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PCR DETECTION OF TRICHOMONAD SPECIES IN THE SEMEN OF BULLS

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Summary

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Trichomoniasis is a sexually transmitted disease of cattle caused by *Tritrichomonas foetus*. Other species of trichomonads have been recently isolated from the prepuce of virgin bulls. It is not clear whether these non-*T. foetus* isolates are also present on the prepuce of breeding bulls. The traditional diagnostic test for *T. foetus* involves collection of preputial or vaginal samples followed by culturing and microscopic examination. Recently, polymerase chain reaction (PCR) techniques have been described for use as diagnostic assays. The objective of this study was to use a PCR assay for detecting *T. foetus* in bull's semen samples. In a PCR assay, six out of 45 (13.3%) semen samples showed a 372 bp amplicon following PCR with primers TFR 1–2 but no amplicon when primers TFR 3–4 were used. The observed amplicons were related to non-*T. foetus* organisms. Our study demonstrated that the PCR assay was an effective method for differentiation of *T. foetus* from other trichmonad species.

Key words: bull, PCR, semen, trichomoniasis

INTRODUCTION

Bovine trichomoniasis is a sexually transmitted disease caused by the flagellated protozoan parasite *Tritrichomonas foetus*. Non-*T. foetus* parasites such as *Tetratrichomonas* spp. or *Pentatrichomonas hominis* have been recently isolated from the prepuce of virgin bulls. It is not clear whether these non-*T. foetus* isolates are also present on the prepuce of breeding bulls (BonDurant, 1997). The prevalence of trichomoniasis among Alabama (US) beef bulls was 1.2% by microscopic method (Rodning *et al.,* 2008), while a cross sectional study conducted in Costa Rica showed a herd prevalence rate of 15.1% (Perez *et al*., 1992). The parasite is transmitted from infected bulls to heifers or cows during mating. Bulls often develop persistent asymptomatic infections and become parasite vectors. In contrast, the parasite may generate various types of diseases in cows, ranging in severity from mild vaginitis or cervicitis to endometritis abortion and early embryonic death (BonDurant, 1997; Parker *et al*., 2001). In the United States, the estimated disease-related economic loss was 665 USD per infected cow. Greatest losses were caused by infertility (Goodger & Skirrow, 1987).

While cows tend to clear the infection, bulls can remain carriers for life and act as the main reservoir of the parasite (Clark *et al*., 1974). Normally, bulls infected by *T. foetus* are asymptomatic. Semen quality and sexual behaviour are not affected and the organism is found only on the penis

and membranes inside the sheath. It localizes in the smegma, or secretions of the penis, sheath and end of the urethra (Felleisen *et al*., 1998).

Since the disease has no effective treatment, timely and accurate laboratory diagnosis of trichomoniasis is of key importance in disease control. Various methods have been developed to accurately diagnose *T. foetus* in cattle while simultaneously attempting to minimize costs. Original techniques included a serological assay for *T. foetus* antibodies, and light microscopic and molecular-based analyses of preputial washings from bulls and cervicovaginal secretions from female cattle. The identification of *T. foetus* isolates is predominately based on morphologic criteria. Although, trichomonads other than *T. foetus*, probably of intestinal origin, can be isolated from the bovine preputial cavity and may confuse the diagnosis of bovine trichomoniasis (Levine, 1985; Felleisen *et al.,* 1998; Walke *et al.,* 2003). In addition, the morphologic distinction between *T. foetus* and other bovine trichomonads may not be reliable enough and thus, a more sensitive test as the polymerase chain reaction (PCR) assay seems necessary. Diagnosis of *T. foetus* has traditionally relied upon serology and microscopic identification of key morphological characteristics in various culture media. However, accurate microscopic identification of *T. foetus* can be complicated by the presence of other trichomonadid protozoa, non-specific antibodies and may also be limited by decreased sensitivity when compared to molecular-based assays such as PCR (Taylor *et al.,* 1994; Felleisen *et al.,* 1998; Cobo *et al.,* 2003; Hayes *et al.,* 2003).

Diagnostic tests for *T. foetus* using PCR on purified culture and preputial samples have been described (BonDurant & Honigberg, 1994; Ho *et al*., 1994). PCR-based assays are able to detect the presence of small numbers of parasites and differentiate various species of trichomonads.

The objective of this study was to use PCR for screening of bull semen samples from a commercial bull stud in Tabriz, Iran in order to detect the prevalence of *T. foetus* organisms.

MATERIALS AND METHODS

Samples

Forty-five semen samples were obtained from bulls aged 3 to 5.5 years, of eighteen different herds that referred to the Artificial Insemination Centre in Tabriz, Iran. The collected semen samples were mixed into a 3 mL tube containing approximately 1 mL of phosphate-buffered saline (PBS, pH 7.2), and sent to the veterinary diagnostic laboratory (Institute of Parasitology, Faculty of Veterinary Medicine, University of Tabriz). PBS suspensions were immediately centrifuged (10,000×*g*, 10 min), the supernatants were discarded, and resuspended pellets were used for DNA extraction.

Molecular detection

Genomic DNA from semen samples was extracted according to the Chomezynski extraction method. DNA concentration was measured at 260 and 280 nm (Biophotometer plus, Eppendorf, Germany). Electrophoresis of each DNA sample on 2% agarose gel in 1× Tris/Borate/EDTA (TBE) buffer was undertaken to check the integrity of the DNA. An aliquot of total DNA was produced from each sample and stored at –20 °C until analysis. A polymerase chain reaction assay, employing primers specific for sequences in the

Table 1. Primer pairs used in the study

No	Primer sequence	Target	Amplicon size (bp)
1*	TFR1:5-TGCTTCAGTTCAGCGGGTCTTCC-3 TFR2:5-CGGTAGGTGAACCTGCCGTTGG-3	5.8S rRNA gene sequences and internal transcribed spacer (ITS) regions	372
2**	TFR3:5-CGGGTCTTCCTATATGAGACAGAA-3 TFR4:5-CTGCCGTTGGATCAGTTTCGTTAA-3	5.8S rRNA gene	347

* Felleisen *et al.* (1998); ** Grahn *et al.* (2005).

5.8S ribosomal RNA gene and flanking internal transcribed spacer (ITS) regions of trichomonads was performed on all samples, as described by Felleisen et al. (1998). Two pairs of commercially (Takapouzist CO, Iran) synthesized primers (Table 1) were used.

All PCR reactions were performed in a 20 µL volume containing 1 µL of sample containing 100 ng DNA, 0.4 µL of 0.2 mM dNTPs mix, 1.4 µL of 3.5 mM $MgSO_4$, 2 µL of PCR buffer, 0.12 µL of 0.6U of Platinum Taq polymerase, 0.4 µL of 0.2 mM of each primer. PCR was performed in a primus 96 thermocycler (Techgene-Techne, Germany) with the following conditions: initial denaturation at 95 ˚C for 5 min, followed by 35 cycles at 94 ˚C for 1 min, 65 ˚C for 1 min and 72 ˚C for 2 min, with a final extension of 72 ˚C for 10 min. A non template control (water blank) was used as negative control in PCR. DNA from *Trichomonas vaginalis* served as a positive control in the first step of PCR assays, while nucleic acid from *T. foetus* was used as the positive control in the second step. Amplification products were analyzed on 2% agarose gels, stained with ethidium bromide, and photographed using ultraviolet light transillumination. As the PCR products of the TFR 1–2 and TFR 3–4 pairs of primers are of similar size (372 and 347 bp, respectively), separate PCR and electrophoretic procedures were conducted for the amplicon of each primer pair.

Gel electrophoresis

PCR amplicons were separated on 2.5% agarose gels and 6% polyacrylamide gels to visualize products. To evaluate the efficacy of agarose separation, 10 mL of amplified sample was electrophoresed for 1.5 h at 100 V. Images were visualized and photo documented on an Alpha Imager (Alpha Innotech Corp., San Leandro, CA).

RESULTS

Because of the long distance between sampling location and the laboratory, we were not able to test the samples morphologically. In PCR detection, 6 samples out of the 45 tested bulls showed a 372 bp amplicon following PCR with primers TFR 1–2, and no amplicon when primers TFR 3–4 were used (Fig. 1).

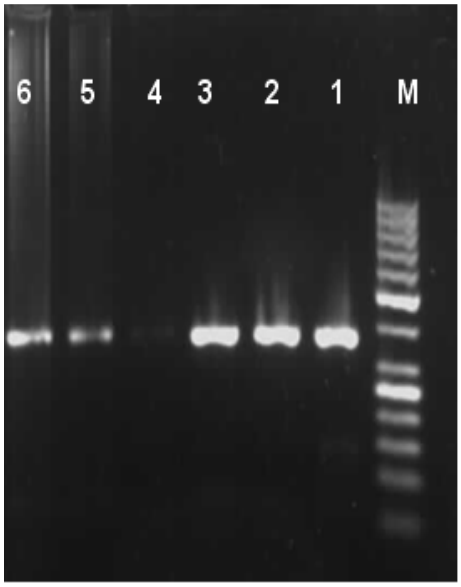

Fig. 1. Gel electrophoresis image showing PCR products: Lane M: molecular size marker; lane 1: positive control; lanes 2 to 6: positive samples; lane 4: negative control.

DISCUSSION

T. foetus remains a very important reproductive tract infective agent in domestic cattle. Control strategies focus on vaccination of females, identification and elimination of infected bulls (Corbeil, 1994; Grahna *et al*., 2005). Therefore, strict surveillance of bulls by sensitive and specific methods of diagnosis is still the most effective measure for the prevention of bovine trichomoniasis (Riley *et al*., 1995).

The infection is currently diagnosed on the basis of morphological identification of viable organisms isolated from infected animals and cultured. Sampling may occur under harsh climatic conditions, and it often involves transportation from remote areas. Thus, an evaluation of tests that can detect parasite artefacts such as DNA, even when the parasite is outside the host has started. PCR based techniques have increased sensitivity and specificity, but some lack the necessary internal controls for determining false negatives and therefore may be unable to most effectively manage the transmission of *T. foetus*. Furthermore, PCR is faster than culture and may detect trichomonads whose survival is uncertain after removal from the host (Hayes *et al*., 2003).

This study described the use of a molecular assay for *Tritrichomonas* infection in bull's semen, which was a reliable and valid method for the identification of *T. foetus*. At the first step of PCR, six samples were positive using specific primers for *Trichomonas* spp. and in the second step, there was no positive reaction (Morgan & Hawkins, 1948). The use of two sets of PCR primer pairs, one that reacts with a broad spectrum of trichomonads and one that is apparently *T. foetus*-specific, provides a procedural control. This study also confirmed the occurrence of non-*T. foetus* trichomonads in bull's semen that were first suggested half a century ago by Morgan & Hawkins in preputial scrapings, and more recently described in North America (BonDurant *et al*., 1999) and Argentina (Campero *et al*., 2003), and which is, to the best of our knowledge, the first report of such trichomonads in Iran. The origin of the non-*T.foetus* trichomonads was not sought in this study, but it seems highly likely that these organisms are lower bowel residents (Castella *et al*., 1997). It could be suggested that these presumably gastrointestinal inhabitants could have reached the prepuce by sodomy, a common practice among young bulls. The finding of the same non-*T. foetus* organisms in ''mature'' breeding bulls suggests that either the organisms survive for a much longer

time in the prepuce than their *in vitro* fastidiousness (BonDurant *et al*., 1999) would predict, or that they may arrive in the prepuce by a route other than sodomy. Improper sample collection could result in contamination and serve as a source for these trichomonads recovered from semen samples. Trichomonads other than *T. foetus* found in the intestinal tract differ morphologically from *T. foetus* by the number of anterior flagella present, size and shape of the body, length and position of axostyle, and presence or length of a posterior flagellum. However, because of the long distance between sampling location and laboratory we couldn't test the samples morphologically, the specific morphologic features can be hardly recognized by light microscopy alone (BonDurant *et al*.,1999), and confirmatory diagnostic tests using a species-specific PCR assay are necessary for the accurate identification of trichomonads from bovine samples (Walke *et al*., 2003).

In summary, our study demonstrated that the PCR assay was an effective method for the differentiation of *T. foetus* from non-*T. foetus,* which could be used as a laboratory diagnostic tool for reliable detection of the parasite in the absence of other diagnostic techniques such as cultivation, microscopy and serology.

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