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Molecular Diagnosis of Bovine Genital Mycoplasmopsis and Ureaplasma Infections by PCR

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Abstract: Genital Mycoplasmas (*Mycoplasma* spp. and *Ureaplasma diversum*), commensals of the genital tract, cause economic loss in cattle enterprises by mainly reproductive failure at the farm level. The productivity loss is due to genetic and environmental factors, stress, wrong antibiotic treatment, and immune system failure. This study aimed to determine the presence of bovine genital *mycoplasma* and *ureaplasmas* in healthy and suspected infertile cattle by PCR for the first time. Conventional methods are also used to examine test results. A comparison of results was conducted in both tested methods. For this aim, 356 bovine genital samples of healthy and suspected infertile cows and bulls of various herds were collected from Ankara and the provinces of Türkiye. Positivity in the genital sample of 221 healthy and 135 suspected infertile cattle was 61.2% by cultural methods and 67.7% by PCR, respectively. In comparison to culture, although PCR gave accurate results in a shorter time, findings show that proper results may be obtained when both methods are used together.

Keywords: *Cattle, Mycoplasmopsis, PCR, Ureaplasma.*

Sığırların Genital Mikoplazma ve Üreplazma İnfeksiyonlarının PCR ile Moleküler Teşhisi

Özet: Genital sistem kommensalleri olan genital mikoplazmalar (*Mycoplasma spp*. ve *Ureaplasma diversum*) büyükbaş hayvan işletmelerinde özellikle çiftlik düzeyinde üreme yetersizliği ile ekonomik kayıplara neden olmaktadır. Verimlilik kaybının başlıca nedenleri genetik ve çevresel faktörler, stres, yanlış antibiyotik tedavisi ve bağışıklık sistemi yetmezliği ile ilişkilidir. Bu çalışmanın amacı, sağlıklı ve infertil olduğundan şüphelenilen sığırlarda sığır genital mikoplazma ve üreplazmaların varlığının ilk defa PCR ile belirlenmesidir. Test sonuçları ayrıca geleneksel yöntemlerle de incelenmiştir. Test edilen her iki yöntemde de sonuçların bir karşılaştırması yapılmıştır. Bu amaçla, Ankara ve Türkiye'nin illerinden çeşitli sürülere ait sağlıklı ve kısır olduğundan şüphelenilen inekler ve boğalardan toplam 356 adet sığır genital örneği toplandı. 221 sağlıklı ve 135 şüpheli infertil sığırın genital materyalinde pozitiflik sırasıyla kültürel yöntemlerle % 61,2 ve PCR ile % 67,7 olarak bulundu. Kültüre göre PCR daha kısa sürede doğru sonuçlar vermesine rağmen bulgular her iki yöntemin birlikte kullanılması durumunda uygun sonuçların alınabileceğini gösterdi.

Anahtar Kelimeler: *Büyükbaş hayvan, Mikoplazmopsis, PCR, Üreplazma.*

Introduction

The *Mycoplasmataceae* family, includes *Mycoplasmopsis*, *Ureaplasma*, and *Mycoplasma* genera and is generally associated with urogenital tract infections, reproductive failure, neonatal morbidity, and mortality. *Mycoplasmopsis bovis (M. bovis)*, *Mycoplasmopsis bovigenitalium* (*M. Bovigenitalium)*, and *Ureaplasma diversum (U. diversum)* are bacteria that are pathogenic for the bovine genital tract without clinical signs (Razin et al., 1998). This situation causes economic losses by affecting meat and milk production in cattle enterprises. However, although the studies on these are limited, pathogenicity is still an issue under investigation.

M. bovis is the largest *Mycoplasmopsis* species isolated from cattle. It causes mastitis, reproductive disorder, infertility, oophoritis, seminovesiculitis, synovitis, endometritis, salpingitis, salpingoperitonitis, seminal vesiculitis, and epididymitis as with many diseases in bovine animals. *M. bovigenitalium* also causes infertility, necrotic endometritis, epididymitis, seminal vesiculitis, and low spermatozoa motility, while among the *ureaplasmas,* mainly *U. diversum* causes reproductive disorders in cattle (Cassell et al., 1989). The transmission of these bacteria whose virulence is mainly dependent on the cellular immune response, is a factor that may adversely affect both domestic and international animal trade. Bovine mycoplasmas are detected in the laboratory using molecular, serological, and cultural techniques (Cardoso, 2000a). For rapid molecular identification of bovine mycoplasmas with restricted biosynthetic capabilities, polymerase chain reaction (PCR) is typically utilized to detect these agents by hybridization of randomly cloned DNA fragments and in vitro amplification of the 16S rRNA gene. Studies have shown that PCR is a sensitive method for detecting genital mycoplasmas in clinical samples (Abele-Horn et al., 1996).

In this study, the detection of *Mycoplasmopsis* and *Ureaplasma* spp. from genital clinical samples was carried out on healthy and infertile cattle in Ankara and other cities (Adana, Erzurum, Kars, Van, Diyarbakır, Erzincan) of Türkiye. At the same time, culture and molecular methods were compared to detect the agents from the clinical samples.

Material and Methods

Ethical approval: This study is not subject to HADYEK permission by Article 8 (k) of the ‘‘Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees’’.

Bacterial isolates

Standard Strains: The Turkish Cattle Breeders' Central Association and the local cattle breeders in Ankara provided frozen healthy and suspected infertile semen samples. And, preputial washes of bulls with suspected infertility were obtained from Etlik Central Veterinary Control and Research Institute, Türkiye.

Clinical samples

A total of 356 genital samples from cattle older than two years old, including 165 cows and 191 bulls, were detected for reproductive failure. Bovine animals were deemed infertile despite at least three attempts at insemination. To detect genital mycoplasmosis, vulvar swabs from heavily discharged animals, cervical swabs from the elderly, and vaginal mucus from other cows without antibiotic treatment were collected. Also, the bulls' semen and preputial fluid were collected. Bulls that lacked clear clinical indicators were suspected of being infertile. Genital samples of infertile 76 cows and 59 bulls and healthy 89 cows and 132 bulls were analyzed. The genital samples of the cows were transferred to Amies transport medium and delivered to the laboratory in a cold chain within 24 hours. All samples were evaluated by cultural methods and PCR.

Culture

Mycoplasmopsis and Ureaplasma species from bovine genital samples are cultivated in the Eaton and Ureaplasma agar mediums. These spesific mediums allow glucose fermentation, arginine hydrolysis, phosphatase activity, and tetrazolium reduction tests to identify *Mycoplasmopsis* spp. As Davidson et al. (1994) suggested, both solid and liquid media were used for detection. The liquid media was dissolved in the Koch device, and the pH was adjusted to 6.0 and sterilized in an autoclave at 121°C for 15 minutes. Following the cooling of SLM (Standard Liquid Medium) to 56 ºC, pH was adjusted to 6.8-7.0, which is the appropriate pH range for both *Ureaplasma* and *Mycoplasmopsis* identification. Culture isolates were maintained by filtration through a sterile filter with a diameter of 0.2 µm (Schleicher & Schuell, BR0534-2) to enrich the composition of the media. Eaton and Ureaplasma agars were divided into petri plates at an average of 6 ml, and SLM agars were divided into tubes at 1.8 ml in sterile conditions. Cultured broths were stored at -20 ºC for detection by PCR. Biochemical tests were conducted on colonies grown on standard liquid media with color changes and on Eaton and Ureaplasma agars. The isolated strains gave %100 negative results in glucose fermentation and arginine hydrolysis tests. However, digitonin sensitivity and urease tests were 100% positive in *U. diversum* cultures. Isolated *M. bovis* and *M. bovigenitalium* strains gave strong positive results, especially in the phosphatase tests. The main growth inhibition test results were considered in the differentiation of the two strains. Since *ureaplasmas* prefer microaerophilic conditions, a color change is usually observed at the bottom of SLM tubes. All positive and negative samples were stored at 20 ºC to be detected by PCR.

Molecular Identification

Detection By PCR: The study aimed to detect *Mycoplasmopsis* and *Ureaplasma strains* using conventional methods and real-time PCR. Results were compared through the detection rate of the agent on SLM. Conventional methods examined 356 genital samples, and PCR identified a serial of SLM dilutions. For DNA extraction, SLM positive and negative aliquots of the genital swab and preputial washes were performed on direct FK (Phenol Chloroform). And semen was applied directly to cetyltrimethylammonium bromide (CTAB, Cetyl Trimethyl Ammonium Bromide).

Positive and negative isolates were further examined by PCR amplifying the 16S rRNA gene using *Mycoplasmopsis* and *Ureaplasma*- specific primers.

Positive control strains: *M. bovis* (NCTC 1031), *M. bovigenitalium* (NCTC 10122), *M. canadense* (NCTC 10152), *M. arginini* (NCTC 10129), and *M. dispar* (NCTC 10125) were obtained from Turkish Ministry of Agriculture and Forestry, Pendik Animal Diseases and Research Institute Mycoplasma Laboratory; and *U. diversum* (NCTC 11709) in lyophilized form from the Institute of Bacteriology, Faculty of Veterinary Medicine, Vienna.

In detecting *M. bovis*, *M. bovis genitalium,* and *U. diversum* by PCR, the standard strain DNA samples known to give positive results obtained from Maristela V. Cardoso (Instituto Biologico, Brazil) were used.

Primers Used in PCR

Primers were synthesized at 0.2 µmol synthesis scale in Biogen (Sao Paulo, Brazil) and obtained in the lyophilized form to be diluted to a concentration of 10 pmol, then vortexed and stored at -20 ºC. Primers are presented in Table 1,2,3.

Table 1. Primers Used in the Diagnosis of Bovine Genital *Mycoplasmopsis* and *Ureaplasma* by PCR.

Mikoplazma türü ile ilgili sekansı simgeleyen gen adı			Sekans Dizilimi	1. döngüde görüntülenen PCR ürünü (bp) Standart PCR	2. döngüde görüntülenen PCR ürünü (bp) Nested PCR
Mycoplasma bovis (uvrC)		*uvrCF*	5´-TTA CGC AAG AGA ATG CTT CA-3´	1626	–
		uvrCR	5´-TAG GAA AGC ACC CTA TTG AT-3´		
Mycoplasma bovigenitalium (Mbg)		*MbgF*	5´-CGT AGA TGC CGC ATG GCA TTT ACG G-3´	312	–
		MbgR	5´- CAT TCA ATA TAG TGG CAT TTC CTA C-3´		
Ureaplasma diversum (UD)	NESTED PCR	*UD1F*	5´-CCG GAT AAT AAC ATT TAC TTG-3´	986	–
		UD2R	5´-CCT TGC GGT AGC AGT ATC GA-3´		
		UD3F	5´-AAT GTC GGC TCG CTT ATG AG-3´	–	215
		UD4R	5´-GCG GAG GTT AAC AAT ATG ACA GG-3´		

DNA Extraction: To detect the presence of *Mycoplasmopsis* and *Ureaplasma* by PCR in the genital samples of healthy and infertile cows and bulls with suspected infertility, inoculated standard broth and preputial fluid samples were treated with FK according to the protocol determined by Csaikli et al. (1998) And, CTAB method was applied to fresh and frozen semen according to the protocol defined by Murray and Thompson (1980).

Table 2. Mixtures Used in PCR Protocols (Total Volume: 50 µl).

Mikoplazma türü ile ilgili sekansı simgeleyen gen adı			Sekans Dizilimi	1. döngüde görüntülenen PCR ürünü (bp) Standart PCR	2. döngüde görüntülenen PCR ürünü (bp) Nested PCR
Mycoplasma bovis (uvrC)		*uvrCF*	5´-TTA CGC AAG AGA ATG CTT CA-3´	1626	–
		uvrCR	5´-TAG GAA AGC ACC CTA TTG AT-3´		
Mycoplasma bovigenitalium (Mbg)		*MbgF*	5´-CGT AGA TGC CGC ATG GCA TTT ACG G-3´	312	–
		MbgR	5´- CAT TCA ATA TAG TGG CAT TTC CTA C-3´		
Ureaplasma diversum (UD)	NESTED PCR	*UD1F*	5´-CCG GAT AAT AAC ATT TAC TTG-3´	986	–
		UD2R	5´-CCT TGC GGT AGC AGT ATC GA-3´		
		UD3F	5´-AAT GTC GGC TCG CTT ATG AG-3´	–	215
		UD4R	5´-GCG GAG GTT AAC AAT ATG ACA GG-3´		

Table 3. PCR Protocol.

Siklus	*M.bovis*		*M.bovigenitalium*		*U. diversum*			
	I. Döngü		I.Döngü		I.Döngü		II.Döngü	
Denaturasyon	95°C 15 dk		94 °C 9 dk		94°C 5dk		94°C 5dk	
Annealing (Bağlanma)	35 Döngü	95 °C 30 sn 53 °C 1,3 dk 72 °C 1,5 dk	37 Döngü	94 °C 30 sn 60 °C 1 dk 72 °C 1 dk	35 Döngü	94 °C 1 dk 55 °C 1 dk 72 °C 30 sn	35 Döngü	94 °C 1 dk 67 °C 1 dk 72 °C 20 sn
Elongasyon (Uzama)	72 °C 10 dk		72 °C 7 dk		72°C 10dk		72°C 10dk	

Detection of Nucleic Acids

The electrophoresis device was operated at a constant current of 96 Volts for 1 hour and the PCR products amplified in the Thermal Cycler were run on a 1.5% agarose gel and examined by a UV transilluminator. DNA bands of the samples were evaluated by comparing the DNA marker with other bands of positive and negative controls.

Expected DNA amplifications in positive samples were 1626 bp for *M. bovis* (Subramaniam et al., 1998), 312 bp for *M. bovigenitalium* (Kobayashi et al., 1998), and 215 bp for *U. diversum* (Cardoso et al., 2000a; Cardoso et.al., 2000b).

Statistical analysis

The chi-square (x^2) analysis method was used to interpret the data obtained in the study (Grunert & Valentine, 1998).

Results

In the study, 356 genital samples of 165 cows and 191 bulls were examinedby cultural and identification methods. The gender, health status, and sample type of the cattle included in the study were considered. Samples were collected from cattle over two years of age, declared to have infertility problems, and from healthy cattle for control purposes. Although inseminated at least three times, cows that did not show pregnancy were considered suspected infertile. A single sample was taken from each animal. Vulvar samples from cows with heavy discharge, cervical swab samples from the elderly, vaginal mucus samples from others, and semen and preputial fluid were collected from bulls. It was noted that antibiotics had not been given to these cattle recently. Of the cows, 76 were suspected of infertile, 89 of healthy; genital samples of 135 suspected were infertile and 221 healthy cattle, 59 of which were suspected infertile and 132 healthy, were examined.

Culture

In the study, 56 (73.6%) of 76 infertility-suspected cows and 71 (79.7%) of 89 healthy cows were found to be positive for *Mycoplasmopsis* and *Ureaplasma*. And bacteria were isolated in 48 (81.3%) of 59 suspected infertile bulls and 43 (32.5%) of 132 healthy bulls. 46 (70.7%) of 65 vaginal mucus, 18 (81.8%) of 22 cervical swabs and, 63 (80.7%) of 78 vulvar swabs were determined as positive. 44 (83%) of 53 preputial fluids belonging to infertile bulls, 42 (32.3%) of 130 frozen semen belonging to healthy bulls, 1 of 2 healthy and 4 of 6 fresh semen belonging to infertility- suspected bulls were found as positive for *Mycoplasmopsis* and *Ureaplasma*. The difference between positivity in the frozen semen and preputial fluid samples analyzed was significant (>P 0.05).

PCR Results

Standard PCR for *Mycoplasmopsis* and nested PCR for *Ureaplasma* suspicious samples were applied , and DNA samples were extracted from cultures of these samples from healthy cattle with suspected infertility. In amplified PCR products, *M. bovis* bands were detected at 1626 bp and *M. bovigenitalium* at 312 bp, while bands of *U. diversum* were detected at 986 bp and 215 bp in nested PCR. All of the samples found positive for *Mycoplasmopsis* and *Ureaplasma* by the cultural method gave positive results by PCR. No positivity was found in 115 (32.3%) of the examined samples of bovine genital *Mycoplasmopsis* and *Ureaplasma*. Each positivity was close to the other in both methods, and the difference between the groups was statistically insignificant (P>0.05). The positivity rate in PCR was 87.1% in healthy cows and 65.3% in infertility-suspected animals. The difference was found to be statistically insignificant (P>0.05). By PCR, positivity for *Mycoplasmopsis* and *Ureaplasma* was determined in 241 (67.6%) of the DNA belonging to a total of 356 samples, 137 (83%) in cows and 104 (54.4%) in bulls. Based on the condition of the cow from which the genital sample was collected, 57 (75%) of 76 suspected infertility cows and 80 (89.8%) of 89 healthy cows were found to be positive for *Mycoplasmopsis* and *Ureaplasma*. According to the type of genital sample evaluated in the study, 51 (78.4%) of 65 vaginal mucus, 19 (86.3%) of 22 cervical swabs, 67 (85.8%) of 78 vulvar swabs were found positive by PCR while positivity in frozen semen from healthy bulls and preputial

fluid samples from bulls with suspected infertility were found to be 38.4% and 92.4%, respectively, in PCR. In the PCR results of fresh semen samples, the data for statistical analysis were insufficient. The difference between positivity in frozen semen and preputial fluid samples was insignificant (P> 0.05).

Discussion and Conclusion

Mycoplasmopsis species, known to be mainly responsible for infertility in cattle, cause significant economic losses in cattle breeding worldwide, especially in Europe and North America.Various studies have been conducted in different parts of the world to determine the role of genital *Mycoplasmopsis species* in urogenital tract infections in cattle. However, the role of *Mycoplasmopsis species* in genital infections is still unclear. Stress, immune system failure, incorrect antibiotic treatment, animal transportation, and artificial insemination with infected semen are essential factors in increasing clinical cases (Vegi et al., 2021). Studies conducted in worldwide have proven that *Mycoplasmopsis strains* can colonize the urogenital tract without causing any disease (Erno et al., 1967; Lein, 1986). The unique phenotypic variability and antigenic variation seen in many *Mycoplasmopsis* species make it difficult for them to be recognized by the host immune system. And the agent becomes resistant to treatments with antibiotics such as tetracycline, tilmicosin, and spectinomycin.

In Türkiye, this infection is thought to cause significant economic losses in cattle breeding as in other countries. Studies on *Mycoplasmopsis species* in Türkiye are generally on detecting CCPP for many years. In this regard, there has been no comprehensive research conducted in our country for the identification and Detection of *Mycoplasmopsis* and *Ureaplasma* causing genital infections in cattle by PCR yet. Today, PCR, one of the molecular methods that give accurate results quickly, is used instead of serological methods with a high probability of false positive results with time-consuming routine biochemical tests. Many studies have reported that PCR is more sensitive than other methods (Razin, 2002).

In this study, the method specified by Genovez et al. (1989) was used in the detection by the cultural method and in the examination of the collected samples. Accordingly, solid media was incubated for 15 days at 37 ºC in a microaerophilic jar, and liquid media were incubated at 37 ºC for five days in an aerobic environment. Accordingly, this information was considered in detecting the agent by PCR. In the evaluation based on the origin of the examined sample, positivity by the conventional method was determined as 70.7% in vaginal mucus samples, 80.7% in vulvar swabs, and 81.8% in cervical swabs. In PCR, these results were determined as 78.4 %, 86.3 %, 85.8 %, respectively. Based on the comparison of the number of samples collected from cows, the positivity obtained by both methods was the highest in cervical and vulvar swab samples and less frequently in vaginal mucus samples. However, the difference between the groups was insignificant according to the chi-square analysis method (P>0.05). In addition, when the evaluation was made according to the health status of the cows from which the samples were obtained, the positivity of *Mycoplasmopsis* and *Ureaplasma* detected by conventional methods and PCR in the cervical swab of healthy cows was found to be 12.8% higher than in the cows with suspected infertility. However, the difference was considered statistically insignificant according to the number of samples examined (P>0.05). On the other hand, the difference between *Mycoplasmopsis* and *Ureaplasma* positivity rates detected in healthy and infertile cows in the genital samples of cows examined by both methods was also found to be statistically insignificant (P>0.05). In addition, 96 (44%) *Mycoplasmopsis* and 122 (55.9%) *Ureaplasma* were isolated from healthy cows and bulls with suspected infertility by cultural methods. A total of 56 *Mycoplasmopsis* was isolated from 29 (38.1%) infertility- suspected cows and 27 of healthy cows. 71 *Ureaplasmas* were detected, including 27 (35.5%) cows with suspected infertility and 44 (49.4%) healthy cows. Accordingly, it was observed that *Mycoplasmopsis* growth in cows was higher in infertility suspects and ureaplasma growth in healthy cows. However, the difference was considered statistically insignificant (P>0.05).

In another study, Cardoso et al. (2000a) examined infected bovine semen. The presence of *Mycoplasma* spp. and *U. diversum* in semen were found to be 1.02% and 1.32% higher, respectively. In another study conducted by Gregory et al. (2012) in sheep, the rate of *mycoplasma*-infected semen resulted in higher results than that of Cardoso et al. (2000a).

According to the data obtained in the study, the findings related to the health status of the cows and the type of sample examined were partially compatible. High positivity in healthy animals suggests that the animals may be under stress or in the initial stage of infection. A definite conclusion could not be reached with the results obtained on the role of *Mycoplasmopsis strains* in infertility. The reason for this was thought to be important for the pathogenicity differences between *Mycoplasmopsis* strains. Although the difference between conventional methods and PCR results was found to be statistically insignificant in the study, it was determined that PCR gave more accurate results in a shorter time than conventional methods in the early detection of *Mycoplasmopsis strains* in reproductive disorders in cattle. The high rate of *Mycoplasmopsis* and *Ureaplasma* positivity detected in the tested samples of healthy and infertile cattle was associated with the fact that these microorganisms are also normal flora agents in the genital tract. However, it is thought that the risk of contamination is high during sample collection in the genital tract of cows, and this may affect the results of detection. In addition, this study highlighted the importance of further research on the pathogenicity of *Mycoplasmopsis* and *Ureaplasma*.

Ethical Approval

This study is not subject to Hadyek's permission within the scope of "the regulation on the working principles and procedures of animal experiments ethics committees"

published in the official newspaper dated February 15, 2014 and numbered 28914. The authors declared that they comply with the Research and Publication Ethics.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Similarity Rate

We declare that the similarity rate of the article is 9% as stated in the report uploaded to the system.

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Explanation

(International Mycoplasma Congress- Cambridge-London) It was presented as a summary paper at the International Microbiology Congress (2006). (Extracted from the author's doctoral thesis of the same name).

Author Contributions

Motivation / Concept: KSD
Design: BO, KSD
Control/Supervision: KSD
Data Collection and / or Processing: BO
Analysis and / or Interpretation: BO, KSD
Literature Review: BO
Writing the Article: BO
Critical Review: KSD

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