RESEARCH ARTICLE Indian Journal of Animal Research, Volume 55 Issue 8: 924-929 (August 2021)

Investigation of the Effect of Intravaginal Device-type and Treatment-duration on Vaginal Features in Ewes and Determination of Antibiotic Susceptibility

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10.18805/ijar.B-1244

ABSTRACT

Background: Seasonal anestrus, which is controlled by the photoperiod in ewes, limits the ability to the fertility of ewe every time. Therefore, using hormones by vaginal devices is the most effective way of increasing fertility in order to form oestrus during the anestrus period and obtain a high rate of pregnancy. But, vaginal devices may cause a change in the profile and number of bacteria in the uterus and vagina, and thus create an environment suitable for infection. For this reason, identification of bacteria that are the source of infection can be achieved by performing antibiotic susceptibility tests and interpreting them correctly. The purpose of this study was to characterize the effect of intravaginal device-type and treatment-duration on vaginal bacteria count and type and determine the antibiotic susceptibility of vaginal bacterias in ewes after a program of oestrus synchronization.

Methods: A total of 90 clinically healthy Kivircik ewes were selected. In order to provide oestrus synchronization during anestrus season (June), CIDR was inserted intravaginally to 45 ewes, the other 45 ewes were progestogen sponges inserted. In both groups remained inserted for 5, 9 and 13 days. While vaginal devices were withdrawal both groups ewes were exposed to intramuscular 350 IU PMSG injection. The mucous samples were collected from all ewes 0, 5, 9 and 13 days at time the IVS or CIDR withdrawal. In samples, bacterial concentration, bacterial identification and antibiotic susceptibility were determined.

Result: The changes in the vaginal flora caused by the vaginal devices favored the development of opportunistic microbiota that could potentially be pathogenic. In the study, after vaginal device withdrawal, the bacteria species most frequently observed in both IVS and CIDR groups were *E. coli*. According to the results of this study, the first choice of antibiotics for vaginal infections caused by G- agents in ewes should be any of AXC, CEP and CTZ compared to other antibiotics.

Key words: Antibiotics, Bacteria number, Ewe, Intravaginal devices, Vaginal microbiota.

INTRODUCTION

Increasing the fertility in ewes and does may be provided by regular and on time matings in the herd. Seasonal anestrus, which is controlled by the photoperiod in ewes, limits the ability to achieve the global target of three lambs per ewe every 2 years. Therefore, using hormones by vaginal devices is the most effective way of increasing fertility in order to form oestrus during the anestrus period and obtain a high rate of pregnancy (Kusina *et al*., 2000; Xiao *et al.,* 2013; Saxena *et al*., 2015). The most common traditional method is progestogen impregnated sponges (FGA-fluorogestone acetate), (MAP-medroxyprogesterone acetate) and CIDR, which is progesterone containing hard medical silicone (Dellal and Cedden, 2002; Emsen and Kosum, 2009; Rowe *et al*., 2009; Souza *et al*., 2011; Machiya *et al.,* 2012; Zohara *et al*., 2014).

Normal vaginal flora members in ewes prevent overgrowth of pathogen microorganisms and increase the resistance of the body against disease formation by providing ecological balance in that region (Levinson and Javetz, 1996; Zhao *et al.,* 2014). As well as, normal vaginal flora is known to increase sexual attractiveness (Ungerfeld and Silva, 2005). But, when the environment is suitable, the opportunistic bacteria among microflora members may take a role in the development of infections in genital organs like vaginitis (Root, 2006; Martins *et al*., 2009a). *Staphylococcus aureus* (*S. aureus*), is the most common microorganism that is isolated from vaginitis of ewes. *Escherichia coli* (*E. coli*), is known as an opportunistic secondary agent for bacterial vaginitis (Sargison *et al*., 2007). Vaginal devices have been accused of local inflammation that promotes the growth of opportunistic bacteria as a predisposing factor that translocate external microorganisms into the reproductive system (Martins *et al*., 2009; Oliveira *et al*., 2013). Vaginal devices may cause a change in the profile and number of

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How to cite this article: Altıncekic, S.O., Cilo, B.D., Duru, S. and Koyuncu, M. (2021). Investigation of the Effect of Intravaginal Device-type and Treatment-duration on Vaginal Features in Ewes and Determination of Antibiotic Susceptibility. Indian Journal of Animal Research. 55(8): 924-929. DOI: 10.18805/ijar.B-1244.

Submitted: 11-01-2020 **Accepted:** 24-04-2020 **Online:** 22-08-2020

bacteria in the uterus and vagina and thus create an environment suitable for infection (Padula and Macmillan, 2006). The vaginal devices cause both mechanic irritation to the vagina and the progesterone hormone within their structure reduce lymphocyte reproduction and PGF2α production leading to susceptibility to infections (Manes *et al*., 2010).

Antibiotic resistance of pathogens is a problem in the treatment of bacterial infections and reproductive losses occur in animals due to treatment failures (Bengtsson and Greko, 2014). For this reason, identification of bacteria that are the source of infection can be achieved by performing susceptibility tests and interpreting them correctly.

The purpose of this study was to i) characterize the effect of intravaginal device-type and treatment-duration on vaginal bacteria count and type ii) determine the antibiotic susceptibility of vaginal bacterias in Kivircik ewes after a program of oestrus synchronization.

MATERIALS AND METHODS

Location and experimental conditions

This study was conducted at the Application and Research Farm of the Agricultural Faculty, Bursa Uludag University, a Province in the northwestern region of Turkey, during June 2017. The farm was located at the humid lowland tropics at an altitude of 100 m above sea level and at longitude 29°E and latitude 40°N. The region has average minimum and maximum temperatures of 3.2°C and 30.7°C, respectively. The annual rainfall of the region was 620.8 mm.

Animal management

A total of 90 clinically healthy Kivircik ewes of 2~3 years old, 50~55 kg body weight and have birth at least once were used for this study. In order to provide oestrus synchronization during anestrus season (June), CIDR was inserted intravaginally to 45 heads of ewes which were randomly chosen from the herd. Each CIDR contained 0.3 g progesterone (Eazi-Breed, Pfizer). Other 45 heads ewes were syncronised using 20 mg FGA (fluorogestone acetate) progestogen sponges inserted intravaginally (Chronogest, grey sponges, Intervet). In both groups remained inserted for 5, 9 and 13 days. The day of insertion was noted as day 0 in all the groups. While vaginal devices were withdrawal on the 5th, 9th and 13th days, both of groups ewes were exposed to intramuscular 350 IU PMSG injection. Research on animals was conducted in accordance with Bursa Uludag University Experimental Animals Ethics Committee Instructions under the report with reference number: 2015/ 12-05. The ewes were maintained on natural grazing along with concentrate mixture (wheat 57,5%, sunflower oilcake 25%, corn 15%, limestone 1.3%, salt 1% and vitamin-mineral premix 0.2%) during the experimental period. Water was supplied *ad libitum*.

Collection of the vaginal mucous samples

The mucous samples were collected from the posterior vaginal region using a sterile cotton swab. Before the IVS or CIDR insertion, mucous samples were collected from all ewes (n=90, D0). Other samples both of groups ewes were collected on the 5th (n=30), 9th (n=30) and 13th (n=30) days at time the IVS or CIDR withdrawal. The samples were maintained at 4°C until the microbiological tests were performed.

Bacterial count and identification

The samples were transferred to the laboratory in transport medium (Stuart's medium, Budapest, Hungary), total aerobic bacteria were counted as described by Suárez, *et al.* (2006). Briefly, the swabs were vigorously vortexed (maximum speed) in 1 mL sterile PBS, pH 7.4, for 1 min in order to suspend the bacteria. The resultant suspension was serially diluted and the bacteria were counted on 5% sheep blood agar (BD, BBL, Germany) plates and incubated for 48 h at 37°C. If bacterial growth was apparent after 24 or 48 h of incubation, smears were made, Gram-stained and examined by microscopy. Bacteria were grown subcultured on blood (BD, BBL, Germany) and Eosin Methylene Blue Agar (BD, BBL, Germany) to obtain pure cultures. Bacterial identification was performed by Gram staining, biochemical tests and using automated BD Phoenix 100 (Becton Dickinson, USA) bacteria identification system with BD Phoenix^{TM} PMIC/ID-87 and Phoenix^{TM} NMIC/ID-400 panels.

Antibiotic susceptibiliy test

Antibiotic susceptibiliy tests were conducted using BD Phoenix 100. All antibiotic resistance values were determined based on current European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimal inhibitor concentration (MIC) values. *E.coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as the quality control organisms.

Statistical analysis

The data from the bacterial concentration (low and high concentration) assays and the test for grouping the Gram-positive bacteria (G^+) and Gram-negative bacteria (G^-) using the samples taken at different times relative to were compared using the chi-square (χ^2) test (Minitab, 2014).

RESULTS AND DISCUSSION

Bacterial count

Prevalence of bacteria isolated from ewe's vaginas at different days in Table 1 presents. After insertion of the vaginal devices for oestrus synchronization to the ewes, the number of CFUs increased significantly compared to D0. In CIDR group, the number of CFUs increased until day 5th and 9th days and not changed up to the 13th day. According to the result of the chi-square analysis, the effect of treatment-duration on the number of CFUs was significant in CIDR group (P<0.05). The number of bacteria, in ewes' vagina subjected to synchronized with IVS, continued to increase from D0 to D13. However, in IVS group, the difference between 5th, 9th and 13th days was not significant the number of CFUs. But, the number of CFUs, possibly due to longer stay duration of sponge, was highest on the 13th day (93.3%). These changes in the CFUs may be attributed to the physical effects of the intravaginal sponge and to the constant absorption and retention of vaginal secretions by the intravaginal sponge, which establishes an environment that favors bacterial growth. The present study results showed that the use of intravaginal devices increased the bacterial number to them despite being stayed in the vagina for different durations. Manes *et al.* (2013) stated no significant difference between devices' in vagina stay durations in terms of the number of vaginal bacteria. Al-Hamedawi *et al.* (2003) stated some changes in the number of vaginal bacteria as a result of the physical actions exerted by the devices that retained and absorbed vaginal secretions throughout their stay in the vagina. Vasconcelos *et al.* (2016), reported that vaginitis six days after the intravaginal devices were inserted, with typical clinical signs such as mucopurulent discharge, erythemas and increased local sensitivity, besides a considerable increase in bacterial count, independently of the type of device and the presence of progesterone. According to Vasconcelos *et al.* (2016), the agents of the vaginitis were coliforms, which were replaced by the original microbiota 48 hours after implant was removed. However, researchers (Suarez *et al.,* 2006; Yesilmen *et al.,* 2008; Oliveira *et al.,* 2013) have reported that CFUs number increased with the device's insertion but decreased after the device's withdrawal. Martins *et al.* (2010) reported that the reduction in CFU/ml resulted from both the withdrawal of the irritant material from the body and the rise of the local immune response during the oestrus. Amin (1996) determined that the number of bacteria in the vagina returned to the previous levels two days after the withdrawal of the vaginal devices. Even some researchers, concluded that not affect the reproductive parameters of the duration that the intravaginal devices stayed in the ewes. Additionally, they reported that the occurrence of oestrus, interval to the onset of oestrus and pregnancy rate were similar between regardless of duration that the intravaginal devices stayed in the ewes (Ungerfeld and Rubianes, 2002; Suárez *et al.,* 2006; Manes *et al.,* 2010). In the other hand, Martins *et al.* (2010) recommend that the use of human vaginal tampons impregnated with progesterone instead of IVS and CIDR. Because, according to them, human vaginal tampons are affected highly hygienic, practical and effective as a low-cost alternative for oestrus synchronization and artificial insemination in ewes. Even if we did not encounter vaginitis in this study, when using this technique under field conditions, the adoption of stringent hygienic procedures particularly during vaginal device insertion should be considered.

Bacterial identification

The results of this study revealed that in both IVS and CIDR groups, the predominant bacterial flora before intravaginal device insertion (D0), were G^+ and the genus most frequently isolated was *Staphylococcus spp. Staphylococcus spp.* are members of the normal vaginal microbiota of ewes, it is necessary to prevent the growth of opportunistic microorganisms such as *E. coli* (Oliveira *et al.,* 2013). Martins *et al.* (2009b) confirmed the presence of *Staphylococcus* isolates in the vaginal microflora of clinically healthy ewes. Another type of bacteria, predominant in the normal vaginal flora of the ewes, was the *E. coli*, G^- bacteria (Table 2). In agreement with the findings of several researchers in ewes, *Staphylococcus spp*. and *E. coli* were the most common isolates in the vaginal flora of the ewes and does (Martins *et al.,* 2010; Oliveira *et al.* 2013; Penna *et al.,* 2013; Vasconcelos *et al.,* 2016; Mohammed *et al.,* 2017). Unlike these findings, Swartz *et al*. (2014) identified that *Aggregatibacter spp.* and *Streptobacillus spp.* types as the most common species in vagina of the ewes. Manes *et al.* (2013) reported that G^+ bacteria as the dominant colony before insertion of the vaginal devices and *Bacillus spp.* as the most commonly observed species. The bacteria colony was predominantly G^- after on the 6th and 11th days of the insertion of the vaginal devices and that the most common isolated type of bacteria was *Arcanobacterium pyogene* (Manes *et al.* 2013). Sawyer (1977) and Manes *et al.* (2010) isolated the *Bacillus spp., Corynebacterium spp., Escherichia spp., Staphylococcus spp.* and *Streptococcus spp.* species frequently in the ewes. Mulu *et al*. (2015) identified that the most frequent isolates from ewes' vagina were *E.coli, Pseudomonas spp.* and *S.aureus.* Donders *et al*. (2002) stated *S. aureus* was the most common microorganism isolated from ewes with vaginitis.

In this study, when the vaginal devices were withdrawn, regardless of the stay duration of the devices in the vagina, we noticed that bacteria of the *Enterobacteriaceae* family were prevalent. This finding is in agreement with those of Suarez *et al.* (2006) and Martins *et al.* (2009a) have been attributed to the local inflammatory effect of the device promoting the growth of opportunistic bacteria. Similarly,

Table 1: Number of bacteria isolated at different days from ewe's vaginas.

	IVS*				CIDR*			
	Low CFU		High CFU		Low CFU		High CFU	
	N	%	N	%	N	%	N	%
D0	40	88,9	5	11,1[a]	43	95,6	2	4,4[a]
D5	5	33,3	10	66,7[b]	10	66,7	5	33,3[b]
D9	3	20,0	12	80,0[b]	3	20,0	12	80,0[c]
D13	1	6,7	14	93,3[b]	3	20,0	12	80,0[c]

CFU, colony-forming unit;D0, before from IVS or CIDR insertion;

D5, 5 day after IVS or CIDR withdrawal; D9, 9 day after IVS or CIDR withdrawal;

D13, 13 day after IVS or CIDR withdrawal.

some researchers reported a significant increase particularly in the number of G^- *Enterobacteriaceae* in the vaginal flora composition of the ruminants due to vaginal device insertion (Manes *et al*., 2010; Gatti *et al*., 2011). Sargison *et al.* (2007) defined *E. coli* as an opportunistic agent causative to bacterial vaginitis. *E. coli* has been reported as an opportunistic agent of bacterial vaginitis, not only in ewes (Martins *et al.,* 2009a), but also in other ruminants (Padula and Macmillan, 2006; Oliveira *et al.,* 2013). Furthermore, Manes *et al.* (2010) and Oliveira *et al.* (2013) reported that intravaginal sponges employed in oestrus synchronization protocols cause changes in the vaginal microbiota of ewes and goats for a short period but after removing the sponges, there was a rapid re-establishment of the normal microbiota. In this study, results demonstrated that the use of intravaginal devices (IVS or CIDR) for synchronizing oestrus, induced important changes in the type of bacterial in ewes' vaginal flora.

Antibiotic susceptibility

Table 3 presents, percentages of various antibiotic-susceptibility of bacteria isolated from ewes' vaginas. Vaginal device insertion have an important role in contamination of the vagina with fecal bacteria. When description for the bacteria with detected significant reproduction (10^5 CFU/ml) was made in the study, the fecal species such as *E. coli* (78) being in the first place and *Proteus mirabilis* (2), *Pseudomonas aeruginosa* (1) were isolated. *P. aeroginosa* ve *P. mirabilis* strains were found to be sensitive to all tested antibiotics in this study including healthy animals without previous history of antibiotic use. *E. coli* strains were detected to resistant to AMP, CIP, PIP and TMP/SXT.

Table 2: Bacterial colony type isolated at different days from ewe's vaginas.

	Gram-positive bacteria	D0	D5	D9	D13	$\Sigma\chi^2$
	Staphylococcus spp.	46.7% (21)	-	-	13.3% (2)	
	Pediococcus pentosaceus	-	-	-	-	
	Bacillus spp.	8.9% (4)	-	-	-	
	Total of gram-positive bacteria	**55.6% (25)**	**0**	**0**	**13.3% (2)**	
IVS	**Gram-negative bacteria**					
	Escherichia coli	44.4% (20)	73.4% (11)	86.7% (13)	86.7% (13)	
	Pseudomonas aeruginosa	-	26.6% (4)	13.3% (2)	-	
	Cardiobacterum hominis	-	-	-	-	
	Neisseria animaloris	-	-	-	-	
	Proteus mirabilis	-	-	-	-	
	Total of gram-negative bacteria	**44.4% (20)**	**100% (15)**	**100% (15)**	**86.7% (13)**	
	χ^2	0.02	17.9	17.9	10.2	**46.1**
	Gram-positive bacteria	**D0**	**D5**	**D9**	**D13**	
	Staphylococcus spp.	64.4% (29)	-	-		
	Pediococcus pentosaceus	-	-	-	6.65% (1)	
	Bacillus spp.	4.5% (2)	-	13.3% (2)	6.65% (1)	
	Total of gram-positive bacteria	**68.9% (31)**	**0**	**13.3% (2)**	**13.3% (2)**	
CIDR	**Gram-negative bacteria**					
	Escherichia coli	31.1% (14)	73.4% (11)	80% (12)	86.7% (13)	
	Pseudomonas aeruginosa	-	-		-	
	Cardiobacterum hominis		13.3% (2)	-	-	
	Neisseria animaloris	-	-	6.65% (1)	-	
	Proteus mirabilis		13.3% (2)	-	-	
	Total of gram-negative bacteria	**31.1% (14)**	**100% (15)**	**86.7% (13)**	**86.7% (13)**	
	χ^2	0.2	28.5	18.1	18.1	**65.0**

D0, before from IVS or CIDR insertion; D5, 5 day after IVS or CIDR withdrawal;
D9, 9 day after IVS or CIDR withdrawal; D13, 13 day after IVS or CIDR withdrawal.

Table 3: Antibiotic susceptibility of strains of bacteria isolated from the vagina of ewes.

Bacteri type	AMP	AXC	GEN	AMC	CEP	CTZ	CEF	CIP	PIP	TMP/SXT
Escherichia coli (n=78)	71.80% (56)	100% (78)	100% (78)	100% (78)	100% (78)	100% (78)	100% (78)	78.20% (61)	71.80% (56)	76.90% (60)
Pseudomonas aeruginosa (n=1)	-	100%	-	-	100%	100%	-	100%	100%	-
Proteus mirabilis (n=2)	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

AMP(ampicillin); AXC(amoxicilin); GEN(gentamicin); AMC(amikacin); CEP(cefepime); CTZ(ceftazidime); CEF(cefuroxime); CIP(ciprofloxacin); PIP(piperacillin); TMP/SXT(trimethoprim-sulfamethoxazole)

In the present study, all strains isolated were susceptibility to at least one tested antibiotic. Although the number of strains isolated is small *P. aeruginosa* and *P. mirabilis* strains were susceptible to all antibiotics tested. However, AMP, CIP, PIP and TMP/SXT antibiotics resistance were found for *E. coli* strains. AXC, CEP and CTZ were the most effective drugs to treat infections by *G*$^-$ bacteria.

There is a paucity of studies regarding antibiotic susceptibility of bacteria isolated from the vagina of ewes and it may vary not only due to the primary incriminating factor but also with the region where the study was conducted (Martins *et al.,* 2009). Aziz *et al.* (2017) reported that cephalosporins such as cefamandole and ceftriaxone showed very good activity, but improper use of cephalosporins lead to resistance development, which might negatively affect animal and human health. Penna *et al.* (2013) reported that the strains isolated from ewes vagina were only sensitive to tetracycline (TET) and GEN. While Suárez *et al.* (2006) recommended using CEP and GEN to prevent the increase in the vaginal bacteria, Manes *et al.* (2013) suggested the use of antibiotic types such as AXC, AMP and streptomycin. Martin *et al.* (2009) identified antibiotic sensitivity of normal vaginal flora members in healthy animals with no prior antimicrobial treatment history as PEN 60%, AMP 73,4%, AXC 80% and GEN 86.7%. Along with all strains isolated from the vagina of healthy ewes, the most effective antibiotic against *Staphylococcus spp.* was CIP. Mulu *et al.* (2015) reported CIP sensitivity of *E. coli, Pseudomonas spp.* and *S. aureus* bacteria to be 80%, 67% and 100% respectively. The GEN sensitivities to these strains were 73,3%, 67% and 100% respectively. Mohammed *et al.* (2017) reported benefit of CIP+TMP/SXT application simultaneously with vaginal sponge to reduce the undesired effects caused by the sponge. Oliveira *et al.* (2013) stated that most of the bacteria showed resistance to antibiotic types PEN and TET which were the most commonly used ones by breeders. These antibiotics were not suitable to be included in the vaginal devices. In many studies, GEN and CIP had been stated as the most effective antibiotics for the species isolated from vagina due to their wide range of effect spectrum. In line with this study, the species isolated from the vagina could show different resistance profiles in many studies including healthy animals without a history of antibiotic use.

CONCLUSION

The results of this study demonstrated that changes in the prevalent bacterial isolate and the number of CFUs in the vaginal flora of ewes subjected to a short-mid or long term protocol of oestrus synchronization using IVS or CIDR occurred. The changes in the vaginal flora caused by the vaginal devices favored the development of opportunistic microbiota that could potentially be pathogenic. In our study, after vaginal device withdrawal, the bacteria species most frequently observed in both IVS and CIDR groups were *E.coli.* In conclusion, it was concluded that intravaginal devices do change microflora in the vagina but may not cause vaginitis. For the oestrus synchronization in ewes, obeying hygiene rules during vaginal device insertions will prevent fecal bacteria from entering the vagina and this will provide benefits in terms of minimizing contamination.

Increased use of antibiotics induces the development of resistance. Therefore, understanding the type of bacteria colonizing in vagina and determination of their antibiotic susceptibility will facilitate the management of genital infections in ewes. According to the results of this study, the first choice of antibiotics for vaginal infections caused by G- agents in ewes should be any of AXC, CEP and CTZ compared to other antibiotics.

ACKNOWLEDGEMENT

This study was financially supported by the the Uludag Universiy project (No. 2016/4).

Conflicts of interest

None of the authors have any conflict of interest to declare.

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