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MOLECULAR DIAGNOSIS AND CHEMOTHERAPY OF *TRYPANOSOMA EVANSI* IN NILI-RAVI BUFFALOES AT DISTRICT OKARA (PAKISTAN)

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ABSTRACT

A total of 144 blood samples were collected from apparently emaciated and anemic Nili-Ravi buffaloes in and around Livestock Experiment Station Bahadurnagar Okara during December, 2008 to May, 2009 including all age groups and either sex. They were screened for the presence of trypanosomes through stained (Hemacolor™, Merck) thin blood smears. The cases were confirmed as *T. evansi* through polymerase chain reaction (PCR). DNA of the trypanosomes was isolated using trizol reagent. The assay employed primers ESAG 6/7 specific for 237 bp fragment from *T. evansi* genomic DNA which was subsequently electrophorised and analyzed on UV transilluminator. Eight samples (5.5%) were positive microscopically while 16 samples (11.11 %) were found positive through PCR, indicating the specificity and sensitivity of PCR for surveying the disease in epidemiological studies. All the diseased buffaloes were treated with diminazene aceturate (FA.TRY.BANIL R.T.U., FATRO, Italy @ 3.5 mg/ kg body weight). The treated animals were found negative for the parasite both microscopically as well as through PCR, 48 hours post injection indicating the sensitivity of this drug to *T. evansi* in buffaloes.

Key words: *T. evansi*, Nili Ravi buffalo, PCR, Diminazene aceturate.

INTRODUCTION

Haemoparasitic diseases are the major constraints to the development of livestock industry in developing countries, like Pakistan. Factors such as climatic conditions, lack of information regarding disease transmitting vectors and knowledge on part of livestock farmers regarding parasitic transmission play role in endemic status of haemoparasitic diseases.

Buffalo population in Pakistan is 32.7 millions (Anonymous, 2011).Pakistan is the 2nd largest buffalo milk producer in the world with 22279000 metric tons during 2010 (FAO, 2010). Punjab province is the home tract of the world‘s renowned Nili Ravi buffalo with 65% of total buffalo population of the country (Livestock Census, 2006).

Trypanosomiasis commonly known as surra caused by *Trypanosoma evansi* is most widely distributed in Asia, Africa and central and South America affecting domesticated livestock Konnai *et al*. (2009). The disease is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Recurrent episodes of fever and parasitaemia occur during the course of disease (Luckins, 2004). Abortions and infertility have been reported in buffaloes in Asia Lohr *et al.* (1986); Davison *et al.*(1999).The hemoparasite causes immunodeficiency Onah *et al.* (1998). The incidence of Trypanosomiasis has also been reported in humans Prashant *et al.*(2005).Vectors involved in the spread of disease are Muscoid and Tabanid biting flies Vijay *et al*.(2002).

At the moment no information is available about Trypanosomiasis in Nili-Ravi buffaloes and its molecular diagnosis through polymerase chain reaction in Pakistan. One Nili-Ravi buffalo maintained at Livestock Experiment Station (LES) Bahadurnagar Okara showed signs and symptoms of progressive anaemia, rapid weight loss among animals, rapid decrease milk production, persistent fever up to 105 °F, circling, uncoordinated gait and in terminal stage lying on ground in lateral recumbancy with kink neck and finally death. Stained blood smear examination showed the presence of trypanosomes under the microscope. On the basis of this finding, a research study was designed to investigate the prevalence of Trypanosomiasis in buffaloes at this area. This study describes the prevalence of Trypanosomiasis in genetically superior Nili- Ravi buffaloes in and around LES Bahadurnagar, district Okara, its molecular diagnosis through polymerase chain reaction and its treatment with diminazene aceturate (FA.TRY.BANIL R.T.U., FATRO, Italy).

MATERIALS AND METHODS

One hundred and forty four blood samples were collected from Nili-Ravi buffaloes maintained at LES Bahadurnagar, district Okara and also from animals surrounding LES during December, 2008 to May, 2009.

Animals of all age groups and either sex were included in this study.

For microscopic examination, a drop of fresh blood from ear vein of each animal was placed on one end of clean (wax free) microscopic glass slide and a thin film were drawn out using another fresh clean slide. The blood smear was air dried and stained (HemaColor, Merck) in the lab according to the manufacturer's instruction. The slides were examined under microscope at magnification 100x (oil immersion) for presence of trypanosomes (Anonymous, 2004).

Five ml blood was also collected from jugular vein in 10ml clean sterile vacutainers containing ethylene diamine tetra acetic acid (EDTA), after cleaning the surface with alcohol and then drying it. Separate vacutainers and needles were used for each animal. Samples were brought to lab immediately after collection in an ice container and were protected from direct sunlight. These blood samples were stored at 4 ºC till DNA extraction for PCR amplification.

Extraction of DNA from Blood Samples: Whole blood was used for extraction of total genomic DNA using commercially available DNA isolation reagent (TRIREAGENT®, Ohio, USA) according to the manufacturer's instruction. Briefly, 300 µl whole blood was mixed with 1 ml of TRIREAGENT in a 1.5 ml sterile eppendrof tube. After 5 minutes incubation at room temperature (15-30 °C) chloroform (200 µl) was added, shaked vigorously for 15 seconds and re-incubated at room temperature for 10 minutes, followed by centrifugation at 12000g for 15 min at 4 °C. After centrifugation upper aqueous phase was removed and interphase was transferred to new 1.5ml eppendrof tube. The DNA was precipitated by mixing 300µl of 100% ethanol, incubated at room temperature for 2-3 minutes, followed by centrifugation at 2000g for 5 min at 4 ºC. The supernatant was discarded and DNA pellet was washed twice in solution containing 0.1M Tri-sodium citrate in 10% ethanol by adding 1ml and incubating at 15-30 ºC for 30 minutes with periodic mixing and re-centrifugation at 2000g for 5min at 4 ºC. The DNA pellet was then suspended in 75% ethanol (1ml), incubated at 15-30 ºC for 20 minutes with periodic mixing and then re-centrifuged at 2000g for 5min at 4 ºC. The DNA pellet thus obtained was air dried for 15 minutes in an open tube then dissolved in 300ml of 8mM NaOH. DNA concentration was determined spectrophotometer at 260 nm wave length and then stored at -20 ºC until used for PCR.

Primers Selection: The primer pair ESAG 6/7 consisted of a forward 21-mer primer (5′-ACA TTC CAG CAG GAG TTG GAG -3′) and a 21-mer reverse (5′ -CAC GTG AAT CCT CAA TTTTGT-3′). Trypanosoma specific (Braem, 1999) were used in this study for the amplification of a 237 bp fragment from *T. evansi* genomic DNA.

Polymerase Chain Reaction: All PCR amplification reactions were carried out in a final volume of 25 µl containing DNA template and 12.5 µl of commercially available PCR master mix (PyroStart™ Fast PCR Master Mix (2X), Fermentas, Canada). The primers were used at a concentration of 10 ρmols /µl.

Amplification was carried out with thermo cycler (PeqSTAR, PeqLab, Germany) and cycling conditions were; first a denaturation step of 4 minutes at 94 °C followed by 35 cycles consisting of 1 minute denaturation at 94 ºC, 1 minute primer-template annealing at 55 °C and 1 minute polymerization at 72 ºC. The last extension step of 5 minutes at 72 °C was performed to polymerize all remaining single strand DNA fragments.

10 µl of the PCR product were electrophorised on a 1 % agarose gel (1hr at 90V) with a 100bp ladder as size marker. The gels were stained with ethidium bromide (2 µl/50ml gel) and were analysed on a UV trans-illuminator (Dolphin-Doc, Wealtec, USA).

Chemotherapeutic Trail: All diseased buffaloes were treated with diminazene aceturate (FA.TRY.BANIL R.T.U., FATRO, Italy @ 3.5 mg/ kg body weight). Blood samples were collected at day 0 (pre-medication) and also on days 1, 2 and 3 (post-medication) for microscopic examination and PCR assay.

RESULTS

Blood Smear Examination: The stained blood films examined microscopically under oil immersion lens 100x revealed trypanosomes as long slender form (Fig. I), although short stumpy and intermediate forms were also seen in between the cells with length from 17 to 30 µm and width 1.5 to 3 µm and they were identified based on their morphology (Smyth 1996). Abnormalities in erythrocyte structure were not observed in Trypanosoma positive smears. Eight out of 144 (5.5 %) was the number of positive cases recorded for *T. evansi* based on blood smear examination.

Polymerase Chain Reaction Test: Data recorded for extracted DNA concentration of each sample by spectrophotometer analysis showed the ratio between 260 nm and 280 nm in the range of 1.5 - 2.1. The 237 bp fragment (Fig. II) was generated in all positive samples tested with ESAG 6/7 primers (Braem, 1999). Sixteen out of 144 (11.11%) blood samples were found positive for *T. evansi* based on PCR test.

Comparison of Blood Smear and PCR Test: Through microscopic examination 5.5 % (8 / 144) cases were detected while with PCR test 11.11 % (16 / 144) cases

were found positive for trypanosoma during present study. The samples positive through blood smear examination were also positive by PCR while out of 16 samples, 8 samples positive by PCR test were negative by microscopic examination.

Chemotherapeutic Results: The treated animals were found negative for the parasite both microscopically as well as through PCR, 48 hours post injection indicating the sensitivity of diminazene aceturate (FA.TRY.BANIL R.T.U., FATRO, Italy @ 3.5 mg/ kg body weight) to *T. evansi* in Nili- Ravi buffaloes.

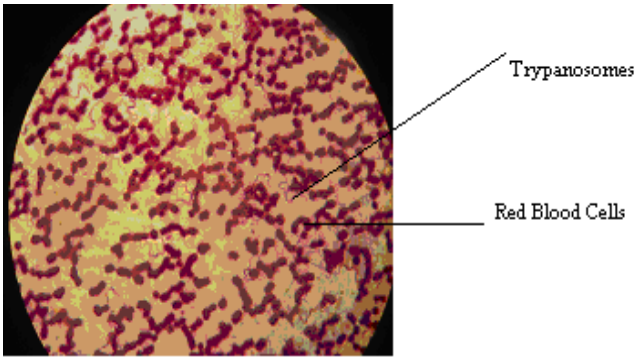

Figure-1 Trypanosomes in a blood Smear of buffalo stained with HemaColor™ (Original magnification 100x)

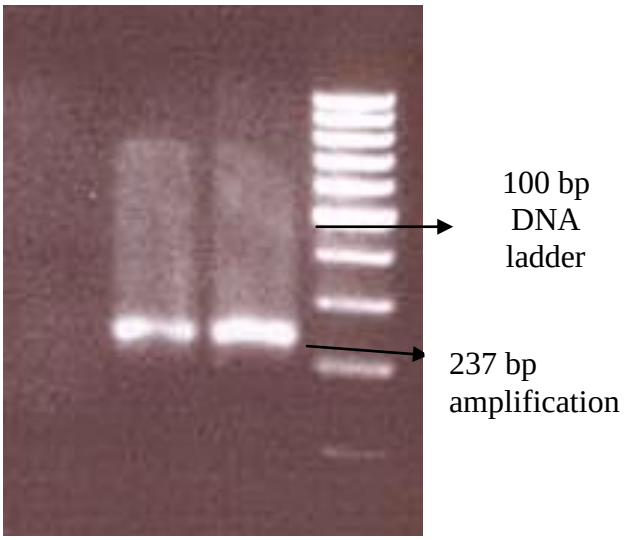

Figure 2. A 237 bp fragment from *Trypanosoma evansi* genomic DNA with ESAG 6 / 7 Primers

DISCUSSION

PCR has been successfully used in detecting infection with *T. evansi* in buffaloes Omanwar *et al*. (1999) and Holland *et al*. (2001), horses Clausen *et al*. (2003) and in camels Masiga *et al*. (2001). So far, there is no comprehensive data on the use of PCR for detecting infection in Nili-Ravi breed of Buffaloes. Microscopic examination of blood smear remains the one of the major diagnostic technique in the area. Several methods developed for the detection of *T. evansi* infection including microscopy, card agglutination test Guterrez *et al*. (2000); microhaematocrit centrifugation technique (Woo, 1970); enzyme linked immunosorbent assay Indrakamhang *et al*. (1996); DNA hybridization Viseshakul *et al*. (1990); and polymerase chain reaction Omawa *et al*. (1999). The PCR provided high sensitivity among these all methods and it had been reported that it could detect *T. evansi* even three days earlier than microscopy Ijaz. *et al*. (1998).

In this study eight samples out of total 144 were found positive for trypanosomes through microscopic examination. Morphological characteristics of trypanosomes as long slender form, with length from 17 to 30 µm and 1.5 to 3 um in width are in accordance as described previously by (Soulsby, 1982), (Luckins, 1992) and (Smyth, 1996).

Sixteen out of 144 samples were found positive for hemoparasite through PCR. The efficacy of microscopic examination revealed was 5.5 % while efficacy of PCR test was 11.11 % .In the present study blood smear examination proved to be of limited value in diagnosis of Trypanosomiasis. According to (Herbert and Lumsden, 1976) when less than 2,500,000 parasites/ml are present in blood sample, microscopy detection is not feasible. While PCR assay are well documented with detection level to a minimum of 5 trypanosomes Artama *et al*. (1992). Amplification of repetitive *T. evansi* infection is possible even with DNA of single trypanosome (Viseshakul and Panyim, 1990).

The samples positive for blood smear were also positive by PCR test but out of 16 samples, 8 samples positive by PCR test were not detected by microscopic examination. Therefore PCR test showed high sensitivity as compared with microscopic examination. Ravindran *et al*. (2008) has also found a higher prevalence of *T. evansi* in camels (34.4 %), dogs (7.7%) and in donkeys (6.8 %) through PCR than blood smear examination indicating PCR more sensitive technique. Holland *et al.* (2001) compared different parasitological tests and PCR for *T. evansi* diagnosis in experimentally infected water buffalo and found that PCR was most sensitive method than other methods to detect trypanosomiasis.

Samples negative through PCR were also negative through slides indicates, specificity of PCR is satisfactory. The ESAG 6/7 primer that is *T. evansi* specific does not give cross-reaction with e.g., *T. theilerie* (Braem, 1999). And due to absence of tsetse flies in Southeast Asia which are required for cyclic transmission of *T. b. brucei*, cross- reactions in water buffalo is not considered important. No data for this primer set is available on possible cross reaction with other related

micro-organisms such as Anaplasma spp., Theileria spp. and Babesia spp. Holland *et al*. (2001).

Nasir *et al.* (1999) reported the prevalence of trypanosomes in Jersey and Frisian cows at livestock experiment station Bhunikey, Kasur, Pakistan which is hardly forty kilometres away from present study station. According to his studies 32.5 % (14/43) jersey cows and 23% (11/47) Frisian cows were positive for Trypanosomiasis. The higher prevalence may be attributed to small sample size or indicates the higher prevalence in cattle than in buffaloes in the region.

A variety of mammalian hosts are known to be susceptible to *T. evansi* infection with many domestic and wild mammals vulnerable to infection Prashant *et al.* (2005). In another study Shah *et al.* (2004) reported 13.72 % prevalence of *T. evansi* in camels in Sind, Pakistan. Similarly Hasan *et al*. (2006) reported 3.3% and 4 % camels positive at parasitological and serological examination, respectively. Prevalence of *T. evansi* has also been reported in four Himalayan black bears from Faisalabad and Jhang districts of Punjab Pakistan by Muhammad *et al*. (2007). All these studies indicate the prevalence of *T. evansi* in different hosts thus supporting prevalence of *T. evansi* in this area.

No information is available so far on the non-tsetse transmission of Trypanosomiasis in Pakistan. The season might be important as there is flowering season of guava and brassica during January to April and is quite suitable for breeding and feeding of Tabanid flies.

The diseased buffaloes were treated with diminazene aceturate and blood samples were collected for another 3 days. Microscopic examination and PCR assay on these samples were negative after 48 hours post treatment, which confirms earlier findings with *T. evansi* in buffalo Holland *et al*. (2001) and *T. evansi* in albino mice Ahmad *et al*. (2005).

In conclusion the PCR for detection of *T. evensi* is highly specific and sensitive. The main advantages of this test are very low false-positive and insensitive to other hemoparasite species. This test is suitable for tracing of carrier animals and provides a quantitative validated measure that is beneficial for epidemiological survey and the following of drug treatment in Nili-Ravi buffaloes. In addition, it is also useful for designing rational Trypanosomiasis control program in the endemic area. Further diminazene aceturate @ 3.5 mg / kg body weight is quite effective for the treatment of *T. evansi* in Nili-Ravi Buffaloes.

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