Research in Veterinary Science 94 (2013) 555–561

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

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

Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle

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ARTICLE INFO

Article history:
Received 18 June 2012
Accepted 21 October 2012

Keywords:
Cattle
Molecular diagnostics
Nigeria
PCR
Trypanosomes

ABSTRACT

Microscopy and polymerase chain reaction (PCR) were used to survey pathogenic trypanosome infection in naturally infected Nigerian cattle. In 411 animals sampled, microscopy detected 15.1% positive infection of at least one of *Trypanosoma brucei*, *Trypanosoma congolense* or *Trypanosoma vivax*, while PCR detected 63.7% positive infections of at least one of those species and *Trypanosoma evansi*. PCR detected 4.4%, 48.7%, 26.0% and 0.5% respectively of *T. brucei*, *T. congolense*, *T. vivax* and *T. evansi* infections. All of the *T. congolense* detected were savannah-type, except for two forest-type infections. Prevalence of mixed infections was 13.9%, being primarily co-infection by *T. congolense* and *T. vivax* while prevalence of mixed infections by *T. evansi*, *T. vivax* and *T. congolense* was 1.5%. Microscopy showed poor sensitivity but specificity greater than 94%. Infection rates were much higher in Southern than in Northern Nigeria. Infections were lowest in N'dama compared to Muturu, Sokoto Gudali and White Fulani breeds. Animals with *T. vivax* monoinfection and mixed infections showed significantly lower packed cell volume (PCV) values. Those infected with any *Trypanosoma* species with <200 parasites/μl showed higher PCV values than those infected with >200 parasites/μl. The new finding of savannah- and forest- type *T. congolense* in Nigeria and the relatively high abundance of mixed infections are of significant clinical relevance. This study also suggests that *T. congolense* is the most prevalent species in Nigeria.

1. Introduction

Trypanosomosis is a complex infectious disease of animals caused by a range of extra-erythrocytic protozoan parasites of the genus *Trypanosoma*, responsible for production losses, morbidity and sometime mortality in infected herds (Abenga et al., 2002). The clinical signs of trypanosomosis depend on the species and strain of the infecting trypanosome, breed of the animal involved (Anene et al., 1991a,b; Matioli et al., 1998) and the prevalence of vectors (Leak et al., 1990; Onyiah, 1997; Merkuria and Gadissa, 2011). Clinical signs include anemia, intermittent fever, parasitaemia, lymphadenopathy, jaundice, progressive emaciation, loss of production, weakness and death, if left untreated (Akinwale et al., 1999; Merkuria and Gadissa, 2011). While Muturu and N'dama are considered trypanotolerant breeds because they strive well under the pressure of trypanosome infections, they act as reservoirs of the infection for other animals (Moloo et al., 1992).

In Nigeria, diagnosis of bovine trypanosomosis largely depends on parasitological and immunological methods. Parasitological techniques have significant limitations exemplified by inability to differentiate between *Trypanosoma brucei* and *Trypanosoma evansi* except through the molecular composition of their kinetoplast DNA (kDNA) (Artama et al., 1992; Feng-Jun et al., 2007). Within species, parasitological methods can identify *Trypanosoma congolense* but not sub groups of the parasite. Hence, this technique lacks the sensitivity and the precision required for the purpose of adequate therapeutic and prophylactic control measures, exacerbated by a high proportion of false negative results. Immunological techniques (i.e. enzyme linked immunosorbent assays, card agglutination and fluorescent antibody tests) on the other hand are good for large scale epidemiological studies (Greiner et al., 1997) but not sensitive enough to detect and differentiate between current and previous infections, also leading to false positive results (Desquesnes and Tresse, 1996). Molecular technique such as polymerase chain

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http://dx.doi.org/10.1016/j.rvsc.2012.10.018

reaction (PCR) has shown to be more sensitive and precise than the aforementioned techniques (Moser et al., 1989; Pinchbeck et al., 2008). This technique, though expensive and relatively new to certain parts of Africa, is so sensitive that parasitaemia as low as 10 parasites per milliliter of blood can be detected using PCR (Desquesnes and Davila, 2002; Delespaux et al., 2003). Due to its sensitivity, it has been used in some parts of Africa to ascertain the incidence, prevalence and characterization of trypanosome strains (Solano et al., 1999; Mugittu et al., 2001; Simukoko et al., 2007; Balmer and Caccone, 2008; Cordon-Obras et al., 2009). However, only *Trypanosoma vivax* Y58 strain, a field isolate with unknown isolation year (Feng-Jun et al., 2007) has been characterized in Nigeria (Morlais et al., 2001).

The prevalence of trypanosomosis has been extensively studied using parasitological and immunological methods in Eastern and Northern parts of Nigeria (Daniel et al., 1993; Kalu, 1995; Kalu and Lawani, 1996; Abenga et al., 2004; Oluwafemi et al., 2007; Ezeani et al., 2008; Qadeer et al., 2008; Enwezor et al., 2009; Kamani et al., 2010). The only recent records of trypanosomosis in the Western part of the country are 3.9% and 36.8% prevalence in Ogbomoso, Oyo and Ogun states, respectively (Ameen et al., 2008; Sam-Wobo et al., 2010). The use of PCR as a better diagnostic tool to ascertain the incidence and prevalence of trypanosomosis has been advocated (Desquesnes and Tresse, 1996; Miyamoto et al., 2006; El-Metanaway et al., 2009) but has not yet been applied in Nigeria. The present study was designed to determine the prevalence and characteristics of trypanosome species and strains in Nigerian cattle using PCR for the first time.

2. Materials and methods

2.1. Study population and sample collection

Random sampling was not possible due to lack of data on national reference census of nomadic herds. Therefore, working with owners, sampling was carried out on selected cattle herds in areas where cattle converge to rest during migration. Two major abattoirs were used for sampling. The animals to be sampled were selected by systematic random sampling technique whereby the sampling interval (j) is computed as the study population size divided by the required sample size and the first study subject is chosen randomly from among the first j study subjects, then every jth study subject after that is included in the sample (Dohoo et al., 2009).

A total of 411 cattle (129 males and 282 females) ranging in age from 9 months to 6 years consisting of Muturu (112), N'dama (31), Sokoto Gudali (68) and White Fulani (200) breeds were sampled in Ogun and Kaduna states (Southern and Northern regions respectively). Animals aged one and under were considered young calves, while those over one year were regarded as adults. Animals with histories of recent trypanocidal treatment and those from institutional farms were excluded from the study. Age was determined by dentition and for the purpose of this study the body conditions were assessed and scored as described by Nicholson and Butterworth (1986). Blood samples were collected from the jugular vein of each animal into 5 ml tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The samples were transported in a mobile refrigerator to the laboratory within 3 h of collection, and were stored at 4°C prior to DNA extraction.

2.2. Parasitological diagnosis

From each tube containing anticoagulant, blood was transferred into three capillary tubes which were sealed at one end with plasticine. The capillary tubes were spun in a microhaematocrit centrifuge at 10,000 rpm for 3 min. After centrifugation, the packed cell volume (PCV) was determined. The buffy coat and upper most layers of red blood cells of one capillary tube were extruded onto a microscope slide and examined with a phase-contrast microscope at 400× magnification (Murray et al., 1977) for the presence of motile trypanosomes. At least 50 fields were examined before positive or negative was declared for each sample. Positive samples were further processed as thin smear stained with Giemsa for trypanosome species identification. Thick blood smears were also prepared, stained with Giemsa and examined with 100× oil immersion objective lens (1000× magnification). Parasitaemia was determined as described by Hebert and Lumsden (1976).

2.3. DNA extraction

DNA was extracted from the blood using Quick-gDNA™ Mini-Prep (Zymo Research Corporation, Irvine, CA, USA) as described by the manufacturer. Briefly, 400 µl of genomic lysis buffer was added to 100 µl of blood, thoroughly mixed and incubated at room temperature for 5–10 min. The mixture was transferred to a spin column in a collection tube and centrifuged at 10,000 × g for 60 s after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. A volume of 200 µl of prewash buffer was added to the spin column and centrifuged at 10,000 × g for 60 s, after which 500 µl of genomic DNA wash buffer was added to the spin column and centrifuged at 10,000 × g for 60 s. The soluble DNA was eluted into 50 µl nuclease free water from the spin column into a clean 1.5 ml microcentrifuge tube, incubated at room temperature for 2–5 min and centrifuged at 16,000 × g for 30 s. Quantification of DNA yield and assessment of quality were done using Nanodrop ND-100 UV/Vis Spectrophotometer (Nanodrop Technologies, Inc., DE, USA). The eluted DNA was stored at −20 °C until use.

2.4. Primer sets and optimization

Polymerase chain reaction (PCR) primers were selected for optimization based on previously published work. These primers were optimized with DNA extracted from the blood of cattle parasitologically positive for *T. vivax*, *T. congolense*, *T. brucei* and *T. evansi* which led to final selection of six primer sets for this study. Details of primer sets are presented in Table 1.

2.5. Trypanosome detection by PCR

PCR amplification was performed in 20 µl final reaction volume containing equivalent of 20 ng of genomic DNA, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primers and 1unit of *Taq* DNA polymerase (Bioneer, Inc. Alameda, CA USA). The reactions were placed in a C-1000 series thermocycler (Biorad, Hercules, CA, USA). The reaction conditions were as follows: *T. brucei* and *T. evansi*; Initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 5 min. *T. congolense*; initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C at 30 s, 60 °C for 30 s and 72 °C for 30 s with final extension at 72 °C for 5 min. *T. vivax*; initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s followed by final extension at 72 °C for 5 min. Ten microliters of the PCR products were electrophoresed through 1% agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid 1 mM EDTA) at 90 V for 80 min. along with 10 µl of GENEMate Quanti-Marker 100 bp DNA ladder (BioExpress, Kaysville, UT, USA). Gels were stained with GelRed® Nucleic Acid Stain (Phenix Research Products, Candler, NC, USA) at 5 µl/100 ml of the agarose gel suspension. After electrophoresis, PCR products were