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Comparative Evaluation of Methods for the Diagnosis of Bovine *Tritrichomonas foetus* Infection

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ABSTRACT

Tritrichomonas foetus is the causative agent of bovine tritrichomonosis, a primarily sexually transmitted disease leading to infertility and abortion in cattle. To date, detection of bovine *T. foetus* infection in routine diagnostics is performed by parasitological methods involving microscopial examination of samples directly or after in vitro cultivation in a suitable culture medium like Diamond's medium or the commercially available InPouch™TF test. These methods may be hampered by contamination of the samples with intestinal or coprophilic microorganisms, furthermore they exhibit limited technical sensitivity. Recently, two different molecular diagnostic tests based upon the polymerase chain reaction (PCR) have been developped. In the present study, the performance characteristics of these two tests were determined using diagnostic and clinical samples, and were comparatively assessed with in vitro cultivation methods. The high suitability of a PCR test based upon ribosomal RNA gene unit sequences for routine diagnostic application was thus confirmed.

INTRODUCTION

Bovine tritrichomonosis, caused by the protozoan parasite *Tritrichomonas foetus*, is a sexually transmitted disease leading to infertility and abortion (Levine 1973). Major route of transmission is the transfer from asymptomatic bulls to heifers or cows during coitus. Prevalence may be considerably high under

natural breeding conditions. Thus, infected bulls were found in 15.8% of beef herds in California, USA (BonDurant et al. 1990) and in 26.7-44.1% of ranches in Nevada, USA (Kvasnicka et al. 1989); in Saskatchewan, Canada, 6% of tested bulls were infected with *T. foetus* (Riley et al. 1995). Comparable prevalence rates were reported for Australia (Dennett et al. 1974), and Costa Rica (Perez et al. 1992). Speer and White (1991) estimated the annual losses to the US beef industry at $650 million. The widespread use of artificial insemination and the incident intensive testing of breeding animals, however, resulted in very effective control of tritrichomonosis in many European countries. For example, no cases of *T. foetus* infection have been reported till 1994 following a period of 20 years in Switzerland (Felleisen et al. 1996), and only two cases of bovine tritrichomonosis have been found in the United Kingdom in the same period (Taylor, Marshall and Stack 1994). However, a prevalence of still 2.9% was reported in herds with alternating use of natural mating and artificial insemination in Northwestern Spain (Martín-Gómez et al. 1996).

Conventional parasitological diagnosis of bovine tritrichomonosis is performed by direct microscopical examination of preputial washings from bulls and cervicovaginal secretions from female cattle. Methodical sensitivity of detection can be considerably increased by in vitro cultivation of the parasites from diagnostic samples (Thomas, Harmon and White 1990; Borchardt et al. 1992; Appell et al. 1993; Schönmann et al. 1994). An important obstacle for parasitological diagnosis, however, is the potential contamination of samples with coprophilic or intestinal trichomonadid protozoa which might be mistaken for *T. foetus* (Levine 1985; Eckert et al. 1992; Taylor, Marshall and Stack 1994). These methods can thus be time consuming and laborious, and may require special experience in the morphological identification of the parasite. When the sample contains only low parasite numbers, an accurate and species-specific diagnosis may be difficult. Alternatively, two different molecular diagnostic tests for the detection of *T. foetus* based upon polymerase chain reaction (PCR) have been independently established (Ho et al. 1994, Felleisen et al. 1998). Both PCR tests were described to be methodically very sensitive and specific.

In the present publication, we report the comparative evaluation of the two available molecular diagnostic tests with clinical and diagnostic samples, and the comparison of their diagnostic performance with in vitro cultivation techniques.

MATERIAL AND METHODS

Protozoa

A Swiss reference strain for *T. foetus* and a human *T. vaginalis* isolate were

from the Institute of Veterinary Bacteriology, University of Bern, and Dr. Mai Nguyen, Institute of Medical Microbiology, University of Bern, respectively. Different other isolates of trichomonadid protozoa were obtained from American Type Culture Collection (ATCC), Rockville MD, USA with the following reference numbers: *T. foetus* (30003, 30166, 30231, 30232, 30924, 50151, 50152); *Tritrichomonas suis* (30167, 30168, 30169); *Tritrichomonas mobilensis* (50116); *Tetratrichomonas gallinarum* (30097); *Pentatrichomonas hominis* (30098); *Trichomonas vaginalis* (30240, 30241); *Trichomonas gallinae* (30230).

In vitro cultivation of trichomonads

Trichomonads obtained from ATCC were cultivated in vitro in Diamond's medium (Diamond 1957) supplemented with 5% heat inactivated horse serum at 37°C. The InPouch™TF test for cultivation of diagnostic samples was obtained from Biomed Diagnostics (San Jose, CA, USA), and was performed according to the manufacturer's instructions.

Samples and groups of animals

Diagnostic samples were used for the comparative testing of the two PCR assays and in vitro cultivation: preputial washings were obtained from 123 bulls free of *T. foetus* infection kept in quarantine by the Swiss Association for Artificial Insemination (group A), and vaginal mucus was collected from 20 healthy cows at the Clinic for Large Animals, University of Bern (group B). Furthermore, preputial wash fluids were selectively obtained from seven different bulls with a suspected *T. foetus* infection (group C), comprising the first cases of bovine *T. foetus* infection documented in Switzerland since 1972. In the farms where these bulls originated from, additional samples were collected from 16 male (group D) and 15 female (group E) cattle with potential contact to the infected animals. Finally, 14 samples from cows were obtained from breeding farms where problems of sterility or frequent abortion have been documented over a longer period (group F).

Sampling of diagnostic material and isolation of genomic DNA.

Preputial washings and vaginal mucus were collected as described previously (Felleisen et al., 1998). Identical aliquots of the samples were in vitro cultivated either in Diamond's medium or in the InPouch™TF test, or were subjected to DNA isolation by a modified proteinase K digestion method as described previously (Felleisen 1997). Preparation of genomic DNA from in vitro

cultivated parasites in logarithmic phase of growth was performed using the same method.

Diagnostic polymerase chain reaction (PCR)

The TF-PCR (Ho et al. 1994) and the TFR-PCR (Felleisen et al. 1998) assays evaluated in this study were carried out under the same conditions as originally described, using AmpliTaq DNA polymerase (Perkin Elmer Rotkreuz, Switzerland). Primers TFR3, TFR4, TFR8-Bio, TF1, and TF2, were synthesized through Gibco-BRL, Basel, Switzerland. Uracil DNA glycosylase (UDG) was obtained from the same company. Amplification was done in a Perkin Elmer thermal cycler model 480. Aliquots of the amplification reactions then were analysed on 10% polyacrylamide and 2% agarose gels (Sambrook, Fritsch and Maniatis, 1989). As DNA molecular weight standard, bacteriophage ΦX174 DNA digested with restriction enzyme *Hae* III was used. Detection of PCR amplification products of TFR3/TFR4-PCR by a DNA enzyme immuno assay (DEIA) was performed as described by Felleisen et al. (1998).

Subcloning and sequencing

The amplification product of the TF-PCR (Ho et al. 1994) obtained with genomic DNA of the Swiss reference strain was isolated from 1.5% agarose gels using a prep-A-gene purification kit (BioRad, Glattbrugg, Switzerland) and subcloned into the unique *Eco* RV site of plasmid vector pBluescript KS^+. Sequencing was performed with Sequenase 2.0 (USB-Amersham, Zürich, Switzerland) and α-^{35}S dATP following the manufacturer's protocol. Sequencing primers KS and SK located adjacent to the polylinker region were used.

RESULTS

As a first step, the methodical sensitivity of the in vitro cultivation methods employing Diamond's medium (Diamond 1957) and the commercially available InPouch™TF test (Biomed Diagnostics) were evaluated under diagnostic sample conditions and with defined numbers of *T. foetus* organisms (Fig. 1). In general, Diamond's medium turned out to be more sensitive than the InPouch™TF test. Inoculation with ten individual cells was sufficient to result in positive cultures, independent of the sample type analyzed. In contrast, sensitivity of the InPouch™TF test was very much dependent on the sample composition. While cells added to preputial wash fluids displayed growth characteristics comparable to those grown in Diamond's medium, parasites grown in the presence of vaginal mucus and in the absence of diagnostic substrates died during cultivation.

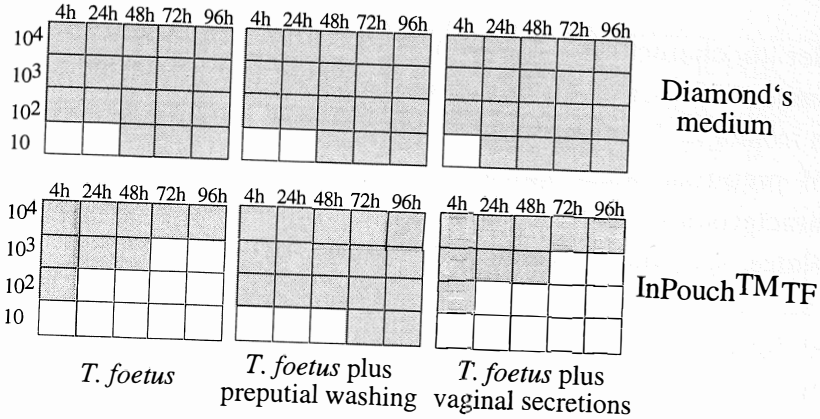

Fig. 1: Methodical sensitivity of in vitro cultivation methods. Defined numbers of *T. foetus* organisms (reference strain) were added to 1 ml of a preputial washing pool (from 11 healthy bulls) or 1 ml of a vaginal mucus pool (from 10 uninfected cows), and cultivated in Diamond's medium and the InPouch™TF test. In parallel, parasites were cultivated without the addition of diagnostic substrates. Cultures were inspected at time points indicated. Filled squares indicate cultures where parasite growth was observed, in cultures with open squares no living parasite cells were found.

Consequently, the time point of microscopic examination of the InPouch™TF test turned out to be very important with these cultures (Fig. 1). Thus, inoculation with 10^4 parasites was necessary to generate a positive result after 4 days of cultivation, while cultures inoculated with less parasites were positive after cultivation for few hours only (Fig. 1).

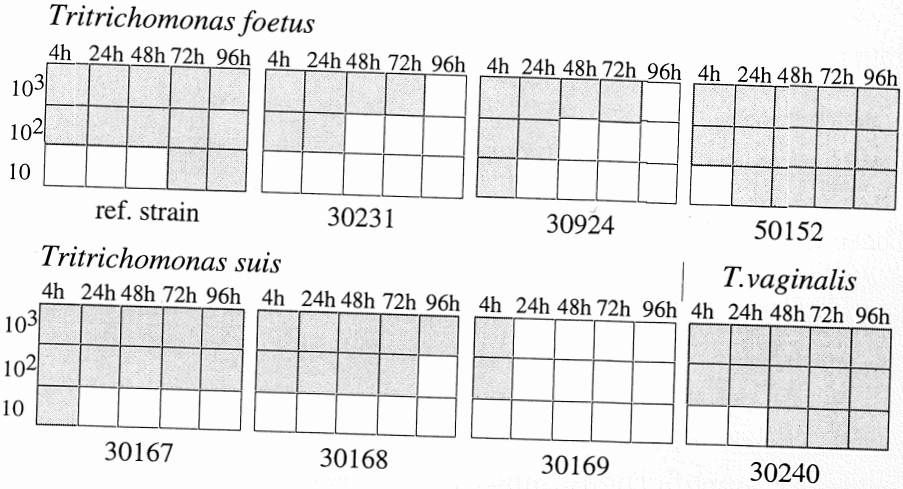

Fig. 2: In vitro cultivation in the InPouch™TF test. Serial dilutions of different isolates were added to 1 ml of a preputial washing pool (from 11 healthy bulls). Cultures were inspected by microscopy at times indicated. Filled squares represent cultures with, and open squares cultures without living organisms detected.

In order to characterize potential variability in growth characteristics, defined numbers of parasites representing various isolates of *T. foetus* and *T. suis*, and an isolate of *T. vaginalis* were inoculated in the InPouch™TF test in the presence of preputial wash fluids (Fig. 2). A remarkable variability in the growth characteristics of the different *T. foetus* isolates was observed. While certain isolates seemed to adapt well to the InPouch™TF culture medium (reference strain and 50152), other strains died out soon in this culture medium (30231 and 30924). The same variability was observed with the *T. suis* strains studied. The diagnostic sensitivity of the InPouch™TF test thus appeared very much dependent on the parasite isolate encountered. The InPouch™TF test obviously was not selective for tritrichomonads, but supported also the growth of other trichomonadid organisms, such as *T. vaginalis* which was well proliferating in this medium.

For a further evaluation, the two in vitro cultivation techniques were comparatively assessed with diagnostic substrates obtained by routinely applied sampling techniques (Table 1). Frequent problems with bacterial or fungal contaminations were encountered when Diamond's medium was used for in vitro cultivation: In total, 47.8% of these samples (17.7% cows/56.1% bulls) could not be analyzed due to bacterial or fungal contamination (Table 1). In contrast, no contamination problems were observed with the InPouch™TF test.

Table 1: Comparison of methods for in vitro cultivation of diagnostic samples.

sample type	Diamond's medium			InPouch™TF test			total
(group)	neg	pos	cont.	neg	pos	cont.	
(A) bulls (controls)	54	0	69	123	0	0	123
(B) cows (controls)	18	0	2	20	0	0	20
(F) cows (diagnostic)	10	0	4	14	0	0	14

These and additional diagnostic samples were comparatively assessed with the two different PCR assays (Table 2): about one third of the control samples (groups A and B; 31% and 33% of bulls and cows, respectively) gave rise to an amplification product with the TF-PCR (Ho et al. 1994). Most of these fragments upon detection by agarose gelelectrophoresis were slightly larger than the expected diagnostic band. We thus assumed that these fragments were derived from unspecific amplifications. Similar, size-different products were also found when culture negative diagnostic samples were analyzed (groups D, E, and F).

The use of the InPouch™TF test resulted in the detection of flagellated protozoa in seven samples (group C). However, except for one sample where subsequently these organisms could be morphologically identified as *T. foetus* by

Table 2: Performance of the different tests with diagnostic samples.

sample type	InPouch™TF test		TF-PCR		TFR-PCR		total
(group)	neg.	pos.	neg.	pos.	neg.	pos.	
(A) bulls (control)	123	0	84[c]	38[a]	121[d]	0	123
(B) cows (control)	20	0	15	5[a]	20	0	20
(C) bulls (diagnostic)	0	7	2	5	1[b]	6	7
(D) bulls (diagnostic)	16	0	14	2[a]	16	0	16
(E/F) cows (diagnostic)	29	0	20	9[a]	29	0	29

[a] PCR product larger than diagnostic band; [b] slightly larger PCR-product, negative in DEIA.
[c] due to inhibition, one sample not analyzed, [d] due to inhibition, two samples not analyzed.

a French reference laboratory, all other samples contained not enough organisms to allow unambigous morphological identification. All other control and diagnostic samples showed no detectable growth of protozoa. From the seven culture positive samples, five reacted positive in the TF-PCR (group C).

In the TFR-PCR, in contrast to the TF-PCR, no unspecific amplicons were observed with genomic DNA of the control samples (groups A and B) and culture negative diagnostic samples (groups D, E, and F). From the seven culture positive bulls (group C), six reacted clearly positive in the test. In one sample, a slightly larger PCR product was found which, in contrast to the six positive samples, reacted negative in the DEIA assay upon confirmation. Of the 195 samples tested in total, only one and two samples, respectively, could not be analyzed by the two PCR assays due to inhibition of the amplification reaction.

The target sequence of the TF-PCR has not been published yet. In order to identify putative restriction sites located in the amplified fragment which could allow to discriminate between products of specific and unspecific primer annealing by restriction enzyme digestion of PCR products, the TF-PCR product of the Swiss reference strain was subcloned and its sequence determined (Fig. 3).

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CATTATCCCAAATGGTATAACATTTAGGACTTAAAGCTATGCAATTATCACCTTGTTTTT    60
GTAATAGGGTTTACCATATTGTAAATCCTGAATTTCGATACGTTAATAGTGGAACAAAAA

CAACAGCAAGACCTAATATTTTCTTTTCATCATTAATGCCTTTTGATGGATCAGGCAACC   120
GTTGTCGTTCTGGATTATAAAAGAAAAGTAGTAATTACGGAAAACTACCTAGTCCGTTGG

ATTTATAAAATATGTTCATTATAGAATTTATGTACTTAATGAC     162
TAAATATTTATACAAGTAATATCTTAAATACATGAATTACTG
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Fig. 3: Sequence of the PCR fragment of the TF-PCR test (Ho et al., 1994). Primer-derived sequences are underlined, the unique *Sau* 3A restriction site (GATC) is highlighted.

The sequence contained a unique restriction site for *Sau* 3A (Fig. 3) which upon digestion of the TF-PCR product generated two fragments of 109bp and 53bp which could be readily distinguished by polyacrylamide gel electrophoresis. However, the slightly larger PCR products could not be digested by *Sau* 3A (data not shown), thus pointing towards a lacking identity with the *T. foetus*-specific sequence.

For decades, the taxonomy within the genus *Tritrichomonas* was the subject of controversal discussion (see literature in Felleisen 1997): it was speculated that *T. foetus* and *T. suis*, the tritrichomonads from cattle and swine, respectively, belong to the same species. Recently, this open question could be mostly clarified by comparative sequencing of the 5.8S rRNA genes and the flanking internal transcribed spacer regions ITS1 and ITS 2 (Felleisen 1997) and by random amplified polymorphic DNA (RAPD) analyses of the two species in question (Felleisen 1998), which unambiguously demonstrated genomic identity of these two parasites. Consequently, the TFR-PCR which is based upon sequences of the rRNA gene unit recognized isolates from both parasites. To clarify if the TF-PCR assay (Ho et al. 1994) was also suitable for the detection of genomic DNA from *T. suis*, we comparatively evaluated the specificity of the two PCR assays with genomic DNA purified from different *T. foetus* and *T. suis* isolates (Fig. 4). Furthermore, other related trichomonadid protozoa were included.

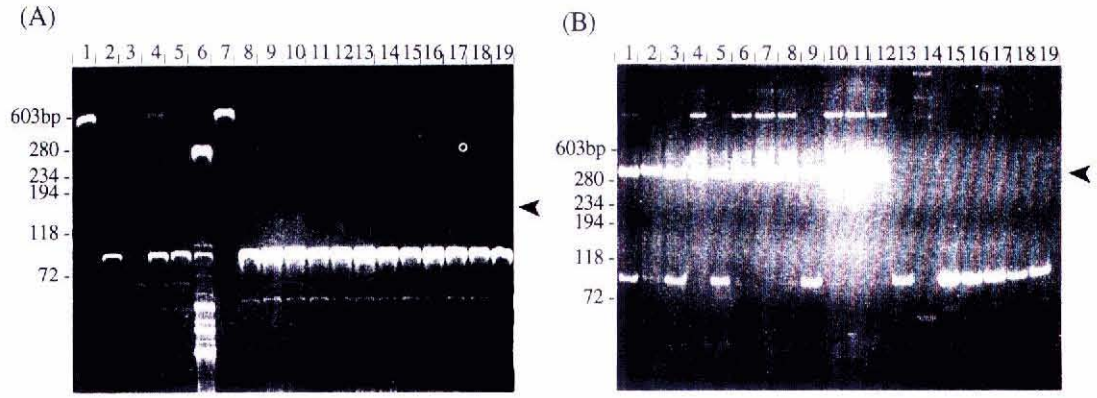

Fig. 4: Comparative analysis of specificity of (A) TF-PCR and (B) TFR-PCR with DNA from *T. foetus* (1-8) and related trichomonads: *T. suis* (9-11), *T. mobilensis* (12), *T. gallinarum* (13), *P. hominis* (14), *T. vaginalis* (15-17), and *T. gallinae* (18). Lane 19: Negative control. Positions of diagnostic bands are indicated by arrows (all others are diagnostically irrelevant).

With both tests, all eight *T. foetus* strains analyzed gave rise to amplification products of the expected size. Furthermore, for both assays amplicons of the same size as with *T. foetus* were obtained using DNA of *T. suis* and *T. mobilensis* as a template (Fig. 4). With the TFR-PCR, no amplification was

observed with genomic DNA from all the other related trichomonads (Fig. 4B). However, with the TF-PCR, week but clearly detectable amplification products were observed with genomic DNAs from *Trichomonas vaginalis, T. gallinae, T. tenax,* and *Pentatrichomonas hominis* (Fig. 4A). Of the trichomonads tested, only *Tetratrichomonas gallinarum* reacted negative.

DISCUSSION

Strict surveillance of breeding-bulls with sensitive and accurate diagnostic methods is still one of the most effective measures for the prevention of bovine tritrichomonosis to date. In the present study, we comparatively assessed conventional parasitological and novel molecular techniques for the diagnostic detection of *T. foetus* infections in cattle.

The in vitro cultivation methods have the advantage of a direct etiological proof for the presence of the parasite in diagnostic samples. However, suitability of the Diamond's medium (Diamond 1957) for primary in vitro cultivation seemed to be limited by relatively frequent contaminations of diagnostic samples with bacteria or fungi. In contrast, problems with contaminations were relatively rare with the commercially available InPouch™TF test. The methodical sensitivity of this in vitro culture system, however, seemed to be limited and in addition very much dependent on the parasite isolate and biological sample composition. Nevertheless, the InPouch™TF test may be the medium of choice for diagnostic in vitro cultivation.

In contrast, the methodical sensitivity of both PCR assays evaluated in the present study principally is very high. Both PCR assays could not discriminate between the three *Tritrichomonas* species analyzed, *T. foetus, T. suis* and *T. mobilensis*. However, a differentiation of these species with respect to bovine infection seems not to be necessary, because, based on molecular biological analyses, it appeared evident that *T. foetus* and *T. suis* represent the same species (Felleisen 1997; 1998). On the other hand, *T. mobilensis* was described as a parasite of the Bolivian squirrel monkey (Pindak et al. 1985; Culberson et al. 1986), and thus its occurence in cattle appears to be very unlikely.

The TF-PCR (Ho et al. 1994) was frequently hampered by unspecific amplicons. Potentially, these unspecific PCR products may be explained by the presence of contaminating coprophilic or intestinal protozoa in these diagnostic samples which might be occasionally occuring (Eckert et al. 1992; Taylor, Marshall and Stack 1994). Furthermore, comparative evaluation of the specificity of the TF-PCR assay in our study revealed the presumable presence of the respective primer sequences also in other trichomonadid protozoa. We could

show that identification of the specific amplicons of TF-PCR and discrimination from products of unspecific primer annealing by restriction enzyme digestion and electrophoretic analysis is in principle possible, but these methods are laborious and time consuming.

In contrast, using the TFR-PCR (Felleisen et al. 1998), only one of 195 samples analyzed showed an amplification product which had a size differing from that of the actual diagnostic fragment. The respective amplicon could be identified unambigously as unspecific by a DNA enzyme immuno assay (DEIA), thus demonstrating the helpfulnes of that technique in the confirmation of positive PCR results. The absence of unspecific products may be due to a higher specificity of the primer sequences used for TFR-PCR which is reflected by the absence of amplification with genomic DNA of trichomonadid protozoa related to *T. foetus*. The slightly larger unspecific product of the TFR-PCR was obtained with a sample which was positive by in vitro cultivation with the InPouch™TF test. This sample turned out to be also negative in the TF-PCR assay, and furthermore, also the in vitro culture sediment was negative in both PCR assays (data not shown). It is thus reasonable to assume that other flagellated protozoa growing in the respective culture may have been mistaken for *T. foetus* by light microscopy. Consequently, the PCR assays can act as a reliable and specific confirmation test for in vitro grown organisms.

As a preventive measure, the Uracil DNA glycosylase (UDG) system, which represents an important and helpful tool to avoid carry-over contaminations through products of previous amplification reactions (Longo et al. 1990), is included in the TFR-PCR assay. Adaptation of the TF-PCR to this system would be recommendable. The methodical sensitivity of both PCR assays in principle is considerably high. However, it was not possible to draw a definite conclusion from our experiments about the diagnostic sensitivity of the two PCR assays because the number of proven culture positive samples was not large enough to allow statistical evidence.

In conclusion, the InPouch™TF test is clearly superior to Diamond's medium for diagnostic in vitro cultivation. Both PCR assays may be applied as confirmation test for in vitro culture sediments of samples with protozoa growing in the InPouch™TF test. In principle, they are also suitable as a direct screening test for *T. foetus* in diagnostic and clinical samples. However, the TF-PCR is hampered by frequently observed unspecific amplicons. Thus this technique potentially may cause difficulties in routine diagnosis. In contrast, the TFR-PCR exhibited a good diagnostic performance, and we suggest to further evaluate it for routine veterinary diagnostic application.

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