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Quantitative PCR detection of *Theileria equi* using laboratory workflows to detect asymptomatic persistently infected horses

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ABSTRACT

Equine piroplasmosis is the most important tick-borne disease of horses. Regulations on movement of horses into disease-free countries are in place to preserve international trade. Introduction of infectious disease, such as equine piroplasmosis, into non-endemic countries remains a substantial risk owing to the wide-spread distribution of vectors. Identification and restriction of movement of *Theileria equi* persistently infected horses is an integral part of control strategies, because persistently infected horses with low parasitaemia are an important reservoir. We used real-time PCR for diagnosis of *T. equi* DNA in clinically healthy horses in an equine piroplasmosis endemic area. The sensitivity was assessed using a synthetic plasmid DNA and a laboratory workflow was developed to maximise detection of persistently infected horses. The detection limit was 10 rDNA copies of the plasmid DNA. Assuming that each red blood cell contains a single *T. equi* genome the detection limit corresponded to 2.5 *T. equi*/μl of total blood and parasitaemia as low as $2–3.8 \times 10^{-5}$%. A laboratory workflow was developed and assessed on samples from Saudi Arabia. The laboratory workflow focused on samples returning no or single positive result in duplicate PCR. In total, we obtained 42% (59/141; 95% confidence interval: 33.85–50.15) *T. equi* positive samples, 26% (37/141) negative for *T. equi* samples. The remaining 45 samples were judged as suspect with no definitive diagnosis made. The Saudi Arabia's *T. equi* small subunit ribosomal DNA (SSU rDNA) sequencing ($n = 16$) demonstrated A clade ($n = 15$) as the dominant *T. equi* clade. Clade B was sequenced in a single case. We present an approach for diagnostic workflow to detect *T. equi* in clinically healthy but persistently infected horses. Results from Saudi Arabia confirm that *T. equi* is widespread in the Middle East region. High proportion of horses with low parasitaemia calls for caution with results based on a single blood sample. Understating of the fluctuation of the parasitaema in persistently infected horses in endemic areas is needed to establish the required sample numbers for reliable detection of *T. equi*.

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1. Introduction

International trade of horses, whether for sport or breeding has amplified over the years to a multimillion dollar industry (Friedhoff et al., 1990). Regulations and restrictions on movement of horses into disease-free countries such as North America, Australia, New Zealand and Japan are in place (Bose et al., 1995; Bruning, 1996; Kappmeyer et al., 2012). However, even with such control in place, introduction of infectious disease such as equine piroplasmosis, into non-endemic countries remains a substantial risk owing to the wide-spread distribution of tick vectors and trade with endemic neighbouring countries (Short et al., 2012).

Equine piroplasmosis caused by *Theileria equi* and *Babesia caballi* is considered the most important tick-borne disease of horses in tropical and subtropical regions of the world (Schein, 1988). Transmission of infection relies on the presence of tick vectors from within the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* (Wakler et al., 2000; Jongejan and Uilenberg, 2004). The disease is characterised by fever, anaemia, hemoglobinuria leading to death, with *T. equi* infections usually more severe than infections with *B. caballi* (Kuttler et al., 1988; Sigg et al., 2010).

Horses acutely infected with *T. equi* eventually recover but remain persistently infected with low *T. equi* parasitaemia (Ueti et al., 2008; Kappmeyer et al., 2012). Clinical signs of equine piroplasmosis are absent in persistently infected horses (Ueti et al., 2005). Ticks feeding on persistently infected horses with low *T. equi* parasitaemia (2×10^3 to 10^6 ml^{-1} of blood) have successfully transmitted the parasites to naïve horses (Ueti et al., 2005, 2008). Since persistently infected horses are an important reservoir for *T. equi* transmission, identification and the restriction of movement of *T. equi* persistently infected horses is an integral part of control strategies.

Diagnosis of *T. equi* is traditionally based on serological methods, including competitive-inhibition ELISA (cELISA) test, indirect fluorescent antibody test (IFAT) and the complement fixation (CF) test developed for the detection of specific antibodies (Donnelly et al., 1982; Knowles et al., 1991; Bruning et al., 1997; Alanazi et al., 2012). In an attempt to overcome diagnostic limitations in persistently infected horses, molecular diagnostic methods capable of detecting low *T. equi* parasitaemia from peripheral blood samples have been developed (Alhassan et al., 2005; Kim et al., 2008; Bhoora et al., 2010). Owing to its inherent sensitivity, quantitative real-time PCR technology is increasingly becoming a diagnostic standard for equine piroplasmosis from peripheral blood of horses (Kim et al., 2008; Bhoora et al., 2010; Kappmeyer et al., 2012).

The objective of this study was to establish the sensitivity and specificity of a real-time PCR workflow for diagnosis of *T. equi* DNA in clinically healthy horses in an equine piroplasmosis endemic area. The sensitivity was assessed using a synthetic plasmid DNA and a laboratory workflow was developed to maximise detection of persistently infected horses. The laboratory workflow was assessed on samples from equine piroplasmosis endemic area in Saudi Arabia.

2. Materials and methods

2.1. Horse blood samples

Blood samples were collected from 141 clinically healthy adult sporting horses from the city of Riyadh, Saudi Arabia (latitude 34–38° north and longitude 43–46° east; June 2012 to September 2013). Sample were collected (5–10 ml) from the jugular vein into vacuum tubes without anticoagulant (BD Vacutainer® Tube, Gribbles Pathology, VIC, Australia) and transported to the Laboratory of Parasitology, Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University for DNA extraction. Blood collection was approved by the Ethical and Human Research Committee, Shaqra University.

Total genomic DNA (gDNA) was isolated using the DNeasy Blood and Tissue kit (Qiagen, Australia) and eluted in 50 or 100 μl of elution buffer following the manufacturer's instructions. An aliquot of gDNA from each sample was storage at −80 °C prior to being sent to the Parasitology Laboratory, Faculty of Veterinary Science, University of Sydney for real-time PCR analysis.

2.2. Plasmid construction and real-time PCR standard curve

A 2674 bp plasmid with a backbone (pMA-T) and the small subunit ribosomal DNA (SSU rDNA) belonging to *T. equi* was synthesised (GeneArt, Life Technologies, Australia). The plasmid was transformed into α-gold competent cells (BioLine, Australia) for clone amplification. Three transformed colonies were chosen at random and plasmid extractions were carried out using the QIAprep Spin miniprep kit (Qiagen, Australia) according to the manufacturer's instructions. For quality control purposes, plasmid concentration was quantified using a Nanodrop 1000 (Thermo, Australia) and the insert was sequenced using M13 primers (Macrogen Ltd., Seoul, Korea) and verified by an *in silico* alignment.

For PCR, the plasmid was linearised using *Kpn*I. Plasmid copy numbers were calculated and 10× serial plasmids dilutions (10^7–10^{-1}) to produce standard curves for copy number quantification. The sensitivity *in vitro* using synthetic plasmid DNA should be interpreted as experimental because primer access to the target site on the plasmid and genome can be different.

2.3. Assessment of the real-time PCR assay

Genomic DNA isolated from horse blood samples were tested for the presence of horse DNA using a real-time PCR assay developed by the European Union Reference Laboratory for Animal Proteins (EURL-AP), Walloon Agricultural Research Centre (Gembloux, France). The EURL-AP protocol features a TaqMan probe that targets horse mitochondrial DNA (mtDNA) to amplify a 87 bp fragment. The PCR mix contained the forward primer (5′-CGA TCC CCT ATC AGC C-3′), the reverse primer (5′-TCC TTA GAT AGA TGG TGT TGG-3′), a probe (5′-TTC TGG TGT TGA CAA CAT GAC TAC TG-3′) and the reporter dye (FAM – position 5′ of the probe) along with a quencher dye (BHQ1 – position 3′ of the probe).