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BRIEF COMMUNICATION

Prevalence and molecular diagnosis of *Trypanosoma evansi* in Nili-Ravi buffalo (*Bubalus bubalis*) in different districts of Punjab (Pakistan)

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Abstract The prevalence of *Trypanosoma evansi* was investigated in 1,250 Nili-Ravi buffaloes of mixed age and sex by polymerase chain reaction (PCR) for the first time in Pakistan. DNA of the trypanosomes was isolated with TRIREAGENT®. The assay was employed using primers ESAG 6/7, specific for a 237-bp fragment from *T. evansi* genomic DNA. The samples were screened for the presence of *T. evansi* also by stained thin smear. Forty-four (3.5%) samples were positive by microscopy, while 97 (7.7%) samples were identified by PCR, indicating the high sensitivity of PCR for surveying the disease in epidemiological studies.

Keywords Trypanosomiasis · Bovine · Polymerase chain reaction · *T. evansi*

Introduction

Hemoparasitic diseases like trypanosomiasis present major constraints to the development of the livestock industry in developing countries such as Pakistan. The disease commonly known as surra, caused by *Trypanosoma evansi*, is of great economic importance in South Asian countries (Konnai et al. 2009). Domestic and wild mammals are susceptible to *T. evansi* infection (Prashant et al. 2005). Trypanosomiasis is manifested by pyrexia directly associated with parasitemia, together with progressive anemia, loss of condition, and lassitude. Recurrent episodes of fever and parasitemia occur (Luckins 2004) and spontaneous abortions and infertility have been reported in buffalo in Asia (Lohr et al. 1986; Davison et al. 1999).The buffalo (*Bubalus bubalis*) population in Pakistan is reported as 29.9 million (Anonymous 2008) and it is the second largest buffalo milk-producing country in the world with 20372000 t (Food and Agriculture Organization of United Nations 2007). Punjab province is the home of the Nili-Ravi buffalo, a breed that represents 65% of the buffalo population of the country (Anonymous 2006). Currently, no information is available on molecular diagnosis through polymerase chain reaction (PCR) of *T. evansi* in Nili-Ravi buffalo in Pakistan. A few Nili-Ravi buffaloes maintained at the Livestock Experiment Station Bahadurnagar, district Okara, showed progressive anemia, rapid weight loss, rapid decrease in milk production, persistent fever up to 40.5°C, circling, and uncoordinated gait, culminating in lateral recumbency with kinked neck and, finally, death. Blood smears revealed the presence of *T. evansi*, which was later confirmed through PCR. This study was initiated to investigate the prevalence of *T. evansi* through two diagnostic tools in Nili-Ravi buffalo in Okara and surrounding districts, Sahiwal, Lahore, Faisalabad, and Kasur.

Materials and methods

Blood samples were collected from the ear veins of 1,250 Nili-Ravi buffaloes from five districts, 250 from each, from

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January to June 2009. Animals of all age groups and both sexes were included in this study. Smears were air dried, stained (HemaColor, Merck), and examined under oil immersion for the presence of *T. evansi*. A 5-ml blood sample was also collected from the jugular vein of each buffalo and placed in 10 ml clean sterile vacutainers containing ethylenediaminetetraacetic acid. They were brought to the laboratory and stored at −20°C until used for DNA extraction for PCR amplification. Whole blood was used for the extraction of total genomic DNA using a commercially available DNA isolation reagent (TRIREAGENT®, Ohio, USA) according to the manufacturer's instructions. The nucleic acid was extracted also from *Trypanosoma* positive known blood samples (mice) and known negative samples (40 healthy buffaloes maintained at the Livestock Production Research Institute Bahadurnagar, Okara). DNA concentration was determined spectrophotometrically at 260 nm and the samples were stored at −20°C till further use. 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 primer (5′-CAC GTG AAT CCT CAA TTTTGT-3′). These *Trypanosoma*-specific primers (Braem 1999) were used for the amplification of a 237-bp fragment from *T. evansi* genomic DNA. All PCR amplification reactions, including control (positive and negative) samples, were carried out in a final volume of 20 µl containing DNA template and 10 µl commercially available PCR master mix (PyroStart™ Fast PCR Master Mix-2X, Fermentas). Twenty microliters of the PCR product were sized by electrophoresis on a 1% agarose gel (1 h at 90 V) with a 100-bp ladder as size marker. The gels were stained with ethidium bromide (2 µl 50 ml^{-1} gel) and analyzed in a UV transilluminator (Dolphin-Doc, Wealtec, USA).

Results

The stained blood films revealed long slender forms and short and intermediate thicker forms identified as *T. evansi* based on their morphology (Smyth 1996). Forty-four (3.5%) positive samples were recorded microscopically. Data recorded for extracted DNA concentration by spectrophotometric analysis showed optical density values of 1.89 and 0.84 at 260 and 280 nm wavelengths, respectively. The 237-bp fragment was generated in all positive samples tested with ESAG 6/7 primers (Fig. 1). No such amplicon was detected in control negative samples. Ninety-seven (7.7%) blood samples were found positive for *T. evansi* through PCR, whereas 44 (3.5%) animals were found positive for trypanosomiasis through microscopic examination. All samples positive by blood smear examination were also positive by PCR, whereas, out of the 97 samples positive by PCR test, 53 were negative by microscopy.

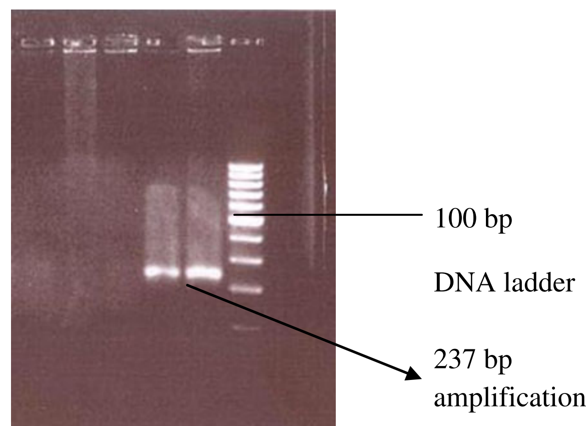

Fig. 1 The 237-bp fragment from *T. evansi* genomic DNA is shown after PCR of two samples of buffalo blood DNA with ESAG 6/7 primers

Discussion

Several studies in Pakistan revealed that *T. evansi* is endemic in horses, camels, and bears (Murtaz et al. 2006; Muhammad et al. 2007). Throughout the country, there has been no reported case of buffalo being infested with this parasite. This is the first report about the prevalence of *T. evansi* in buffalo in Pakistan. However, Nasir et al. (1999) reported 32.5% (14 out of 43) Jersey cows and 23% (11 out of 47) Friesian cows positive for trypanosomes in herds at a livestock experimental station at Bhunikey, Kasur, Pakistan in the same area where the present study was conducted. In that study, the species of *Trypanosoma* was not confirmed. The higher prevalence of trypanosomiasis in this previous study cannot be compared with the low prevalence reported in the present study in the same area due to host species variation as well as a gap of one decade. The disease was diagnosed through symptoms and microscopy in earlier work. In this study, a molecular technique (PCR) was used for the first time to diagnose this parasitic infestation in any animal species in the country. Microscopy of blood smears is an easy and economic diagnostic technique, although this technique proved to be less sensitive as compared to PCR. Another limitation of microscopy is that it cannot differentiate different species of *Trypanosoma* while the molecular technique does so. The results of the present study are in agreement with the findings of Holland et al. (2001) who compared several parasitological tests with PCR in experimentally infected water buffalo and found PCR to be the most sensitive method for *T. evansi* diagnosis. Similar observations were made by Ravindran et al. (2008) who found a higher prevalence of *T. evansi* in camels, dogs, and donkeys through PCR than with blood smear examination. Samples negative by PCR were also negative by microscopy, indicating that the specificity of PCR is satisfactory. In

conclusion, PCR for the detection of *T. evansi* is specific and sensitive. The test is suitable for tracing carrier animals and provides a quantitative validated measure that is useful in epidemiological surveys and follow-up for drug treatment in Nili-Ravi buffalo. In addition, it would be useful for designing trypanosomiasis control programs in endemic areas.

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