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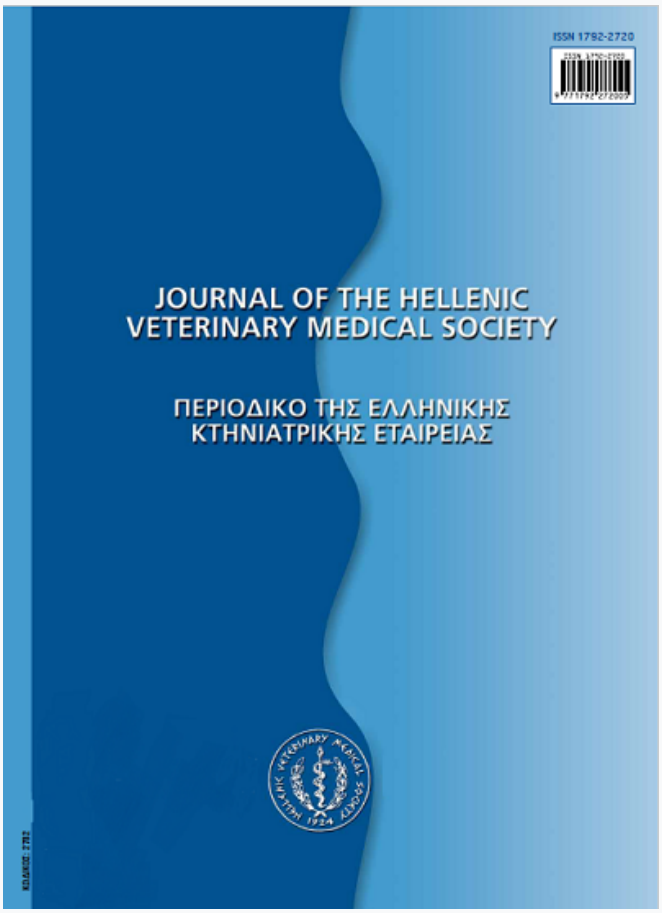

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Research article
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Comparison of the Vaginal Cytological and Microbiological Results in the Detection of Normal Microflora of Pregnant Ewes

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ABSTRACT. The aim of this study was to carry out a cytological and microbiological comparative investigation of vaginal microflora in pregnant ewes. The subjects for the study comprised of 39 healthy curly fleeced breed ewes (n=39), approximately 3 years old, at 2-4 months of pregnancy. Two vaginal samples were taken for cytological and microbiological examinations from each ewe in a sterile manner. Hemacolor® was used in cytological examination, while microbiological analysis were completed by conventional techniques. In cytological examination, slides were evaluated to detect lactobacilli, other bacteria, "clue cell" formation and presence of neutrophils. Microbiological investigation was carried out to detect possible pathogens. Cytological results compatible with bacterial vaginosis were obtained in 10 cases. Microbiologically, single type bacteria in 27 cases and more than one bacterium in 12 cases were isolated. The most common isolated pathogen was *Escherichia coli*. Comparing the cytological and microbiological results, 7 out of 27 cases were compatible with the bacterial vaginosis. In 3 cases of bacterial vaginosis non-pathogenic agents were revealed. In conclusion, it was proven that utilising the cytological examination provides more reliable results for detection of normal vaginal microflora of pregnant ewes.

***Key words*:** Vaginal microflora, microbiology, cytology, ewe.

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INTRODUCTION

The genital tract microflora in ewe contains many useful bacteria, which do not affect the reproductive performance (Ungerfeld and Silva, 2005; El-Arabi et al., 2013). These bacteria form sexual stimulation during estrus period in animals, and prevent the growth of the other pathogen bacteria (Shallali et al., 1987; Ungerfeld and Silva, 2005). Lactobacilli located in genital tract microflora lead to clearance of the pathogenic bacteria in the reproductive tract by releasing lactic acid, which maintains low vaginal pH (<4.5) (Shallali et al., 2001; O'Halan et al., 2013). However, these bacteria may be involved in the disease process along with some commensal bacteria when the appropriate conditions occur (Larsen and Monif, 2001). Reductions of vaginal lactobacilli result in an elevated vaginal pH, which is a feature of bacterial vaginosis (Li et al., 2012). Contamination of the female genital tract by fecal flora may enhance vaginal bacterial colonization, non-specific infections and sometimes abortions in pregnant animals (Shallali et al., 1987; Shallali et al., 2001). Opportunistic infections result in a variety of bacteria that worldwide are major causes of endometritis and low fertility (El-Arabi et al., 2013). When an infection forms in the genital tract, bacterial components lead to infertility by forming a direct negative impact on movement and life of the spermatozoa (Gorga et al., 2001).

Various microbiological studies have been carried out in order to determine the vaginal microflora in ewes (Shallali et al., 2001; El-Arabi et al., 2013), however, lack of studies concerning the local pathogenic effects of the bacteria upon vaginal microflora were confirmed. In some circumstances, bacterial vaginosis can occur in some clinical diseases characterised as homogenous vaginal discharge that has positive amine results, presence of "clue cells", higher pH values (>4.5) and normal clinical appearance. Cytological examination results of the discharge can be the only indicator for determining bacterial vaginosis (Demirezen, 2003). Therefore, in this study, we evaluated examination of vaginal bacteriological culture and vaginal smears (infiltration of inflammatory cells, 'clue cell' formation, presence of lactobacilli and other bacteria) as a diagnostic tool for distinguishing presence of normal microbial flora from bacterial vaginosis in pregnant ewes.

MATERIALS and METHODS

This study was evaluated and approved by the the local ethics committee of Uludag University, Bursa-Turkey (no:B.30.2.ULU.0.8Z.00.00/131). The subjects of the study were 39 healthy Curly-fleeced breed ewes (n=39), about 3 years old. They were housed in a semi-extensive barn, and their gestation periods ranged between 2 to 4 months.

All clinically healthy ewes (without any systemic disease, normal general examination results with no vulvar discharge or any signs of vaginal disease) were restrained, their tails held up, and vulvas cleaned with water and benzalkonium chloride. Using a sterile speculum, vaginal samples were collected with sterile swabs. Two vaginal smear samples were prepared for each ewe (totally 78 samples), one smear was used for vaginal cytology and the other was used for microbiological. The smear samples were stained with Hemacolor® (Merck, Germany), air dried and fixed with methanol. These samples were then examined under a light microscope (Olympus®, U-D03, Tokio, Japan).

Cytological evaluation was used to investigate bacterial vaginosis in the prepared smear samples. In cytological examination, slides were evaluated to detect lactobacilli, other bacteria except lactobacilli, "clue cell" formation as well as the presence of neutrophils. The presence of neutrophils was evaluated using a four category scoring system: "absence (-)", "mild (+)", "moderate (++)" and "intense (+++)"

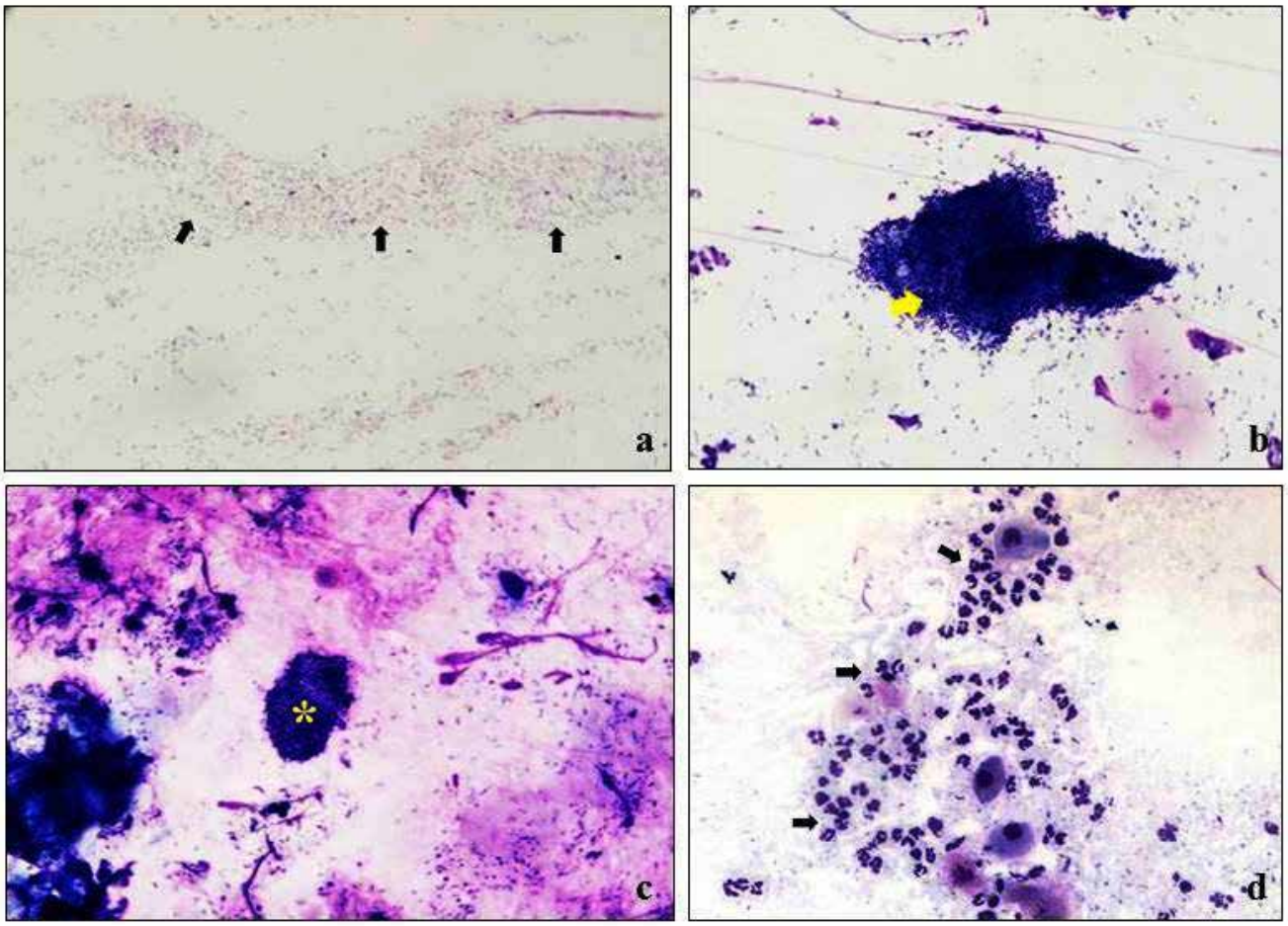
For microbiological analysis, all swab samples were incubated at 37°C for 24 hours in fluid thioglycollate (BBL; 221196). On the next day part of the initial culture was transferred in blood agar (BBL; 297876) and EMB agar (Eosin-Methylenblue-Lactose-Saccharose) (BBL; 221355) and incubated at 37°C for another 24 hours. According to colony morphology and Gram color features, identified colonies were quantitatively assessed. For the identification of the bacteria, their direct cultures were performed using BBL Crystal (Becton-Dickinson, Sparks, USA) Gram-Positive and Gram-Negative ID system kits and its computer program.

Table 1.Cytological and microbiological results of vaginal samples.

Case No	Lactobacilli	Other bacteria	Neutrophil leucocytes	Clue cells	Microbiological culture results
1	+	++	++	+	*Bacillus subtilis, Lactococcus* spp.
2	+++	+	++	-	*Escherichia coli, Bacillus subtilis*
3	+++	-	-	-	*Escherichia coli*
4	+++	-	-	-	*Bacillus subtilis, Lactococcus* spp.
5	+	-	+	-	*Bacillus subtilis*
6	++	-	-	-	*Escherichia coli*
7	+	+++	++	+	*Escherichia coli*
8	++	-	-	-	*Bacillus licheniformis*
9	+	+	-	+	*Escherichia coli, Bacillus pumilus*
10	-	-	-	-	*Escherichia coli*
11	++	-	-	-	*Escherichia coli*
12	++	-	-	-	*Escherichia coli*
13	+	+	++	-	*Bacillus subtilis*
14	-	+	-	-	*Escherichia coli, Streptococcus bovis II (Strep. group D)*
15	-	++	-	+	*Escherichia coli, Pantoea (Enterobacter) agglomerans*
16	+++		++	-	*Escherichia coli*
17	-	++	-	+	*Escherichia coli*
18	+	+	-	+	*Pantoea (Enterobacter) agglomerans, Bacillus licheniformis, Corynebacterium propinquum*
19	-	+++	-	+	*Escherichia coli*
20	+++	+	++	-	*Escherichia coli, Bacillus subtilis*
21	+	++	+	+	*Bacillus cereus, Bacillus subtilis, Enterococcus faecium*
22	-	-	+	-	*Escherichia coli*
23	-	+	+	-	*Pantoea (Enterobacter) agglomerans*
24	+	-	-	-	*Escherichia coli, Bacillus subtilis*
25	-	-	-	-	*Bacillus subtilis*
26	-	-	-	-	*Bacillus subtilis*
27	-	-	-	-	*Escherichia coli*
28	-	+	-	-	*Escherichia coli*
29	-	-	-	-	*Escherichia coli*
30	-	-	+++	-	*Pediococcus pentosaceus*
31	+++	+	-	-	*Escherichia coli*
32	-	+	-	-	*Bacillus cereus*
33	-	+++	+	+	*Bacillus subtilis*
34	-	-	-	-	*Bacillus licheniformis*
35	+++	-	-	-	*Escherichia coli*
36	+++	-	-	-	*Bacillus subtilis*
37	+	++	-	-	*Bacillus cereus, Pediococcus pentosaceus*
38	-	++	-	+	*Escherichia coli, Proteus spp.*
39	-	+	+	-	*Bacillus subtilis*

Absence: (-), mild: (+), moderate: (++), intense (+++)

Figure legend
Figure 1. Vaginal cytology smear, a: lactobacilli (black arrows), b: other bacteria (yellow arrow), c: clue cell formation (asterisk) and d: neutrophil leucocytes (arrows) (X400).



RESULTS

Microbiological isolations obtained from all 39 vaginal samples taken from the 39 ewes are listed in Table 1. In 12 of cases (30.8%), isolations included more than one bacteria species. In mix cultures, *Bacillus subtilis, Lactococcus* spp*., Escherichia coli, Bacillus pumilus, Streptococcus bovis II (Strep. Grup D), Pantotea (Enterobacter) agglomerans, Bacillus licheniformis, Corynebacterium propinquum, Enterococcus faecium, Bacillus cereus, Pediococcus pentosaceus* and *Proteus* spp. were isolated. *Escherichia coli* was the only identified pathogenic bacterium, with regards to the opportunistic pathogens, *Streptococcus bovis II (Strep. Grup D), Bacillus cereus, Enterococcus faecium* and *Proteus* spp. were identified (Table 1).

Microscopically, 6 of 39 preparations were considered not diagnostic due to low cellular and bacterial density. The presence of lactobacilli was observed in 12 of 33 samples, these samples results were evaluated as normal floral bacterium (Fig. 1a). Presence of a moderate neutrophil population in 3 of the above 12 examples was evaluated as a non-specific inflammatory reaction. In 10 of the examined samples, low numbers or absence of lactobacilli together with varying degree increase of the other bacteria (Fig. 1b) and bacteria filled epithelial cells (clue cells) (Fig. 1c) were observed. Four samples had also mild to moderate neutrophil infiltration (Fig. 1d). Based on these findings, diagnosis of bacterial vaginosis in 25.6% of samples was concluded (Table 1). This ratio was determined at 30.3% when inadequate samples were

not included. Samples was rejected due to low cellular and bacterial density. In the comparison of the cytological and microbiological findings, only 7 of the 23 samples containing pathogenic and opportunistic pathogenic bacteria were consistent with bacterial vaginosis. Bacterial vaginosis was determined based on the "clue cell" existence and reduction of lactobacilli with or without neutrophilic infiltrations. Bacterial vaginosis was not diagnosed in case of neutrophilic infiltration alone without clue cell existence and reduction of lactobacilli.

DISCUSSION

The reproductive effectiveness is very important in sheep, due to the limited number of lambs produced per ewe on an annual basis (El-Arabi et al., 2013). Particularly in pregnant ewes, vaginal microflora changes and bacterial vaginosis are crucial due to abortion risk (Shallali et al., 1987; Shallali et al., 2001). Bacterial microflora of the lower genital tract is not yet fully understood and it is an example of dynamic and complex microbial colonization (Larsen and Monif, 2001). Vaginal flora consists of many bacteria while some of the pathogenic ones could be the result of contamination or may be resident in a tolerable number (El-Arabi et al., 2013; Zaid, 2009). Zaid (2009). Vaginal microflora investigations in different reproductive periods acquired a 55.6% rate of bacterial growth in pregnant sheep. These results, *Enterobacter species*, *Escherichia coli* and *Lactobacilli* were the most isolated bacteria (Zaid, 2009). Swartz (2014) characterized vaginal microbiota of ewes and reported that *Aggregatibacter* spp. and *Streptobacillus* spp were the most abundant agents (Swartz et al., 2014). In this study, we planned to determinate normal microbial flora in pregnant ewes based on vaginal cytology and microbiology. Bacteria was observed in all vaginal cultures. *Escherichia coli* was the most commonly isolated bacterium encountered, however, its presence could be due to fecal contamination.

Bacterial vaginosis is the most common causes of vaginitis. In humans, the density of lactobacilli and other bacterial agents, presence of "clue cells" and neutrophils are the main findings of Papanicolaou-stained smears (Pap-smear) (Demirezen, 2003).. Lactobacilli are found predominantly in the normal bacterial flora and have a crucial function (Shallali et al., 1987). Colonised vaginal bacteria as a result of contamination can lead to bacterial vaginosis (Shallali et al., 1987; Larsen and Monif, 2001). In our study, it was observed that there were decreased lactobacilli population and increased other bacterial density together with the presence of "clue cells" in 10 cases. It has been reported that the presence of neutrophils cannot be considered as evidence of bacterial vaginosis (Demirezen, 2003) because they can be seen in vaginal cytological examinations during pregnancy (Doğaneli et al., 1979). Considering this, diagnosis of bacterial vaginosis was established in 10 cases. The culture results in three of these cases revealed non-pathogenic bacteria.

Vaginal microbial culture was used in the past as a primary diagnosis of bacterial vaginosis, but it has lost its diagnostic importance because of the wide variety of the bacteria identified, as well as the false positive results of non-pathogenic bacteria in the normal flora (Money, 2005). Therefore, more reliable results can be attributed to both clinical and cytological examinations as well as microbiological isolations (Demirezen, 2003). Unlike routine techniques, this study presents bacterial vaginosis with Hemacolor® stained cytology smears and the effectiveness of cytological examinations in the determination of the normal microbial flora. When compared with cytological examinations (10 accurately positive results), microbiological examinations demonstrated 16 false positive and 3 false negative cases.

In conclusion, comparative results of cytological and microbiological investigation can lead to more reliable data concerning the vaginal flora; thus, these should be performed together in order to determinate the normal microbial flora of ewes.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest of any author of this article.

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