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Comparative studies on the sensitivity of polymerase chain reaction (PCR) and microscopic examination for the detection of *Trypanosoma evansi* in horses

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Abstract: The lack of reliable diagnostic methods is a setback in the effective control of diseases. Diagnosis of *Trypanosoma evansi* infection (surra) in livestock is of considerable economic importance. Detection of trypanosomes in blood has been the "gold standard"; however, finding the organisms or establishing patency of parasitaemia has not always been possible even in symptomatic infections. As a consequence of low dose infection, the pre-patent period in *Trypanosoma evansi* may be longer and, even when symptoms have developed, trypanosomes may still not be demonstrable in blood, thus delaying treatment and thereby increasing morbidity and mortality. *Trypanosoma evansi*, a protozoan parasite in the blood of horses, is routinely diagnosed by finding the flagellates in the stained smear of blood examined under a microscope. Although specific, this method is not sensitive at the early stages of infection. Using a specific pair of primers we tested the use of polymerase chain reaction (PCR) in comparison with microscopic examination, i.e. Giemsa stained smear method, for the diagnosis of *Trypanosoma evansi* infection (surra) in horses. The PCR method was sufficiently sensitive; using this method out of 100 blood samples examined from horses in Lahore, Pakistan, 16 (16%) showed a positive reaction (a 164-bp fragment) as visualized by agarose gel electrophoresis and 5 (5%) were positive by microscopic examination. These findings suggest that PCR is a useful diagnostic tool for detecting *Trypanosoma evansi* infected horses in the very early stages where microscopic examination is equivocal. Application of PCR to field diagnosis is therefore clearly indicated by the results. Field application of PCR would not only ensure early diagnosis and treatment in individual animals but would also help eliminate the animal reservoir of infection and thus the threat to equine and camel herds that are grazed and housed together and where blood sucking mechanical fly vectors are ever present.

Key words: Trypnosomiasis, horses, parasitemia, PCR, detection

Introduction

Trypanosoma evansi (*T. evansi*) was the first trypanosome shown to be pathogenic for mammals. Transmission is by biting flies such as *Tabanus*, *Stomoxys*, and *Lyperosia*. *Trypanosoma evansi* affects a wide range of hosts including horses, camels, dogs,

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buffaloes, elephants, pigs, cats, tapirs, capybaras, and deer. Laboratory rodents such as mice, rabbits, rats, and guinea pigs are readily infected. The clinical disease varies according to the strain of parasite and the species of host. The most severe disease occurs in horses, camels, and dogs. The classical disease entity in the Indian subcontinent occurs in horses and is known as "surra", a Hindi word meaning rotten as described by Soulsby (1).

Waheed et al. (2) have reported that surra is distributed in Pakistan, India, Sri Lanka, China, the Philippines, Vietnam, Indonesia, Malaysia, Brunei, some areas of the Middle East, and Central and South America. Trypanosomiasis has received intense consideration and attention due to its disastrous effects on healthy working animals. *Trypanosoma evansi* makes an animal unable to perform its duty in a proper manner; slowly and gradually the victim moves towards death.

Trypanosomosis is never pathognomonic and suspicion has to be confirmed by other means, i.e. laboratory methods. A variety of diagnostic tests are available and researchers are still trying to improve existing tests and to develop new ones. Current diagnostic tests vary in their sensitivity and specificity. The simplest techniques are examination of wet, thick or thin films of fresh blood; the diagnostic sensitivity of these methods is generally low but depends on the examiner's experience and the level of parasitaemia. Others include parasite concentration techniques (microhaematocrit centrifugation technique, dark-ground/phase-contrast buffy coat technique, anion exchange, and in-vitro cultivation); parasite concentration techniques rely on the detection of motile, live trypanosomes, and identification of trypanosome species is difficult. Regarding the animal inoculation test, the method is not practical, it is expensive, and diagnosis is not immediate. Tests to detect trypanosomal antigen include serological tests (indirect fluorescent antibody test, antibody-detection enzyme-linked immunosorbent assay, and card agglutination test); their species specificity is generally low, and they detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. The others include DNA amplification tests such as polymerase chain reaction (PCR) as described in OIE (3) and DNA probes as suggested by Uilenberg (4). The PCR procedure is extremely sensitive as even minute quantities of parasite DNA can be amplified into a detectable quantity if the number of cycles is sufficiently high.

The present study was undertaken to determine the reliability of PCR as compared with the microscopic examination, i.e. Giemsa stained smear method, for the diagnosis of *Trypanosoma evansi* infection (surra) in horses.

Materials and Methods

A total of 100 horses of either sex and all ages suspected for *Trypanosoma evansi* infection (surra) were selected from different localities including, Brooke hospital Lahore, Society for Prevention of Cruelty to Animals (S.P.C.A.), and the outdoor clinic of the University of Veterinary and Animal Sciences Lahore (Pakistan).

Under aseptic condition, 5 mL of blood from each animal was collected with the help of a disposable syringe. A drop of blood was taken in the center on one end of a clean glass slide for smear preparation as suggested by Benjamin (5); the remaining blood was transferred to a sterile EDTA coated vacutainer and stored in a deep freeze at -20 °C for PCR, as suggested by Ijaz et al. (6).

The blood smears were prepared and dried by waving in the air. The blood smears were fixed in absolute methyl alcohol for 3 min and stained with Giemsa stain (2 parts commercial Giemsa stain solution diluted in 4 parts distilled water) method of staining as described by Benjamin (5). A drop of cedar wood oil was placed on the stained smear and the slide was examined under the oil immersion lens (100×) of the microscope for the presence of *Trypanosoma evansi*; it was monomorphic in character, slender in shape, having an undulating membrane with a well developed free flagellum present outside the cell as reported previously by Soulsby (1).

For the extraction of total genomic DNA, 500 µL of whole blood was collected in an Eppendorf tube, 166 µL of 1N NaOH was added to it and vortexed. The sample was boiled in a water bath for 5 min. An equal

volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to it and vortexed. The mixture was centrifuged at 13,000 rpm for 10 min and the aqueous phase was collected. The aqueous phase was mixed with 2.5 volumes of absolute ethanol (kept at -20 °C) and was incubated at -20 °C for 30 min for the precipitation of genomic DNA. The DNA was concentrated by centrifugation at 13,000 rpm for 10 min. The tube was inverted and drained on clean absorbent paper; the DNA pellet was obtained and allowed to air dry for 10-15 min. The DNA was dissolved in sterile deionized water and kept at -20 °C until used for amplification by PCR.

The PCR was carried out on the blood of the same suspected horses to evaluate its efficacy in the diagnosis of *T. evansi* infection and to compare its diagnostic value against the microscopic examination method currently in use. The primers, TBR1 and TBR2, as described previously by Ijaz et al. (6) used in these experiments were commercially prepared and are presented in Table 1. The PCR reaction was performed in a 50 µL reaction mixture containing 1× Taq buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 2.5 U/µL Taq polymerase, 4 µM of each primer, 2 µL of DNA extracted, and 31.5 µL of DNase-free deionized water. The tubes containing the mixture were subjected to 30 cycles of amplification in a thermocycler. During each cycle the sample of DNA was denatured at 93 °C for 30 s, annealed at 45 °C for 30 s, and extended at 72 °C for 1 min. Prior to the cycling and at the end of cycling the mixture was subjected to incubation at 93 °C for 3 min and final extension at 72 °C for 5 min, respectively. PCR product was then characterized by 2.5% agarose gel electrophoresis.

To detect the amplified product 50 µL of each amplified sample was mixed with 8 µL of 6× loading dye (40.0% w/v sucrose, 0.25% xylene cyanol FF, 0.25% bromophenol blue), (final conc. 1× in 50 µL sample) and loaded in a gel containing 2.5% agarose with ethidium bromide (at a final concentration of 0.4 µg/mL). The electrophoresis was carried out at a constant voltage of 90 V for 1.5 h. Following electrophoresis the gels were visualized under UV illumination and photographed (Figure).

Results

One hundred horses with suspected *Trypanosoma evansi* infection (surra) were examined by microscopic examination, i.e. Giemsa stained smear method, and PCR. With the stained blood smear method, 5 out of the 100 horses were found positive for *T. evansi* infection (Table 2). For PCR total genomic DNA of horse blood samples were amplified using TBR1 and TBR2 primers. A DNA fragment of 164 bp was amplified. The PCR revealed 16 positive cases out of the 100 above-mentioned suspected cases (Table 2). These 16 positive cases diagnosed by PCR also included animals diagnosed by the stained blood smear method. These results indicate clearly that the PCR assay is a much more sensitive detector of *Trypanosoma evansi* infection than microscopic examination of Giemsa stained smears.

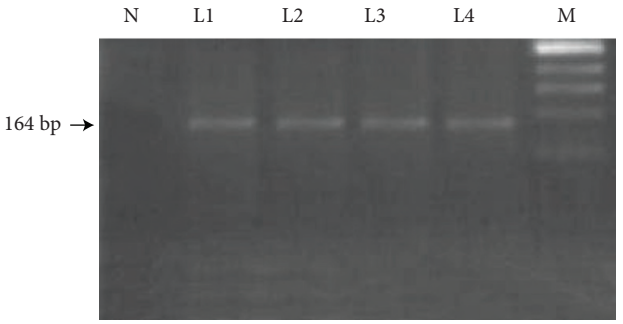
Discussion

Microscopy of stained blood smears is the least sensitive method of detecting infections with hemoprotozoa. Introduction of the HCT method of hematocrit centrifugation of blood followed by buffy

Table 1. Primer sequences and predicted amplification product size.

Primers	Primer sequences*	Expected size
TBR 1	GAATATTAAACAATGCGCAG	
		164bp
TBR 2	CCATTTATTAGCTTTGTTGC	

*Primer sequences are shown in the 5′-3′ orientation



N = Negative control.
L1, L2, L3, L4 = Experimental samples.
M = Marker (1 Kb ladder).

Figure. Detection of Trypanosoma evansi in horse blood by PCR.

coat examination as described by Woo (7) and elution of blood through an ion exchange column as suggested by Lanham and Godfrey (8) and miniature ion exchange as described previously by Lumsden et al. (9) increased the sensitivity of the microscopic methods manifold. Indirect methods of diagnosis based on the demonstration of specific antibodies or parasite antigens in the host blood or tissue have higher sensitivity and greater variability. Therefore, the latter tests are more difficult to interpret in terms of disease status. Franke et al. (10) have shown that the variable sensitivity of the most advanced methods of detecting antibodies (Ab ELISA, CATT/*T. evansi*) or antigens (Ag ELISA) vary during the phase of infection at the time of examination.

Application of minute amounts of nucleic acid down to a single organism by various modification of the PCR has been demonstrated for a number of infectious organisms including trypanosomes by a number of researchers including Panyim et al. (11), Wuyts et al. (12), Almeida et al. (13), Basagoudanavar et al. (14,15), Wang-YunFei et al. (16), Omanwar et al. (17), Holland et al. (18), Masiga et al. (19), Chansiri et al. (20), Clausen et al. (21), Davila et al. (22), Herrera et al. (23), Njiru et al. (24), and Singh et al. (25), and the primers used have the sufficient specificity to detect *T. evansi*. The PCR procedure is extremely sensitive, as even minute quantities of parasite DNA can be amplified into a detectable quantity if the number of cycles is sufficiently high as reported by Uilenberg (4). As a consequence of low dose infection, the prepatent period in *Trypanosoma evansi* may be longer and, even when symptoms have developed, trypanosomes may still not be demonstrable in blood, thus delaying treatment and thereby increasing morbidity and mortality.

Surra has been prevalent in Punjab since 1880 as reported by Evans (26), Lal (27), Jaffery et al. (28), Khan (29), Saeed (30), Ahmad (31), Rauf (32), Waheed et al. (2), and Shehzad (33). The present study has confirmed the prevalence of the disease in the area.

From the results it is evident that PCR is more sensitive than the microscopic examination of blood for diagnosis of surra in horses as a higher percentage of positive cases (16%) was detected as compared to microscopic examination, which revealed a positive percentage of 5%.

It was concluded that for the diagnosis of *Trypanosoma evansi* infection PCR could be used in horses as a quick and reliable test for the detection of trypanosomiasis, especially in cases where the

Table 2. Efficacy of the microscopic examination and polymerase chain reaction (PCR) for the detection of *Trypanosoma evansi* in horses.

Test performed	No. of suspected horses examined	No. of horses found positive	Percent efficacy
Microscopic examination	100	05	05.0
Polymerase chain reaction (PCR)	100	16	16.0

parasitemia is low. This test could be used in other species of animals, especially camels, where the disease is more chronic and difficult to confirm by other routine methods. Field application of PCR would not only ensure early diagnosis and treatment in individual animals but would also help eliminate the animal reservoir of infection and thus the threat to equine and camel herds that are grazed and housed together and where blood sucking mechanical fly vectors are ever present.

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