Veterinary Parasitology 207 (2015) 329–334

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

differentiation. Three negative samples were tested to detect potential false positive results. The two animals with double infections were counted as cases for each group (not an extra-group for double infections). The following calculations were performed to describe the relevance of the clinical and laboratory parameters (Bossuyt et al., 2003a,b): diagnostic sensitivity = [TP/(TP + FN)] × 100; diagnostic specificity = [TN/(TN + FP)] × 100; positive predictive value (PPV) = [TP/(TP + FP)] × 100; negative predictive value (NPV) = [TN/(TN + FN)] × 100; diagnostic accuracy = (TP + TN)/(TP + TN + FP + FN) × 100; TN, true negative; TP, true positive; FN, false negative; FP, false positive.

2.5. Amplification, sequencing of 18S and 16S rRNA genes and data analysis

All of the PCR-positive samples were confirmed by DNA sequencing of partially amplified 18S and 16S rRNA genes. Briefly, after electrophoresis of PCR products, the bands were cut out and purified using the Agarose Gel Extraction Kit (Jena Bioscience GmbH) according to the instructions of the manufacturer. Cycle sequencing of the 18S and 16S rRNA gene fragments was done with primers RLB-R2 and AM-F and the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. Sequencing products were analyzed with a Genetic Analyzer ABI PRISM 3130 (Applied Biosystems). Identification was carried out by a BLAST search (http://www.ncbi.nlm.nih.gov/blast).

2.6. LCD Array hybridization and detection

The LCD-Array Kit B.T.A. 1.0 (Chipron GmbH, Berlin, Germany) was designed for the detection of DNA of *B. bigemina, B. bovis, B. divergens, B. major, T. annulata, T. mutans, and T. parva* based on 18S rRNA gene sequences as targets especially for this study. All primer and probe sequences have been designed within a 304–365 bp (species dependent) fragment of the 18S rRNA genes (e.g. accession number EU083801 for *T. annulata*). Each LCD chip contained eight identical arrays in rectangular reaction chambers, which can be addressed individually. All species-specific capture probes were positioned as vertical

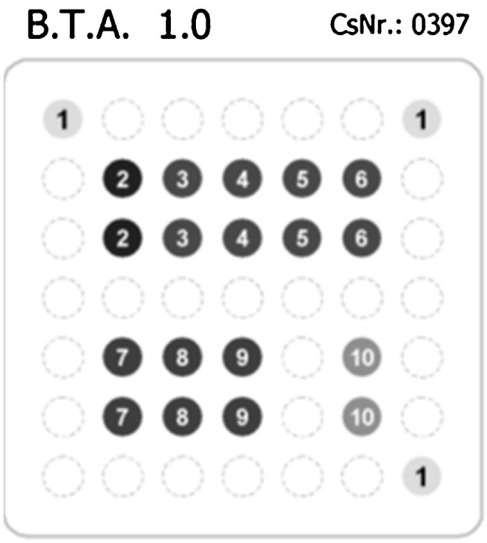

Fig. 1. Arrangement of the probes of LCD-microarray system B.T.A. 1.0 (Chipron GmbH, Berlin, Germany) designed for this study: 1 hybridization-control, 2 *Piroplasmidae*, 3 *Babesia* (*B.*) *bovis*, 4 *B. divergens*, 5 *B. bigemina*, 6 *B. major*, 7 *Theileria* (*T.*) *mutans*, 8 *T. annulata*, 9 *T. parva*, 10 not used.

Table 1
Clinical and laboratory findings of infections with *Piroplasmidae* and *Anaplasma marginale* in relationship to identified blood parasites.

	B. bovis (*n* = 11)	*A. marginale* (*n* = 33)		*Babesia* and *Anaplasma* (*n* = 2)
	Acute infection (*n* = 11)	Acute infection (*n* = 10)	Carriers (*n* = 23)	Acute infection (*n* = 2)
Microscopy positive (*n*)	9	7	–	2
PCV [%]	18.27 ± 3.5	20.10 ± 3.87	32.08 ± 2.44	13.30 ± 0.57
Rectal temperature [°C]	40.75 ± 0.38	40.40 ± 0.75	38.30 ± 0.32	40.96 ± 0.05
Heart rate [min^{-1}]	113.8 ± 0.4	112.5 ± 0.7	61.91 ± 5.15	119.0 ± 0.1
Respiratory rate [min^{-1}]	35.45 ± 3.67	33.20 ± 4.34	15.73 ± 1.74	40.0 ± 3.46
Cough (*n*)	11	9	–	2
Dyspnea (*n*)	8	7	–	2
Mucous membranes (*n*)				
• Bright red	–	–	17	–
• Pale	5	3	6	–
• Icteric	6	7	–	2
Lymphadenopathy (*n*)	2	3	–	–
Oculonasal discharge (*n*)	11	10	–	2
Hemoglobinuria (*n*)	6	1	–	2
Bloody feces (*n*)	–	6	–	2
Appetite (*n*)				
• Normal	–	–	23	–
• Inappetance	–	1	–	–
• Anorexia	11	9	–	2
Nervous signs	5	–	–	1
Posture (*n*)				
• Normal	9	10	23	1
• Recumbency	2	–	–	1
Frothy salivation (*n*)	1	1	–	1

duplicates (Fig. 1). The arrays of the B.T.A. 1.0 kit harbored 7 × 7 patterns with average spot diameters of 350 μm.

PCR reaction of a total volume of 25 μl consisted of 15.3 μl deionized water, 2.5 μl 10× buffer, 1.0 μl dNTPs, 1.0 μl primer mix Piroplas, 0.2 μl *Taq* DNA polymerase was carried out with the following conditions: 40 cycles of denaturation at 94 °C for 30 s, annealing 52 °C for 45 s, and extension at 72 °C for 45 s after initial denaturation at 95 °C for 5 min and a final extension at 72 °C for 2 min. Five μl of extracted DNA were added as template to each reaction tube. During this amplification, the generated PCR fragments were labeled with biotin. Hybridization, washing, and visualization by staining of specifically bound amplicons after incubation with streptavidin-peroxidase conjugate were performed according to the instructions of the manufacturer. Reading of the results was carried out using a CHIP Scanner PF7250 (Chipron GmbH).

3. Results

The initial presumptive diagnosis of bovine babesiosis and anaplasmosis was done based on case history, clinical symptoms, and microscopy, while confirmation was done using PCR assays, analysis of gene sequences, and a DNA microarray. In this study, 71 out of 164 cattle showed acute illness, whereas 93 were apparently healthy. In acutely-ill cattle, *Babesia*/*Theileria* species ($n = 11$) and *A. marginale* ($n = 10$) were detected. Mixed infections were present in two further cases. *A. marginale* infections were also detected in clinically healthy carriers ($n = 23$). All samples that were positive in the *Babesia*/*Theileria* PCR gave positive results using the LCD microarray system. No false positive results were observed in three PCR negative samples. Species identification via microarray resulted in detection of *B. bovis* ($n = 12$) and *B. bigemina* ($n = 2$). In one blood sample, DNA of *B. bovis*, and *B. bigemina* were identified. A second mixed infection was found with *B. bigemina* and *A. marginale*.

Table 1 shows the clinical findings of investigated cattle in relationship to the identified blood parasites. Cattle with babesiosis were likely to have hemoglobinuria ($n = 6$) and nervous signs ($n = 5$) when compared to patients with anaplasmosis that frequently had bloody feces ($n = 6$). All animals suffering from acute babesiosis or *A. marginale* infections had PCV % values < 24, rectal temperature > 39.5, a heart rate > 110 per min, a respiratory rate > 28 per min, and oculo-nasal discharge. However, only 23 animals out of 71 with these symptoms actually suffered from babesiosis or anaplasmosis (2 animals had mixed infection). No other clinical findings were observed in cattle that yield negative results with PCR. All carrier animals and most of the acutely ill patients responded to medical treatment ($n = 39$), nevertheless, seven cattle infected with *Babesia* perished. Table 2 gives the relationship between typical clinical signs of babesiosis and anaplasmosis and the detection of their causative agents.

4. Discussion

Tick-borne infections caused by *B. bovis*, *B. bigemina* and *A. marginale* are important diseases of cattle in tropical and

Table 2
Relationship between significant clinical signs of babesiosis and anaplasmosis and the detection of their causing agents.

	DNA array	Acute signs of infection				*Babesia bovis*/*bigemina*			*Anaplasma marginale*	
Diseased animals (n)			71				13		12	
PCR positive (n)	13	PCV% (value < 24)	Heart rate (>110 min^{-1})	Respiratory rate (>28 min^{-1})	Oculo-nasal discharge	Hemoglobinuria	Microscopy	Nervous signs	Bloody faeces	Microscopy
True positive	13	23	23	23	23	8	11	6	8	9
False negative	0	0	0	0	0	5	2	7	4	3
False positive	0	48	48	48	48	1	0	0	0	0
True negative	3	93	93	93	93	151	151	151	152	152
Diagnostic sensitivity	100	100.0	100.0	100.0	100.0	61.5	84.6	66.7	66.7	75.0
Diagnostic specificity	100	66.0	66.0	66.0	66.0	99.3	100.0	100.0	100.0	100.0
PPV	100	32.4	32.4	32.4	32.4	88.9	100.0	100.0	100.0	100.0
NPV	100	100.0	100.0	100.0	100.0	96.8	98.7	95.6	97.4	98.1
Diagnostic accuracy	100	70.7	70.7	70.7	70.7	96.4	98.8	95.7	97.6	98.2

subtropical regions (Jonsson et al., 2008; Kocan et al., 2010). Animals suffering from acute babesiosis or anaplasmosis can have a variety of symptoms such as fever, oculo-nasal discharge, increased heart rate, increased respiratory rate, abnormal mucous membrane color, and low PCV values. Although these symptoms are very typical, they are not pathognomonic, and animals with chronic infections can be asymptomatic carriers. Carrier animals without clinical symptoms are considered an important reservoir of infection for ticks that can transmit the infection to other susceptible animals (Jonsson et al., 2008; Kocan et al., 2010).

In Egypt, these infections are considered one of the major constraints to livestock improvement programs and cause serious health problems resulting in reduced animal productivity and economic losses. In this study, the rate of animals infected with *B. bovis*, *B. bigemina*, and *A. marginale* was 7.3%, 1.2%, and 21.3%, respectively. Ibrahim et al. (2013) reported a prevalence of *B. bigemina* and *B. bovis* in cattle in Beheira and Faiyum, Egypt of 5.30% and 3.97%, respectively. Adel (2007), Nayel et al. (2012) and El-Fayomy et al. (2013) reported that *Babesia* spp. were detected in 11.31%, 8.15%, and 23% of cattle in Gharbia, Menofia and Port Said Governorates, Egypt, respectively. Younis et al. (2009) detected *A. marginale* in cattle with a rate of 3.68%. Farm management, micro-climate pattern, tick distribution, breeds, and the sampling condition may explain variation in prevalence rates.

Bloody feces were found to be associated with anaplasmosis, while hemoglobinuria was typical for babesiosis with a diagnostic accuracy of 97.6% and 96.4%, respectively. These typical clinical signs were present in only 32.4% of the cases (23/71). It was also possible to identify the causative agents of the infection using microscopy in 87% of cases with acute symptoms (11/13 babesiosis and 9/12 anaplasmosis) with more than 98% diagnostic accuracy for both. In this study, we used a novel DNA microarray based assay in combination with PCR assays to better identify and discriminate these infections and to detect co-infections with different pathogenic agents. These LCD chips proved to be suitable for identification of different *Babesia* spp., but further investigations including larger numbers of animals are warranted for a clinical validation.

5. Conclusion

Clinical examination in combination with microscopy are still very helpful for the diagnosis of acute cases of babesiosis and anaplasmosis, but a combination of molecular biological diagnostic assays will detect asymptomatic carriers that are an important reservoir of infection. In perspective, parallel detection of *Babesia*/*Theileria* species and *A. marginale* infections using a single microarray system will be a valuable improvement.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This work is in part funded by Science & Technology Development Fund, Egypt (STDF). The authors would like to thank Prof. Lygia Friche Passos, PhD, Universidade Federal de Minas Gerais (UFMG), Escola de Veterinária, Belo Horizonte, Brazil for providing reference DNA of *Anaplasma marginale*, and K. Zmuda for excellent technical assistance. Sincere gratitude is expressed to Prof. Dr. Ahmed El-Sawalhy, the director of African Union and Interafrican Bureau for Animal Resources, for his kind help in revising the manuscript. Special thanks are extended to Dr. Gamal Badawy and Dr. Mohamed Mosbah El-Diasty, clinical veterinarians, for their kind help during sample collection.

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