Veterinary Parasitology 207 (2015) 329–334

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Short Communication

# Molecular biological identification of *Babesia*, *Theileria*, and *Anaplasma* species in cattle in Egypt using PCR assays, gene sequence analysis and a novel DNA microarray

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ARTICLE INFO

*Article history:*
Received 21 August 2014
Received in revised form
17 December 2014
Accepted 18 December 2014

*Keywords:*
Bovine piroplasms
*Babesia*
*Anaplasma*
Cattle
PCR
Microarray

ABSTRACT

In this preliminary study, a novel DNA microarray system was tested for the diagnosis of bovine piroplasmosis and anaplasmosis in comparison with microscopy and PCR assay results. In the Dakahlia Governorate, Egypt, 164 cattle were investigated for the presence of piroplasms and *Anaplasma* species. All investigated cattle were clinically examined. Blood samples were screened for the presence of blood parasites using microscopy and PCR assays. Seventy-one animals were acutely ill, whereas 93 were apparently healthy. In acutely ill cattle, *Babesia*/*Theileria* species ($n = 11$) and *Anaplasma marginale* ($n = 10$) were detected. Mixed infections with *Babesia*/*Theileria* spp. and *A. marginale* were present in two further cases. *A. marginale* infections were also detected in apparently healthy subjects ($n = 23$). The results of PCR assays were confirmed by DNA sequencing. All samples that were positive by PCR for *Babesia*/*Theileria* spp. gave also positive results in the microarray analysis. The microarray chips identified *Babesia bovis* ($n = 12$) and *Babesia bigemina* ($n = 2$). Cattle with babesiosis were likely to have hemoglobinuria and nervous signs when compared to those with anaplasmosis that frequently had bloody feces. We conclude that clinical examination in combination with microscopy are still very useful in diagnosing acute cases of babesiosis and anaplasmosis, but a combination of molecular biological diagnostic assays will detect even asymptomatic carriers. In perspective, parallel detection of *Babesia*/*Theileria* spp. and *A. marginale* infections using a single microarray system will be a valuable improvement.


## 1. Introduction

Tick-borne diseases (TBDs) hamper the growth of the livestock sector and impose serious constraints on the health and productivity of domesticated cattle in tropical and sub-tropical regions of the world (de Castro, 1997). Tropical theileriosis, bovine babesiosis and anaplasmosis are among the economically most important diseases.

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http://dx.doi.org/10.1016/j.vetpar.2014.12.025
0304-4017/

Babesiosis in cattle is caused mainly by *Babesia* (*B.*) *bovis* and *B. bigemina*, which are responsible for high mortality rates (up to 50%) in susceptible herds (Antoniassi et al., 2009). Bovine anaplasmosis is caused by *Anaplasma* (*A.*) *marginale* which affects the breeding of herds and causes low annual yields of milk per cow (Kocan et al., 2010). Theileriosis is caused by the protozoan parasite *Theileria* (*T.*) *annulata*. Animals which recover from acute infections can become carriers with long-term persistent infections that are microscopically undetectable (Brown, 1990). Piroplasma infections are usually diagnosed by microscopy of blood smears, but carrier animals remain undetected (Bono et al., 2008; OIE, 2008). To overcome this drawback, conventional PCR assays in combination with sequencing of the amplicons have been used for the sensitive and specific detection of several piroplasma species and *A. marginale* (Almeria et al., 2001; Carelli et al., 2007; Kim et al., 2007; Ramos et al., 2011). To date, no microarray assays have been developed for diagnosis of bovine TBDs. Thus, the present study was conducted to assess the potential diagnostic value of a novel DNA microarray chip in comparison with microscopy and PCR assay for the diagnosis of bovine piroplasmosis and anaplasmosis.

## 2. Materials and methods

### 2.1. Animal population and clinical presentation

During the summer of 2012 and 2013, 164 Holstein Friesian cattle (129 from 6 dairy farms; 35 from smallholders) from farms located in Dakahlia Governorate, Egypt, were clinically and parasitologically examined for the presence of piroplasms and *Anaplasma* infections. For cattle in dairy farms, the age ranged between 1 and 4 years and between 6 months and 2 years for the animals of small-holders. Animals of four dairy farms ($n = 49$) as well as those of small-holders ($n = 35$) had recent clinical cases of piroplasm infections and a history of tick infestation as well as sporadic cases of sudden deaths in the respective herds. These cattle typically had pyrexia, anorexia, abnormal mucous membrane color, increased respiratory rate, and oculo-nasal discharge. Some rare cases showed enlarged superficial lymph nodes and others had discolored urine; while the other two dairy farms ($n = 80$) had a previous outbreak, but the animals were apparently healthy upon clinical examination.

### 2.2. Sampling and microscopy

Blood samples were drawn from the jugular vein of each cattle into Eppendorf tubes containing EDTA for DNA extraction and for determination of the packed cell volume (PCV %). Blood smears were prepared from the ear vein of each cattle. After drying the slides in ambient air, the blood smears were quickly fixed in methanol (99%) for 5 min and stained with 10% Giemsa staining solution (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) for 30 min. The slides were examined under an oil immersion lens at a total magnification of 1000 for the presence of piroplasms. After examining more than 50 microscopic fields of blood smears, the parasitemia was quantified and expressed as the percentage of infected erythrocytes. The remaining blood samples were kept frozen until further processing.

### 2.3. DNA extraction of blood samples

DNA was extracted from whole blood using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Positive control samples were kindly provided by A. Hildebrandt (Institute of Medical Microbiology, Friedrich-Schiller-University, Jena, Germany). DNA concentration was measured by using a NanoDrop™ ND-1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

### 2.4. PCR amplification

For amplification of an approximately 430 bp fragment of the 18S rRNA genes of *Theileria* and *Babesia* species, the primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) were used (Gubbels et al., 1999). For PCR reaction, a total volume of 50 μl was used containing 5 μl of 10× buffer, 2 μl of mixed dNTPs (Carl Roth GmbH, Karlsruhe, Germany), 10 pmol of each primer (Jena Bioscience GmbH, Jena, Germany) and one unit *Taq* DNA polymerase (Jena Bioscience GmbH). Five μl of extracted DNA were added to each PCR reaction. PCR assays were performed using a Mastercycler personal (Eppendorf, Hamburg, Germany) under the following conditions: after an initial denaturation at 96 °C for 60 s, 35 cycles followed with denaturation at 96 °C for 15 s, annealing at 60 °C for 1 min, extension at 72 °C for 30 s, and a final extension step at 72 °C for 1 min.

Species-specific PCRs for *A. marginale* with primer pair AM-F (5′-TTG GCA AGG CAG CAG CTT-3′), and AM-R (5′-TTC CGC GAG CAT GTG CAT-3′) (Carelli et al., 2007) and *Anaplasma centrale* with AC316 (5′-TCC AGT AAC AAG CAG TTC-3′) and AC716 (5′-AAC CCA CGC GGG CAG CTT GA-3′) (Decaro et al., 2008) were performed in a total volume of 50 μl per reaction with 5 μl of 10× buffer, 2 μl of mixed dNTPs (Carl Roth GmbH), 10 pmol of each primer (Jena Bioscience GmbH), one unit *Taq* DNA polymerase (Jena Bioscience GmbH) and five μl of DNA extract. *A. marginale* specific PCRs were carried out using the equipment described above under the following conditions: initial denaturation at 96 °C for 60 s, 35 cycles with denaturation at 96 °C for 15 s, annealing at 53 °C for 1 min, extension at 72 °C for 20 s, and final extension step at 72 °C for 1 min. For *A. centrale* an annealing temperature of 53 °C was chosen and extension at 72 °C was done for 30 s. PCRs resulted in 95 bp (*A. marginale*) and approximately 400 bp (*A. centrale*) products which were subjected to electrophoresis in 2.5% and 1.5% agarose gels, respectively. After staining with ethidium bromide PCR products were visualized under UV light. Documentation was done using a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

The PCR assay targeting 18S rRNA genes of *Theileria* and *Babesia* was regarded as reference test (Table 2). Samples that were positive for the respective 18S rRNA gene sequences were further tested using the DNA array for