BIOTECHNOLOGY APPLIED TO THE DETECTION OF INFECTIOUS AGENTS IN CATTLE
Diagnosis of *Babesia bovis* by PCR

C. J. AZAMBUJA[1], V. GAYO[2], M. SOLARI[2], M. SUAREZ[2] & M. STOLL[1]

(1) Unidad de Biotecnologia, Estación Experimental Las Brujas, Rincón del Colorado, Uruguay; (2) División de Parasitologia, Dirección de Laboratorios Veterinarios "Miguel C. Rubino", Montevideo, Uruguay.

SUMMARY: A PCR diagnostic test for *Babesia bovis* has been developed in our laboratory. A pair of primers used for the amplification were designed from the BBMER 60 sequence of a gene that encodes for a surface merozoite protein of *B. bovis*, and should allow an amplification of a 1056 bp fragment. Four splenectomized calves were experimentally infected with *B. bovis* uruguayan vaccinal strain, *B. bovis* field strain, *B. bigemina* field strain, and *Anaplasma marginale* field strain. DNA from blood of those calves and from a non-infected one was purified, and amplified by PCR. DNA of the expected size was amplified from the samples containing vaccinal and field strains of *B. bovis*, and no amplified product was observed with *B. bigemina*, *A. marginale*, or DNA from a non-infected calf, demonstrating the specificity of the reation. To make sure that the amplified product was the expected one, we digested it with restriction enzymes, Hinf I and Taq I, in order to compare the restriction map of the sequence with the bands obtained after digestion. The identity of the amplified fragment was then corroborated. The results obtained combined with the simplicity and speed of PCR, suggests that this technique can be used for routine diagnosis of *B. bovis*.

KEY WORDS: *Babesia bovis*, PCR.

INTRODUCTION

The use of biotechnological techniques in the control of infectious diseases is steadily increasing. These techniques offer some advantages over conventional ones; they are more specific, sensitive and rapid.

Traditionally, pathogens are identified by either their shape or biochemical properties. These characteristics are the result of the expression of their genetic information. Molecular biology allows the identification of pathogens by direct analysis of their genetic material.

There are DNA sequences caracteristic of species and it is possible to evidence the agent by the amplification of one of those sequences using the Polymerase Chain Reaction (PCR) (SAIKI *et alii*, 1985).

Another advantage when using PCR as a diagnostic method is the simplification of samples remission to the laboratory because it is not necessary to preserve the viability of the agent by refrigeration. (KAWASAKI, 1990).

Besides, DNA stands better than proteins temperature changes and dessication.

Diagnosis of different diseases that affect our farms, are many times difficult due to transportation problems existent in a great area of our country. A method that allows the analysis of samples that have sttod for several days at room temperature, will solve this problem.

In this report the pathogen to diagnose was *Babesia bovis*, one of the intraerythrocytic protozoan parasites causing haemolytic disease in cattle.

B. bovis is an hemoparasite of cattle transmitted by *Boophilus microplus*.

Babesiosis is considered one of the most important diseases in South America (MONTENEGRO-JAMES, 1992). In Argentina, in enzootic areas, a damage of U$S 6,2 per animal per year was estimated due to babesias (GUGLIELMONE, 1991).

In Uruguay these economic losses are not yet determined, but taking into account the above mentioned data, an estimation of U$S 20 to U$S 40 million per year will not be far away from reality.

Babesiosis affects animal production not only because of the deaths but for the weight losses that causes either in the accute or the chronic phase of the disease (SOLARI, 1992).

MATERIAL AND METHODS

Design of primers: We searched for sequences of *B. bovis* in the GEN BANK release 6.7, SWISS-PROT 23, EMBL 31 and UGenBank 72-31 using the PC/GENE software (Intelligenetics, Inc. Mountain View, CA, USA).
From the 9 sequences found we chosed the BBMER60 GB number that encodes for a surface merozoite, protein of *B.bovis* (SUAREZ *et alii*, 1991). Using the PCRPLAN program we designed a pair of primers of 25 nucleotides of length called BBMER#1 and BBMER#2, that amplify a DNA fragment of 1056 base pairs.
The primers were synthetized at Biodynamics SRL (Bs. As., Argentina).

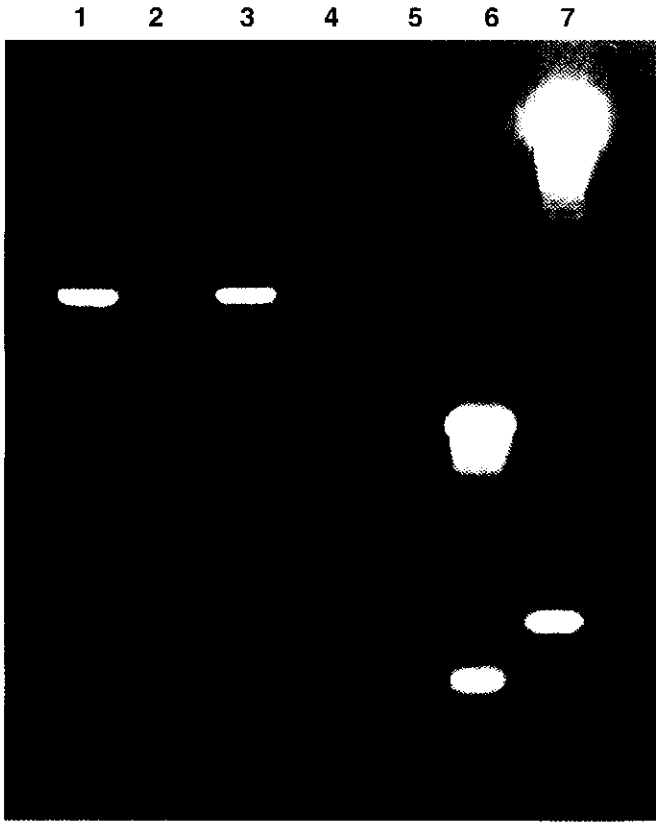

Fig.1. Analysis of PCR products by 1,4% gel electrophoresis.
Lanes: 1, purified DNA of *B. bovis*; 2 and 4, empty wells; 3, DNA from blood containing *B. bovis;* 5, no template DNA (negative controls); 6, amplified product of lambda bacteriophage used as a positive control of reagents; 7, 123 bp ladder markers (Bethesda Research Laboratories).

Amplification of BBMER60 from purified parasitic DNA: From four splenectomized calves experimentally infected with vaccinal strain of *B. bovis*, field strain of *B. bovis*, *B. bigemina*, and *Anaplasma marginale*, blood was taken and the erythrocytes separated from white cells by centrifugation. Afterwards, by hypotonic lysis we recovered the parasites from the erythrocytes.

The purification of parasitic DNA was effected by digestion with proteinase K and Tween 20 for two hours, followed by phenol-chloroform extraction (SAMBROOK *et alii*, 1989).
Normal bovine blood was collected from a calf donor, and DNA purified from nucleated cells of peripherical blood.
The program used for the amplification by PCR are described bellow.
Initial denaturation 94°C -2 minutes 35 cycles of:
Cyclic denaturation 94°C -1 minute
Annealing to the primer 53°C -1 minute
Extension of the primer 72°C -2 minutes
Final extension 72°C -5 minutes
The amplificated products were analyzed by electrophoresis on 1,4% agarose gel stained with Ethidium Bromide. Bands were visualized by transilumination with U.V. light.
The identity of the amplified fragment was corroborated by comparison with the restriction map of the sequence obtained from the GENE BANK, by digesting the amplified product with the restriction enzymes, Hinf I and Taq I.

RESULTS

The 1056 bp was present in the different samples of blood infected with *B. bovis* as shown in Fig. 1.
The fragments obtained after digestion of the amplified product with restriction enzymes did coincid with the ones expected according to the restriction map (Fig.2a and Fig.2b).
To ensure that this fragment was not present in bovine DNA, nor in the other pathogens above mentioned, we tried to amplify DNA of *B. bigemina*, *A. marginale*, and normal bovine blood.
The systems specificity was demonstrated after no amplification was observed with *B. bigemina*, *A. marginale*, and bovine DNA (Fig.3).

DISCUSSION

We have developed a PCR based method for *B. bovis* detection through the *in vitro* amplification of DNA sequence of this parasite.
Such method can be used to differentiate *B. bovis* from *B. bigemina* and from other bovine's hemoparasites due to its absolute specificity.
The results' interpretation is not technician-dependant which diminishes greatly error and also permits a major processing of samples. FIGUEROA *et alli* (1992), using a similar system for the diagnosis of *B. bigemina*, obtained a sensitivity a million times higher than that obtained with Giemsa stained smears.
Actually, we are trying to make the same comparison with *B. bovis*.

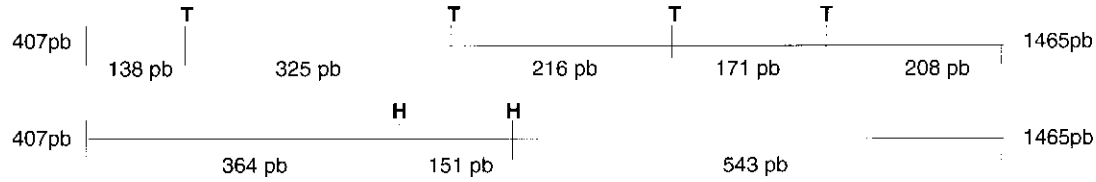

Fig. 2. Restriction analysis of BBMER60 sequence.
Fig.2a. T and H restriction sites of enzymes Taq I and Hinf I respectively. Between each restriction site is indicated the lenght of the originated fragment; 1 and 2, expected band pattern when digesting the 1056 bp fragment between bases 407 and 1465 with Tq I and Hinf I respectively.

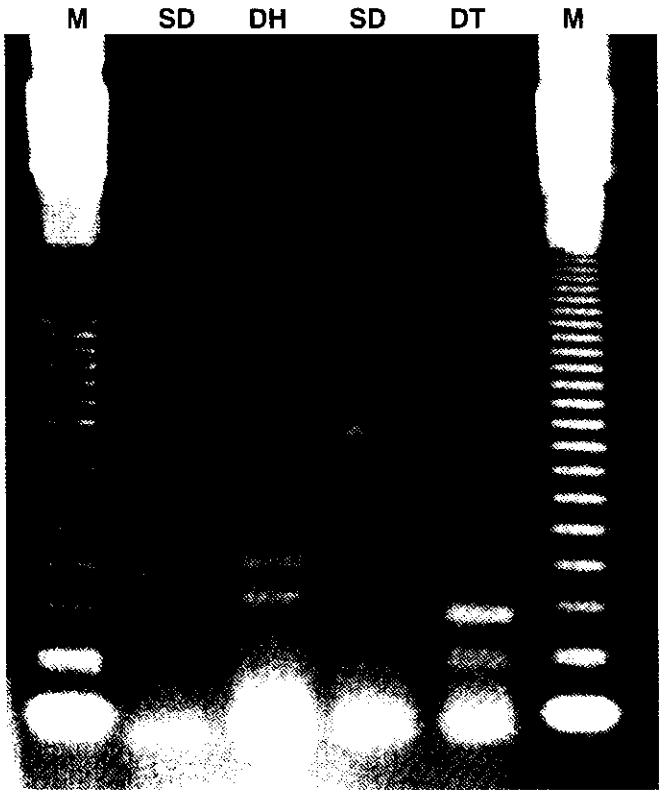

Fig.2b. M, 123bp ladder markers (Bethesda Research Laboratories); SD, 1056 bp fragment without digestion; DH, digestion with Hinf I; DT, digestion with Taq I.

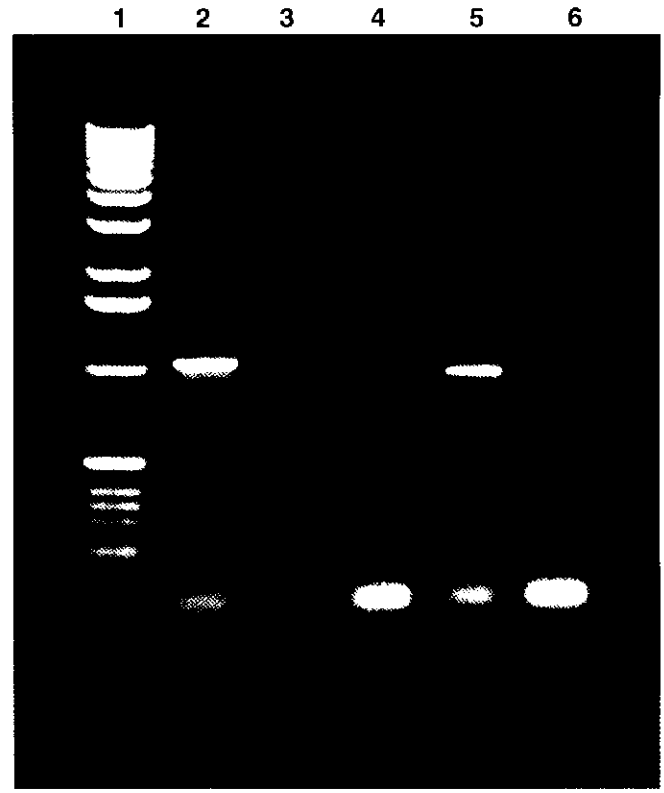

Fig.3. Constatation of the system's specificity.
Lanes: 1, 1Kb ladder markers (Bethesda Research Laboratories); 2, 3, and 4, amplified product of purified parasitic DNA from *B. bovis*, *B. bigemina*, and *A. marginale* respectively; 5, amplification obtained from bovine blood infected with *B. bovis*; 6, PCR product from bovine DNA free from hemoparasites.

We are also working in the simplification of the proceedings in order to use it in the field.

Treatment of the outbreakes will be more specific because actually infected animals are treated without knowledge of the exact cause.

Likewise, programmed epidemiological studies of the integral dynamic of the tick-hemoparasite-bovine system will be possible in order to optimize the strategy control programs.

During this project performance, the Biotechnology Unit of INIA together with the Parasitology Division of DILAVE have developed skills in PCR based diagnostic technics. This technology is being internationally applied to the diagnosis of bovine Leucosis, Tuberculosis, Brucelosis, Leptospirosis, Toxoplasmosis, Pseudorrabia, Blue tongue, and other infectious diseases.

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