Veterinary Parasitology 211 (2015) 293–299

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Multiplex PCR for detection of *Trypanosoma evansi* and *Theileria equi* in equids of Punjab, India

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ARTICLE INFO

Article history:
Received 31 December 2014
Received in revised form 20 May 2015
Accepted 22 May 2015

Keywords:
Trypanosoma evansi
Theileria equi
Multiplex PCR
Prevalence
Risk factors
Punjab

ABSTRACT

Multiplex PCR for simultaneous detection of *Trypanosoma evansi* and *Theileria equi* in single-step reaction was optimized and employed on 108 equids (99 horses and 9 donkeys/mules) blood samples collected from two agro-climatic zones (Sub-mountain undulating zone and Undulating plain zone) of Punjab to evaluate the status of concurrent infection and associated risk factors. The amplification products of 257 and 709 bp targeting repetitive nucleotide sequence of variable surface glycoproteins of *T. evansi* and 18S rRNA gene of *T. equi*, respectively expressed high fidelity of the primer pairs with sequence homology to neighboring geographic isolates. The overall prevalence of *T. evansi* and *T. equi* was 3.7 and 1.85%, with Undulating plain zone at higher infection risk for *T. equi* (OR = 3.24, 95% CI = 0.28–83.65); and Sub-mountain undulating zone (OR = ∞, 95% CI = 0.25–∞) for *T. evansi*. Multiplex PCR revealed higher risk of infection of both *T. equi* (OR = 6.75, 95% CI = 0.58–175.38) and *T. evansi* (OR = 2.11, 95% CI = 0.05–80.36) in the farms with inappropriate management system. The risk factor associated with the type of host species had an odds ratio of 12.35 (95% CI = 0.29–508.37) for donkeys/mules versus horses for *T. evansi* infection. This group was also at higher risk of infection with Odds ratio (OR) of 4 (95% CI = 0.14–53.99) for *T. equi*. The current investigation brings out various commodities at risk of infection pertaining to equid trypanosomosis and theileriosis evaluated by a rapid and sensitive multiplex PCR assay.

1. Introduction

Trypanosomosis and theileiriosis are two economically important vector-borne diseases of equids (horses, donkeys and mules) in tropical and subtropical parts of the world including India. In the Indian subcontinent trypanosomosis caused by *Trypanosoma evansi* [Kinetoplastid; haemoprotozoan] is known as 'surra', (a Hindi word meaning '*rotten*') (Soulsby, 1982). Surra is characterized by a fluctuating parasitaemia with periods of paroxysms and intermissions, anemia, edema of the legs, dehydration, fever, abortion, and incoordination, followed by paralysis of the hind limbs (Gill, 1991). Theileiriosis, caused by an apicomplexan parasite (*Theileria equi*), is clinically characterised by fever, haemolytic anemia, icterus, petechial haemorrhages of the mucous membranes etc., but these are variable and are non-specific (Baptista et al., 2013). The disease renders negative effects on the health of equids, decreasing their productivity and work efficacy. *T. equi* infection lasts for 7–12 days, but can be peracute with death occurring in 1–2 days, or can be chronic, lasting for weeks. Mortality rate associated with this disease can vary for 10–50% (Taylor et al., 2007). Imidocarb dipropionate @ 4 mg/kg intra-muscularly on four occasions at 72 h intervals eliminates *T. equi* from horses (Berlin et al., 2010) but this dose is lethal to donkeys (Soulsby, 1982). A single dose of diminazene aceturate @ 3.5 mg/kg intra-muscularly eliminates *T. evansi* from equids (Berlin et al., 2010). *T. evansi* can also be successfully managed with Quinapyramine sulphate and a chloride combination [Triquin] (Kumar et al., 2012).

In northern India, *T. evansi* is transmitted mechanically by biting hematophagous flies (Tabanids) (Sumba et al., 1998) and *T. equi* is mainly transmitted by longistrate tick, *Hyalomma anatolicum anatolicum*, causing significant morbidity and mortality in equids (Kumar et al., 2007). As the vectors of both the diseases are habitual to hot plain areas of south-western Punjab, the epidemiological status of these infections in north-eastern parts of the province still remains unexplored.

T. evansi and *T. equi* are routinely diagnosed by conventional parasitological techniques (stained blood smears), serological (CATT/*T. evansi*, LATEX/*T.evansi*, IFAT, ELISA, etc) and molecular techniques.

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http://dx.doi.org/10.1016/j.vetpar.2015.05.018
0304-4017/

For the demonstration of parasite in blood, examination of stained thin blood smears is not a sensitive method, as the parasite has a periodically cryptic nature. Serological methods proffer the limitation of cross reactivity and failure to differentiate between past and current infection. For the diagnosis of latent infection, molecular techniques (PCR) give a promising result with greater levels of sensitivity and specificity (Bashir et al., 2014). Since both *T. equi* and *T. evansi* infections have almost similar types of signs and their vectors co-exist in suitable tropical climatic conditions, as in Punjab, it becomes necessary to optimize an economical, specific, and sensitive technique for simultaneous detection of these two diseases in suspected equines. Further, to evade the expenses on epidemiology of two important haemoparasites in comparatively quiescent zone, their simultaneous detection in a single reaction by multiplex PCR will be both time and cost effective. Hence, the purpose of the present study was to employ multiplex PCR assay for one-step detection of clinical, and latent forms of *T. evansi* and *T. equi* infections, for assessing the prevalence of these infections in a Sub-mountain undulating zone and Undulating plain zone of Punjab state and to further evaluate the risk factors contributing to the prevalence of these infections in equids for their proper control strategies.

2. Materials and methods

2.1. Ethical aspects

The study has the approval (IAEC/2014/46-73) of the ethics committee for animal experiments duly constituted by the Guru Angad Dev Veterinary and Animal Sciences University. Blood samples were collected in a humanly manner, so as to avoid any accidental injury to the equids. A prior consent of the equines keepers was also sought.

2.2. Study area and sampling

The province of Punjab covers a total area of 50,362 square kilometres between 2930′N to 3232′N latitude and 7355′E to 7650′E longitudes. In Punjab state lie areas between altitudes 213 meters and 959 meters above sea level (http://punjabjudiciary.gov.in/index.php?trs=gurdist). There are about 34,000 equids which are at risk of infection due to haemoparasite in Punjab (Fazili and Kirmani, 2011). The present study was conducted in north-eastern Punjab comprising of Sub-mountains undulating zone (SMZ) and Undulating plain zone (UZ) lying in the foothill areas of the Zaskar Range, the Pir Panjal Range, and part of the Siwalik Range. To study the status of molecular prevalence of the concurrent infection of *T. equi* and *T. evansi*, the expected prevalence was considered 10% with confidence limits of 95% and a desired absolute precision of 5% to collect maximum number of samples (Thrusfield, 2005). The number of samples thus calculated was adjusted for finite population and was correlated with 108 samples (99 horses and 9 donkeys/mules) that were collected for 36 discrete premises of four districts of Punjab (Table 2). Blood (~3 ml) was drawn from jugular vein of each animal into anticoagulant-coated vacutainers for microscopy and nucleic acid extraction. The information related with sampled animal (species, age, sex, health status and purchase history, if any) and to the farm (management, and presence of vectors and other in contact domestic ruminants) was collected from the owner on pre-designed questionnaire. The equine keepers following improper managemental practices of rearing their stock *viz.* stables with kacha floor, poor sanitation and unbalanced feeding program were classified as 'unorganised farms' while those pursuing appropriate scientific managemental schedule were considered 'organised farms'. The animals were thoroughly screened for the presence of ticks especially in area around inner flap of ears, brisket, groin and dock regions.

2.3. Blood film

Two thin blood films of each blood sample were prepared, dried and fixed with absolute methyl alcohol for 1–2 min. These blood smears were immersed for 30–45 min into diluted Giemsa stain, and thereafter washed with distilled water to remove excess of stain. The slides were air dried and examined under oil immersion lens (Coles, 1986) for the presence of any haemoprotozoan parasite (*T. evansi* and *T. equi*).

2.4. Micro Aerophilus stationary phase technique (MASP) for Theileria equi

The laboratory at ICAR-National Research Centre on Equines, Hisar, India has developed the facilities for *in-vitro* propagation of *T. equi* by MASP technique (Avarzed et al., 1998; Kumar et al., 2013) and *T. equi* positive cultured red blood cells were collected and processed for DNA extraction. This DNA was used in the present study as a reference *T. equi* positive DNA template in PCR reaction. The detailed description of MASP technique for *T. equi in vitro* cultivation has been described elsewhere (Kumar et al., 2013).

2.5. Culturing for Trypanosoma evansi

Blood was collected from a *T. evansi* clinically infected equid and transfused intraperitonially to Swiss albino mice. Mice were sacrificed when parasitaemia was maximum (10^7–10^9 parasites ml^{-1}) and *T. evansi* parasites were purified from the collected blood by adsorbing it on to DEAE (Diethyl amino ethane cellulose) columns chromatography (Lanham and Godfrey, 1970; Bal et al., 2012). DNA was extracted from purified *T. evansi* parasites and was used as reference positive control in this study.

2.6. Oligonucleotide primers for multiplex PCR

The details of oligonucleotide primers used for establishing multiplex PCR are mentioned in Table 1.

2.7. DNA extraction and multiplex PCR amplification

Genomic DNA was extracted from the equine blood samples as per the protocol of HiPura™ Blood Genomic DNA Miniprep Purification Spin Kit. A total of 25 μl PCR reaction mixture was prepared which contained 12.5 μl of master ready mix (1X containing KAPA2G Fast HotStart DNA polymerase, KAPA 2G Fast HotStart PCR buffer, 0.2 mM dNTP each, and 1.5 mM $MgCl_2$); 1.5 μl of each 10 pmol BeqF1/ BeqR1 primers (*T. equi* specific), 1.25 μl of each 10 pmol TR3/TR4 primers (*T.evansi* specific), additional 1 mM $MgCl_2$ suspended in nuclease free water and 5 μl DNA template of field equid samples. Positive control reaction was set up by taking, 2.5 μl each of *T. evansi* and *T. equi* refrence DNA as template instead of field sample DNA. Negative control reaction was also put with DNA obtained from a day old horse foal. The no-template-control reaction contained 5 μl of nuclease free water in place of DNA template. The PCR suspension was pre-heated for 5 min at 94 °C to activate the T*aq* polymerase, and 32 cycles were repeated – denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C, extension for 1 min at 72 °C; and a final extension for 10 min at 72 °C. The amplified DNA samples were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light using gel documentation instrument (UVP Instruments, USA) for observation of 257 bp and 709 bp PCR products in the samples.