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Use of a multiplex polymerase chain reaction-based assay to conduct epidemiological studies on bovine hemoparasites in Mexico

FIGUEROA (J.V.), ALVAREZ (J.A.), RAMOS (J.A.), VEGA (C.A.), BUENING (G.M.). Utilisation d'un test multiple basé sur la réaction de polymérase en chaîne pour des enquêtes épidémiologiques sur les hémoparasites des bovins au Mexique. *Revue Elev. Méd. vét. Pays trop.*, 1993, 46 (1-2) : 71-75

Une étude a été effectuée sur la possibilité d'appliquer la technique de la réaction de polymérase en chaîne (PCR) pour la détection simultanée des hémoparasites bovins *Babesia bigemina*, *B. bovis* et *Anaplasma marginale*. Des échantillons de sang de bovins ont été récoltés dans des ranches d'une zone endémique identifiée au préalable dans la péninsule de Yucatan au Mexique, et ont été préparés pour analyse par PCR. Ils ont été soumis à l'amplification de l'ADN dans un tube à réaction contenant des amorces d'oligonucléotides spécifiques pour l'ADN de chaque espèce d'hémoparasite. Les produits de la PCR ont été détectés par hybridation en Dot-Blot de l'acide nucléique utilisant des sondes d'ADN non-radioactives, spécifiques, marquées à la digoxigénine par PCR. Quatre cent vingt échantillons analysés par le test multiple PCR-sonde ADN ont montré des taux de prévalence de 66,7 p.100, 60,1 et 59,6 p.100 pour *B. bigemina*, *B. bovis* et *A. marginale*, respectivement. L'analyse multiple par PCR a montré que des animaux ayant des infections simples, doubles ou triples pouvaient être détectés par les sondes d'ADN spécifiques. La procédure est proposée comme un outil de valeur pour l'analyse épidémiologique dans les régions où ces espèces d'hémoparasites infectent les bovins simultanément.

Mots clés : Bovin - *Babesia bigemina* - *Babesia bovis* - *Anaplasma marginale* - Épidémiologie - Enquête pathologique - Dot-Blot - Sonde à ADN - Mexique.

INTRODUCTION

One of the most economically important diseases of cattle in tropical and subtropical areas of the world, is bovine babesiosis (24). In America, two species have been recognized in cattle, *B. bovis* and *B. bigemina* (21). Both *Babesia* species share the tick *Boophilus* spp. as the vector for their biological transmission to cattle (5, 24). Clinically the disease is manifested as fever, anorexia, dullness, weakness, ataxia, hemoglobinuria, icterus, anemia, and presence of intraerythrocytic parasites (5, 24). Bovine anaplasmosis is a biologically and mechanically arthropod-transmitted disease caused by the rickettsia *Anaplasma marginale* which is widely distributed throu-

ghout the world (5, 25). Clinically, bovine anaplasmosis is at times very similar to bovine babesiosis. However, anaplasmosis is manifested as a progressive anemia (usually without hemoglobinuria) associated with the presence of intraerythrocytic inclusion bodies (5, 15, 25).

Under field conditions, the three organisms may be involved in the causation of disease (7, 24, 25, 30) since they can be transmitted by a common tick vector. Traditionally, peripheral blood smears have been utilized to detect the presence of intraerythrocytic bodies (5, 15, 24, 25). However, microscopic examination of Giemsa-stained smears often does not detect low levels of parasites because the parasitemia fluctuates during the carrier state in cattle (5, 17).

The reported use of the polymerase chain reaction technique (26) with its high sensitivity for detection of infectious organisms (1, 12, 19, 28) led to the development of an assay for the simultaneous detection of the hemoparasites *B. bigemina*, *B. bovis* and *A. marginale* in carrier cattle (13). In this study, we test the applicability of the assay to determine the distribution of the 3 hemoparasites in cattle from the Yucatan State of Mexico.

MATERIALS AND METHODS

Hemoparasite-infected bovine erythrocytes

Babesia bigemina and *B. bovis* Mexico isolates were cultivated as previously described (18, 29). *Anaplasma marginale*-infected blood (USA isolate) was collected from an experimentally infected calf at peak of parasitemia. Percentage of parasitized erythrocytes (PPE) was estimated by light microscopy of Giemsa-stained smears (5) and dilutions of infected blood were prepared in normal blood to serve as positive controls in the PCR assay (13).

Primers

Six sets of oligonucleotide primers were utilized in the PCR assays and their nucleotide sequences have previously been reported (13). Three sets (external primers, each set species specific) were used to amplify DNA from genomic hemoparasite templates present in the blood samples. Expected size of amplified fragments was 280 bp, 350 bp and 200 bp for *B. bigemina*, *B. bovis* and *A.*

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marginale DNA, respectively (13). Three more sets of oligonucleotides (internal primers) were used to prepare nonradioactive DNA probes specific for each species of hemoparasite as described below.

PCR DNA amplification

For blood sample analysis, 20 μ l of packed and washed bovine erythrocytes were processed as previously described (12, 28). Briefly, pelleted erythrocytes were resuspended in saponin lysis buffer (28). After one wash in reaction mixture buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin, without dNTPs and primers), pellets were directly resuspended in 99.5 μ l of reaction mixture buffer containing 200 μ M of each dNTP and 1 μ M of each external primer and incubated at 100 °C for 10 min.

After a brief centrifugation, 2.5 U of Taq polymerase (Promega Co., Madison, WI) was added to each tube and the PCR tubes were mixed and placed in a TempCycler 60 (Coy, Ann Arbor, MI) for thermocycling. The first cycle consisted of 2 min template denaturation at 95 °C, 1 min primer annealing at 60 °C, and 1.5 min primer extension at 73 °C. The second to 36th cycle were as above except that the denaturation step was decreased to 1 min. A final extension of 15 min at 73 °C was included in the amplification reaction. To avoid amplicon contamination, individual steps were performed in separate rooms and dedicated pipets were utilized as recommended (16).

Experimental animals

This study was carried out in southeast Mexico, in the state of Yucatan. Yucatan, a "dry" tropical area, with a warm, subhumid climate Aw (14), has a total cattle population of 646,371 head (23). The sample size ($n = 903$) was determined according to the exact population equation (8), considering the total number of head in the region as the population "N", regardless of age. An error of 0.2 and a confidence level of 95 % were assumed, based on a previous estimation of a 60 % serological prevalence for bovine babesiosis. Collected bovine blood samples were then placed into five groups, considering the age in months of the animals: Group A, < 3; Group B, 3 to 9; Group C, 9 to 18; Group D, 18 to 36; and Group E, > 36 months (23). For this experiment in particular, blood samples from 421 randomly selected animals in the region were processed for multiplex PCR assay analysis.

Sample collection and preparation

Peripheral blood was aseptically obtained from cattle's jugular or tail veins into evacuated glass tubes containing EDTA (Vacutainer, Becton-Dickinson de México, S.A. de C.V.). Blood samples were processed as previously described (11). Briefly, blood samples were washed three

times by centrifugation with TEN buffer (0.1 M Tris-HCl, pH 8.0; 0.15 M NaCl, 10 mM EDTA); supernate and buffy coat were discarded each time. Packed erythrocytes were stored frozen at -20 °C in 500 μ l aliquots. Blood was maintained at -20 °C until analyzed. After two freeze-thaw steps 20 μ l aliquots were removed and the PCR procedure performed as described above.

Preparation of PCR-labeled probes

Purified pBbi55 plasmid DNA (12), purified p60 DNA (27) kindly provided by T.F. McELWAIN (Washington State University, Pullman WA, USA), and purified p25 plasmid DNA (1) served as templates for the synthesis of probes via PCR using the internal set of primers. The procedure was carried out essentially as described (13) in which digoxigenin-dUPT is incorporated into the newly synthesized DNA (9). The size of DNA probes are 170 Bp, 291 Bp and 160 Bp for *B. bigemina*, *B. bovis* and *A. marginale*, respectively (13).

Analysis of PCR products

Aliquots from the amplification reaction were analyzed by dot-blot nucleic acid hybridization-chemiluminescent detection (13). Briefly, three 20 μ l aliquots of the PCR reaction were spotted onto 3 different nylon membranes using a dot-blot apparatus. After DNA denaturation, neutralization and fixation to the nylon membrane, dot-blots were each hybridized with a parasite-specific nonradioactive probe (100 ng/ml hybridization solution) as suggested by the supplier (3). DNA hybrids were detected by the alkaline phosphatase-based chemiluminescent reaction as described (12, 13).

RESULTS

Estimation of the sample size indicated that a total of 942 samples was sufficient for determination of hemoparasite prevalence rates by serology (23); however, since this study was subsampled, the total number of blood samples analyzed was 421.

The multiplex PCR assay detected, in clinically apparently healthy cattle, animals carrying one, two or the three hemoparasites. Figure 1 shows the picture of a representative autoradiograph obtained by doing the PCR assay on the bovine blood samples. The positive responses in the multiplex PCR assay were 281, 253 and 251 for an overall prevalence infection rate of 66.7, 60.1 and 59.6 % for *B. bigemina*, *B. bovis* and *A. marginale*, respectively.

Percentages of distribution of single- or multiple-infected cattle are shown in table I. Cattle carrying the three hemoparasites showed the highest prevalence (34.9 %), followed by *B. bigemina* infected cattle (12.2 %) and

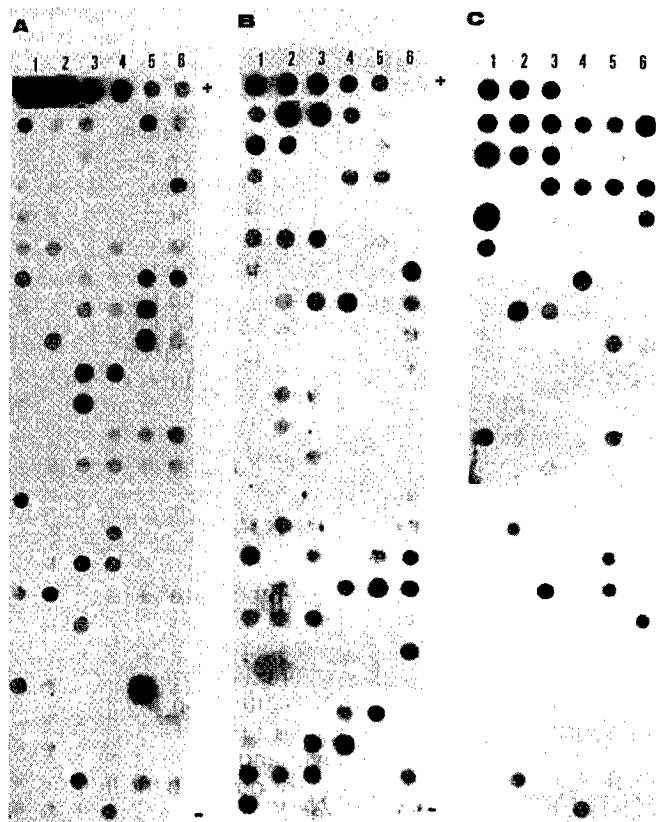


Figure 1 : Analysis of PCR products by dot blot nucleic acid hybridization from bovine blood field samples. First row: Numbers 1-6) positive controls (0.1 % to 0.000001 % infected erythrocytes). Next rows : Blood samples from Yucatan, Mexico. Last row: Numbers 5-6) negative controls (normal bovine blood). Panel A: Dot blots hybridized with *B. bigemina*-specific DNA probe. Panel B : Dot blots hybridized with *B. bovis*-specific DNA probe. Panel C : Dot blots hybridized with *A. marginale*-specific DNA probe.

TABLE I PCR-reactors distribution of bovine blood samples.

	<i>B. bigemina</i>	<i>B. bovis</i>	<i>A. marginale</i>	Total	%
1)	+	+	+	147	34.9
2)	—	+	+	41	9.7
3)	+	—	+	36	8.5
4)	+	+	—	47	11.3
5)	—	—	+	27	6.4
6)	—	+	—	18	4.2
7)	+	—	—	51	12.2
8)	—	—	—	54	12.8

+ = specific hybridization with species specific DNA probe.

B. bigemina/*B. bovis*-infected cattle (11.3 %). Cattle harboring both protozoan and rickettsial organisms showed a prevalence rate of 9.7 % for *B. bovis*/*A. marginale*, and 8.5 % for *B. bigemina*/*A. marginale* combination. Cattle singly infected with *B. bovis* or *A. marginale* had the lowest prevalence values (4.2 % and 6.4 %, respectively). Only 12.8 % of the cattle tested were PCR-assay negative for all of the three hemoparasites detected in this study.

According to the different age groups the prevalence rates for *A. marginale*, *B. bigemina* and *B. bovis* are summarized in tables II, III and IV, respectively. The results indicate that the number of positive reactors to *A. marginale* was very similar for cattle aged < 3 months to 36 months (around 50 % prevalence rate), whereas the prevalence rate to the rickettsia increased to 76 % in adult animals over 3 years of age.

High prevalence rates of *Babesia* infection (60-70 %) were observed for young cattle regardless of the infecting protozoan species. The prevalence of *Babesia bigemina* infection in adult cattle slightly declined; however, a relative

TABLE II Response distribution to *Anaplasma marginale* of blood samples PCR-analyzed by age in months.

Group	PCR (—)	PCR (+)	Total	% (+)
1) < 3 months	37	42	79	53.1
2) 3-9 months	36	46	82	56.1
3) 9-18 months	43	47	90	52.2
4) 18-36 months	30	39	69	56.5
5) > 36 months	24	77	101	76.2

TABLE III Response distribution to *Babesia bigemina* of blood samples PCR-analyzed by age in months.

Group	PCR (—)	PCR (+)	Total	% (+)
1) < 3 months	22	57	79	72.1
2) 3-9 months	28	54	82	65.8
3) 9-18 months	25	65	90	72.2
4) 18-36 months	28	41	69	59.4
5) > 36 months	37	64	101	63.3

Table IV Response distribution to *Babesia bovis* of blood samples PCR-analyzed by age in months.

Group	PCR (-)	PCR (+)	Total	% (+)
1) < 3 months	31	48	79	60.7
2) 3-9 months	30	52	82	63.4
3) 9-18 months	24	66	90	73.3
4) 18-36 months	24	45	69	65.2
5) > 36 months	59	42	101	41.5

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very low prevalence rate for *Babesia bovis* (41.5 %), was determined for cattle over 3 years of age.

When the results were analyzed to determine the Daily Probability Infection (DPI) rate adjusted to 9 months of age for bovine babesiosis, it was found that DPI was 0.004 for *B. bigemina*, whereas for *B. bovis* the DPI was 0.003, indicating a field situation of enzootic instability for bovine babesiosis (17).

DISCUSSION

It has been postulated that use of DNA based-assays for the direct detection of hemoparasites in bovine blood samples would have many advantages over serologic techniques. For example, carrier animals would be identified based upon the presence of an organism (2, 10).

A colorimetric DNA probe-based assay to detect *B. bigemina* was recently reported (11). The DNA probe utilized was able to detect parasitemias of 0.001 % in 200-500 μ l volumes of packed cells. It was found, however, that the analytical sensitivity of the *B. bigemina* DNA probe appeared to be too low for its utilization in widespread epidemiological surveys (23), since carrier cattle infected with *B. bigemina* may have peripheral blood parasitemia levels below the limit of detection of this nonradioactive DNA probe (12). The same situation could be applicable for the *B. bovis*-infected carrier cattle (13, 17), and *A. marginale*-infected carriers (1, 4, 10). A PCR-based assay with an elevated analytical sensitivity could overcome some of the previously reported constraints. Moreover, detection of multiple infective agents could be accomplished and facilitate the use of a PCR-DNA based assay in epidemiological studies (13). Overall prevalence infection rates for *A. marginale*, *B. bovis* and *B. bigemina* (77, 44 and 60 %, respectively) have been serologically determined in bovine serum samples obtained from the same cattle of the region (22). There were discrepancies between prevalence infection rates determined in this study using the PCR assay, compared to those reported using serological procedure (22). The Multiplex PCR assay identifies hemoparasite DNA in blood samples, whereas the ELISA (*A. marginale*) and the IFAT (*Babesia* sp.) assays identify antibody in serum samples. However, the overall prevalence rate determined for bovine babesiosis in this study (> 60 %), was similar to the 69 % obtained 14 years ago in a herd of cattle (20). This observation confirmed the endemic status of bovine babesiosis in the area.

One of the advantages of the Multiplex PCR assay over the conventional serological assays was the identification of the presence of the hemoparasites in the youngest group of animals. Detection of colostral antibodies reactive in serological tests, preclude the utilization of this type of assays to determine the infection rate in calves 3-4 months old (17). The estimated *Babesia* infection rates for animals less than 9 months old obtained in this study

indicate that the risk of the occurrence of bovine babesiosis outbreaks exists in animals of this geographical region (17). In addition, the concept of inverse age resistance in bovine babesiosis (6) is corroborated in this study, because despite a relatively large number of calves already infected at or around 3 months of age, the presentation of clinical babesiosis in this group was not observed (22). Furthermore, the identification, at the species level, of *Babesia*-infected animals within a population, would facilitate the control measures decision-making process by animal health officers. Thus, this information is very useful when selecting a procedure for specific immunization of cattle, particularly in zones in which a *Babesia* species may be considered more important in terms of causing disease outbreaks (5, 17, 30).

CONCLUSION

In conclusion, the Multiplex PCR procedure for the detection of *B. bigemina*, *B. bovis* and *A. marginale* infection in carriers is proposed as a valuable tool for epidemiological studies especially in regions where the three hemoparasite concomitantly infect cattle. Cattle from the Yucatan state of Mexico were determined to have high infection rates for *B. bigemina*, *B. bovis* and *A. marginale*. This study confirmed the endemicity of bovine babesiosis and bovine anaplasmosis in the area.

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FIGUEROA (J.V.), ALVAREZ (J.A.), RAMOS (J.A.), VEGA (C.A.), BUENING (G.M.). Use of multiplex polymerase chain reaction-based assay to conduct epidemiological studies on bovine hemoparasites in Mexico. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 71-75

A study was conducted to test the applicability of a Polymerase Chain Reaction (PCR)-based approach for the simultaneous detection of the bovine hemoparasites *Babesia bigemina*, *B. bovis* and *Anaplasma marginale*. Bovine blood samples from cattle ranches of a previously determined enzootic zone in the Yucatan Peninsula of Mexico, were collected from peripheral blood and processed for PCR analysis. Blood samples were subjected to DNA amplification by placing an aliquot in a reaction tube containing oligonucleotide primers specific for DNA of each hemoparasite species. The PCR products were detected by Dot-Blot nucleic acid hybridization utilizing nonradioactive, species-specific, digoxigenin PCR-labeled DNA probes. Four hundred twenty one field samples analyzed by the multiplex PCR-DNA probe assay showed 66.7 %, 60.1 % and 59.6 % prevalence rates for *B. bigemina*, *B. bovis* and *A. marginale*, respectively. The multiplex PCR analysis showed that animals with single, double or triple infection could be detected with the parasite specific DNA probes. The procedure is proposed as a valuable tool for the epidemiological analysis in regions where the hemoparasite species are concurrently infecting cattle.

Key words : Cattle - *Babesia bigemina* - *Babesia bovis* - *Anaplasma marginale* - Epidemiology - Serological survey - Dot-Blot - DNA probe - Mexico.

FIGUEROA (J.V.), ALVAREZ (J.A.), RAMOS (J.A.), VEGA (C.A.), BUENING (G.M.). Uso de ensayos basados en reacciones en cadena de polimerasas múltiples para estudios epidemiológicos en hemoparásitos de bovinos, en México. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 71-75

Se llevaron a cabo estudios para probar la utilidad de los métodos de reacciones en cadena de polimerasas (PCR), en la detección simultánea de *Babesia bigemina*, *Babesia bovis* y *Anaplasma marginale*, hemoparásitos de los bovinos. Se colectaron muestras de sangre periférica, de bovinos originarios de ranchos escogidos en zonas enzooticas de la Península de Yucatán, en México, las cuales se analizaron por PCR. Las muestras sanguíneas se sometieron a una amplificación del ADN, mediante una reacción en tubo de un alícuota con un primer de un oligonucleótido específico para el ADN de cada especie de hemoparásito. Los resultados de las PCR se detectaron gracias a la hibridación de ácidos nucleicos por Dot-Blot, con probadores de ADN marcados con digoxigenina-PCR, no radioactivos, específicos para cada especie. El análisis de las cuatrocientas veintinueve muestras mostró prevalencias de 66,7 p.100, 60,1 y 59,6 p.100 para *Babesia bigemina*, *Babesia bovis* y *Anaplasma marginale*, respectivamente. El análisis por PCR múltiple mostró que los animales con infecciones únicas, dobles o triples pueden ser detectados con los probadores específicos de ADN. Este procedimiento podría ser valioso para los análisis epidemiológicos en aquellas zonas en las cuales las infecciones de ganado son frecuentes.

Palabras claves : Bovino - *Babesia bigemina* - *Babesia bovis* - *Anaplasma marginale* - Epidemiología - Encuesta serológica - Dot-Blot - Sonda de ADN - México.