Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87*, Suppl. III, 207-211, 1992

DETECTION OF *BABESIA BIGEMINA* INFECTION: USE OF A DNA PROBE - A REVIEW

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The development of a repetitive DNA probe for Babesia bigemina *was reviewed. The original plasmid (p(Bbi)16) contained an insert of* B. bigemina *DNA of approximately 6.3 kb. This probe has been evaluated for specificity and analytical sensitivity by dot blot hybridization with isolates from Mexico, the Caribbean region and Kenya. A partial restriction map has been constructed and insert fragments have been subcloned and utilized as specific DNA probes. A comparison of* ^{32}P *labelled and non-radioactive DNA probes was presented. Non-radioactive detection systems that have been used include digoxigenin dUTP incorporation, and detection by colorimetric substrate methods. Derivatives from the original DNA probe have been utilized to detect* B. bigemina *infection in a) experimentally inoculated cattle, b) field exposed cattle, c) infected* Boophilus microplus *ticks, and d) the development of a PCR amplification system.*

Key words: *Babesia bigemina* – DNA probe – detection – diagnosis – protozoan

Bovine babesiosis remains a major cattle disease in the world's tropical and subtropical areas. The causative agents in North, South and Central America are *Babesia bovis* and *B. bigemina*. For epidemiological studies it is desirable to be able to detect in carrier animals the specific species. Ambrosio & DeWall (1990) reviewed recent advances in the diagnosis of parasitic diseases. They emphasized the importance of having available highly sensitive and specific tests. Properly constructed DNA probes would fulfill these two requirements.

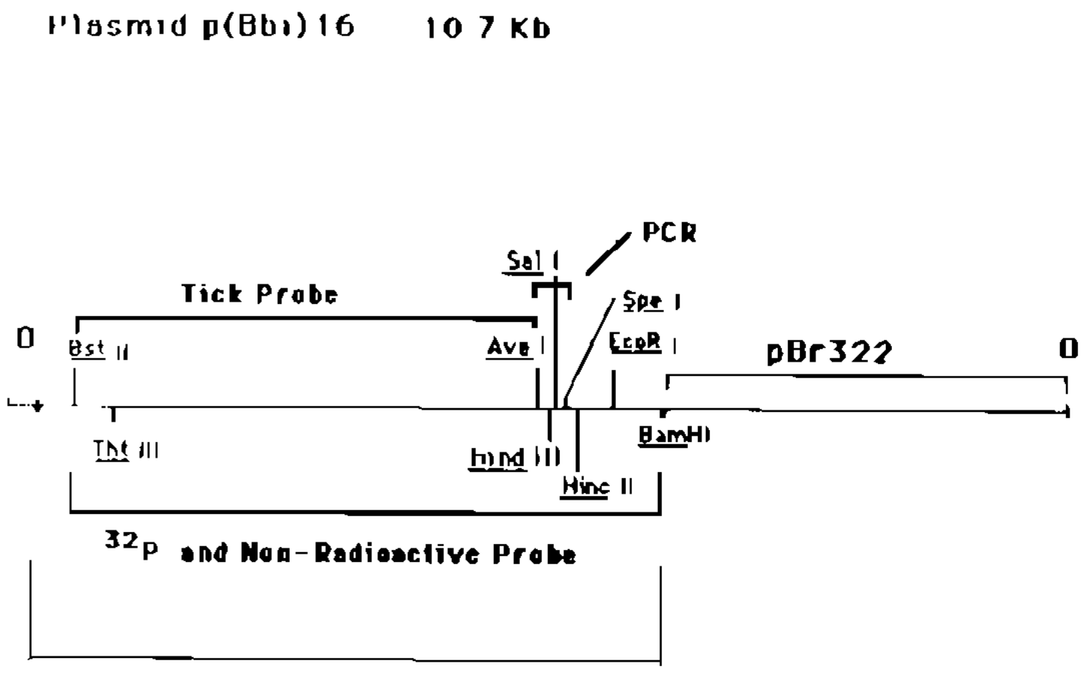
Several investigators (McLaughlin et al., 1986; Holman et al., 1989; Jasmer et al., 1990) have reported the detection of *B. bovis* in blood by DNA hybridization procedures. Sensitivities ranged from 12 pg (Holman et al., 1989) to 100 pg (McLaughlin et al., 1986; Jasmer et al., 1990). Recently investigators have reported that selected *B. bovis* nucleic acid probes can discriminate among different isolates of *B. bovis* (Dalrymple, 1990: Jasmer et al., 1990) and the detection of variations of virulence of subpopulations (Carson et al., 1990).

Supported in part by USAID Grant DAN-4178-A-00-7056-00.

Buening et al. (1990) reported the construction and partial characterization of a *B. bigemina* specific probe. The objective of this paper is to summarize the present status of this candidate probe and discuss additional applications in the detection of *B. bigemina* infections.

REVIEW OF ORIGINAL REPORT

Buening et al. (1990) reported the characterization of a repetitive DNA probe for *B. bigemina*. The plasmid (p(Bbi)16) contained an insert of *B. bigemina* DNA of approximately 6.3 kb in size. A partial restriction map was constructed (Fig.). The ^{32}P labelled probe was shown to be species specific and only hybridized with *B. bigemina* DNA and not DNAs from *B. bovis*, bovine leukocytes, *Trypanosoma brucei* or *Anaplasma marginale*. The probe specifically hybridized with *Babesia* isolates from Mexico, the Caribbean and Kenya. The DNA probe detected as little as 10 pg of *B. bigemina* DNA. A simple whole blood dot blot procedure was utilized to demonstrate that the probe would detect as few as 150 *B. bigemina*-infected erythrocytes diluted in saline. Subsequent studies (Buening, unpublished data) were done diluting *in vitro* cultivated *B. bigemina* infected erythrocytes (Vega et al., 1985) into known quantities of normal bovine erythrocytes. DNA was extracted by standard procedures and



A partial restriction map of plasmid p(Bbi)16. The ^{32}P and non-radioactive probe used for the detection of infected erythrocytes was derived from a subfragment obtained by *Bst*EII/*Bam*HI digestion. The probe used in the tick study was a subfragment obtained by a *Bst*EII/*Ava*I digestion. The construction of the PCR-based assay was based upon the subfragment obtained by a *Spe*I/*Ava*I digestion.

blotted on nitrocellulose membranes. These membranes were hydridized with the ^{32}P-labelled probe. Positive hybridizations were observed with samples containing at least 10^3 infected erythrocytes. This observation would indicate that large concentrations of normal erythrocytes in samples resulted in a 10 fold decrease in sensitivity.

Since this candidate probe hybridized with *B. bigemina* isolates from Texas, Mexico, Puerto Rico, St. Croix, Costa Rica and Kenya, it could be used in worldwide epidemiological studies. For epidemiological studies it is desirable to have a nucleic acid probe that would detect carrier animals which have a very low parasitemia.

COMPARISON OF ^{32}P LABELS AND NON-RADIOACTIVE DNA PROBES

Radiolabelled (^{32}P) DNA probes had been shown to be highly sensitive and specific. However, major disadvantages are that they can be hazardous, expensive, and have short half-lives. These disadvantages have encouraged researchers to develop non-radioactive DNA labelling and detection methods. Such probes have the advantage in that they can be stored for long periods without the loss of sensitivity and require no special safety or disposal precautions. Such probes may be ideally suited to be utilized in developing countries.

It has been previously reported that a ^{32}P labelled *B. bigemina* DNA probe derived from the p(Bbi)16 would detect 10 pg of purified *B. bigemina* DNA (Hodgson et al., 1992; Buening et al., 1990) and the DNA extracted from 3,000 *B. bigemina* merozoites (Hodgson et al., 1992).

Figueroa et al. (1992) evaluated a derivative of the p(Bbi)16 as a non-radioactive DNA probe. The *B. bigemina* insert was isolated by restriction enzyme *Bam*HI and *Bst*EII digestion and purified (Fig.). This DNA was labelled using the random prime technique for the incorporation of ^{32}P or digoxigenin-labelled deoxyuridine-triphosphate (Boehringer Mannheim GmbHBiochemica. 1989. Genius™ non-radioactive DNA labeling and detection kit. Applications Manual). Experimental details were presented elsewhere.

Four different procedures were evaluated for the preparation of target *B. bigemina* DNA from infected erythrocytes (see Table I). Culture *B. bigemina* infected erythrocytes were harvested and the percentage of parasitized erythrocytes (PPE) estimated. The samples were adjusted to 1 PPE and 10 fold serial dilutions were made in normal bovine erythocyte suspensions. Each series of dilutions was extracted by each procedure. The results were as follows: Procedure 1 (the erythrocyte lysate spot) could be used with the ^{32}P probe (Buening et al., 1990). However, with the non-radioactive system the presence of large amounts of hemoglobin interfered with substrate color development. Procedure 2 (deproteinization of samples by phenol/chloroform extraction) increased the sensitivity of the non-radioactive probe to 0.01 PPE. Procedure 3 involved deproteinization and additional DNA purification by the use of sodium iodide DNA preferential binding and elution from silica beads (Goff, personal communication). Procedure 4 was the same as Procedure 3, without deproteinization. The last two procedures increased the sensitivity of the non-radioactive probe to 0.001 PPE. It was elected to use Procedure 4 because of simplicity. In this study, it was reported that the non-radioactive *B. bigemina* insert DNA probe would detect 1 ng of genomic DNA and 37,500 infected erythrocytes. This study reported that the probe reacted with equal intensity against seven geographically different *B. bigemina* isolates.

Six intact experimental animals were inoculated with aliquots of blood containing *B.*

bigemina-infected erythrocytes (Figueroa et al., 1992). Four animals received a Puerto Rican isolate, one animal a Mexican isolate, and one animal a Texas isolate. Peripheral blood samples were collected on various dates from - 1 to day 86 post-inoculation. Blood samples were prepared for DNA purification by Procedure 4. The presence of infected erythrocytes varied between sampling dates and animal to animal. Blood samples were examined by light microscopy of Giemsa-stained smears and by DNA hybridization. Positive hybridization reactions were recorded in some animals from 10 to 72 days post-inoculation. There was not a complete correlation between light microscopy and the hybridization assay. The nucleic acid hybridization assay detected more positive samples than light microscopy examination (Table II).

FIELD EXPOSED CATTLE

The methodology developed by Figueroa et al. (1992) has been transferred to scientists in Mexico (INIFAP-SARH) to be utilized in an epidemiological survey. The objective of this study was to evaluate the methodology under field conditions. For details of the experimental design and preliminary results refer to the paper presented at the IV International Congress on Malaria and Babesiosis by Ramos et al. (1991).

INFECTED *BOOPLHILUS MICROPLUS* TICKS

Hodgson et al. (1992) presented preliminary results of a study to detect *B. bigemina* infected *B. microplus* ticks . The ^{32}P labelled DNA probe was a 4.8 kb *Ava*I and *Bst*EII fragment of the *B. bigemina* insert (Fig.). Hodgson et al. (1992) reported that the probe was specific for *B. bigemina* DNA, for it did not hybridize with *B. microplus* DNA, bovine DNA or *B. bovis* DNA. The sensitivity was reported to be 10 pg of *B. bigemina* DNA and the amount of DNA which could be extracted from 10^3 *B. bigemina* merozoites. It was concluded from this study that the probe could be utilized to quantitatively analyze *B. bigemina* infection of *B. microplus* ticks. The technique was utilized to identify conditions for the maximum production of infected *B. bigemina* organisms in ticks.

DEVELOPMENT OF A POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION SYSTEM

The results of the previous study with experimentally inoculated animals indicated that a non-radioactive *B. bigemina* probe would detect carrier animals if the PPE was at least 0.001. Some carrier animals would have PPE below 0.001. Therefore, it was desirable to develop a highly sensitive polymerase chain reaction (PCR) based assay for detecting lower

TABLE I

Comparison of preparation to extract *Babesia bigemina* DNA from infected erythrocytes

Procedure	Freeze Thawed: 2X Washed in TE: 3X	Triton X 100 Proteinase K[a]	Phenol Phenol-Chloroform Extraction	"Genecleaned"[b]	Material spotted	Sensitivity
1	Yes	Yes	No	No	lysate supernatants	0.1 PPE or 150,000 infected erythrocytes
2	Yes	Yes	Yes	No	aqueous phases	0.01 PPE in 400 µl sample
3	Yes	Yes	Yes	Yes	eluates	0.001 PPE in 200 µl sample ~ 30,000 infected erythrocytes
4	Yes	Yes	No	Yes	eluates	0.001 PPE in 250 µl sample ~ 37,500 infected erythrocytes

a: incubated at 65°C for 2 h.
b: modified procedure - Geneclean Kit, BIO101, Inc., San Diego, CA.
PPE: percent parasitized erythrocytes.

TABLE II

A comparison of light microscopy and hybridization reaction on blood samples derived from experimentally inoculated animals

Animal ID	175		68		NT		59		182		335	
	LM	HR	LM	HR	LM	HR	LM	HR	LM	HR	LM	HR
	0[a]/23[b]	0/23	5/23	13/23	5/23	7/23	2/23	15/23	5/23	10/23	0/22	0/22
CF	–		1:40		1:160		–		1:40		1:10	
IFA	–		1:1280		1:1280		–		1:1280		ND	

a: number of positive observations.
b: number of observations.

LM: light microscopy of Giemsa-stained blood smears. At least 10 min per slide were spent for examination.
HR: hybridization reaction obtained when DNA from samples were tested with the nonradioactive p16 Bbi DNA probe.
CF: Complement Fixation test. IFA: Indirect Fluorescent Antibody test. ND: not done.

numbers of *B. bigemina*-infected erythrocytes.

Figueroa et al. (1991) developed a PCR based assay. A *SpeI/Ava*I fragment (~ 0.3 kb) was isolated from p(Bbi)16 (see Fig.). This fragment was subcloned and sequenced by the dideoxy-mediated chain termination method. The sequence data was used to design two sets of primers for the PCR assay. Primer set I was used to amplify a 278 Bp fragment of the *B. bigemina* genomic target DNA. The second set of primers bound to the fragment at internal sites to primer set one. The second set of primers was utilized to incorporate by the polymerase chain reaction, digoxygenin-dUTP into a 174 Bp fragment using p(Bbi)16 or p(Bbi)55 as template DNA. The PCR amplified nonradiactive probe was then used in studies of specificity and sensitivity.

Laboratory experiments conducted to evaluate the sensitivity and specificity of the PCR-based assay demonstrated that this nonradiactive PCR-based assay could detect as little as 100 fg of *B. bigemina* genomic DNA. This assay would amplify genomic DNA from seven different *B. bigemina* isolates, but did not amplify *B. bovis*, *A. marginale*, or bovine leukocyte DNA. Preliminary experiments utilizing Procedure 4, for the preparation of *B. bigemina* DNA from infected erythrocytes indicated that this PCR based assay would detect a parasitemia as low as 0.000001% in a 200 µl sample. This would be equivalent to approximately 30 *B. bigemina* infected erythrocytes. Additional details concerning this assay were presented in a poster at the IV International Congress on Malaria and Babesiosis.

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