8%). The other saprophytic bacteria were *Streptococcus lutetiensis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* were present in a percentage from 94.61%, 93.25%, 80.5% and 78.69%. respectively. The study concluded that there are many types of Saprophytic bacterial proliferation during estrus cycle inside vagina and uterus of cows with a variation of different countries, breed and age.

**Keywords** | Saprophytic bacteria, Estrus cycle, Cows, PCR, Clinical isolation

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## INTRODUCTION

During the normal estrus cycle of cows, the presence of saprophytic bacteria in the reproductive tract does not significantly affect reproductive efficiency (Qodri *et al.*, 2020). Studies have shown that the uterine microbiota composition differs between cows exhibiting estrus and non-estrus cows (Madison *et al.*, 2022; Dong-Yan *et al.*, 2022). Additionally, shifts in the intestinal microbiota have been observed during estrus synchronization in Simmental cows, influenced by reproductive hormones

(Madison *et al.*, 2022). Abnormal cervical mucus discharge (A-CMD) in cows and heifers has been associated with specific pathogens affecting fertility, emphasizing the importance of evaluating cervical mucus discharge for infertility issues (Ayhan *et al.*, 2010). Overall, while the reproductive tract microbiota may vary during the estrus cycle, the presence of saprophytic bacteria does not seem to hinder the reproductive performance of cows. Saprophytic bacteria have been isolated from the vaginal flora of cows in various studies. The vaginal microbiota of Criollo Limonero cows revealed the presence of Gram-positive

bacteria like *Staphylococcus aureus* and *Arcanobacterium pyogenes* (Julio *et al.*, 2010). Additionally, research on crossbred cows identified *Bacillus*, *Corynebacterium*, and *Staphylococcus* as predominant vaginal bacteria during different physiological states (Patel *et al.*, 2019). Furthermore, studies on postpartum cows showed the presence of *Bacillus*, *Streptococcus*, and *Escherichia coli* in the vaginal flora, with varying antimicrobial resistance profiles (Yvonne *et al.*, 2013). These findings highlight the diverse bacterial populations present in the vaginal environment of cows, including saprophytic species, which play a role in the overall reproductive health and microbial balance in these animals (Al-Mutar, 2017).

The normal flora of the cow vagina consists predominantly of Gram-positive bacteria, including *Arcanobacterium pyogenes*, *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *Erysipelothrix rhusiopathiae*, *Bacteroides* spp., and *Peptostreptococcus* spp. (Sunny *et al.*, 2011; Jeffrey *et al.*, 2014; James, 2005). Additionally, *Streptococci* and *Staphylococci* are the dominant bacteria in the cow's vaginal microbiota, with low levels of *Enterobacteriaceae* and lactobacilli present (Otero *et al.*, 2000). The vaginal microbiota of cows and ewes exhibit differences in terms of  $\alpha$ -diversity, with both being mainly colonized by *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria*, along with the presence of *Archaea*, mainly *Desulfurococcales* (Mateus *et al.*, 2015). The microbial community in the cow's vaginal tract is influenced by factors such as hormonal changes, pregnancy status, and potential interactions with the gastrointestinal microbes.

Genetic detection of saprophytic bacteria from the vaginal tract of cows can be achieved through molecular techniques like PCR (Laith *et al.*, 2018). Studies have successfully utilized PCR assays for specific identification of bacteria like *Staphylococcus saprophyticus* in different contexts (Marta *et al.*, 2022; Ibraheem and Abbas, 2015). Similarly, in the context of bovine genital *campylobacteriosis*, a real-time PCR assay coupled with high-resolution melting analysis was developed for the identification of *Campylobacter fetus* subspecies in bull preputial samples (Fernanda *et al.*, 2016). Furthermore, research on *Leptospira* colonization in cows utilized PCR for detecting leptospiral DNA in vaginal fluid samples (Maryam and Ezzat, 2023; Rahawy and Al-Mutar, 2021). These studies highlight the effectiveness of genetic detection methods, such as PCR, in identifying specific bacteria in the vaginal tract of cows, showcasing the potential for accurate and sensitive detection of *Saprophytic bacteria*.

## FACTORS EFFECTING GROWTH OF SAPROPHYTIC BACTERIA

The growth of saprophytic bacteria in the vagina of cows is influenced by various factors. Research indicates that the

normal microbial flora of a cow's vagina is predominantly composed of *Streptococci* and *Staphylococci* (Biao *et al.*, 2012; Brooke *et al.*, 2017). Additionally, lactobacilli levels increase during the cow's growth, potentially due to hormonal influences (Friedrich, 2010). Factors such as age, stage of the sexual cycle, and pH value do not seem to directly correlate with the bacterial flora in the cow's vagina (Diana *et al.*, 2020). Moreover, the presence of specific microbes in the reproductive organs of cows, including the uterus and vagina, can impact reproductive success (Gloria and Morales, 2015). Understanding these microbial communities and their dynamics is crucial for managing reproductive inefficiencies in cattle operations and ensuring overall productivity. The death of saprophytic bacteria in the cow genital system can be influenced by various factors. For instance, *Staphylococcus saprophyticus*, a common bacterium found in ticks, has been identified as a causative agent of pathological symptoms in fully engorged female ticks, leading to their death (Estefhan *et al.*, 2010). Additionally, bacterio-spermia caused by bacteria like *S. saprophyticus* and *Escherichia coli* can impact sperm parameters and clinical outcomes in semen samples, potentially affecting assisted reproductive techniques and reducing the likelihood of clinical pregnancy (Francisco *et al.*, 2018). Moreover, the presence of bacteria like *E. coli* and *Staphylococcus* coagulase-negative in semen samples can lead to infections and impact fertility outcomes (Luis *et al.*, 2005). Therefore, the interaction of saprophytic bacteria with the reproductive system can have significant implications for the health and fertility of cows.

## MATERIALS AND METHODS

### VAGINAL SWABS

A vaginal swab collected from 43 sexually matured cows randomly. These samples consist of 11 swabs collected from cows in proestrus phase, 10 in estrus phase, 10 metestrus phase and 12 in diestrus phase. Then the swabs samples are flood in Stuart transport media, after that planted in blood agar media to identify the number of colonies counted, followed by gram staining and biochemical tests then the results were presented descriptively.

### BLOOD SAMPLES

A 3-5 ml of blood samples were collected form jugular vein and stored in EDTA collecting tube in very sterile conditions.

### TEMPLATE PREPARATION AND DNA EXTRACTION

In compliance with the Manufacturer's instructions, the DNA of cow used in the study were extracted using a kit for extraction DNA (kit no. 17045). The augmented product of the *16s* gene was exposed to (2%) agarose gel electrophoresis.

## POLYMERASE CHAIN REACTION (PCR) REACTION

Through amplification of the *16s* gene, the prolactin receptor (PRLR) was identified by PCR. forward primer (5'-AGAGTTTGA TCCTGGCTCAG-3') and reverse primer (5'-GGTTACCTTGTTACGACTT-3') (1250 bp), (each 10 Picomoles/ $\mu$ L) and the annealing (56°C for 35 sec.) (MacKichan *et al.*, 2015).

## DNA SEQUENCES

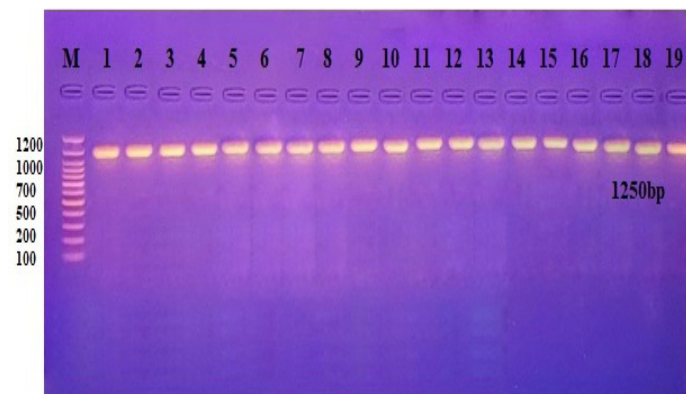
All the samples products of PCR from these primers (1250bp) obtained from the Iraqi cows were used. The PCR product was sequencing using online BLASTNCBI and BioEdit.

## STATISTICAL ANALYSIS

By using the statistical SPSS-USA-v.23 software, statistical analysis of the data was performed. These data were track using (HWE) and Fisher's-exact test (Raymond and Rousset, 1995).

## RESULTS AND DISCUSSION

Results of conventional PCR for detection of extracted DNA of *16s* RNA gene: Gel electrophoresis revealed bands of extracted nucleic acid of *16s* gene (Figure 1).

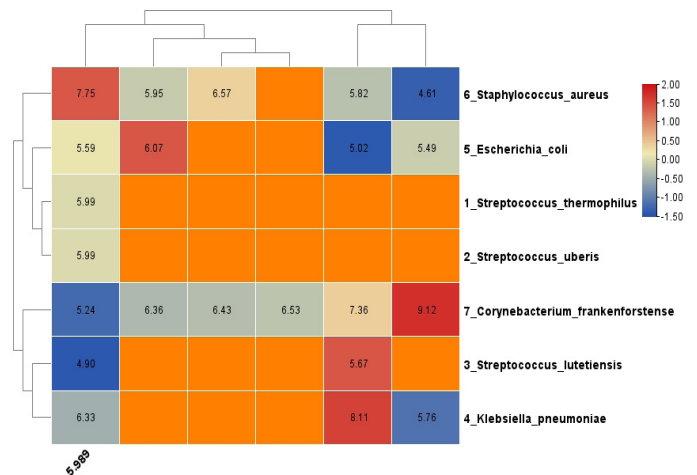


**Figure 1:** Product of PCR, the size of band was 1250 bp. The product electrophoresis on 2% agarose for 1:20 hrs. M: DNA ladder (100).

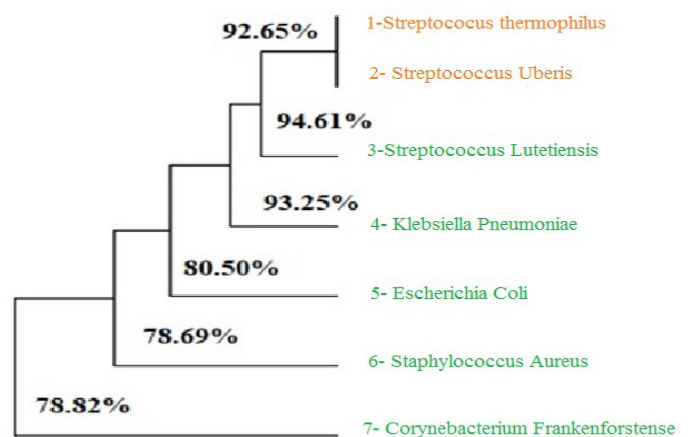
The result show as present in Figures 2 and 3 that the most more bacterial present in bovine vagina were *Streptococcus thermophilus* and *Streptococcus uberis* in percent 92.65% while the lowest percent of bacteria present in bovine vagina was *Sorynebacterium frankenforstense* (78.8%). The other saprophytic bacteria were *Streptococcus lutetiensis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* were present in a percent of 94.61%, 93.25%, 80.5 % and 78.69%, respectively.

According to Table 1, the results show high bacterial proliferation *Klebsiella pneumoniae* (50 %) in estrus, less of these in metestrus (30 %) and diestrus (33.33 %)

phasein comparison with proestrus phase (9.09). In case of *Escherichia coli* present in vagina of cows, the percentage of proliferation was highest in proestrus, diestrus and metestrus phases (45.45%, 41.66% and 40%, respectively) while there was no bacterial proliferation of *Escherichia coli* estrus phase. The result either show proliferation of *Streptococcus thermophiles* (18.18% and 20%, respectively), *Streptococcus uberis* (9.09% and 20%, respectively) and *Streptococcus pneumoniae* (18.18% and 20%, respectively) in proestrus and estrus phases while there were no proliferation of these bacteria in metestrus and diestrus. Opposite of that, the result shows no proliferation of *Streptococcus lutetiensis* bacteria in bovine vaginal mucosa in proestrus and estrus phase while there was low proliferation (20%) of this bacteria in metestrus phase and (25%) in diestrus and phase. Finally, the *Corynebacterium frankenforstense* found in vaginal mucosa in percentage of 10% in metestrus only while not presented in the other phases of estrus cycle (proestrus, estrus and diestrus). The study concluded that the highest percentage of bacterial normal flora present in pro-estrus, estrus, metestrus and dieasrus were (*Escherichia coli* 45%, *Klebsiella pneumoniae* 50%, *Escherichia coli* 40% and *Escherichia coli* 41%, respectively).



**Figure 2:** The heat map of the differentially expressed genes.



**Figure 3:** The phylogenetic tree of the type of Bacteria.



**Table 1:** Percentage of bacteria present in vagina of cow during phases of estrus cycle.

| Diestrus phase (%) | Metestrus phase (%) | Estrus phase (%) | Proestrus phase (%) | Bacteria                                |
|--------------------|---------------------|------------------|---------------------|-----------------------------------------|
| 33.33              | 30                  | 50               | 9.09                | <i>Klebsiella pneumoniae</i>            |
| 41.66              | 40                  | 0                | 45.45               | <i>Escherichia coli</i>                 |
| 0                  | 0                   | 20               | 18.18               | <i>Streptococcus thermophilus</i>       |
| 0                  | 0                   | 20               | 9.09                | <i>Streptococcus uberis</i>             |
| 0                  | 0                   | 10               | 18.18               | <i>Streptococcus pneumoniae</i>         |
| 25                 | 20                  | 0                | 0                   | <i>Streptococcus lutetiensis</i>        |
| 0                  | 10                  | 0                | 0                   | <i>Corynebacterium frankenforstense</i> |

It was long believed that the healthy uterus and vagina are a sterile environment. However, the microbial population in the uterus and vagina of cows has been characterized thanks to recent advancements in sequencing technology. The result of bacterial present in vaginal mucosa agreed with (Qodri *et al.*, 2020; Madison *et al.*, 2022) they found that there are many of saprophytic bacteria in vaginal mucosa during estrus such as *Escherichia*, *Streptococcus*, *Corynebacterium* and *Staphylococcus* bacteria. While (Dong-yan *et al.*, 2022) reported that *Firmicutes*, *Clostridia*, *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Ruminiclostridium*, *Oscillibacter*, *Romboutsia*, *Lachnoclostridium*, *Coproccoccus*, *Ruminococcus*, *Clostridium leptum*, and *Dorea* spp. were significantly increased, while *Bacteroidetes*, *Bacteroidia*, and *Bacteroidales* were significantly decreased in vaginal mucosa. The result also agreed with (Ayhan *et al.*, 2010) they found *K. pneumoniae*, *A. pyogenes*, *S. aureus*, present in vaginal mucosa in low percentage ranged from (3%–17%). *Bacillus* spp. were the most frequently isolated bacteria during the follicular phase of the estrous cycle, followed by *Corynebacterium* spp., *Staphylococcus* spp., and *Streptococcus* spp. In contrast, *Staphylococcus* spp. were the most frequently isolated bacteria during the luteal phase, followed by *Corynebacterium* spp., *Bacillus* spp., *E. coli*, and *Streptococcus* spp. In descending order, the most common vaginal bacterial isolates found during pregnancy were *Bacillus*, *Streptococcus*, *E. Coli*, *Klebsiella*, and *pseudomonas*. While the main isolates in endometritis were *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *E. coli*, *Salmonella*, and *Corynebacterium*, the vaginal aspirates of acyclic cows contained *Streptococcus* spp., *Bacillus* spp., and *Staphylococcus* spp., *Corynebacterium* spp., and *Micrococcus*. Rich bacterial diversity was found in the vagina of crossbred calves with a range of physiological disorders (Patel *et al.*, 2019).

## CONCLUSIONS AND RECOMMENDATIONS

The study concluded that there are many types of Saprophytic bacterial proliferation during estrus cycle inside vagina and uterus of cows with a variation of different countries, breed and age.

## ACKNOWLEDGMENT

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## NOVELTY STATEMENT

This study introduces an innovative approach to obtaining clinical and molecular bacterial isolation from pregnant and non-pregnant in cow

## AUTHOR'S CONTRIBUTION

This work was carried out in collaboration between all authors. HAHM-M: Designed the study, performed the statistical analysis. HAHM-M, BJH, KAH, and SOH: Wrote the protocol, and wrote the first draft of the manuscript managed the analyses of the study and the literature searches.

## ETHICAL APPROVAL

The research started in Jan 2022, lasting for one year, samples were taken from Baghdad government fields. All samples were examined at the laboratory of Bacteriology in the College of Veterinary Medicine, University of Baghdad.

## CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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