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Full Length Research Paper

Comparison of reverse line blot and β-tubulin targeted nested polymerase chain reaction (PCR) techniques in the detection of *Theileria* and *Babesia* piroplasms in cattle in Uganda

Kalenzi David Atuhaire[1], Kokas Ikwap[1], Samuel Eyanu[1], Frank Norbert Mwiine[1*], Micheal Ocaido[1], William Olaho-Mukani[2], Margaret Saimo[1] and George William Lubega[1]

[1]College of Veterinary Medicine, Animal resources and Biosecurity, Makerere University, P. O. 7062 Kampala, Uganda.
[2]OIE Regional Commission for Africa, Arusha, Tanzania.

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Three hundred (300) blood samples from apparently healthy cattle were collected in areas around Lake Mburo National Park in Western Uganda. These were used in the comparison of the sensitivity and specificity of reverse line blot (RLB) and β-tubulin targeted nested polymerase chain reaction (PCR) in the detection of tick-borne piroplasms in cattle. The sensitivity of RLB technique for the detection of *Theileria* and *Babesia* species was 71.25% (95% CI: 60.05 to 80.82%) and the specificity was 57.50% (95% CI: 40.89 to 72.960%) while the sensitivity of the beta-tubulin targeted nested PCR was 62.5% (95% CI: 50.96 to 73.08%) and the specificity was 72.5% (95% CI: 56.11 to 85.40%). The positive predictive value using RLB was 77.03% (95% CI: 65.79 to 86.01%) and the negative predictive value was 50% (95% CI: 34.90 to 65.10%) while the positive predictive value using β-tubulin targeted nested PCR was 81.97 % (95% CI: 70.02 to 90.64%) and the negative predictive value was 49.15% (95% CI: 35.89 to 62.50%). The Kappa statistic for level of agreement in detection of tick-borne piroplasms between RLB assay and β-tubulin targeted nested PCR was 0.7984 which indicated substantial agreement between the two tests. The RLB assay allowed the detection of individual species that simultaneously infected the cattle. However, it was not possible to identify the species with the β-tubulin targeted nested PCR.

Key words: *Babesia, Theileria,* β-tubulin, reverse line blot, sensitivity, specificity.

INTRODUCTION

In Africa and specifically in Uganda, tick-borne diseases (TBDs) are considered to be the major constraints to livestock productivity (Young et al., 1988; Ocaido et al., 2005). TBDs affect 80% of the world's cattle population and are widely distributed throughout the world, particularly in the tropics and subtropics. In acute cases, bovine piroplasmosis can be diagnosed by microscopic examination of Giemsa-stained thin blood smears and by clinical symptoms. Nevertheless, following acute infections, recovered animals frequently retain subclinical infections (carriers). Serological methods are employed in diagnosing subclinical infections, but false positive and false negative results are commonly observed due to cross-reaction. Therefore, a more specific and sensitive method for the diagnosis of piroplasms is required. Recently, species-specific polymerase chain reaction (PCR) and PCR-based reverse line blot (RLB) hybridization methods have been developed and used (Schnittger et al., 2004; Aktas et al., 2005).

RLB hybridization is a laborious and expensive technique that has been used for screening of tick-borne infections. Therefore, there is a need to adopt a less laborious and cheap technique that can also simultaneously detect multiple infections. All eukaryotic cells are

*Corresponding author. E-mail: mwiine@vetmed.mak.ac.ug.

structurally and functionally supported by hollow proteinacious organelles known as microtubules. Microtubules are composed of two major proteins, α and β-tubulin, and a third minor species the γ-tubulin (Little and Seeheus, 1988; Oakely and Oakely, 1989). The β-tubulin gene is one of the few apicomplexan genes that are interrupted by one or more introns (Nagel and Boothroyd, 1988). In addition, the position of the first intron is conserved in all the species investigated so far, allowing for a rational design of primers around this region. Introns associated with the β-tubulin gene show extensive variations both in length and in sequence. These features make this region of the genome a good candidate for the development of informative markers, as it has been shown previously for several protozoan parasites (Costa et al., 1997).

A fragment of the β-tubulin gene has been amplified and sequenced from *Theileria* and *Babesia* species. The presence (within the amplified gene fragment) of an intron that varies extensively both in length and in sequence, has allowed the development of an assay to differentiate the species directly on the basis of the specific size of the PCR products or by employing a simple PCR-restriction fragment length polymorphism (RFLP) protocol (Caccio et al., 2000). The ß-tubulin targeted nested PCR falls into this category of simultaneously detecting multiple infections (Caccio et al., 2000) and the detection of the reaction product can easily be done by running an agarose gel or any other method such as optical density measure, hence the necessity to compare it with RLB technique.

This study compared the capacities of ß-tubulin targeted nested PCR and RLB techniques in detection and identification of *Theileria* and *Babesia* species in healthy cattle so that one of the two can be adopted in the screening and epidemiological studies of tick-borne infections in resource poor countries.

MATERIALS AND METHODS

Blood samples

Three hundred (300) blood samples from apparently healthy adult Ankole cattle raised on free range ranging around Lake Mburo National Park (Western Uganda) were collected by jugular venipuncture into Ethylenediaminetetraacetic acid (EDTA) coated vacutainers. Purposive random sampling was used in this study. Upon collection, samples were put on ice packs and transported to the laboratory or thin smears were made and also transported to the laboratory where they were methanol fixed for 5 minutes, Giemsa stained for 30 min and then microscopically examined.

DNA extraction

Eighty (80) of microscopically positive and 40 negative samples were selected and blood aliquoted in 1.5 ml eppendorf tubes andstored at -20°C till used for DNA extraction. DNA was extracted as described by d'Oliviera et al. (1995) with some modifications. Briefly, 200 µl of thawed blood in an eppendorf tube was washed 3 to 5 times by mixing with 0.5 ml Phosphate buffered saline (PBS) (137 mM NaCl, 2.6 mM, KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), followed by centrifugation at maximum speed (13,000 rpm) for 5 min. After the final wash, the cell pellet was resuspended in 100 µl of lysis mixture (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5% Tween 20, 100 µg/ml of proteinase K). This mixture was incubated over night at 56°C, followed by 10 min of boiling to inactivate proteinase K. The mixture was then kept at -20°C until needed for PCR.

PCR amplification of 18s ribosomal RNA (rRNA)

One set of primers was used to amplify a 390 to 430 bp fragment of the 18S rRNA gene spanning the V4 region of *Theileria* and *Babesia* organisms. The forward primer used was RLB-F2 5'-GAC ACA GGG AGG TAG TGA CAA G-3' and the reverse primer was RLB-R2 5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3', as described by Georges et al. (2001). The primers were manufactured by Bioneer, Germany. The reaction constituents in a final volume of 25 µl were as follows: 1× PCR buffer (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), 200 µM each dATP, dCTP, dGTP, 100 µM dTTP (ABgene) and 100 µM dUTP (Amersham), 1.25 U of Taq DNA polymerase (Invitrogen), 0.1 U of UDG (Amersham), 25 pmol of each primer, 2.5 µl of template DNA and sufficient distilled water to top up the reaction (Bekker et al., 2002). Each time, positive control DNA (*T. parva* or *B. bovis*) and negative control (reaction constituents without DNA or with *Trypanosoma* DNA) were included. The reactions were performed using the program described by Bekker et al. (2002). Thereafter, PCR products were kept at -20°C until needed for reverse line blot hybridization.

PCR amplification of beta-tubulin regions

A fragment of the β-tubulin gene was amplified using the forward primer F34 (5'-TGTGGTAACCAGAT(t/c)GG(a/t)GCCAA-3'), and the reverse primer R323 (5‟ -TCnGT(a/g)TA(a/g)TGnCC(t/c)TT(a/g)GCCCA-3'). The reaction mixture and PCR amplification were as described by Caccio et al. (2000). Amplifications were performed on a Perkin-Elmer model 2400 thermal cycler. For nested PCR reactions, a forward primer F79 (5‟ -GA(a/g)CA(t/c)GGnATnGA(t/c)CCnGTAA-3''), and a reverse primer R206 (5‟ -AC(a/t/g)GA(a/g)TCCATGGT(a/t/g)CCnGG(t/c)T-3'') were used. The reactions were run using the same profile as described above for the primary PCR, (Caccio et al., 2000). The PCR products were run on 2% agarose, stained with ethidium bromide and visualized under UV light.

Application of species-specific oligonucleotide probes onto the biodyne C membrane

The species-specific probes were applied to the membrane as described by Gubbels et al. (1999). After activation, the membrane was sealed in a plastic bag containing 5 ml of 20 mM EDTA and stored at 4°C until required.

Reverse line blot hybridization of PCR products

Hybridization of PCR products to the species-specific probes was performed as described by Gubbels et al. (1999). Membranes were then incubated for 1 min at room temperature in 10 ml of mixed Electrogenerated chemiluminescence (ECL) detection liquids 1 and 2 (Amersham). The membrane was placed between two colorless polythene sheets, placed on the intensifying screen in an exposure

cassette with the DNA side up and exposed to an ECL-hyper film (Amersham) for 20 to 25 min in the dark room.

Data analysis

The RLB and nested beta-tubulin data sets were analyzed using the DAG-STAT software program for comparing diagnostic tests and determining the level of agreement between tests (Mckinnon, 2000).

RESULTS

Light-microscopy examination of thin blood smears

Of the 300 samples microscopically analyzed, 120 were positive (intra-erythrocytic parasites observed) and the rest (180) negative (no intra-erythrocytic parasites observed). Some samples (80 positive and 40 negative) were randomly selected and analyzed using the RLB and β-tubulin nested PCR assays.

Reverse line blot analysis

The RLB assay allowed the detection of individual species that simultaneously infected the cattle. Out of the 80 samples positive by microscopy, RLB confirmed 57 (71.25%) positive for at least one species while 23 (28.75%) were negative (Table 1). Of the 57 samples positive by RLB assay, 8 (14.04%) were infected by *T. mutans* only, 47 (82.46%) were simultaneously infected with *T. parva* and *Theileria* spp. (buffalo) but not with any other species, while 1(1.75%) sample was simultaneously infected with *T. parva*, *Theileria* spp. (buffalo) and *B. bovis.* Only 1 (1.75%) sample failed to match with any of the species represented on the probe membrane. On the other hand, out of randomly selected 40 microscopically negative samples, 17 (42.5%) were found to be positive and 23 (57.5%) maintained the negative status. Of the 17 samples that changed status to positive by RLB, 11(64.5%) had mixed infection, while 6 (35.3%) had single infection. All the samples that showed mixed infection included *T. parva* and *Theileria* spp. (buffalo) (Figure 1). Out of the 6 samples showing single infection, 5 samples were positive for *T. parva* while one was positive for *B. bovis*.

Beta-tubulin targeted nested PCR analysis

Eighty (80) samples positive by microscopy were subjected to beta-tubulin nested PCR analysis. Fifty samples (62.5%) tested positive while 30 samples (37.5%) tested negative with beta-tubulin nested PCR analysis (Table 1). The gel (Figure 2) shows some representative samples tested with the beta-tubulin nested PCR analysis. Two to three bands (300 to 400 bp) were identified in some samples, suggesting mixed infection in some samples, though it was not possible to know the specific species in question. Forty (40) samples testing negative by microscopy were subjected to beta-tubulin nested PCR analysis. Eleven samples (27.5%) were found positive and the rest negative. The beta-tubulin primers did not amplify *Trypanosoma* tubulin (Figure 2). The amplicons were obtained using primers targeting the β-tubulin DNA of *Theileria* and *Babesia* species and fractionated on a 2% agarose gel.

Comparison of β-tubulin targeted nested PCR with RLB assay

The sensitivity of RLB technique for the detection of *Theileria* and *Babesia* species was 71.25% (95% CI: 60.05 to 80.82%) and the specificity was 57.50% (95% CI: 40.89 to 72.960%) while the sensitivity of the beta-tubulin targeted nested PCR was 62.5% (95% CI: 50.96 to 73.08%) and the specificity was 72.5% (95% CI: 56.11 to 85.40%). The Positive predictive value using RLB was 77.03 % (95% CI: 65.79 to 86.01%) and the Negative predictive value was 50% (95% CI: 34.90 to 65.10%) while the Positive predictive value using beta-tubulin targeted nested PCR was 81.97 % (95% CI: 70.02 to 90.64%) and the Negative predictive value was 49.15% (95% CI: 35.89 to 62.50%). The Kappa statistic for level of agreement in detection of tick-borne piroplasms between RLB assay and beta-tubulin targeted nested PCR was 0.7984 (95% CI: 0.6925 to 0.9044).

DISCUSSION

The aim of this study was to compare Reverse line blot and beta-tubulin targeted nested polymerase chain reaction in the detection of tick-borne piroplasms of apparently healthy cattle in Western Uganda so that their role in routine diagnosis of clinical cases and in epidemiological studies can be substantiated. The RLB assay is currently used in the detection of tick-borne infections whereas the β-tubulin targeted PCR is not.

Previous studies (Gubbels et al., 1999; Oura et al., 2004; Schnittger et al., 2004) have emphasized the high sensitivity of the RLB assay. However, the RLB assay is expensive and laborious. In this study therefore, the β-tubulin targeted nested PCR was compared with the RLB assay to determine if it can be an easy but still sensitive alternative to use. The RLB permitted simultaneous detection of multiple infections in single animals as previously reported by Muhanguzi et al. (2010). A substantial proportion of the cattle showed mixed infection (84.21%) with the majority (82.46%) infected with two species only; that is, *T. parva* and *Theileria* spp(buffalo) while eight cattle (14.04%) were infected with *T. mutans* alone while one animal had mixed

Table1. Comparison of reverse line blot and the beta-tubulin targeted nested PCR with microscopy as the reference test.

TEST		Microscopy (n = 120)	
		Positive (80)	Negative (40)
RLB	Positive (74)	57	17
	Negative (46)	23	23
ß-tubulin nested PCR	Positive (61)	50	11
	Negative (59)	30	29

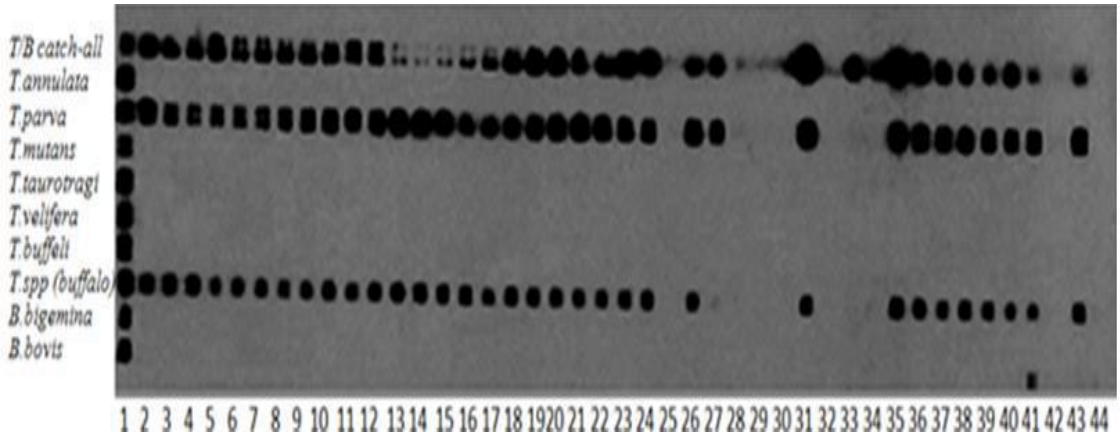

Figure 1. Reverse line blot analysis, species-specific oligonucleotide and theileria/babesia catch-all were applied to the horizontal rows of the RLB as shown. Slot 1 is the membrane positive control (Isogen, The Netherlands) and slot 27 is the positive control(*T. parva*). The remaining slots are test samples.

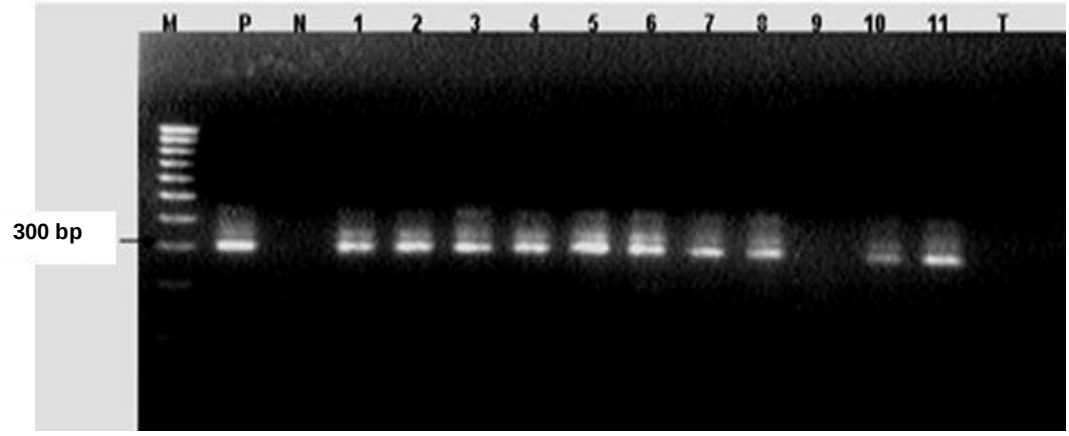

Figure 2. Agarose gel analysis of the β-tubulin PCR amplicons. Lane M; 100 bp sigma molecular weight DNA marker, lane P; positivecontrol, lane N; negative control, lanes 1 to 11,test samples and lane T; *Trypanosoma* DNA.

infection with three species that is, *T. parva*, *Theileria* spp (buffalo) and *B. bovis.* However, *Theileria* spp (buffalo) and *B. bovis* presented with weak signals. Both *T. parva* and *B. bovis* are pathogenic to cattle. The beta-tubulin nested PCR was able to determine the genus but it was not possible to determine the species. This is because the sizes of PCR amplicons of most species have not been established. The previous study by Caccio et al. (2000) used known species of *Theileria* and *Babesia* and the sizes of their PCR products were determined. The beta-tubulin primers did not amplify *Trypanosoma* tubulin DNA. This showed that the primers were specific to

Theileria and *Babesia* tubulin and probably not to any other parasite tubulin.

The beta-tubulin targeted nested PCR compared very well with the RLB assay and with very high sensitivity and specificity. Since the two tests showed substantial agreement, we strongly believe that the less laborious beta-tubulin targeted nested PCR can be used for diagnosis of clinical cases. However, for field epidemiological studies in which establishment of the species are required, the RLB would be preferable. Therefore, more research needs to be done on the beta-tubulin targeted nested PCR using known species of *Theileria* and *Babesia* such that the species-specific sizes of amplicons can be established.

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