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RESEARCH ARTICLE

Use of Immunoperoxidase Technique in Smears Prepared from Vaginal Secretions in Early Diagnosis of Listerial Abortions in Cattle [1]

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Summary

Listeriosis is a sporadic disease of ruminants that causes meningoencephalitis, septicemia and abortion. In this study, the usefulness of immunoperoxidase technique was investigated in early diagnosis of cattle listerial abortions. For this purpose, 96 smears prepared from vaginal swab samples that were collected from aborting cattle were stained for *L. monocytogenes* by immunoperoxidase technique. Presence of the agent in vaginal swab samples were investigated by bacteriological culture technique and the results were compared to that of immunoperoxidase technique. A total of 7 samples, 4 out 5 bacteriological culture positive smear samples and 3 out of 91 bacteriological culture negative smear samples, were detected to be positive by immunoperoxidase technique. Compared to bacteriological culture technique, sensitivity and specificity of immunoperoxidase technique was calculated as 80% and 96.7%, respectively. In conclusion, immunoperoxidase technique in smears prepared from vaginal swabs can be used in early diagnosis of listerial abortions since it can give results the same day samples collected, however the technique must be supported by bacteriological culture technique which is performed for bacterial isolation and identification of the agent.

Keywords: *Listeriosis, Abortion, Cattle, Immunoperoxidase*

Sığır Listerial Abortlarının Ön Tanısında Vajinal Akıntılardan Hazırlanan Sürme Preparatlarda İmmunoperoksidaz Yönteminin Kullanılması

Özet

Listeriozis; ruminantlarda meningoensefalitis, septisemi ve abortusa neden olan, genellikle sporadik seyirli bir enfeksiyondur. Bu çalışmada sığır listerial abortlarında erken ön tanı amacıyla immunoperoksidaz boyama yönteminin kullanılabilirliği araştırıldı. Bu amaçla atık yapan 96 sığırdan alınan vajinal sıvap örneklerinden hazırlanan sürme preparatlar *L. monocytogenes* antikoru ile immunoperoksidaz boyama yöntemi kullanılarak boyandı. Bakteriyolojik kültür metodu ile vajinal sıvap örneklerinde etkenin varlığı araştırılarak immunoperoksidaz yönteminin sonuçları ile karşılaştırıldı. Bakteriyolojik kültürü pozitif olan 5 adet vajinal sıvap örneğinden hazırlanan sürme preparatların 4'ünde ve negatif sonuçlanan 91 adet örneğin 3'ünde olmak üzere toplam 7 örnekte immunoperoksidaz ile pozitif reaksiyon saptandı. İmmunoperoksidaz metodu kültür metodu ile karşılaştırıldığında duyarlılığı ve özgüllüğü sırasıyla %80 ve %96.7 olarak saptandı. Sonuç olarak vajinal akıntılardan hazırlanan sürme preparatlarda immunoperoksidaz tekniğinin aynı gün içerisinde sonuç alınması nedeni ile listerial abortların ön tanısı amacıyla kullanılabileceği ancak bu yöntemin etken izolasyon ve identifikasyonuna yönelik olarak yürütülen bakteriyolojik kültür yöntemi ile desteklenmesi gerektiği sonucuna varıldı.

Anahtar sözcükler: *Listeriozis, Abort, Sığır, İmmunoperoksidaz*

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INTRODUCTION

Listeriosis is a sporadic, occasionally seen as an enzootic disease of ruminants that causes meningoencephalitis, septicemia and abortion [1]. It can manifest some other prenatal diseases in all ruminants, resulting important economic losses. Being a zoonosis, it might cause drastic problems in humans. Among the known *Listeria* species, namely; *L. monocytogenes*, *L. ivanovii, L. innocula, L. seeligeri, L. welshimeri, L. grayi,* and *L. murrayi*, only *L. monocytogenes* can cause clinical listeriosis [2]. However, there are few studies reporting that *L. ivanovii* might be an etiological agent in some cases [3,4].

Listeria species are Gram positive, non-capsulated, non-spore forming, aerobic to facultative anaerobic, short round edged, rod-shaped bacteria measuring 0.5x0.5x2.0 µm. The bacteria can be observed as single, chained, V- shaped or grouped in parallel forms under microscope [5]. They grow best at 30-37°C, and can replicate well in silage that are not prepared properly. Moreover, the bacteria can survive at 1-45°C, which gives them a wider range of survival chance [6]. *Listeria* species are very common in environment and can be isolated from the infected animals of several species [7]. Soil is a good reservoir for the agent. Since the agent is found in the micro flora of digestive tract of healthy and diseased animals, feces is the natural reservoir [8]. In addition, infected animals spread the bacteria to the environment via milk, urine, aborted fetus, and uteral and vaginal secretions. Contaminated silage and feedings for ruminants, and raw meat, fish, and vegetables, non-pasteurized milk and milk products for humans are the major sources of the agent [9,10].

Definitive diagnosis of listeriosis is made by the agent's isolation and identification by bacteriological culture techniques [11]. Serological means such as agglutination test and ELISA, and PCR as a molecular test are also in use for determination of the bacteria [12,13]. However, all these techniques possess some drawbacks. A simple, reliable and cheap detection technique is therefore still needed. Immunoperoxidase technique was previously shown to be successful in diagnosis of listerial infections [14,15]. However, the technique has not been tried before on fetal tissues or vaginal smears in cattle with Listerial abortion.

In this study, immunoperoxidase technique, as a potentially early detection technique for *L. monocytogenes* was investigated in smears prepared from vaginal swab samples of cattle that had abortion. The technique was also compared to the standard bacteriological culture technique for its sensitivity and specificity.

MATERIAL and METHODS

Material

Vaginal swab samples were collected between February-May 2008 in Kars province from 96 cattle that had abortion in their history. Three vaginal swab samples were obtained from each animal, and placed in Tryptose Broth containing screw capped-tubes, and then sent to Kafkas University, Faculty of Veterinary Medicine, and Department of Microbiology under cold-chain in the same day.

Methods

Microbiological Investigations: Bacteriological isolation of *Listeria* from vaginal swab samples was performed according to the technique suggested by FDA [16]. Vaginal swab samples in Trpytose Broth were transferred to selective supplement (Oxoid, SR141) containing Listeria Enrichment Broth (Oxoid, CM862) and incubated at 30°C for 48 h. Then, they were inoculated onto selective supplement (Oxoid, SR140 containing Listeria Selective Agar (LSA) (Oxoid CM856), and incubated at 30°C for 24 h. Smooth, round, grey black centered colonies were inoculated onto Tryptic Soy Agar-Yeast Extract (TSA-YE), and incubated at 30°C for 24 h. Identification of the bacteria as *L. monocytogenes* were succeeded by Gram staining, catalase and oxidase tests, motility, umbrella-like growth in semi-solid agar, CAMP test, growth in D-mannitol, D-xylose, L-rhamnose, α-methyl-D-mannoside and D-glucose. Negative and positive controls for identification were also performed.

Immunoperoxidase Technique: Smears prepared from total of 96 vaginal swab samples were used in immunoperoxidase staining. The technique was performed broadly as follows; all smears were fixed in alcohol and then dehydrated. Following blocking the endogenous peroxidase activity by treating the smears with 3% H_2O_2 for 30 min, antigen retrieval was performed by microwaving in citrate solution (pH 6.0) for 25 min. Non-specific antibody binding was blocked by a blocking solution for 30 min. Smear samples were then incubated for 60 min at room temperature by anti-Listeria polyclonal antibody (LSBio, LS C122061) diluted 1:1000. Biotinylated secondary antibody and Streptavidin peroxidase complex (Zymed Histostain Plus Bulk Kit, Cat No: 85-9043) were consecutively applied for 30 min each with three times phosphate buffer saline, pH 7.4 (PBS) washings between. With the use of substrate, 3,3-diaminobenzidine H_2O_2, antibody binding was visualized. Background staining was provided by Harris hematoxylin. Finally, immunmount was applied and smears were observed under a microscope. Negative controls, at which primer antibody was not applied, and positive controls of smears prepared from *L. monocytogenes* standard strains and smears prepared from 5 isolates of swab samples that were determined to be *L. monocytoges* positive by microbiological means were also performed.

Quantitative Assessment of Immunoperoxidase Technique: Sensitivity and specificity of the immunoperoxidase technique was evaluated as described by Moore et al.[17].

RESULTS

In bacteriological culture technique, colonies grown in LSA were characterized as *Listeria* spp. based on Gram positivity, non-capsulation, non-spore forming, motility at 20-25°C, and umbrella-shaped growth at 20°C with short rod-like morphology. By microbiological investigation of 96 vaginal swab samples, 5 (5.2%) were determined to be positive for Listeria *(Table 1)*. All *Listeria* spp. were also identified as *L. monocytogenes* by β-hemolysis, positive reactivity with *Staphylococcus aureus* at CAMP test, glucose, methyl-D-mannoside and rhamnose positivity and xylose, mannitol and nitrat reduction negativity.

The results of the immunoperoxidase technique were summarized in *Table 1*. Total of 7 samples were detected positive for listeria by immunoperoxidase technique. Out of 5 samples that were positive by bacteriological culture technique 4 were determined to be positive, and out of 91 samples that were negative by bacteriological culture technique 3 were found to be positive by immunoperoxidase technique. The positive stained bacteria were observed as bacillus shaped and mostly single or in groups of two or forming V-shapes *(Fig. 1a-b)*. Smears prepared from the isolates of cultures were all shown positive immunoreactivity *(Fig. 2)*. While all negative controls in which primer antibody was switched by PBS gave negative results, all positive controls that were prepared from *L. monocytogenes* standard strain (ATTC-7644) showed positive immunoreactivity. According to these results, sensitivity and specificity of the immunoperoxidase technique compared to the bacteriological culture technique were calculated as 80% and 96.7%, respectively.

DISCUSSION

Cases of abortion negatively affect cattle breeding not only in Kars province but also nationwide. Most of the abortions are known to be caused by bacterial agents, and *Listeria* is a significant bacterium in the list [18,19]. Stillbirth, infertility and drops in milk production and quality are the major reasons of economic losses due to Listeriosis. The disease is also a zoonosis, and therefore poses a risk for human health [20].

Listerial abortions in cattle are often sporadic and take place mostly within the last trimester [21]. The agent could be easily detected in fetal tissues as well as in uteral and

Table 1. *Time of abortion, time lapse after abortion until sample collection, and results of the bacteriological culture and immunoperoxidase techniques for Listeria (Total of 88 cases that were negative by both techniques were not shown)*

Tablo 1. *Abort zamanı, abortu takiben örnek toplamaya kadar geçen süre ve Listeria yönünden bakteriyolojik kültür ile immunoperoksidaz yöntemlerinin sonuçları (Her iki yöntemle de negatif sonuçlanan toplam 88 vaka tabloda gösterilmemiştir)*

Case No	Time of Abortion (Months)	Sample Collection (Days)	Bacteriological Culture	Immunoperoxidase
1	7-8	30	+	+
2	6-7	45	+	-
3	6-7	45	+	+
4	8-9	30	+	+
5	8-9	45	+	+
6	7-8	45	-	+
7	7-8	45	-	+
8	8-9	45	-	+

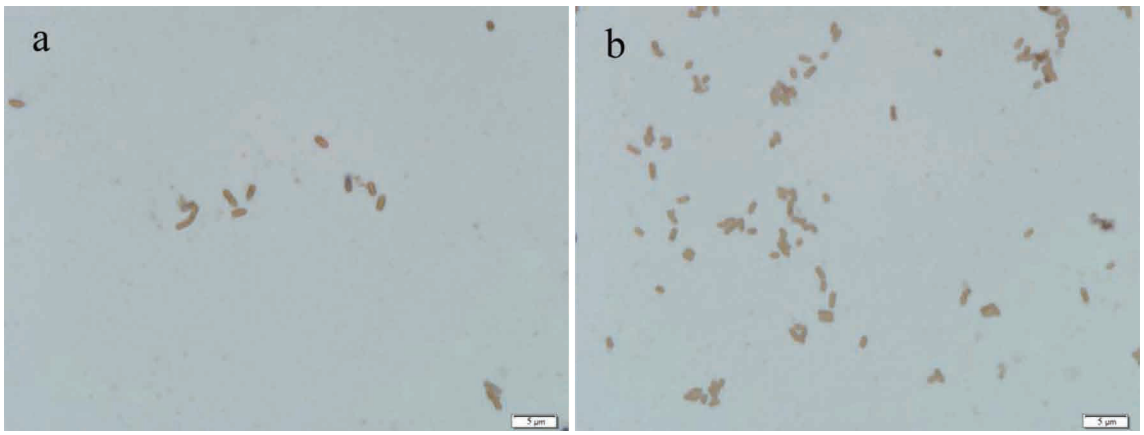

Fig 1. a-b. Listeria agents observed in smears prepared from vaginal swab samples by immunoperoxidase technique

Şekil 1. a-b. Vajinal sıvap örneklerinden hazırlanan sürme frotilerde immunoperoksidaz yöntemi ile gözlenen Listeria etkenleri

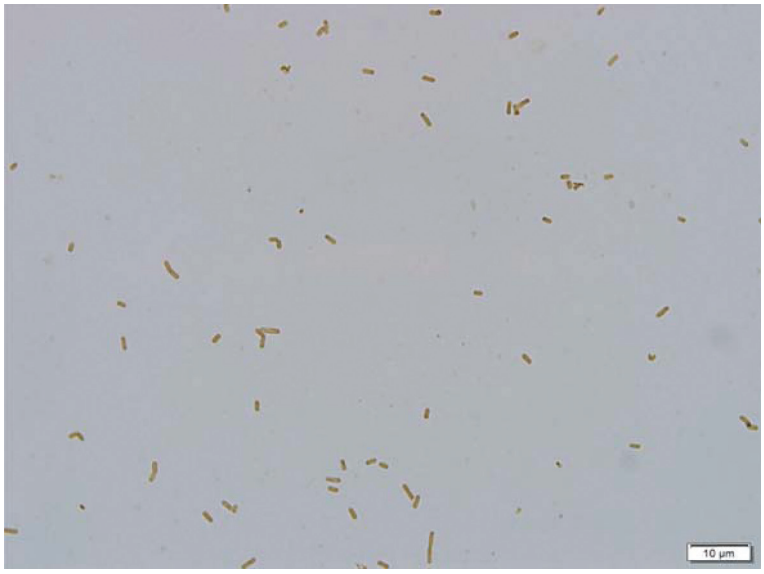

Fig 2. Listeria agents showing positive immunoreactivity in smears prepared from culture isolates

Şekil 2. Kültür izolatlarından hazırlanan sürme frotilerde pozitif immunoreaksiyon gösteren Listeria etkenleri

vaginal secretions after abortion [18,19]. Although it is not known exactly how long the bacteria shade in uteral and vaginal secretions it is thought to last for several months after abortion [22]. Studies regarding to bacteriological isolation of the agent in vaginal secretions are however, quite limited [23-25]. In a study conducted in servico-vaginal swab samples, *L. monocytogenes* was detected at 10% of cattle with genital system diseases [25]. In another study, *L. ivanovi* was isolated in vaginal and fecal swab samples collected from sheep with abortion [24]. Similarly, *L. monocytoges* and *L. ivanovi* were detected in the vaginal swab samples of buffalos at 2.4% and 0.8%, respectively [23]. In Turkey, most studies of listeriosis in animals have aimed to seroprevalence detection [13,26,27]. However, there are limited studies on the bacteriological isolation and identification for *Listeria*. In a study conducted by Erdogan et al.[13] sero-prevalence of listeriosis was determined 78.9% in Kars province. A bacteriological study for isolation and identification of the agent was also performed in Kars [4]. In the current study, *L. monocytogenes* was isolated and identified in 5 vaginal swab samples of 96 cattle that had abortion or stillbirth in their history. In spite of the time lapse and delayed sampling after the abortion the isolation rate seems to be similar to previous studies in the literature [23,25].

Listerial agents are known to spread by aborted fetuses, fetal membranes, vaginal secretions, milk, urine and feces. The agent was also reported to be present in digestive tract of healthy animals as well as humans [28]. Therefore, soil, water, and foodstuffs could be easily contaminated with the bacteria and become a treat for spread to humans. Hence, rapid and reliable detection of the agent is important in taking precautions in epidemics. The definitive detection of listeriosis can only be possible with isolation and identification of the agent by bacteriological means. Although bacteriological culture technique is the reliable and standard method it takes time and require equipped laboratory environment and personnel. The technique might also suffer from inadequacies in samplings and low bacterial presence in samples. Possible laboratory spread to laboratory technician is the main drawback of bacteriological culture technique. Moreover, isolation of the bacteria in clinical samples might not be reliable since the agent can be found in brain and feces [28,29]. Therefore, a rapid, simple, and reliable technique is still needed.

Complement fixation, agglutination and ELISA are the commonly used serological techniques to determine listeriosis [13]. They are based on the detection of antibodies that are reactive against somatic (O) and flagella (H) antigens. Although these techniques are simple and sensitive they might be insufficient in diagnosis. The tests might also suffer from cross reactions with other Gram negative bacteria such as *Streptococcus*, *Staphylococcus*, and *Enterococcus* [30].

Polymerase chain reaction and DNA hybridization techniques are some molecular methods used in detection *Listeria* especially in foodstuffs. Virulence factors such as haemolysin and p60 extracellular protein are mostly used for detection. PCR technique has been successfully used in determination of *Listeria* species in milk and vaginal secretions [31]. However, it is comparably expensive and requires equipped laboratory equipment and personal. Optimization problems and longer time requirement to obtain results renders the use of molecular techniques [12].

Pathological findings in aborted fetuses are not specific for *Listeria*, and therefore definitive cause in abortions could not be easily determined in listerial abortions. Rapid and reliable determination of the causative agent however could be succeeded by peroxidase-antiperoxidase technique [32,33]. The technique has also been shown to provide better detection results compared to the bacteriological culture techniques in certain studies, though it was stated that the technique must be supported by other laboratory detection means [33].

In the current investigation, 4 out of 5 samples that were determined to be positive for *Listeria* by bacteriological culture technique showed positive immunoreactivity by immunoperoxidase technique. Therefore, 20% false negativity in comparison to the bacteriological culture technique was observed. Low number of bacteria in the collected samples might be the cause of the false negativity in one sample. Use of enriched broth agar, therefore would provide better results for isolation of the agent, and hence might be superior to immunoperoxidase technique in such cases. In addition, presence of low number of *Listeria* cases studied in the current study might be the cause of high false reactivity for immunoperoxidase technique. Moreover, 3 out of 91 negative samples by bacteriological culture technique showed positive immunoreactivity for *Listeria*. Therefore, immunoperoxidase technique yielded 3.3% false positivity. This false positivity might be caused by cross reactivity with other bacterial agents and/or possible contamination during the sample staining. According to the results obtained, sensitivity and specificity of the immunoperoxidase technique was calculated as 80% and 96.7%, respectively. In a previous study for *Brucella*, similar results were also obtained [34].

In conclusion, immunoperoxidase technique was shown to be a useful tool in rapid, easy, relatively inexpensive and fairly reliable detection of *Listeria* in smears prepared from vaginal secretions of cattle which have abortion. However, the results of the technique should be evaluated carefully keeping in mind that the listerial agents could be found in environment and feces of healthy animals and possible contamination could occur during sampling. Finally, it was concluded that the immunoperoxidase technique might provide some help in early determination of *Listeria* cases; however the results should be supported by some other detection techniques.

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