

Comparative Evaluation of Parasitological, Serological and DNA Amplification Methods for Diagnosis of Natural Trypanosomal Infection in Equines

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Abstract.- The study was conducted to evaluate and compare the parasitological (micro-haematocrit method, MHCT), serological (enzyme-linked immunosorbent assay, ELISA) and molecular (polymerase chain reaction, PCR) methods for diagnosis of trypanosomal infection in equines. Blood samples, taken from 500 animals (horses and donkeys), were primarily screened with formol gel test. Of 500 samples examined, 120 samples were found positive with formol gel test. Later, these samples were further processed for the parasitaemia with MHCT, detection of antibody against trypanosomal antigens positive ELISA and trypanosomal DNA using PCR. Results showed that higher number of positive samples ($p < 0.05$) were detected with PCR (30.8%) compared to either ELISA (21.6%) or MHCT (17.5%). The sensitivity and specificity of ELISA were 85.7% and 79.5%, respectively compared to PCR which were found to be 100% and 58.97%, respectively. To conclude PCR test was found to be a superior test over MHCT and ELISA for the diagnosis of trypanosome in equines and can be used in field conditions.

Key words: Trypanosomiasis, PCR, MHCT, ELISA, equines.

INTRODUCTION

Trypanosomiasis, also known as surra, is a most widely distributed disease caused by different species of pathogenic trypanosomes affecting livestock in Asia, Africa and South America (Luckins, 1988). Cattle, buffaloes, horses and camels are principally affected, along with other species including wild ruminants. The disease produces negative effects on health that decreases the productivity of horses. The disease has been reported in the different regions of Pakistan (Hasan *et al.*, 2006, Khan *et al.*, 1987; Waheed *et al.*, 1998; Saeed, 1986; Bano and Jan, 1986). Generally, the disease is diagnosed based on the clinical evidences augmented with some parasitological or serological tests. However, the clinical signs like emaciation, fever, anaemia, lacrimation, corneal opacity and diarrhoea (Chaudhary and Iqbal, 2000) are not sufficient for diagnosis. On the other hand, the detection of parasites in the blood is difficult

because parasitaemia is often low and fluctuating, particularly, during the chronic stage of infection (Mahmound and Gray, 1980; Nantulya, 1990). Serological diagnostics based upon the detection of either antigen or antibodies were developed but were unable to resolve the problem completely and showed un-satisfactory results (Lantulya and Lindqvist, 1989; Olaho-Mukani *et al.*, 1993). Furthermore, serological diagnostic based on antibody detection lacks specificity and are unable to differentiate on-going infection from previous infection. Attempts to develop an antigen-enzyme-linked immunosorbant assay (Ac-ELISA) have been made (Nantulya and Lindqvist, 1989), but still lacks sensitivity and is not normally recommended (Tony Luckins, International Atomic Energy Agency, Vienna, Austria. Personal communication). Consequently, there is a need to devise more sensitive test for the detection of trypanosomiasis in animals.

Recently, molecular diagnostic techniques particularly, polymerase chain reaction (PCR) assays appear to be a promising technique for the diagnosis of trypanosomal infection based on the detection of trypanosomal DNA in the blood samples. The PCR is a highly sensitive and specific

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method that has been widely used for the detection of trypanosomes. Primers targeting different subgroup of trypanosomes have been developed. (Moser *et al.*, 1989; Wuyts *et al.*, 1994; Masiga, 1994; Ventura *et al.*, 2002). Though PCR has been reported to be more sensitive than conventional parasitological techniques (Wuyts *et al.*, 1995; De Almeida *et al.*, 1997; Desquesnes, 1997; Masake *et al.*, 1997), however, most of PCR assays have not been validated under field conditions for the diagnosis of natural infection (Wuyts *et al.*, 1995; Clausen *et al.*, 1998; Donelson *et al.*, 1998).

Therefore, the present study was conducted to evaluate the PCR technique to detect the trypanosomal antigens in the naturally infected equines, using two sets of primers (TBR1/2), and to compare the PCR results with the parasitological (microhaematocrit centrifugation, MHCT) and serological (ELISA) techniques.

MATERIALS AND METHODS

Study area

The study was conducted in the Lahore city. Lahore is lying between 31°15' and 31°45' north latitude and 74°01' and 74°39' east longitude, The River Ravi flows on the northern side of the Lahore city. The city covers a total land area of 404 km² and is still growing. A total of 500 animals (250 each of horses and donkeys) of either sex and ages were selected randomly that were presented to the Outdoor Hospital, University of Veterinary and Animal Sciences, Lahore, Pakistan and various clinics situated in and around the Lahore city for the treatment of different ailments.

Sample collection

The study was conducted during three months (July to September, 2007). Two blood samples were collected from the jugular vein of the same animals. One in a tube containing an anticoagulant, ethylene diamine acetic acid (EDTA), while others without anticoagulant. These blood samples were kept in ice and were processed on the day of collection. The blood samples with EDTA were used for the parasitological diagnosis and extraction of trypanosomal DNA for PCR amplification. The other blood samples were processed for serum

collection. Blood samples were centrifuged at 5,000 rpm for 15 minutes to separate serum. The serum samples were stored at -20°C until further used for ELISA and Formol gel test.

Diagnostic tests

The formol gel test was used as a screening test for the detection of trypanosomal infection (Radostits *et al.*, 2000). The test was performed as described earlier (OIE, 1996). Briefly, serum samples were transferred to the smaller tubes and two drops of concentrated formalin solution [37% formaldehyde (w/v)] was added. The test was defined positive if the serum was coagulated immediately and turned white or otherwise negative if serum remained unchanged or coagulation appeared after 30 minutes.

Micro-haematocrit centrifugation technique

This technique was employed for examination of parasitaemia. Briefly, after centrifugation of capillary tube (75 x 1.5) at 5,000 rpm for 5 minutes, the capillary tubes were broken 1 mm above the buffy-coat layer. The contents of this piece were partially expelled onto a slide and stained with Giemsa stain. The parasites were examined under a microscope (100 X).

Polymerase chain reaction

The blood samples destined for PCR were processed as described by Ijaz *et al.* (1998).

For extraction of trypanosomal genome approximately 1 ml of each blood sample was mixed with 1 ml of solution 1 (10 mM Tris, 10 mM KCl, 10 mM MgCl₂) and 25 ml of Triton X followed by centrifugation at 2,000 g for 10 minutes. To the pellet, 500 ml of NET buffer (0.5 M EDTA, 1 M Tris-HCl, 5 M NaCl), 500 ml of lysis buffer (TES buffer, SDS) and 5 ml of Proteinase K (100 mg/ml) was added and incubated at 48-50°C for three hours. Thereafter, the phenol:chloroform:isoamyl alcohol extraction procedure was followed after transfer of the contents in the tubes. The recovered DNA was precipitated by adding 95% cold ethanol and keeping it overnight at -20°C. It was, then, spun at 10,000 x g for 30 min at 4°C. Pellet was then washed twice in 70% chilled ethanol and air dried.

Later, the DNA was re-suspended in 50 ml of TE buffer (pH 8.0) and kept at 4°C. One microliter of this extract was used as a template for PCR amplification.

The PCR amplification was performed using a set of oligonucleotide primers (TBR 1 and TBR 2) for *Trypanosoma brucei* as described earlier (Ijaz *et al.*, 1998). Both TBR1 (GAATATTAACAATGCGCAG) and TBR 2 (CCATTATTAGCTTTGTTGC) contained 164 bp. The PCR reaction was performed in 50 µL reaction mixture containing 20mM Tris HCl, 50mM KCl, 1.5mM MgCl₂, 250nM each of dNTP, 0.125% glycerol, 1.5 units of Taq DNA polymerase, 250 nM of primer sets and approximately 0.20% (1.0 µL) of extracted DNA. The reaction mixture was placed in a thermal cycler (Eppendorf Master Cycler Gradient, Hamburg, Germany) and pre-incubated at 95°C for 4 minutes to completely denature the DNA. This was followed by 30 cycles of 1 minute at 93°C (to denature), 1 minute at 48°C (to anneal) and 1.5 minutes at 72°C (to extend). At the end of thermal cycling, the products were cooled to 4°C.

About 2.0µl of amplified product was mixed with 5.0µl of gel loading buffer (40% Sigma-Aldrich, St. Louis, MO, USA) and was loaded in a gel containing 1.5% agarose with ethidium bromide (0.4 µg/ml). The electrophoresis was carried out at a constant voltage of 100 volts for one hour. The genomic DNA was visible on UV gel documentation system and was photographed.

Enzyme linked immunosorbent assay (ELISA)

The tests were conducted using a commercially available kit (FAO/IAEA, Vienna, Austria) to detect the antibodies of trypanosomes in equines (Clausen *et al.*, 2003). The sensitivity of the ELISA was based on the detection of trypanosome antibodies (Wernery *et al.*, 2001). Trypanosomal ELISA was conducted using the FAO/IAEA Trypanosome ELISA kit, courtesy to the FAO/IAEA Joint Division for Nuclear Techniques, Vienna, in accordance with the kit manual. The monoclonal antibodies contained in these kits were of *T. brucei* (TBR 7 IgM type).

The sensitivity and specificity of PCR and ELISA was evaluated by considering the MHCT as a gold standard test (Thrusfield, 2006).

Statistical analysis

To compare between MHCT, ELISA and PCR, chi-square test was employed (Statistical Package for Social Science, SPSS for Windows, Inc, USA version 14.0). The level of significance was predetermined at < 0.05.

RESULTS AND DISCUSSION

In the present study, formol gel test was employed as a screening test to determine the prevalence of trypanosomiasis in equine population. Formol gel test is a non-specific biochemical test that has been used to detect the chronic infection in animals. The test depends upon elevated concentration of serum globulins due to infection. Though, this test has been considered as an outdated test, but it has still some use in the field conditions because it is simple to perform (OIE, 1996). Out of 500 blood samples analyzed, 120 (24.0%) samples were found positive. Thereafter, 120 formol-gel positive samples were further analyzed using MHCT, ELISA and PCR. results (Table I) showed that out of 120 samples, trypanosomes were detected in the blood smear of 21 samples. Similarly, antibodies against trypanosomal antigens were detected in 26 (21.6%) samples, while, DNA amplicons of trypanosomes were detected in 37 samples (30.8%). Statistically, the prevalence of trypanosoma was more ($p < 0.05$) when detected with PCR compared to MHCT or ELISA. The higher prevalence rate ($p < 0.05$) detected with PCR was due to the higher sensitivity of the molecular technique, which can detect even less than one trypanosome per milliliter under experimental conditions (Singh *et al.*, 2004).

Various diagnostic techniques demonstrating the presence of trypanosome in the blood samples are widely used. These parasitological techniques are simple, rapid and provide a satisfactory sensitivity. However, the samples must be processed within 4-6 hours after collection, otherwise, parasites are dead and false negative results may be obtained. It, therefore, seems to be inconvenient to use the parasitological techniques in the field. Additionally, some of these techniques can not always detect ongoing infections especially when the level of parasitaemia is either low or fluctuating

(Nantulya, 1990). On the other hand, serological tests based on detection of either antibody or antigens are also used as diagnostic procedures. Antibody-based ELISA assay was included in the current study to compare it with the other tests used. However, the disadvantage of this test is that the results obtained from this assay cannot be taken as an absolute evidence of infection as it may demonstrate the previous exposure of the animals to the trypanosomes.

Table I.- Prevalence of trypanosomiasis using different diagnostic tests.

Test	Total No. of Samples	Positive sample	
		No.	%
Formol gel	500	120	24
Microhaematocrit centrifuge technique	120	21	17.5
Enzyme linked immunosorbent assay	120	26	21.6
Polymerase chain reaction	120	37	30.8

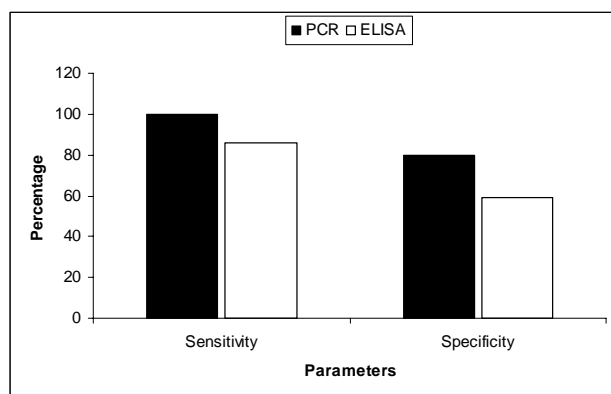


Fig. 1. Sensitivity and specificity of ELISA and PCR tests for the diagnosis of trypanosomal infection in equines.

The diagnosis of trypanosomes was improved since the 1980's due to advancement in the DNA-based molecular techniques. In recent years, DNA-based technologies like PCR had been used for diagnosis of trypanosomal infection in animals. The technique has been found specific and sensitive for large-scale analysis of trypanosome samples (Hide and Tait, 1991). PCR was found to be superior over

the other parasitological or serological test (Fig. 1) as more ($p < 0.05$) number of samples was found positive with PCR compared to MHCT or ELISA (Table I). The sensitivity and specificity of ELISA was 85.7% and 79.5% respectively. Similarly, sensitivity and specificity of PCR was found to be 100% and 58.97% respectively. A number of studies has shown that PCR was more sensitive test compared to other conventional parasitological techniques in cattle (Wuyts *et al.*, 1995, Desquesnes, 1997), water buffaloes (Holland *et al.*, 2001), mice (Ijaz *et al.*, 1998; González *et al.*, 2006), camels (Singh *et al.*, 2004) and other mammalian species (Herrera *et al.*, 2005). Previous studies showed that PCR gave twice as many positive results compared with the parasitological techniques in cattle (Masake *et al.*, 1997; Clausen *et al.*, 1998) and goats (De Almeida *et al.*, 1997). In another study, PCR showed a higher detection rate of trypanosomes that was about two times higher with the ELISA and four times higher than with the parasitological techniques (Singh *et al.*, 2004). PCR has some major advantages over the parasitological technique as the samples do not have to be processed within a short period after collection and can be delayed. The technique can specially be useful when large number of animals need to be sampled during field surveys. Further, PCR could be an important tool for evaluating the efficacy of chemotherapy. Various studies (Wuyts *et al.*, 1994; Njiru *et al.*, 2004) also showed >90% sensitivity of PCR for the detection of trypanosomes that is in accordance with the present study. Generally, the percentage of detection and the sensitivity of the PCR is variable depending upon the primers employed, which are determined by the number of copies and the homology of the primers with the target sequence (Fernández *et al.*, 2009). The 100% diagnostic sensitivity of PCR assay in the current study showed that primers used in this study permitted the identification of parasites far below the detectable limit of microscopic examination as also reported earlier (Omanwar *et al.*, 1999a,b).

In conclusion, PCR test detected more cases of trypanosomal infection in equines compared to MHCT, and ELISA and confirmed its superiority for the diagnosis of trypanosomiasis.

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