International Journal for Parasitology 42 (2012) 207–214

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

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

A highly sensitive DNA bead-based suspension array for the detection and species identification of bovine piroplasms

Amaia Ros-García, Ramón A. Juste, Ana Hurtado *

Department of Animal Health, NEIKER – Instituto Vasco de Investigación y Desarrollo Agrario, Berreaga 1, 48160 Derio, Bizkaia, Spain

ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 7 December 2011
Accepted 8 December 2011
Available online 27 December 2011

Keywords:
Babesia
Theileria
Luminex
Suspension microarray
Cattle
RLB
Tick-borne pathogens
Piroplasmosis

ABSTRACT

Piroplasms are among the most harmful tick-borne pathogens for livestock and sensitive and specific diagnostic methods for rapid detection and identification of the different species are needed for effective control. Reverse Line Blot has been the molecular technique of choice but it is laborious, time-consuming and highly susceptible to subjective variation in the interpretation of the hybridisation signal. Here, an oligonucleotide multiplex suspension microarray (Luminex® microsphere system) was developed for bovine piroplasms. Probes previously used in Reverse Line Blot for *Babesia divergens*, *Babesia bovis*, *Babesia occultans*, *Babesia bigemina* and *Theileria buffeli*, and a catch-all *Theileria* and *Babesia* control probe, were included in the Luminex assay together with newly designed probes for *Theileria annulata* and *Babesia major*. An internal amplification control that was detected with a Luminex probe was included to monitor for inhibition. Serially diluted linearised recombinant plasmids of the different species were used to assess the analytical sensitivity and specificity, and the detection limit of the Luminex assay was determined using serial dilutions of infected blood from an animal with a known level of *T. annulata* parasitaemia. The assay was then validated on 214 bovine blood samples analysed in parallel by Reverse Line Blot and Luminex. The Luminex assay proved to be highly specific and more sensitive than Reverse Line Blot, detecting 0.05 parasites/μl of blood. Technically, the Luminex procedure was rapid, provided high throughput screening, transformed the subjective interpretation of Reverse Line Blot results into numerical objective values, and allowed more flexibility in array preparation than Reverse Line Blot. The method described herein can substantially improve the detection of piroplasm carriers and thus better protect livestock trade and facilitate preventive control programs.

1. Introduction

Piroplasms are tick-borne intracellular apicomplexan parasites classified into two main genera, *Theileria* and *Babesia*. Some piroplasm species are pathogenic and therefore of economic importance while others are considered moderately pathogenic or benign (Preston, 2001; Uilenberg, 2001). Piroplasmosis has a wide distribution and bovine piroplasmosis is among the most serious diseases affecting cattle. Certain *Babesia* spp. can also cause disease in humans; in Europe mainly due to infection with *Babesia divergens*. Laboratory diagnosis of infection with piroplasms was traditionally based on microscopy and serological tests. Nevertheless, both of those have limitations in detecting carrier animals with low numbers of infected erythrocytes and discriminating pathogenic from non-pathogenic species, particularly in mixed infections, and cross-reactivity problems have been described for serology (Bono et al., 2008; OIE, 2008).

To overcome these drawbacks, molecular tests based on PCRs have been used for the sensitive and specific detection of several piroplasms (Figueroa et al., 1993; Almeria et al., 2001). More recently, real-time PCR procedures have been developed for a more rapid detection of the infection with reduced contamination risk and generally with higher sensitivity (Kim et al., 2007; Criado-Fornelio et al., 2009; Ramos et al., 2011). However, the multiplexing capacity of PCR is limited and does not allow for the simultaneous detection of the several *Babesia* and *Theileria* spp. that can infect a single host. For this purpose, Reverse Line Blot (RLB) hybridisation methods were developed for bovine piroplasms in 1999 (Gubbels et al., 1999) and soon extended to other piroplasm species (Schnittger et al., 2004; Nagore et al., 2004a,b). Nowadays, RLB hybridisation has become the molecular technique of choice for the simultaneous detection and identification of several *Babesia* and *Theileria* spp. in hosts and tick vectors (Georges et al., 2001; Oura et al., 2004; Nagore et al., 2004b; Bosman et al., 2007; García-Sanmartín et al., 2008; Niu et al., 2009). Although this assay provides high sensitivity and specificity, it is laborious, time-consuming and interpretation is subjective. The need for technologies that allow rapid, cost-effective, high-throughput detection of infectious agents impelled us to develop an alternative method to RLB hybridisation.

The Luminex xMAP technology (Luminex Corporation, Austin, TX, USA) incorporates 5.6 micron polystyrene microspheres inter-

* Corresponding author. Tel.: +34 944034300; fax: +34 944034310.
E-mail address: ahurtado@neiker.net (A. Hurtado).

0020-7519/$36.00
doi:10.1016/j.ijpara.2011.12.001

nally dyed with two spectrally distinct fluorochromes, giving an array of up to 100 different microsphere sets with specific spectral addresses. Each microsphere set can be labelled with a different molecule (DNA probes or protein antigens) on its surface and combined in a multiplex system that allows measurement of up to 100 different analytes simultaneously in a single reaction tube. A third fluorochrome coupled to a reporter molecule quantifies hybridisation at the microsphere surface. Microspheres are interrogated as they pass by two separate lasers in a microfluidic system. High-speed digital signal processing classifies the microsphere based on its spectral address and quantifies the reaction on the surface (Dunbar et al., 2003). Several Luminex DNA assays have been developed for the detection, identification and genotyping of infectious agents such as *Cryptosporidium*, *Mycobacterium*, *Salmonella*, classical swine fever virus and other pestiviruses (Cowan et al., 2004; Deregt et al., 2006; Bandyopadhyay et al., 2007; Fitzgerald et al., 2007; Li et al., 2010; Leblanc et al., 2010). The technique can also be used for the detection of antibodies (Binnicker et al., 2010; Anderson et al., 2011; Griffin et al., 2011; Shoma et al., 2011).

Here, we describe the results of the development of a multiplexed microsphere-based suspension array platform using the xMAP Luminex system as an alternative to RLB for the simultaneous detection and identification of bovine piroplasms. The assay was validated using 214 DNA samples extracted from blood collected from cattle and analysed in parallel by RLB and Luminex.

2. Materials and methods

2.1. Samples

2.1.1. Positive controls

A total of 25 plasmid DNAs containing inserts corresponding to the V4 variable region of the 18S rRNA gene of the different species within the genera *Theileria* and *Babesia* constructed as described elsewhere (Nagore et al., 2004a), were used as positive controls.

2.1.2. Field samples

DNA extracts from 214 cattle blood samples were used to optimise and validate the Luminex assay. Blood samples were taken from cattle herds with symptoms of piroplasmosis in different geographic regions of Spain.

2.2. DNA extraction

DNA was extracted from 200 μl of cattle blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), including negative extraction controls every 10 samples. DNA yields were determined with a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, DE, USA), and DNA was stored at −20 °C until subsequent analysis.

2.3. DNA amplification

PCR was used to amplify fragments of 385–430 bp corresponding to the V4 hypervariable region of the 18S rRNA gene of *Theileria* and *Babesia* spp. with the forward and reverse primers RLB-F (phosphor-5′-GACACAGGGAGGTAGTGACAAG-3′) and RLB-R (biotin-5′-CTAAGAATTTCACCTCTGACAGT-3′) (Sigma Aldrich, St. Louis, USA) as reported previously (Georges et al., 2001). The reverse primer was 5′-biotinylated to allow conjugation of streptavidin–peroxidase or streptavidin–phycoerythrin for later detection by the RLB and the Luminex assays, respectively. The forward primer was 5′-phosphorylated to allow digestion of the corresponding amplicon strand with Lambda exonuclease (Leblanc et al., 2010). An internal amplification control (IAC), which is co-amplified with the same primers as the piroplasm DNA (see Section 2.5), was also included in each tube. Thus, PCR amplification was performed in a final volume of 50 μl containing 50 ng of genomic DNA, 200 nM of each primer, 1× PCR buffer, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, one copy of the IAC plasmid (see Section 2.5) and 1 U of Taq Platinum Polymerase (Invitrogen, CA, USA). PCR conditions consisted of a first denaturation step of 4 min at 94 °C, a second step of 40 cycles of 30 s at 94 °C, 1 min at 51 °C and 35 s at 72 °C. Extraction controls and PCR negative (water) controls were included in each PCR experiment as negative controls.

2.4. RLB hybridisation

Samples subjected to RLB hybridisation were processed as previously described including a panel of specific probes for 23 piroplasms (García-Sanmartín et al., 2008) and a new probe for *Babesia occultans* (Ros-García et al., 2011). Preparation of RLB membrane and hybridisation were carried out as previously described (Gubbels et al., 1999) with the adaptations reported elsewhere (Nagore et al., 2004a).

2.5. Internal amplification control (IAC) design

An artificially synthesized single stranded DNA fragment of 112 nucleotides (nt) (Sigma) was designed to produce an IAC. The fragment consisted of the IAC probe sequence flanked by the target sequence for the primers RLB-F and RLB-R which amplify the piroplasm 18S rDNA V4 region. A double-stranded DNA amplicon was generated by PCR in 25 μl reactions under the same conditions used to amplify piroplasm DNA as described in Section 2.3. The amplified IAC product was purified with a GenElute™ Gel extraction Kit (Sigma), cloned into a pCR®-TOPO® vector, and transformed into *Escherichia coli* according to the manufacturer's instructions (TOPO TA Cloning® kit for sequencing, Invitrogen). Recombinant plasmid DNA was purified using a plasmid purification kit (FastPlasmid™ Mini Kit, Eppendorf AG, Hamburg, Germany) and the sequence confirmed using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Once sequenced, plasmids were linearised and serially diluted (10^4–1 copies/μl) to test for the optimal number of copies to be included in each test tube for the identification of false negative PCR results caused by PCR inhibition. One copy was considered the optimal IAC amount of IAC plasmid for detection without affecting the piroplasm detection limit.

2.6. Luminex assay

2.6.1. Probe design

The oligonucleotide probes previously designed for bovine piroplasm identification by RLB hybridisation (Gubbels et al., 1999; Georges et al., 2001; García-Sanmartín et al., 2006; Ros-Garcia et al., 2011) were initially tested using Luminex. When low sensitivity or cross-reactions were observed, novel probes were designed using PrimerPlex software (PREMIER Biosoft International, CA, USA) and synthesized by Sigma. All the probes included a C6 amino linker at the 5′ end. Thus, six of the probes used in RLB (the *Theileria–Babesia* conserved catch-all TB probe, *Theileria buffeli*, *Babesia bigemina*, *B. divergens*, *Babesia bovis* and *B. occultans*) proved suitable for use in Luminex, but two (*Babesia major* and *Theileria annulata*) were unsuitable and required redesign. A further probe designed to detect the IAC in the Luminex assay was also included.

2.6.2. Oligonucleotide probe bead coupling

Oligonucleotide probes were conjugated to colour-coded beads (carboxylated microspheres, Luminex Corporation) according to the protocol recommended by the manufacturer. Beads (5×10^5