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NON-IMMUNOLOGIC METHODS OF DIAGNOSIS OF BABESIOSIS

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The diagnosis of tick-borne diseases such as babesiosis still depends on observing the parasite in the infected erythrocyte. Microscopic observation is tedious and often problematic in both early and carrier infections. Better diagnostic methods are needed to prevent clinical disease, especially when susceptible cattle are being moved into disease enzootic areas. This study evaluates two techniques for early diagnosis of Babesia bovis *infections in cattle, DNA probes specific for the organism and fluorescent probes specific for nucleic acid. The radioisotopically labeled DNA probes are used in slot blot hybridizations with lysed blood samples, not purified DNA. Thusfar, the probe is specific for* B. bovis *and can detect as few as 1000* B. bovis *parasites in 10 µl of blood. The specificity of the fluorescent probe depends on the characteristic morphology of the babesia in whole blood samples, as determined microscopically. The fluorescent probe detects as few as 10,000* B. bovis *parasites in 10 µl os blood. The application of each method for laboratory and field use is discussed.*

Key words: babesiosis – DNA probe – fluorescent probe – cattle, tick – bone disease

Tick-borne diseases such as babesiosis seriously affect the economics of livestock production. In many countries in the western hemisphere, while better diagnostic methods have diminished the problems due to viral and bacterial disease agents, the problems with tick-borne diseases such as babesiosis seem to have increased. Babesiosis is of special concern for several reasons. Regions of high potential for cattle production are also favorable for vector *Boophilus* ticks and enzootic babesiosis. Movements of susceptible cattle from tick-free, generally less productive areas into these regions is often disastrous. Since the disease has been eradicated from North America, importation of cattle from the United States and Canada into these regions is equally hazardous. Clearly, control of babesiosis will depend on the thorough understanding of the interrelationships of susceptible cattle with the *Boophilus* tick vector and the reservoir of *Babesia* infection. Thus, better diagnostic methods to prevent clinical disease, to determine the role of the carrier animal in the epidemiology of the disease, and to determine the infectivity of vector ticks are critical elements in an integrated approach to the control of babesiosis.

The diagnosis of *Babesia* infections in cattle still depends on microscopic observation of the parasite in stained blood films, which is a tedious procedure frequently made more difficult by extremely low circulating parasitemias present for very limited periods of time. In very early infection or after establishment of the carrier state microscopic detection of *Babesia* is uncommon due to low numbers or the absence of circulating parasites (Todorovic & Carson, 1981). Such infections may be missed microscopically. The same procedure, with the same limitations, is followed with thick smears prepared from hemolymph obtained from the tick (Purnell, 1981).

Serology is limited in usefulness because antibody activity is a reflection of exposure, but not necessarily indicative of current infection. *B. bovis* specific antibodies are not detected in the serum until 6-8 days after the onset of the parasitemia (Kuttler et al., 1977; Goff et al., 1982). Cattle recently exposed may test negative, and positive responses may persist longer than the ability to transmit the infection (Johnston & Tammemagi, 1969). Thus, carrier animals may test negative both microscopically

and serologically, yet become reservoirs of infection when susceptible cattle are introduced to the pasture and *Boophilus* ticks are present. Because the serological status of a cow may not accurately reflect the infectivity of the animal, the development of a test with high sensitivity and specificity that can directly identify *Babesia* in a blood sample is imperative.

The potential of diagnostic probes for infectious diseases is now being realized. Both sensitivity and specificity appear to be very good with the recently reported DNA probes for diagnosis of hemoparasitic diseases (Barker et al., 1986; McLaughlin et al., 1986; Holman et al., 1989; Jasmer et al., 1991). Moreover, tests based on DNA offer advantages over current serological tests that rely on parasite-derived antigens which may vary in quality and deteriorate under storage (Barker et al., 1986). Diagnostic promise has been shown for DNA probes that have been developed for hemoparasites such as *Plasmodium falciparum* (Barker et al., 1986), *B. bovis* (McLaughlin, 1986; Holman et al, 1989; Jasmer et al., 1991), *B. bigemina* (Buening et al., 1990), *B. equi* (Posnett & Ambrosio, 1989), and for rickettsial infections of cattle such as anaplasmosis (Goff et al., 1982; Ambrosio & Potgeiter, 1987; Goff et al., 1988; Aboytes-Torres & Buening, 1990).

Fluorescent probes, non-specific for the parasite but specific for nucleic acid, also offer a simple and sensitive technique for the rapid diagnosis of hemoparasites (Kawamoto & Kumada, 1987). Microscopic examination of specimens after fluorochrome staining with such compounds as acridine orange, ethidium bromide, propidium iodide or diamidino phenylindole has been shown to be effective in diagnosing infections with parasitic protozoa such as *Cryptosporidium, Entamoeba, Brugia* and *Wucheria* (Kawamoto & Kumada, 1987; Long et al., 1990), and for human infections with hemoparasites such as *Toxoplasma, Trypanosoma* and *Plasmodium* (Kato et al., 1987; Kawamoto & Kumada, 1987; Levine & Wardlaw, 1988; Levine et al., 1989; Moody, 1990). Diagnosis with fluorescent probes depends on the distinctive microscopic morphologic of the protozoa after the staining procedure.

We have developed and evaluated DNA probes specific for the DNA of the *Babesia* organism, and recently evaluated fluorescent probes specific for the nucleic acid of the parasite. Both probes can contribute an accurate test to the diagnosis of babesiosis, the carrier status of the animal, and tick infectivity.

Babesia bovis of Mexican origin was established *in vitro* (Holman et al., 1988). A genomic library of *B. bovis* was prepared using mung bean nuclease digestion (Tripp et al., 1989). The library was screened with various specific

TABLE I

Specificity of *Babesia bovis* DNA probes

	Bb1[a]	Bb3[a]	Bb1 - PCR[b]
Purified DNA:			
B. bovis Mexico	+	+	+
B. bovis Australia	+	+	+
B. bigemina Mexico	–	–	–
B. bigemina Australia	–	–	–
B. bigemina St. Croix	–	–	Not done
B. microti U.S.	–	–	Not done
Bovine WBC	–	–	–
Blood lysates:			
B. bovis Mexico (1987)	+	+	+
B. bovis Mexico (1989)	+	+	Not done
B. bigemina Mexico	–	–	–
B. divergens Ireland	–	–	Not done
B. odocoilei Texas	–	–	Not done

a: results of slot blot hybridization assays.
b: results of PCR amplification using internal primers derived from Bb1 sequence analysis.

antisera, including sera collected from naturally infected cattle in enzootic areas of Mexico (Teclaw et al., 1985). Characterization of two selected genes for probe use has shown that both are absolutely specific for *B. bovis* (both Mexican and Australian isolates) with no hybridization obtained with *B. bigemina* of Mexican, St. Croix (Virgin Islands), or Australian origin, *B. divergens* of Irish origin, *B. odocoilei* from white-tailed deer, *B. microti,* or to bovine leukocyte DNA (Holman et al., 1989; Tripp et al., manuscript in preparation). Both probes were specific in slot blot hybridizations (Table I).

The sensitivity of the *B. bovis* probes was determined using blood samples obtained from cattle infected with tick-derived *B. bovis* stabilate. The sensitivity was approximately 1000 *B. bovis* parasites per 10 µl blood sample, or 0.00005%. These infected blood samples had either extremely low or undetectable parasitemia when Giemsa stained thin smears were examined microscopically. A sample taken nine days after infection, for instance, had less than one parasite infected cell per 10-1000x fields.

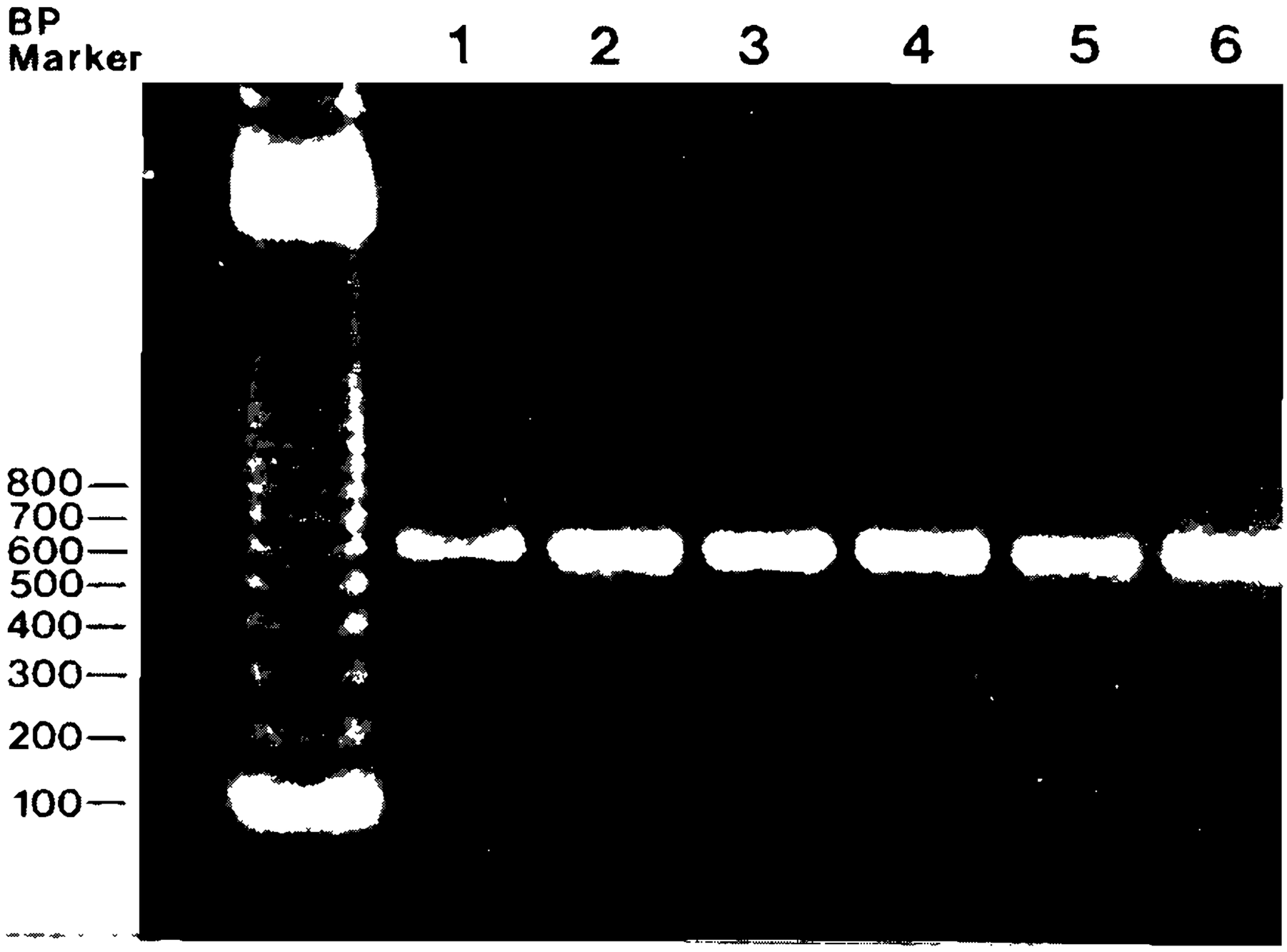
DNA primers selected internally from sequence analysis of one of the *B. bovis* DNA probes specifically amplify an approximately 600 base pair segment of both purified *B. bovis* DNA and *B. bovis* DNA in blood lysates using the polymerase chain reaction (PCR, Holman et al., manuscript in preparation). Preliminary results with purified DNA yielded amplification of *B. bovis,* but not of *B. bigemina* or bovine white blood cell DNA (data not shown). Lysates of *B. bovis* erythrocyte cultures at parasitemias of 0.6 and 0.06% were prepared by a modification of a described protocol (Higuchi, 1989). Amplification of 1 ng control purified *B. bovis* DNA and of a lysate samples containing $4.6X10^3$ or $4.6X10^2$ parasitized erythrocytes occurred as shown in Fig. 1.

Hemolymph collected from *B. bovis* infected *Boophilus microplus* ticks was compared to uninfected tick hemolymph from *Bo. annulatus*. The uninfected hemolymph showed no hybridization to the probes, but the infected hemolymph hybridized with both probes. Further, the probes detected *B. bovis* DNA in a 1:3 dilution of infected hemolymph. Microscopic analysis, on the other hand, revealed *B. bovis* kinetes only in the undilute sample. Similarly, the presence of *B. bigemina* DNA within infected *Bo. microplus* tick tissues has been determined by dot blot hybridization with a *B. bigemina* DNA probe (Hodgson et al., 1991).

The fluorescent probe method we have evaluated takes advantage of an early observation (Worth, 1964) that microhematocrit centrifugation effectively concentrates hemoparasites in the buffy coat region. The method utilizes commercially available microhematocrit tubes (QBC tubes, Becton Dickinson, Franklin Lakes, New Jersey, USA) precoated with anticoagulants (Na heparin and EDTA) and acridine orange. The tubes are supplied with a plastic insert that floats in the buffy coat region after centrifugation. If the blood sample contains *Babesia* - infected erythrocytes, they are 1) stained characteristically by the acridine orange, 2) concentrated near the erythrocyte-leukocyte interface after centrifugation, and 3) displaced toward the inside wall of the tube by the presence of the plastic insert. The parasites are then easily detected by fluorescence microscopy.

The QBC tubes were used according to manufacturers directions. Venous blood samples were obtained from cattle undergoing experimental *B. bovis* and *B. bigemina* infections in tubes containing anticoagulant (EDTA-VACUTAINER, Becton Dickinson). Later, the QBC tubes were filled with blood (approx. 65 µl), stoppered at one end and the plastic insert placed in the open end of the tube. The tubes were inverted to mix the contents and centrifuged at approx. 14,400 Xg for 5 min (PARAFUGE, Becton Dickinson) and observed using a lucite tube holder and 60X fluorescent objective attached to an ordinary light microscope (PARALENS fiber optic illuminator and UV microscope adapter, Becton Dickinson). In some experiments, the QBC tubes were filled directly with blood from a tail stick. Also, several drops of blood (approx. 200 µl) from a tail stick were collected into a clean dry tube. The tube was loosely stoppered and placed in a dry, 37 °C incubator for 72 h to dry the blood. The dried blood was rehydrated in 200 - 300 µl 0.01M phosphate buffered saline solution (PBS) and the QBC tubes filled and examined as above.

Thin blood smears were prepared from each of the above samples on clean microscope slides, air dried, fixed in methanol for 1 min, and stained for 20 to 30 min in Giemsa stain



Lanes 1&2. 1 ng *B bovis* DNA
Lanes 2&5. $4.6x10^3$ *B bovis* infected RBC.
Lanes 3&6. $4.6x10^2$ *B bovis* infected RBC.

7.5 pM each primers B1 and B2 for each reaction.

Fig. 1: agarose gel electorphoresis of 1µl PCR product after 50 amplification cycles. Reaction mixture (100µl total volume) contained either $4.6x10^2$ or $4.6x10^3$ *Babesia bovis* parasitized cells, or 1 ng purified *B. bovis* DNA. 7.5 pM of each primer were used.

diluted 1:10 in 001M phosphate buffer, pH 6.85. After staining, the slides were rinsed in buffer and allowed to air dry. The slides were examined by light microscopy using oil immersion.

In one experiment with *B. bigemina*, an ethidium bromide stain was also tested. After collection, an aliquot of each blood sample was centrifuged at 1,000Xg for 10 min and the plasma removed. The packed cells were washed twice in PBS, with the buffy coat being removed each time. The cells were then resuspended to approx. 50% v/v in PBS containing ethidium bromide, 10 mg/ml. The cell suspension was centrifuged at 13,000Xg for 5 sec. Ten µl of packed cells were collected from the top of the cell layer and mounted on clean microscope slides and examined under UV illumination.

To determine the relative sensitivity of the QBC method, known numbers of *Babesia*-infected erythrocytes from cell culture were added to samples of fresh whole blood collected in anticoagulant from a normal steer. Two-fold dilutions were prepared using additional blood as diluent. QBC tubes were filled with aliquots from each dilution and examined for the presence of parasites. Giemsa stained thin smears were also prepared and examined.

Both *B. bovis* and *B. bigemina* merozoites were observed easily using the QBC method and could be identified by their characteristic paired morphology. The *Babesia* nuclei fluoresced bright green with an orange or reddish-orange cytoplasm. The parasites were concentrated in the part of the erythrocytes layer adjacent to the buffy coat. With careful observa-

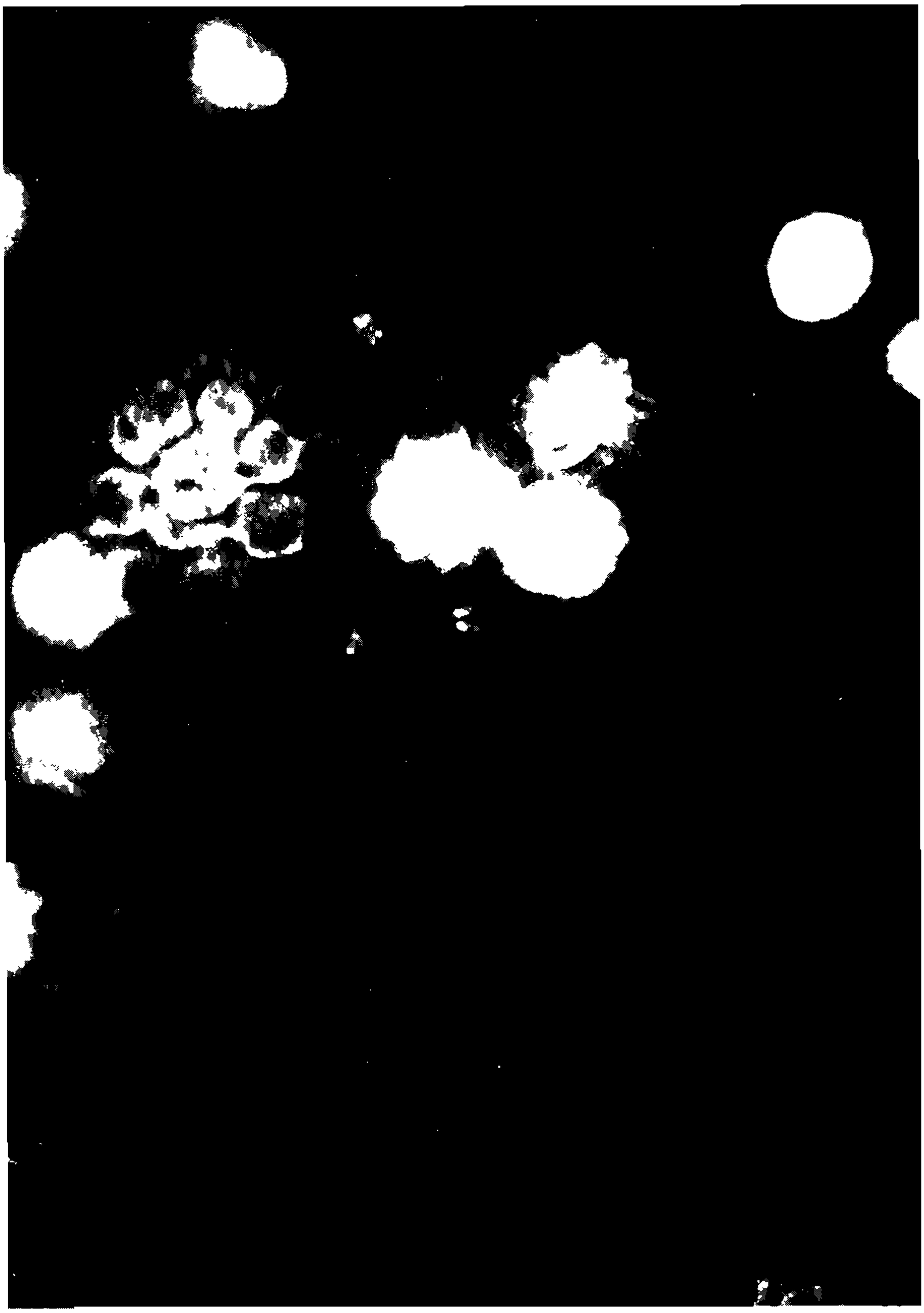


Fig. 2: fluorescence micrograph (X600) of acridine orange stained *Babesia bigemina* in bovine erythrocytes in a QBC tube. Shown is a characteristic *B. bigemina* paired merozoite, and several granulocytes and lymphocytes.

tion, occasional parasites could be seen moving within erythrocytes. Trophozoites could also be identified. The bovine lymphocytes in the preparation were observed to stain light green, platelets, granulocytes and granules from damaged cells stained yellow (Fig. 2). We observed that the bovine granuylocytes tended to be scattered throughout the red cell layer rather than banding as is seen with human samples.

The QBC method was found to be more sensitive in detecting *Babesia* from whole blood compared to other methods used. *Babesia bigemina* parasites were detected in blood samples from one of four infected cattle at two days post infection, two days earlier than conventional Giemsa stained smear examination (Table II). Similarly, *B. bovis* parasitemia was detected two days before Giemsa stained smear

TABLE II

Detection of *Babesia* parasites by QBC, Giemsa stain and ethidium bromide stain

		Days post infection, as detected by		
		QBC	Giemsa	EtBr
Babesia bigemina:				
Calf #	183	3	6	not done
	1938	2	4	3
	1975	3	5	5
	1977	4	5	4
Babesia bovis:				
Calf #	134	7	9	not done

TABLE III

Relative advantages of the DNA probe and the QBC methods for the detection of *Babesia* parasits

	DNA probe	QBC method
Time	days	hours
Ease	less	more
Sensitive	more	less
Specific	yes	no
Equipment	elaborate	simple
Field application	maybe	yes

examination. Ethidium bromide staining detected *B. bigemina* infected cells about one day later than the QBC and one day earlier than Giemsa staining.

The sensitivity of the QBC method was determined using *B. bovis* infected erythrocytes from cell culture mixed with whole blood from an uninfected steer. The sensitivity was approximately 100 to 1000 *B. bovis* parasites per 65 µl blood sample. The experiment was repeated several times, each time with similar results. The normal cows erythrocyte counts ranged from $2x10^8$ to $1x10^9$. The results suggest that the QBC can detect parasitemias of approx. 0.0001%, with about 100 *B. bovis* infected cells the lower limit. The results were affected by the number of merozoites present with characteristic paired morphology; one experiment detected a parasitemia of 0.00004%. As with the sensitivity determination of the DNA probes described above, these samples had either extremely low or undetectable parasitemia when Giemsa stained thin smears were examined microscopically.

Several experiments were conducted with dried blood samples containing *Babesia* infected erythrocytes. These experiments were intended to evaluate the QBC method under less than optimal methods. Although the rehydrated samples contained few intact erythrocytes and much debris, an experienced evaluator had no trouble in identifying intact parasites. However, after centrifugation, the plastic plug was removed and the end sealed with clay to enable viewing of the entire length of the QBC tube in the lucite holder. The only technique similar to this adaptation of the QBC is the thick blood smear method (Mahoney & Saal, 1961). Although the latter method was not used for comparison with the QBC in this study, our experience with thick smear preparation and evaluation suggests that the QBC is much easier and faster.

In conclusion, we found the QBC method for detection of *Babesia* infected etythrocytes in whole blood to be simple and relatively inexpensive compared to the use of DNA probes. There is, of course, no comparison between the two methods for specificity; the DNA probe is absolutely *Babesia* specific, while the QBC is non-specific. Both the QBC and the DNA probe offer the ability to test relatively large amounts of blood in a short time. The QBC instrumentation package can be easily adapted to field use, assuming a source of electricity such as a generator is available. These points are summarized in Table III.

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