

Investigation of *Taylorella equigenitalis* from thoroughbred stallion genital swabs by direct polymerase chain reaction and culture method

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Summary: In this study, classical culture method and a direct polymerase chain reaction (direct-PCR) assay was applied to urethral fossa and sinus swabs taken from 25 thoroughbred stallion racehorses to detect *Taylorella equigenitalis* responsible for contagious equine metritis (CEM). *T. equigenitalis* was not isolated from any of the samples and was not detected by direct-PCR.

Keywords: Culture, direct-PCR, stallion, *Taylorella equigenitalis*

Safkan yarış aygırlarından alınan genital svaplarda direkt polimeraz zincir reaksiyonu ve kültür yöntemi ile *Taylorella equigenitalis*'in araştırılması

Özet: Bu çalışmada 25 safkan yarış aygırından alınan uretral fossa ve sinus svaplarında, direkt polimeraz zincir reaksiyonu (direkt-PCR) ve klasik kültür yöntemleri ile kontagiyöz equine metritis (CEM)'in etkeni olan *Taylorella equigenitalis* varlığı aranmıştır. Alınan örneklerden *T. equigenitalis* izole edilemediği gibi direkt-PCR yöntemi ile de tespit edilememiştir.

Anahtar sözcükler: Aygır, direkt-PCR, kültür, *Taylorella equigenitalis*

Introduction

Contagious equine metritis (CEM) is a highly contagious venereal non-systemic infection of horses. Clinical signs are seen only in mares with a slight to copious mucopurulent vaginal discharge and a variable cervicitis, endometritis and vaginitis findings following temporary infertility which lead to economic loss in horse breeding. Unlike the mares, stallions exposed to CEM do not develop clinical signs but they carry the infection and pass it to the mares during mating (OIE, 2008).

The causative agent of CEM, *T. equigenitalis*, is a Gram negative, microaerophilic, non-motile organism that needs fastidious growth requirements. Bacteriological, serological and recently developed molecular techniques can be used in diagnosis of CEM but definitive diagnosis is isolation of the agent from swab samples taken from urogenital membranes of mares and stallions. Swabs must be sent to the laboratory at 4°C in 48 hours with a transport medium (Amies medium) that includes active charcoal (WATSON, 1997).

These time and temperature settings must be obeyed in order not to decline the number of *T. equigenitalis* on swab samples. The urogenital membranes of stallions or mares are colonized by genital flora bacteria and fungi that inhibits the growth of *T. equigenitalis* on culture agar plates. Amphotericin B, clindamycin and trimethoprim is added to the culture medium to prevent contamination with these commensal microorganisms. After culturing the organism, daily inspection is needed during the incubation period which requires 72 hours to 14 days (OIE, 2008).

Jang et al. (2001) reported a second species, *Taylorella asinigenitalis* within the genus *Taylorella* which they isolated from genital tracts of donkeys. The new species has not been associated with naturally occurring disease. Differentiation through bacterial isolation of these two species is difficult because of the similar colonial appearance and identical biochemical test results.

To overcome the difficulties in isolation and identification of *T. equigenitalis* and its differentiation from *T. asinigenitalis*, several PCR assays

were developed and used in many countries. As well as acutely infected mares, PCR assays can also be used to identify carrier stallions. It was reported that PCR has a much higher rate of detection than bacteriological culture methods and can detect small numbers of *T. equigenitalis* through commensal bacteria from urogenital tract of horses (ANZAI et al., 1999; BLEUMINK-PLUYM et al., 1994; DUQUESNE et al., 2007). PCR is a rapid, specific and sensitive method for identification of *T. equigenitalis*. PCR was also used in the eradication of CEM in Japan and successful results were obtained (ANZAI et al., 2002). In this study, we aimed to identify *T. equigenitalis* from thoroughbred stallion genital swabs by direct-PCR assay.

Material and Method

Isolation and identification: Urethral fossa and sinus swab samples with Amies medium taken from 25 stallions sent to our department in cold chain and cultured immediately. Eugon agar with 5-10% lysed horse blood was used for bacteriological culture. Each swab sample inoculated on agar plates with 3 different compositions including; 1, amphotericin-B (5 mg/l); 2, amphotericin-B (5 mg/l), streptomycin (200 µg/ml); 3, amphotericin-B (5 mg/l), clindamycin (5 mg/l), trimethoprim (1 mg/l) respectively and incubated at 37°C in 5-10% (v/v) CO₂ in air. Plates were inspected daily for 14 days. Colonies became visible in 72 hours were not take into consideration. Suspected colonies were Gram stained and examined with catalase, phosphatase and oxidase tests. *T. equigenitalis* Kentucky 188 (K-188) strain was used as a positive control to check the accuracy of culture method. Cultured swab samples were stored at 4°C for further examination with direct-PCR.

Direct polymerase chain reaction (Direct-PCR):

A swab sample taken from urethral fossa of a stallion was dipped in an ependorf, containing *T. equigenitalis* K-188 strain which was suspended

with 500 µl sterile distilled water in order to test the direct-PCR with an experimental positive control. After being stored at 4°C in Amies transport medium for 24 hours, the positive control swab was suspended in 500 µl sterile distilled water at 37°C for 10 minutes. Other 25 swab samples were suspended and incubated like the positive control swab. DNA was extracted from these cell suspensions using DNeasy Blood & Tissue Kit[®] of Qiagen (product number: 69506) according to the manufacturer directives. Extracted DNA was stored at -20°C.

Te1:CAGCATAAGGAGAGCTTGCTTTTCT and Te2:GTCCATGGTATTAACACAAAC primers were used to amplify a 413-bp 16S rDNA sequence by PCR (DUQUESNE et al., 2007). PCR reaction was performed by using Fermentas Taq DNA Polymerase (recombinant) Kit (product number: EP0402). The 25 µl reaction solution contained 2.5 µl 10 × PCR buffer with KCl, 1.5 µl 25 mM MgCl₂, 0.5 µl 10 mM dNTP (Qiagen), 0.5 µl Taq DNA Polymerase (5 U/µl), 1 µl of each primer (20 pmol/ µl), 16 µl deionized water and 2 µl template DNA. PCR incubations were performed in Sensequest thermal cycler with an initial denaturation at 95°C for 2 min, followed by 35 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. Final extension was done at 72°C for 5 min at the end of last cycle. The amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV transluminator.

Findings

T. equigenitalis was not isolated from swab samples taken from urethral fossa and sinus of 25 stallions in bacteriological culture. DNA extracted from culture and experimental swab sample of *T. equigenitalis* K-188 strain produced a 413-bp amplification fragment at agarose gel electrophoresis. On the other hand, urethral fossa and sinus swab samples taken from 25 stallions were found to be negative in direct-PCR (Figure).

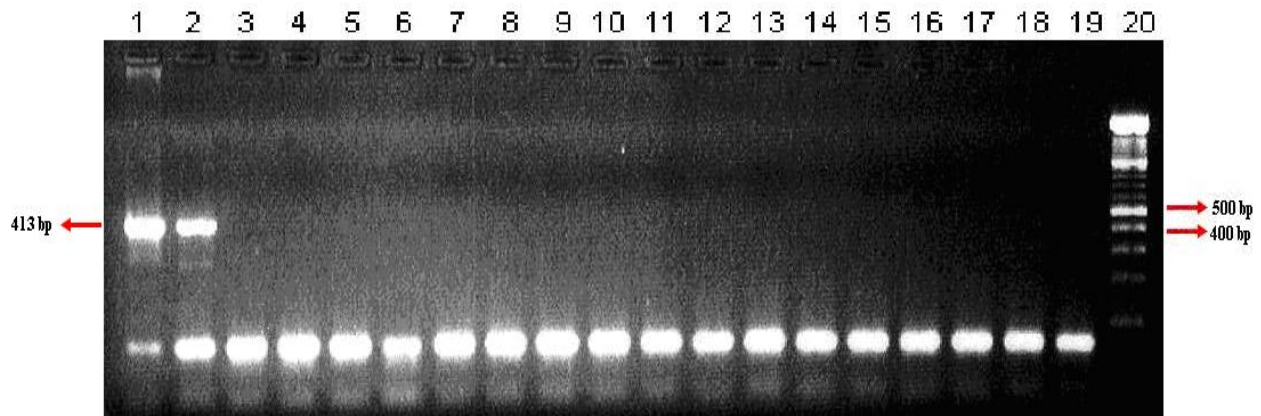


Figure. PCR amplification results. Lane 1: Positive control with *T. equigenitalis* K-188 strain genomic DNA (413-bp); lane 2: Experimental positive control swab sample; lane 3-19: Negative results belong to swab samples taken from urethral fossa and sinus of stallions; lane 20: 100 bp DNA ladder (Fermentas, Latvia).

Discussion and Conclusion

CEM was first described in the United Kingdom in 1977 (CROWHURST, 1977), after which it was spread through a number of countries worldwide. In Turkey; the agent of CEM, *T. equigenitalis*, was first described in 2001 by Ozgur et al. (2001b) from clitoral fossa swabs of 2 mares by bacteriological culture of 81 intrauterine and 39 clitoral fossa swab samples of 120 thoroughbred mares which had either endometritis or an infertility problem.

Although it has many disadvantages, bacteriological culture is the main tool for definitive diagnosis of *T. equigenitalis*. Several PCR assays have already been developed with advantages of rapid and definitive diagnosis of *T. equigenitalis* and its discrimination from *T. asinigenitalis*. In this study, we chose the method and primers that Duquesne et al. (2007) used, because, these primers distinguish *T. equigenitalis* from *T. asinigenitalis*, and do not amplify DNA's of equine urogenital tract commensal bacteria (*Streptococcus zooepidemicus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Serratia liquefaciens* and *Escherichia coli*). Also authors found the sensitivity of PCR assay as 10 CFU which can not be detected by bacteriological methods.

In Turkey, the presence of *T. equigenitalis* was well demonstrated by former studies in which serological tests (ÖZGÜR et al., 2001a; ERDEĞER et al., 2002) and bacteriological culture methods (ÖZGÜR et al., 2001b) were used, but this is the first study that PCR assay was used for investiga-

tion of CEM in Turkey. In this study, we aimed to compare bacteriological culture and direct-PCR results and test the usefulness of method in order to use in our laboratory for routine diagnosis.

We could not detect *T. equigenitalis* with both culture and direct-PCR, but it is understood that the result we obtained is due to the swab material which was low in number and taken from healthy thoroughbred stallions. In order to get valid results and increase the probability of getting positive results we have to plan an extensive study using swab materials taken from suspected carrier stallions and mares which have endometritis or infertility problem. Nevertheless, results obtained from positive controls showed that direct-PCR assay used in this study was successful and it is useful in routine diagnosis of CEM.

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Geliş Tarihi / Received: 14.10.2009

Kabul Tarihi / Accepted: 16.11.2009

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