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Original Research

Comparative Sensitivity and Specificity of Polymerase Chain Reaction Assays for the Detection of *Theileria equi* Coupled With Three DNA Template Extraction Methods

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ABSTRACT

The present study demonstrates the relative sensitivity and specificity of two 18S rRNA gene-based polymerase chain reaction (PCR) assays (designated as PCR_1 and PCR_2) coupled with three genomic DNA extraction methods viz., via Whatman filter paper#1 method (Ext 1), Whatman Flinders Technology Associate (FTA) elute microcards method (Ext 2), and Qiagen DNeasy Blood & Tissue Kits (Ext three3) for the detection of *Theileria equi*. Both PCR assays on the nucleic acid of 72 field blood samples extracted by Ext 1 and Ext 3 revealed an prevalence rate of 2.77 and 18.56%, respectively; whereas in case of Ext 2, PCR_1 and PCR_2 revealed 16.67% and 18.56% prevalence, respectively. Ext 1 showed only 15.38% sensitivity and 100% specificity, whereas Ext 2 displayed 92.3% to 100% sensitivity and 100% specific results with respect to Ext 3. The amplified products were clearly positive with a diminishing signal up to 10^{-6} DNA dilutions in Ext 2 and Ext 3 method in both PCRs. Ext 1 was able to detect only 10^{-3} and 10^{-4} DNA dilution. Based on these results, it was concluded that blood sampled on FTA cards for DNA extraction is the recommended approach with detection ability up to 10^{-6} dilution.

1. Introduction

Equine piroplasmosis (EP) caused by *Babesia (Theileria) equi*, a hemoprotozoan parasite of red blood cells (RBCs) of phylum Apicomplexa, order Piroplasmida, possess a worldwide threat on the horse industry [1–3]. Although more than 35% latent infection have been reported in India [4], Pakistan [5], Mongolia [6], and China [7,8], but only few sporadic clinical case reports are available [9,10].

Theileria equi parasites are commonly revealed in stained blood films during the acute phase of infection [11]. However, during pauciparasitemia (individuals with few parasites detected in the blood) in latent infections, it is tremendously problematic to perceive parasites in blood films [12]. Furthermore, the diagnosis of *T. equi* by recognizing this hemoparasite through classical microscopy is a cumbersome procedure due to diminutive size of the parasite. On the other hand, serologic methods proffer the limitation of crossreactivity and failure to differentiate between the past and current infection. The molecular methods for appropriate recognition of parasitic agents in clinical samples continue to increase in the due course of time and promise to play an imperative role in investigative centers across the world [13]. Many reports have been cited in the literature regarding the nucleic acid–based amplification (polymerase chain reaction [PCR]) targeting this hemoparasite, but reports regarding the parasitic DNA recovery methods for PCR are still lacking. Ample amount of DNA recovery acts as a turning point; as better PCR amplification is always directly proportional to a decent/appropriate quantity of parasitic DNA. Standardization of

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PCR assay for *T. equi* and *Babesia caballi* is difficult as in earlier days many reports had been published targeting different genes (18S ribosomal ribonucleic acid [rRNA], 16S rRNA [14], equine merozoites antigen-1, and rhoptry-associated protein 1) with different methods of sample preparation [15]. However, these protocols have never been simultaneously compared.

In developing country, like India with both hot and cold climatic ranges along with limited amount of financial resources, it is important to consider the challenges linked to create laboratories with PCR facility. Although molecular techniques offer precise diagnostic gadget, however, these approaches proffer various hurdles in the field applications. FTA cards are gaining popularity due to the ease of samples collection and DNA extraction preventing deterioration of DNA in the collected samples. Hence, the present study was planned to demonstrate the relative sensitivity and specificity of PCR assays targeting 18S rRNA gene for detection of EP coupled with three genomic DNA isolation methods viz., Whatman filter paper#1, Whatman FTA elute microcards, and Qiagen DNeasy Blood & Tissue Kits.

2. Materials and Methods

2.1. Ethical Aspects (Consent Statements)

The Ethics Committee for Animal Experiments from the Guru Angad Dev Veterinary and Animal Sciences University granted an approval (GADVASU/2014/IAEC/22/002, on 14-07-2014) for the conduction of work. Prior consent was taken from the owners of the equines. Complete care and measures were taken to avoid any accidental injury to the equine while collecting the blood samples.

2.2. Animal Samples

A total of 72 blood samples from Ferozepur (29 samples), Patiala (21 samples), and Nawanshahr districts (22 samples) of Punjab were collected on filter papers (Whatman filter paper#1), Whatman FTA elute microcards, and ethylenediaminetetraacetic acid (EDTA)-coated vials during the year 2014 to study the status of infection at the expected prevalence of 50% with confidence limits of 95% and a desired absolute precision of 5% [16]. *T. equi* were cultured in horse RBCs in vitro by continuous microaerophilous stationary phase technique (6% parasitemia) and used as positive control, whereas for negative control, nuclease-free water was used. These *T. equi* infected RBCs were obtained from splenectomized experimental horses maintained at NRCE (National Research Centre on Equines). The RBCs were then washed twice in 1 × phosphate-buffered saline, and cell suspensions diluted to 1% parasitemia were transferred to Whatman filter paper#1, Whatman FTA elute microcards, and 2-mL collection vial for DNA extraction procedure Ext 1, Ext 2, and Ext 3, respectively, as mentioned below.

2.3. Genomic DNA Extraction Methods

Whatman filter paper#1 method (Ext 1): 3-mm discs (approximate 25 mg) were punched out followed by protocol of Qiagen DNeasy Blood & Tissue Kits. DNA was finally eluted in 120 μL of elution buffer and then stored at −20°C for further use. Moreover, before storage, the purity of DNA was determined by UV spectroscopy using a Nanodrop 1,000 spectrometer (Thermo Scientific, USA). The recorded purity fell between 1.65 and 1.85.

Whatman FTA elute microcards method (Ext 2): The DNA was extracted as per manufacturer instructions (Whatman; GE Healthcare Life Sciences; cat no. WB120410). The eluted DNA was kept at −20°C till further PCR analysis.

Qiagen DNeasy Blood & Tissue Kits (Ext 3): Genomic DNA was extracted from the EDTA blood vials by using the standard protocol of DNeasy Blood & Tissue Kits (Qiagen Science, Germantown, MD).

2.4. Polymerase Chain Reaction

PCR_1: PCR_1 reactions were performed using primers BeqF1/BeqR1 (Table 1). The reaction mixture composition PCR reaction mixture (25 μL) was constituted by 12.5 μL of KAPA 2G Fast HotStart Ready Mix (2X containing KAPA2G Fast HotStart DNA polymerase, KAPA 2G Fast HotStart PCR buffer, 0.2 mM dNTP each, 1.5 mM $MgCl_2$), 1.5 μL of 10 pmol BeqF1/BeqR1 primers, 5 μL of template DNA (from Ext 1, 2, and 3, separately) and added 4.5 μL of nuclease-free water to complete the volume. The reaction tube was placed in automated thermocycler with the following program: the initial denaturation at 94°C (5 minutes), 35 cycles of denaturation at 94°C (15 seconds), annealing at 57°C (30 seconds), extension at 72°C (1 minute), and the final extension at 72°C (5 minutes).

PCR_2: For PCR_2 reaction, pair of primers Universal Forward Primer (UFP)/EquiR (Table 1) was used. As described in PCR_1, the reaction was carried out, but primer used here was UFP/EquiR. Thermocycler with the initial denaturation at 96°C for 10 minutes, 35 cycles with denaturing at 96°C (1 minute), annealing at 60.5°C (1 minute), extension at 72°C (1 minute), followed by a final extension at 72°C (10 minutes) was followed.

Five-microliter PCR products from each reaction mixture were run on a 1.5% agarose gel with a 1% Tris borate-EDTA buffer at 95 volt for 4 hours. Gels were stained with ethidium bromide and visualized under UV light.

Table 1
Description of oligonucleotides used in PCR assay.

PCR Assay	Oligonucleotide Sequences	Tm (Melting Temperature)	G-C (Guanine-Cytosine) Content	Product Size
PCR_1 [17]	BeqF1: 5′-TTCGTTGACTGCGCTTGGCG-3′ BeqR1: 5′-CTAAGAAGCGGAAATGAAA-3′	57.1°C–73.8°C	37–60	709 bp
PCR_2 [18]	UFP: 5′TCGAAGACGATCAGATACCGTCG-3′ EquiR: 5′TGCCTTAAACTTCCTTGCGAT-3′	55.9°C–62.4°C	43–52	430 bp

Abbreviation: PCR, polymerase chain reaction.