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Detection of *Fusobacterium* Species in Female Dromedary Camels using qPCR and MicroSEQ 500

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ABSTRACT

The goal of this study was to detect *Fusobacterium* spp. in apparently healthy dromedary camels using qPCR and Microseq 500 sequencing. Six pregnant dromedary camels were swabbed for vaginal, rectal, nasal, and ocular discharges. Genomic DNA was extracted. qPCR followed by microseq 500 was used for the detection of *Fusobacterium* spp. The purity of the extracted DNA from various swabs ranged from 1.5 to 2.1. Detection of Fusobacteria using qPCR indicated their presence in 91.7% of the collected swabs. *Fusobacterium* spp. was found in all vaginal, ocular, and fecal swabs, but in 66.7% of nasal swabs. The *Fusobacterium* gene was abundant in vaginal swabs. The isolate was identified by 16S rRNA sequence as *Fusobacterum gastrosuis* CAMSA16 OQ824900. In conclusion, this study sheds light on some naturally occurring microbes in camels that, under specific circumstances, can cause serious diseases. *F. gastrosuis* may have zoonotic potential.

Key words: Female camels, *Fusobacterium*, qPCR, Microseq 500.

INTRODUCTION

Fusobacterium spp. is a Gram-negative, non-spore-forming, non-motile, anaerobic, rod-shaped bacteria (Han 2015). Many reports considered *Fusobacterium* spp. as a normal flora in the gut and oral cavities, however, the current consensus is that *Fusobacterium* spp. are pathogens (Han 2015; Lee et al. 2022). In women, *Fusobacterium* spp. can produce ovarian abscess (Morrall and Schmidt 2022), premature labor and serious uterine infection (Vidaurrazaga et al. 2020), bacteremia and septic shock (Lee et al. 2022), thrombophlebitis of the internal jugular vein (Kherabi et al. 2020), stillbirth, and neonatal sepsis (Han, 2015). In animals, *Fusobacterium* spp. has been associated with abortion (Agerholm et al. 2007); digital dermatitis (Rosander et al. 2022); metritis (Galvão et al. 2019); salpingitis (Sadeghi et al. 2022) and pyometra (Knudsen et al. 2015). In dromedary camels, *Fusobacterium* has been found in cases with cervicovaginal adhesion (Ghoneim et al. 2021) and in the prepuce of infertile males (Waheed et al. 2022).

Despite serving as the first line of defense against pathogens that ascend in the genital system, vagina contains a variety of bacteria that have the potential to cause a disease (Moreno et al. 2021). Additionally, during pregnancy the uterus is not sterile. Cows and mares may become pregnant notwithstanding the existence of few possibly harmful microbes (Karstrup et al. 2017; van Heule et al. 2023).

An essential protocol in microbiology laboratories is the accurate automated identification and sensitivity testing of chemotherapeutic drug pathogens. VITEK 2 (bioMe'rieux) and the Phoenix (Becton Dickinson) are two examples of automated identification systems. Unculturable bacteria cannot be detected by automated methods. However, genomic DNA analysis and identification based on the sequencing of the 16S rRNA gene could be an optional strategy. The Microseq 500 was recommended in several reports for sequencing the 16S rRNA gene in samples that are difficult to access by conventional techniques (Woo et al. 2003; Fontana et al. 2005).

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This study aimed to use qPCR and Microseq[500] sequencing to detect *Fusobacterium* spp. in the vagina, feces, and nasal and ocular discharges of apparently healthy pregnant dromedary camels.

MATERIALS AND METHODS

Compliance with Ethical Standards

This study was approved by the Animal Care and Welfare Committee, Deanship of Scientific Research, Qassim University, Kingdom of Saudi Arabia. The summary for the detection of *Fusobacterium species* in female dromedary camels is illustrated (Fig. 1).

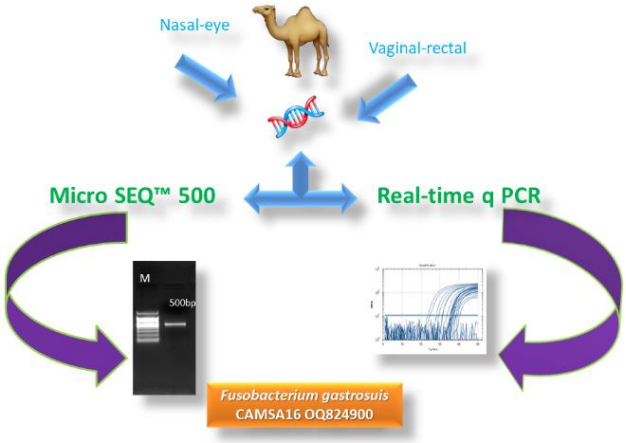

Fig. 1: Summary of protocol for detection of *Fusobacterium species* in female dromedary camels using qPCR and MicroSEQ 500.

Sampling

Follow the Green and Correct Red and Similar

Vaginal, rectal, nasal, and ocular swabs were obtained from six pregnant dromedary camels (aged 6-7 years). Swabs were prepared according to the method described by Barati et al. (2022). A sucrose, phosphate, and glutamate (SPG transport) medium were used to collect each swab. SPG medium contained the following ingredients: streptomycin (0.05g/L), L-glutamic acid (0.721g/L), K2HPO (1.237g/L), KH2PO4 (0.512g/L), and sucrose (74.6g/L). A pH of 7.2 to 7.4 was adjusted. Centrifugation at 3000rpm for 15min was used to clear the samples. For DNA extraction, the supernatant was stored at -20°C.

Genomic DNA Isolation from Prepared Swabs

The DNeasy Blood and Tissue Kit (50) from QIAGEN (catalog number 69504) was used to extract DNA from the swabs. The OD A260/A280 ratio of the purified DNA ranged from 1.7 to 1.9. Thermo Scientific's Nanodrop 2000 spectrophotometer was used to measure the DNA concentration. The 16S rDNA region was amplified using two different methods, qPCR and Microseq500 (Wang et al. 2017).

Real-time Quantitative PCR

The primer and probe set sequences are shown (Table 1; Boutaga et al. 2005). The National Center for Biotechnology Information's (NCBI) blasts search for homology with unrelated sequences to validate primer and probe sequences (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1997). The optimal concentrations of forward and reverse primers, as well as the probe were 300, 300, and 300nM, respectively. Purified genomic DNA from various swabs was used to test the specificity of the probe and primer sets for their target DNA. Tenfold dilutions of DNA were used in series as standard curves. Amplification was carried out as previously designated by Boutaga et al. (2005). Using Biosystem Step One Plus, the results from unidentified genomic DNA samples were automatically plotted on the standard curves of the target positive control.

MicroSEQ™ 500

The technique was carried out using Thermofisher Scientific MicroSEQTM 500 16S rDNA PCR Kit (Catalog Numbers 4348228) and MicroSEQTM 500 16S rDNA Sequencing Kit (Catalog Numbers 4346480) supplied by Thermo Fisher Scientific168 (Waltham, USA). As described by (Fontana et al. 2005), amplification of 527bp fragment of the 16S rRNA gene was performed from the 5' end of swab DNA with suspected bacterial identity.

PCR Reactions

MicroAmpTM reaction tubes were used to prepare the samples and controls. The supplied negative control was mixed with 15 µL of PCR master. Positive control DNA is included in the kit. The 9600 thermal cycle from the GeneAmpTM PCR System's 9700-9600 emulation was used. The thermal cycling conditions were set to start at 95°C/10min. The following steps were included in each of the 30 cycles: melting at 95°C/30s, annealing at 60°C/30s, and extension at 72°C/45 seconds. The final extension lasted 10min at 72°C. The final step is 4°C for an infinite time. For separation, PCR products were loaded onto a 2% agarose gel. A standard ladder is used to estimate the PCR product yield. The actual fragment size depends on the bacterial species. PCR products were purified for sequencing.

Statistical Analysis

The data were presented in mean±SD. Analysis of variance (ANOVA) was used for comparison between means with Fisher's protected least significant difference (LSD) as the post-ANOVA test. IBM SPSS Statistics 21 for Windows was used for analysis. The significance level was set at $P<0.05$.

RESULTS

The extracted DNA purity from the various swabs ranged from 1.5 to 2.1. qPCR detected an increase in the Fusobacterial gene in 91.7% (22/24) of the collected swabs. Cycle quantitation (Cq) values ranged from 23 to 40 (Table 2).

Linear regression was calculated automatically by the Biosystem Step One Plus Instrument. The efficiency of the reaction was 88.15% (Fig.). The amplification plot of the target gene after a 10-fold serial dilution with camel swabs was shown (Fig. 3). *Fusobacterium* spp. was found in all vaginal, ocular, and rectal swabs, but only in 66.7% of nasal swabs. *Fusobacterium* gene was found in high concentrations in vaginal swabs compared to fecal, nasal, and ocular swabs (Table 2).

Table 1: Primers and fluorogenic probes for detection of *Fusobacterium* spp (Boutaga et al. 2005)

	Sequence (5 to 3)	Amplicon size (bp)
Fusobacterium spp.	Forward GGATTTATTGGGCGTAAAGC Reverse GGCATTCCTACAAATATCTACGAA Probe FAM-CTCTACACTTGTAGTTCCG-BHQ	162

Table 2: Cycle quantitation (Cq) values for the *Fusobacterum* spp. gene in vaginal (V), ocular (C), nasal (N), and rectal (F) swabs from pregnant female dromedary camels (n=6)

Cq/Swab,s type	1st	2nd	3th	4th	5th	6th	Mean±SD	*F	P-value
Cq/V	23.64	24.74	31.39	28.1	30	35.62	28.91±4.4[a]	10.75	0.0003
Cq/C	36.97	38.31	36.42	38.45	35.41	37.13	37.11±1.2[b]		
Cq/N	37.01	40.36	33.62	-ve	-ve	39	37.49±2.9[b]		
Cq/F	31.2	35	35.39	35.45	36.18	36	34.87±1.9[b]		

[a-b] Means in the same column differ significantly.

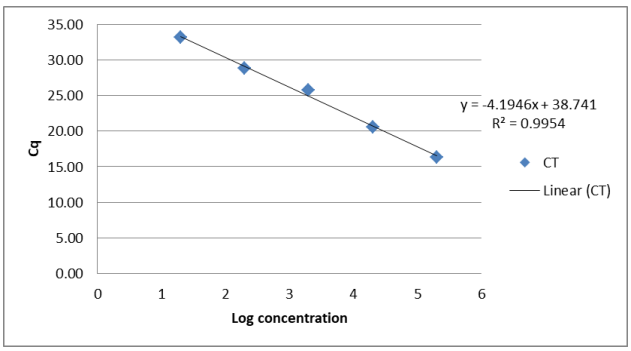

Fig. 2: The cycle threshold (Cq) values were determined by serially diluting a standard positive control, which contained the target gene, tenfold at concentrations of $2x10^5$, $2x10^4$, $3x10^3$, $2x10^2$, and 20 and 2 copies/µL, respectively. The initial number of target genes in the sample is inversely correlated with the Cq values. The Applied Biosystems Step One Plus instrument automatically determined the target gene's copy number. The reaction's efficacy was 88.15%. The linear regression formula was Y = -4.1946x + 38.741. Slope = -4.1946x; Y-intercept = 38.741; R2 = 0.995.

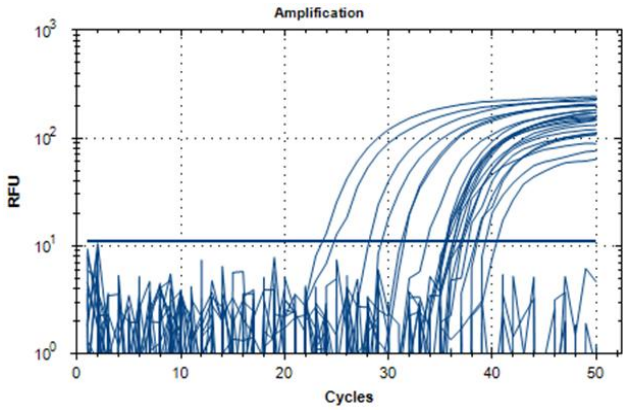

Fig. 3: Target gene amplification plot after a 10-fold serial dilution in the camel swabs. The fluorescent signal grows at each time point, measured in relative fluorescence units (RFU). Baseline was below (equal 10^1), with PCR cycles accumulating with undetected reporter fluorescent signal. When the fractional PCR cycle number (Ct) was 23 points above the threshold, real amplification began.

The MicroSEQ 500 technique is used for direct DNA sequencing (Fig. 4). Sequence data from camel vaginal swabs was submitted to GenBank as CAMSA16, accession number OQ824900. It is similar to the *Fusobacterium gastrosuis* strain.

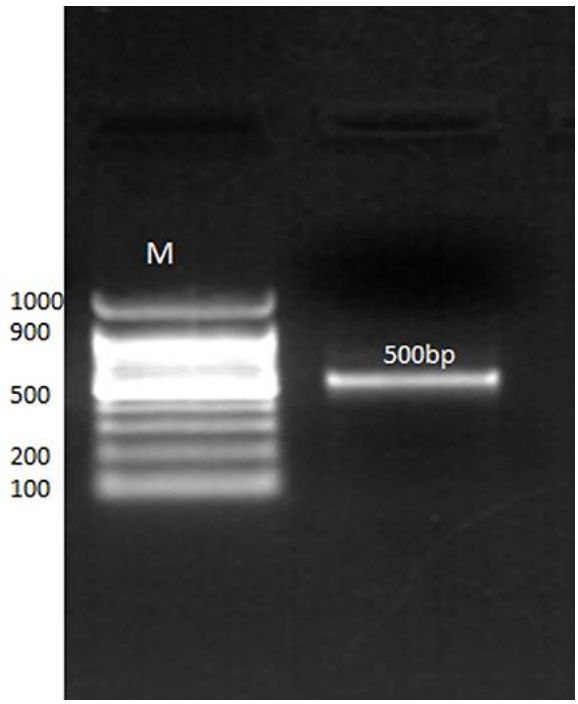

Fig. 4: Band of *Fusobacterum* spp using Microseq500. Ladder 100bp.

DISCUSSION

In this study, a high relative profusion of *Fusobacterium.* spp, in the vaginal, rectal, ocular, and nasal secretion of apparently healthy pregnant camels, which is nearly homologous to *F. gastrosuis*. *F. gastrosuis* is a novel pathogen with the potential to cause gastric ulceration (De Witte et al. 2018). Earlier studies have exposed existence of *F. gastrosuis* in the oral and nasal microbiota of pigs and dogs (De Witte et al. 2019), in the stomach of wild boars (Cortez Nunes et al. 2022) and in the stool of humans (De Paepe et al. 2018), indicating that *F. gastrosuis*, like *F. necrophorum and F. nucleatum*, can inhabit a wide variety of mammalian hosts (De Witte et al. 2018).

Fusobacterium gene with high concentration were detected in vaginal swabs compared with fecal, nasal and ocular swabs. The main uterine pathogens were found in vaginal samples from cattle (Jeon et al. 2017). In addition, in cows, a strong association between fecal microbial populations and vaginal-uterine microbial populations was confirmed (Jeon et al. 2017). Galvão et al. (2019) clarified that *Fusobacterium* has the strongest association with metritis.

Despite its role as the frontline to mechanically guard the genital system, the vagina is rich with microbial flora with diverse commensal bacteria and fungus with potential pathogenicity (Moreno et al. 2021). In addition, *Fusobacterium* has been identified in cervicovaginal adhesion cases of dromedaries (Ghoneim et al. 2021).

F. gastrosuis bacteria clearly induced cell death (Thomson et al. 2012). These cell death findings support a role for *F. gastrosuis* in the development of porcine gastric ulceration. However, it is still possible that *F. gastrosuis* is only transient in camels. Future research is needed to determine whether *F. gastrosuis* is a member of the uterine microbiota and/or whether it can cause genital pathologies in dromedaries. The main sources of uterine contamination are thought to be bacteria from the vagina, feces, or environment (Jeon et al. 2017). It would be fascinating to investigate the effect of *F. gastrosuis* on mucosal explants from non-porcine species (De Witte et al. 2018).

Fusobacterium spp. genome analysis revealed the presence of a broad range of virulence-linked genes, which have been related to cell death (Ang et al. 2014). Furthermore, some *Fusobacterium* spp. can adhere to and invade host cells without the assistance of other factors (De Witte et al. 2018). Fusobacteria can produce lactamase, which protects organisms from lactam antibiotics, as well as proteolytic enzymes, which promote regional vein invasion (Karstrup et al. 2017).

Risks associated Fusobacterium infection and other diseases in camels include environmental stress, immunodeficiency, mucus abrasion, unhygienic obstetrical care, or the presence of other supporting infections microbes (Hussain et al. 2016; Ali et al. 2017; Aqib et al. 2017). When cows are exposed to a comparable number of pathogens, prepartum heat stress disrupts the host immune utility and raises metritis risk (Morrall and Schmidt 2022). Healthy heifers and cows with uterine disease shared the vaginal microbiological league (Moreno et al. 2021). Within 20min of calving, cows have an established uterine microbiome that matches between metritic cows and healthy cows. In cows with metritis, the microbiome deviated two days postpartum in favor of a greater relative abundance of Bacteroidetes and Fusobacteria (Galvão et al. 2019). *Fusobacterium* was isolated from healthy and metritic cow uterine samples (Burfeind et al. 2014).

In conclusion, this study sheds light on a naturally occurring microbe in camels that can cause serious diseases under certain conditions. More research is needed to determine whether *F. gastrosuis* is a member of the uterine microbiota and/or whether it can cause genital pathologies in dromedaries. *F. gastrosuis* may also have zoonotic impending.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author Contributions

Sahar Allam: conceptualization, sampling, methodology; draft writing. Ahmed Ali: writing, reviewing, editing. Derar R. Derar: reviewing, editing. Sahar T.M. Tolba: methodology and reviewing.

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