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MOLECULAR APPROACHES TO MALARIA AND BABESIOSIS DIAGNOSIS

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The development of additional methods for detecting and identifying Babesia *and* Plasmodium *infections may be useful in disease monitoring, management and control efforts. To preliminarily evaluate synthetic peptide-based serodiagnosis, a hydrophilic sequence (DDESEFDKEK) was selected from the published BabR gene of* B. bovis. *Immunization of rabbits and cattle with the hemocyanin-conjugated peptide elicited antibody responses that specifically detected both* P. falciparum *and* B. bovis *antigens by immunofluorescence and Western blots. Using a dot-ELISA with this peptide, antisera from immunized and naturally-infected cattle, and immunized rodents, were specifically detected. Reactivity was weak and correlated with peptide immunization or infection. DNA-based detection using repetitive DNA was species-specific in dot-blot formats for* B. bovis *DNA, and in both dot-blot and* in situ *formats for* P. falciparum; *a streamlined enzyme-linked synthetic DNA assay for* P. falciparum *detected 30 parasites/mm³ from patient blood using either colorimetric (2-15 h color development) or chemiluminescent detection (0.5-6-min exposures). Serodiagnostic and DNA hybridization methods may be complementary in the respective detection of both chronic and acute infections. However, recent improvements in the polymerase chain reaction (PCR) make feasible a more sensitive and uniform approach to the diagnosis of these and other infectious disease complexes, with appropriate primers and processing methods. An analysis of ribosomal DNA genes of* Plasmodium *and* Toxoplasma *identified Apicomplexa-conserved sequence regions. Specific and distinctive PCR profiles were obtained for primers spanning the internal transcribed spacer locus for each of several* Plasmodium *and* Babesia *species.*

Key words: *Babesia* – *Plasmodium* – diagnosis – synthetic peptide – serology – *in situ* detection – chemiluminescence – dot-blot – polymerase chain reaction – internal transcribed spacer – ribosomal DNA

Microscopic examination of Giemsa-stained slides remains the technique of choice for the routine diagnosis of blood stages of Apicomplexa parasites (Anonymous, 1988). However, the development of additional strategies that can reliably detect and identify species of the *Babesia* and *Plasmodium* complexes may lead to improved molecular epidemiology studies and help to implement rational programs of disease management and control (Smith, 1984).

Additional diagnostic methods may be helpful in determining the prevalence of zoonotic infections of *Babesia* in humans. The frequency of human babesiosis is probably low and difficult to estimate using current microscopic, IFA and ELISA technologies (James et al., 1987). Additionally, the tests of recent vaccines for babesiosis and malaria (Cheung et al., 1986; Anders & Brown, 1990) may require techniques that can distinguish vaccination from infection. Serology is of interest because blood is conveniently sampled, and because detec-

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tion is feasible even when the infectious agent is absent or in low abundance. Primary sequence information from selected parasite antigens makes feasible the development of peptide-based serodiagnostic assays which can identify infections in inexpensive and convenient formats, and such assays are well-advanced for malaria (Campbell et al., 1987), but not for babesiosis (James et al., 1987). In the current study, we have explored immune responses to a single *B. bovis* peptide, which was also evaluated as a vaccine.

The development of additional methods for the direct detection of *Babesia* and *Plasmodium* oligonucleotides in animals and humans, has received much recent attention. Isotopically labeled plasmid-borne repetitive DNA probes have been described for *B. bovis* (McLaughlin et al., 1986), several other *Babesia* species (Posnett & Ambrosio, 1989; Ambrosio & DeWaal, 1990), and *P. falciparum* (Franzen et al., 1984). The falciparum malaria hybridization assay has been adapted to nonisotopic, enzyme-linked synthetic DNA detection (McLaughlin et al., 1987, 1988; Lanar et al., 1989), using a rapid lysis and filtration processing procedure (Barker et al., 1989; McLaughlin et al., 1991b). In the current study, we describe preliminary work with *in situ* and chemiluminescence detection (Martin et al., 1990) using the enzyme-linked synthetic DNA probe.

These types of serology or nonisotopic hybridization assays may be helpful for studies of the overall prevalence and densities of disease. Additional techniques are helpful to determine the relationships of independent isolates of the same infectious agent which differ in important phenotypes. It is relevant to the development of such techniques, that lower protozoa recombine at a slow rate, as reflected by the rarity of heterozygotes at many loci; they may, therefore, be considered as clonal with regard to many genetic markers (Tibayrenc et al., 1990, 1991). This is surprising for malaria, where gamete production and fusion is known to occur, but ecological and life cycle restrictions may contribute to inbreeding. A consequence is that at least for epidemiologically relevant time periods, many genetic markers will remain correlated with particular phenotypes such as drug resistance or virulence. The expectation is that even arbitrary parasite markers and disease phenotypes will remain correlated.

The available ribosomal (r)RNA sequence data may provide a rational and well-understood marker for species or strain identification (Stahl, 1986; Olsen, 1988). Ribotyping is an established approach to pathogen identification among bacterial species; pathogen DNA is digested with restriction endonuclease(s), and rDNA band size polymorphisms in the moderately repetitive ribosomal DNA cistrons are detected after hybridization with rRNA or rDNA. Ribosomal DNA fingerprinting is generally useful for typing organisms, and has for instance been used to identify virulent strains of *Pseudomonas* (LiPuma et al., 1988) and *Pasteurella* (Snipes et al., 1990). Similar approaches have been used to distinguish *Anopheles* species which can transmit malaria (Collins et al., 1988), and strains of maize (Rocheford et al., 1990). Ribotyping of closely related isolates or strains depends upon length and sequence polymorphisms in the rapidly evolving long and short spacer DNA between the structural rRNA genes. Currently, ribotyping requires significant amounts of purified pathogen DNA and homologous RNA or rDNA clones, but this requirement can be avoided by adapting the polymerase chain reaction (PCR) (Saiki et al., 1988; Sauvaigo et al., 1990; Persing, 1991; Erlich et al., 1991). The nontranscribed spacer between the 28S ribosomal RNA gene and the beginning of the 18S ribosomal RNA gene is too long (ca 2,000 base pairs) (Rocheford et al., 1990) for effective amplification using PCR (Persing, 1991). We have therefore chosen to evaluate the shorter (ca 500 bp) internal transcribed spacer (ITS) between the 18S and the 5.8S-28S genes. In some ribosomal gene clusters in bacteria, the ITS locus includes tRNA genes, and alterations in this locus are involved with gene regulation (Yamamoto et al., 1976; Bacot & Reeves, 1991). We have previously shown that ITS polymorphisms can be used to distinguish isolates of pathogenic free-living amoeba (Vodkin et al., 1992) and eubacteria (McLaughlin et al., 1993, in press, *Mol. Cell Probes*). The characteristics of the ITS locus may prove useful for PCR fingerprinting studies of *Plasmodium* and *Babesia* species.

In the current study, we describe experiments which preliminarily evaluate the immune responses to a *Babesia*-derived synthetic peptide. We also describe the direct nonisotopic detection of *Plasmodium* in *in situ* and dot-blot formats. Finally, the ITS locus is evaluated for

several *Babesia* and *Plasmodium* species using the PCR.

MATERIALS AND METHODS

Babesia-derived synthetic peptide – A moderately repetitive DNA family (BabR) was observed to rearrange during the attenuation of vaccine strains of *B. bovis* in Australia (Cowman et al., 1984). The published BabR amino acid sequences were examined for hydrophilic regions, which have been shown to favor immunodominant epitopes in a number of proteins (Hopp & Woods, 1981; Atassi & Webster, 1983; Tainer et al., 1984; Jemmerson & Paterson, 1986. A decamaric peptide was selected (DDESEFDKEK) and synthesized. Computer matches of a slightly longer peptide and oligonucleotide region were performed with the available malaria database (Walter and Elisa Hall Institute of Medical Research, 1991 update), and the entire sequence database, using standard matching programs (Devereux et al., 1984). The synthetic oligopeptide was coupled to an equal weight of *Limulus* hemocyanin protein carrier using 0.25% glutaraldehyde, emulsified in Freund's adjuvant, and injected subcutaneously (SC) into rabbits (1 mg) and cattle (4.5 mg), with boosts using Freund's incomplete adjuvant, in a regimen of 3SC injections at weeks 0, 6, and 12. Control animals were identically treated, except for the absence of synthetic peptide. Two weeks after the third immunization, four vaccinated and four control cattle (18-20 months-old) were each challenged with 1.8 x 10^8 virulent Venezuelan *B. bovis* organisms. Prepatent period, days of fever, packed cell volume, and maximum percent parasitemias were monitored. All animals were treated chemotherapeutically when hematocrits dropped below 50% of prechallenge values, and they completely recovered thereafter. Immune reactivity of immunized cattle, cattle infected with *B. bovis*, and control cattle were monitored using indirect immunofluorescence (Sulzer et al., 1969), dot ELISA (Pappas, 1988), and Western blot (Tsang et al., 1983).

In situ detection of Plasmodium falciparum — Thin smears were made on Fisher Probe-On slides and dried at room temperature or on a slide warmer. Slides were immersed in 100% methanol for 1 min and rinsed by dipping in distilled water. Slides were prehybridized in 5X SSC (1XSSC = 0.15M NaCl - 0.015M Na citrate), 0.5% bovine serum albumin, 70 °C for 5 min, rinsed by dipping in phosphate-buffered saline (PBS), 0.075% Brij 35, and denaturating was in 0.3N NaOH, 1 min at 23 °C; slides were rinsed in 1X PBS, 0.075% Brij. Sandwiched slides were hybridized in 200 µl of 5X SSC, 0.5% BSA, 0.075% Brij, 7.5 nM enzyme-linked synthetic DNA probe PFR1-AP (McLaughlin et al., 1987) (Molecular Biosystems,Inc, San Diego) for 20 min in a humidified chamber. Slides were washed twice in 1X SSC at 37 °C for 5 min. Color was developed in NBT/BCIP substrate for 2 hr at 37 °C (McLaughlin et al., 1988). Slides were mounted with permount and examined with oil immersion microscopy.

Chemiluminescence detection using DNA dot-blots – A lysis and filtration procedure for blood processing in a microtiter well format, adapted from a published procedure (Barker et al., 1989), was used to apply 15-µl amounts of lysed blood in 100-µl volumes to filters using a dot-blot apparatus (McLaughlin et al., 1991a). After the 50-µl 3-M Na acetate wash step, subsequent filter treatment in alkaline salt (1.5 M NaCl, 0.5M NaOH), and neutralization in 1M Tris-HCl, pH 8.0, were done by 15-second immersions, rather than the previous 5-or 10-min soaking; blocking time was also shortened to 5 min. After hybridization and washes, in addition to detecting with dye deposition (McLaughlin et al., 1991a), Lumiphos 540 substrate (Martin et al., 1990) was added (3 ml/filter) in heat-sealed bags. Six min exposure times were chosen after initial substrate addition, but 30-sec exposures were optimal after development of maximal signal intensities, 30 min after adding substrate. Film exposure intensities were compared with dye deposition and with parasitemias determined by counts of Giemsa-stained slides.

PCR amplification using rDNA primers — Computer-assisted analysis (Deveruex et al., 1984; Rychik & Rhoads, 1989) of the 16S and 5.8-S sequences from *Plasmodium* and *Toxoplasma* was used to choose a pair of primers (API16.SEQ: TCCCCTAGGAGCTTGTG, and API5.SEQ: A/C CATTGCGTATCGCATTTT) that are predicted to bracket the short internal transcribed spacer (ITS) locus of organisms of the Apicomplexa phylum. DNA was isolated from 50-µl aliquots of human, cattle, or dog blood, by SDS-proteinase K-Tris-EDTA solubilization, phenol extraction, and isopropanol precipitation (Bell et al., 1981). DNA was dissolved in 50 µl water. Amplification was performed using 1-µl aliquots of DNA in 50-µl reaction volumes, using reagents from the

Cetus-Perkin Elmer PCR kit with 2.5 mM magnesium. A three-step amplification process was employed for 38 cycles of denaturation at 94°C for 1.0 min, primer annealing at 55°C for 1.0 min, and polymerization at 74 °C for 1.0 min; a final cycle included a 5-min polymerization step at 72°C to ensure complete extension. Amplification products were resolved using 2% agarose gels and visualized and photographed after staining in 1 μg/ml ethidium bromide (McLaughlin et al., 1991a).

RESULTS

Babesia-derived synthetic peptide – Computer matching with the extended peptide and its corresponding oligonucleotide sequence from the BabR locus of *B. bovis* detected no other significant homologies at the amino acid or nucleotide levels for known *Plasmodium* or *Babesia* sequences except for the homologous peptide (Table I). The conjugated synthetic peptide was used to generate antisera in cattle and rabbits. By immunofluorescence, the antisera detected *B. bovis* piroplasms with a distinct whole-trophozoite fluorescence pattern; *P. falciparum* schizonts were questionably reactive with rabbit antisera. Peak antibody titers (1:113) were observed in vaccinated cattle two weeks following the third immunization (Table II). Using dot-ELISA with 1-ng dried peptide on nitrocellulose, 1:100 dilutions of sera from immunized rabbits and cattle, and from immunized and *Babesia*-infected cattle, were weakly reactive, but sera from unexposed animals was not detected. Using Western blots with *P. falciparum* antigen and antisera from rabbits, doublet 40 kilodalton antigens were detected by Western blot analysis which were not detected with control sera (data not shown). Using *B. bovis* antigens and cattle antisera, a number of more strongly reactive bands was observed, with specific bands at about 150, 50, 40, and 20 kDa (Fig. 1).

To determine whether the immune response to the peptide conjugate was protective, cattle were challenged with virulent parasites and a number of parameters were monitored (Table III). No significant differences were found between control and infected animals in prepatent time, days of fever, maximum packed cell volume, or weight loss, but a significant difference in maximum percent parasitemia was observed (0.01% vs 0.74%, respectively). Control animals required treatment within two

TABLE I

Amino acid computer matching

BBE1	Y	D	D	E	S	E	F	D	K	E	K	L	Q	K
PYMSA	Y	D	T	Y	S	T	Y	K	L	E	R	L	Y	N
PFDHR	N	D	D	K	D	T	C	H	M	K	K	L	T	E
PFHRP	N	D	E	D	E	E	G	K	E	A	L	A	I	K
PFASER	D	D	D	E	D	D	Y	T	E	Y	K	L	T	E
PFGP*185*	Y	L	I	D	G	Y	E	E	I	N	E	L	L	Y

The synthetic peptide (Y) DDESEFDKEK (LQK) with four adjacent amino acids (Y, LQK) from the BabR locus were matched against *Plasmodium* databases, and the top 5 matches are shown, with identical residues underlined. Abbreviations, and the initial oligonucleotide number of the matched regions of these loci in the database (in parenthesis) are as follows: BBE1, *B. bovis* epitope 1 from the BabR locus (393); PYMSA, *P. yoelii* merozoite surface antigen (113), PFDHR, *P. falciparum* dihydrofolate reducatase (8093), PFHRP, *P. falciparum* histidine rich protein (1402), PFASER, *P. falciparum* serine-repeat protein (1254), PFGP185 (716), *P. falciparum* glycoprotein 185 (716). None of the sequence matches is considered significant, and similar matches with larger databases also produced no significant amino acid matches. Matches with the specific oligonucleotides of the sequence YDDESEFDKEKLQK also were not significant, even when the *Babesia* codon usage was modified to that of *P. falciparum* (data not shown).

TABLE II

IFA titers

Week	0	2	3	8	11	13	14
Reciprocal titer	0	34	48	40	28	80	113

IFA titers were identical for each vaccinated animal. IFA titers of control animals remained negative. Immunizations were at 0, 6, and 12 weeks.

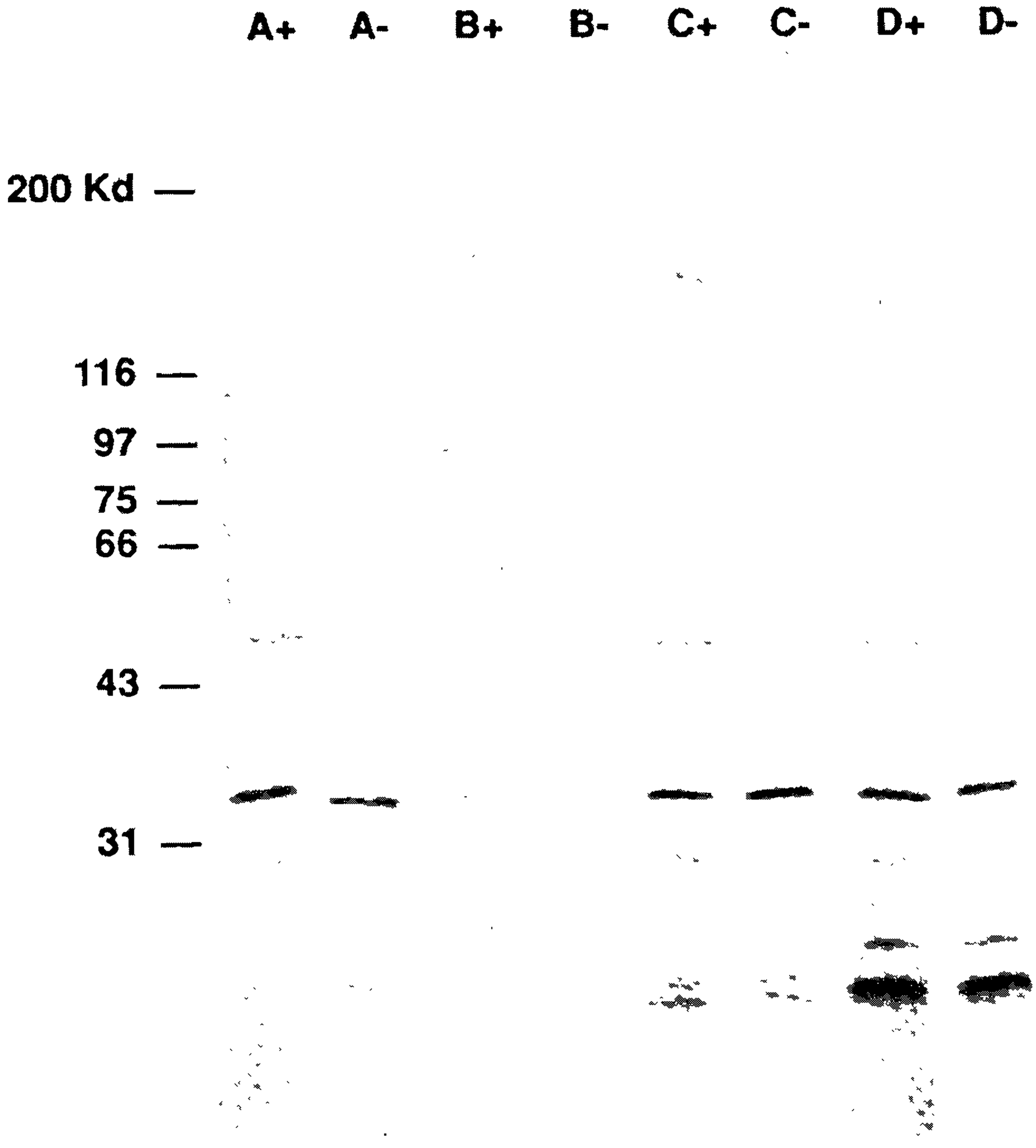

Fig. 1: nitrocellulose strips from a Western blot of *Babesia bovis* proteins incubated with 1:100 dilutions of antisera from 4 cattle (A-D) which had been immunized with hemocyanin-conjugated synthetic peptide (+). Prebleed sera from the same four cattle were processed in parallel (–). Horseradish peroxidase-coupled antibovine antibody conjugated was used for detection.

weeks, when clinical signs became acute. Chronic infections also persisted in vaccinated animals, and drug treatment was used to eliminate these infections after 4 weeks.

In situ detection of P. falciparum – A number of protocols were evaluated for *in situ* detection of *P. falciparum* in blood smears. Frequently, cells detached from the microscope slide during processing. Cell detachment was lessened by acetone treatment of dried blood smears (Hira & Behbehani, 1984) or by precoating slides with 1-5% bovine serum albumin. Using a number of alternative protocols for cell permeabilization, denaturation, hybridization, washes, and color development, strong, specific deposition of dye at *P. falciparum* cell nuclei was observed microscopically. More established slide processing methods for DNA probes, which included paraformaldehyde fixation, heat denaturation, and proteinase K treatment (Van der Loos et al., 1989), gave adequate signals but were laborious (GLM, unpublished observations). The presented protocol was convenient and rapid and gave equivalent or superior signals. Rec-

TABLE III

Clinical parameters

Parameter	Vaccinated[a]	Controls[b]
Prepatent period (Days)	6.0 ± 0.6	5.3 ± 0.3
Days of fever	4.3 ± 0.9	4.5 ± 0.9
Max % PCV decrease	-56 ± 5.8	- 60 ± 2.0

a: synthetic *Babesia bovis* 10-mer peptide.
b: significantly different from control values $P < 0.001$.

ognizable *P. falciparum* cell morphologies remained intact and were outlined by dye deposition peripheral to the nucleus. However, weaker dye deposition was observed from ring-stage nuclei than from nuclei of trophozoite or schizont stages (Fig. 2), perhaps because of higher impermeability of the associated membranes in ring stages. However, near-equivalent parasitemias were observed in blinded counts of 1000 red blood cells in Giemsa-stained and DNA probe-stained slides, using cultured parasites (5.2 and 5.0%, respectively). Also, no dye deposition was observed from white blood cells or from parallel-processed slides containing either *B. Bovis, P. vivax, P. ovale,* or *P. malariae* (data not shown). Preliminary attempts to restain destained thick smears with the current assay were unsuccessful (data not shown).

Chemiluminescence detection using DNA dot-blots Chemiluminescence detection (Fig. 3, top) was compared with dye deposition (Fig. 3, bottom) with blood samples from the MRC clinic, using a streamlined protocol adapted from a previously described protocol (Mc-Laughlin et al., 1991b). After about 30 min, signal intensities from many dots could be directly visualized in the darkroom. Similar detection of *P. falciparum* blood parasitemias was observed with 6-min exposure

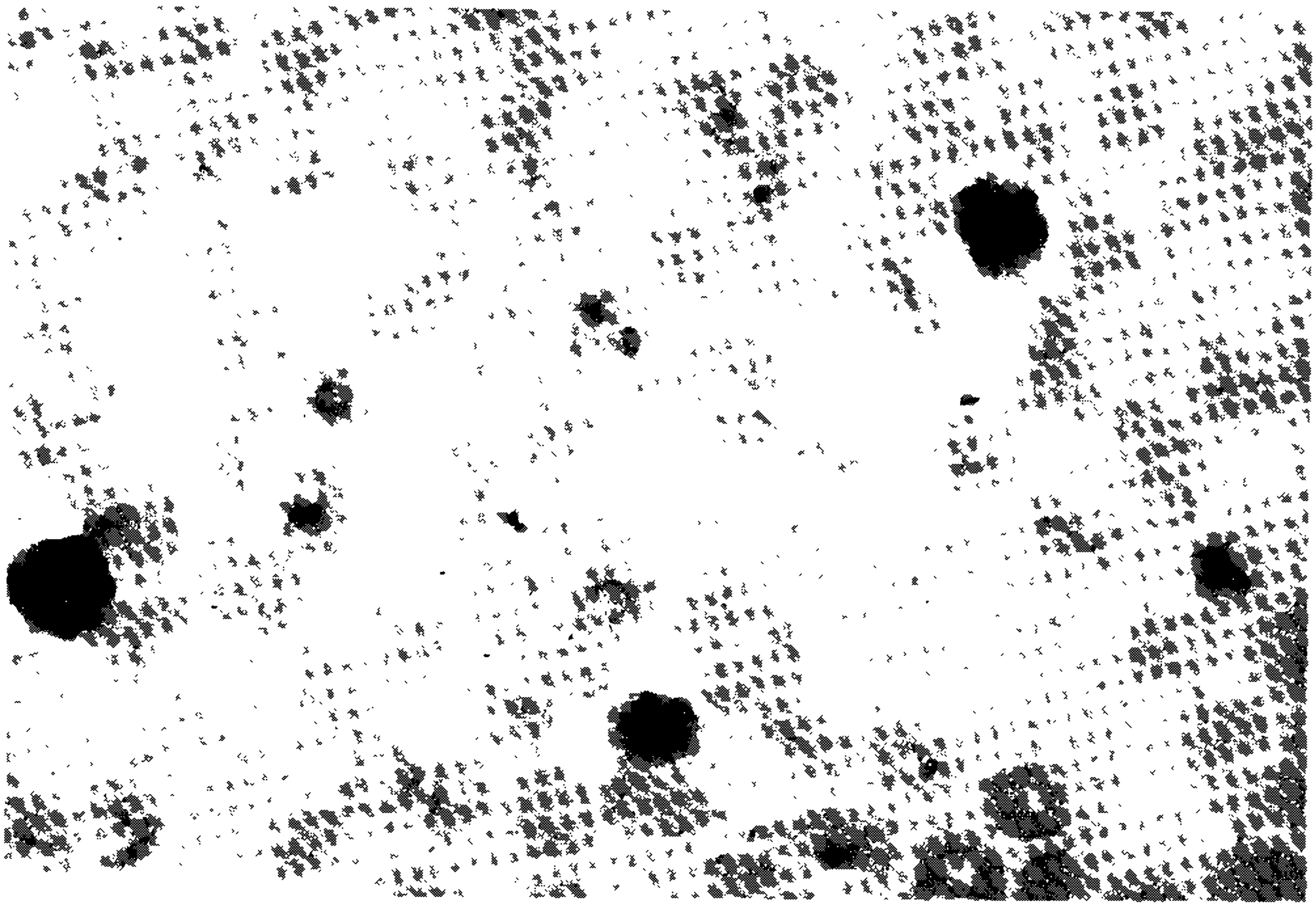

Fig. 2: microscopic detection of blood cultures of *Plasmodium falciparum* after *in situ* processing with enzyme-linked synthetic DNA and dye color development. Parasite cell morphologies were recognizable. Notice that stronger signals were obtained from trophozoite and schizont nuclei than from ring stages.

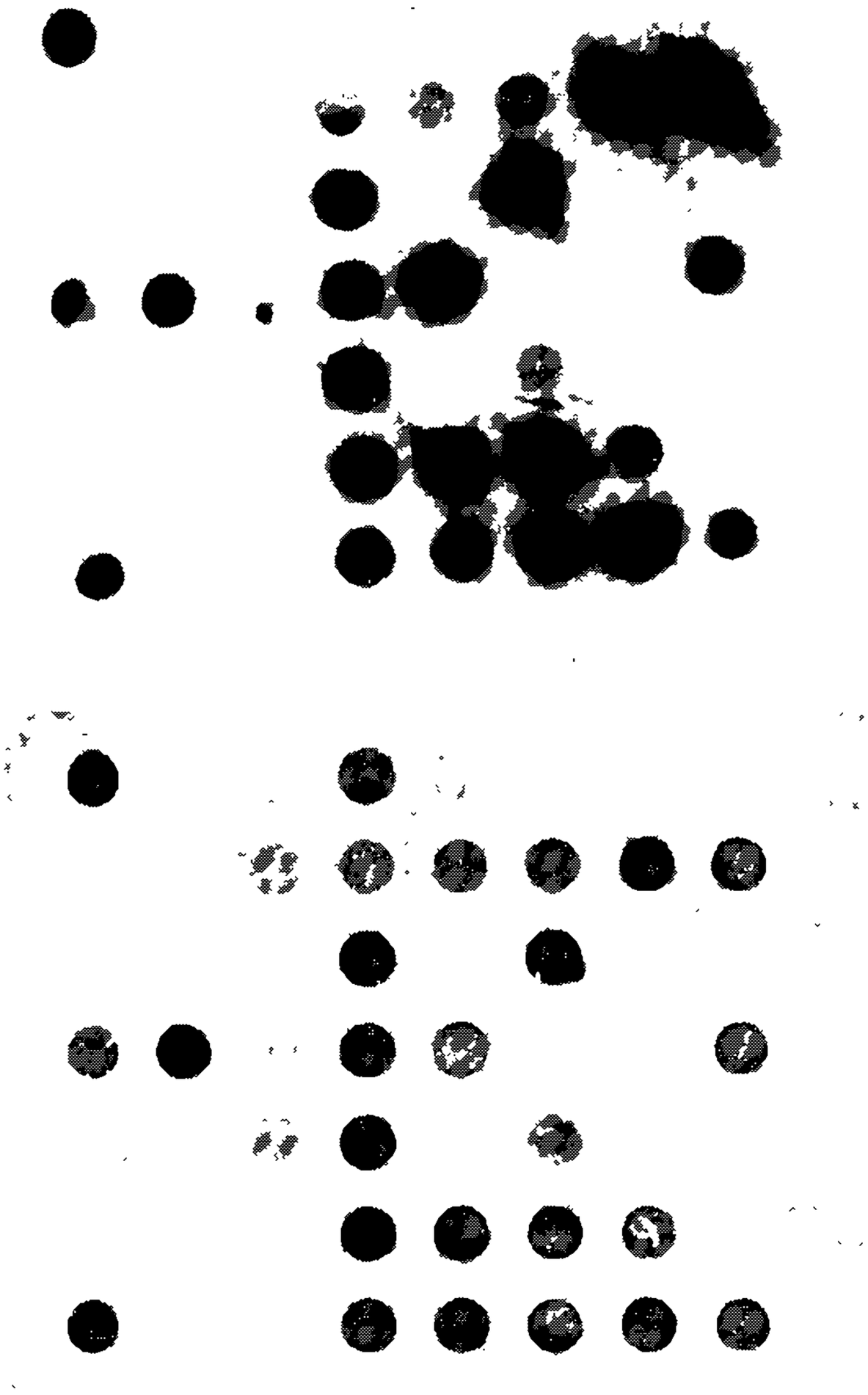

Fig. 3: a battery of 64 patient specimens from the MRC clinic was processed in duplicate for detection on dot-blot microtiter well formats using enzyme-linked synthetic DNA, with detection using either chemiluminescence-exposed X-ray film (top) or dye-deposition on nylon filter (bottom). Corresponding signal intensities of the autoradiogram generally corresponded to the amount of dye deposition on the filter. Some signal variations are attributed to the use of different amounts of the limited volume of available patient. Signal intensities and positive/negative scores were also correlated with patient parasitemias determined by microscopy (data not shown).

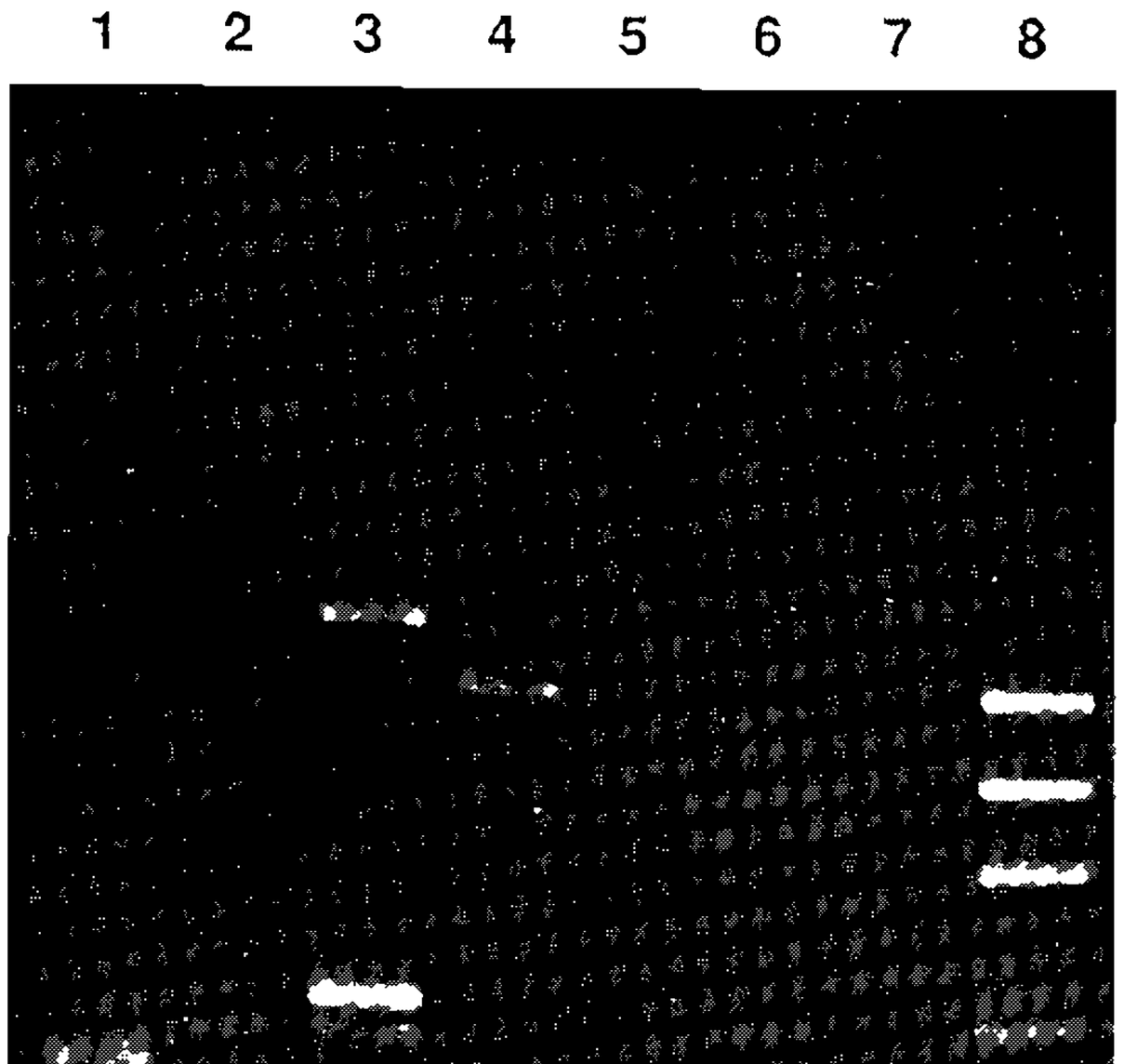

Fig. 4: agarose gel electrophoresis of PCR products after amplification of DNA isolated from cultured human blood infected with a 5% parasitemia of *Plasmodium falciparum* (lane 1); a 5% parasitemia of *B. fragile* from primate blood (lane 2); a 10% parasitemia of *P. knowlesi* from primate blood (lane 3); a 5% infection of *Babesia bovis* in cattle blood (lane 4); a 5% infection of *B. canis* in dog blood (lane 5); and a 0.5% infection of *Babesia canis* in dog blood (lane 6). Lane 7 contains parallel-processed blood from a prebleed of the dog shown in lanes 5 and 6. Lane 8 includes molecular weight markers, (Hae III digestion of Phix DNA), with sizes (top to bottom) of 1353, 1078, 872, 603, 310, 271/281, 234, and 194 bp. Notice a *Babesia*-characteristic band at about 500 bp.

to X-ray film using chemiluminescence (Fig. 3, top), compared with 2-hr color development (Fig. 3, bottom).

PCR amplification using rDNA primers — We used primers derived from computer-assisted analysis of *Plasmodium* and *Toxoplasma* 5.8-S and 16S ribosomal RNA genes to amplify DNA isolated from flood containing seven *Plasmodium* and *Babesia* species (Fig. 4). A single dominant band at about 600 bp and several satellite bands were observed for *P. falciparum*, a weaker 650 bp band was observed for *P. fragile*, two bands 650 and 1500 bp were observed for *P. knowlesi*, and several bands were detected for *Babesia* species, with a conserved band at about 500 bp. Weaker signals, with some shared bands and some bands of differing mobilities, were observed for parallel-processed samples of low and high parasitemias of *B. canis* (Fig. 4, lanes 5 and 6). No bands, or weak bands of different sizes, were observed for uninfected host DNA's processed in parallel (e.g. see Fig.4 , lane 8).

DISCUSSION

A number of novel approaches to the detection and typing of babesiosis and malaria infec-

tions were preliminarily evaluated. The synthetic peptide-hemocyanin conjugate induced antisera with cross-reactivity by IFA and Western blots to *Babesia* (strong signals, multiple antigens) and *Plasmodium* antigens (weak, one antigen). Also, the peptide dot-ELISA indicates that antisera from infected or immunized cattle was specifically, although weakly, detected. One interpretation of our results is that multiple homologous epitopes exist in the Venezuelan isolate of *B. bovis* relative to the Australian isolate, and that similar epitopes exist in *P. falciparum* antigens of about 40 KD size. Similarly, the decreased maximum parasitemias observed with peptide-immunized cattle may be due to a specific immune response to an epitope contained in the decameric peptide. However, small peptides require cross-linking to make adequate immunogens, and this treatment may eliminate or create novel epitopes (Braind et al., 1985). Immune studies using cross-linked peptide antigens and strong adjuvants have been difficult to reproduce for both *B. bovis* and *P. falciparum* (Anders & Brown, 1990), and specific results are difficult to separate from nonspecific stimulation of the immune system. Additional controls, e.g. using heterologous peptides with similar molecular characteristics such as hydrophilicity, would be necessary to distinguish nonspecific and specific immune responses. However, even nonspecific immune responses are of interest, if improved clinical outcomes are observed. It is encouraging that antisera from infected and immunized cattle are specifically detected by dot-ELISA, even though the signals are weak. Peptide-based ELISA serology will require significantly improved methodologies. One approach would be to evaluate polypeptides with independent polymerized epitopes without carrier protein, similar to the antigens which are being evaluated for malaria vaccines (DiMarchi et al., 1986; Patarroyo et al., 1988).

Babesia bovis piroplasms and *P. falciparum* rings resemble each other microscopically, and *P. falciparum* rings are also difficult to distinguish from early ring stages of other *Plasmodium* species in mixed infections (Anonymous, 1988). The *in situ* approach for *P. falciparum* detection using enzyme-linked synthetic DNA may confirm the presence of falciparum malaria in some cases. However, improved protocols for cell attachment to slides, and protocols which also allow analysis of slides previously stained with Giemsa stain are necessary. Theoretically, the *in situ* assay could then confirm the identity of rare parasites first noticed using standard staining procedures. Confirmation of the identity of parasites with altered morphologies caused by drug therapy or immune responses, or to identify mixed-species infections, is also feasible.

The dot-blot protocol for detection of *P. falciparum* asexual blood-stage infections using enzyme-linked synthetic DNA has been significantly shortened by using lysis and filtration blood processing for sample processing, by replacing 5-15 min denaturation, neutralization, and blocking steps with 30-second immersions, and by using 6-min chemiluminescence detection instead of 2-h dye deposition. However, the assay's effective sensitivity remains about 30-50 parasites/mm^3, and although this is a significant improvement relative to earlier assays with this probe (McLaughlin et al., 1988), it still does not match good microscopy, although there are questions regarding routine microscopy (Barker et al., 1989, McLaughlin et al., 1991a). Recently, an antigen-targeted ELISA test has shown promise for falciparum malaria detection, but still with sensitivity that is questionable for carriers (McLaughlin et al., MS in preparation). In summary, microtiter-well formats for falciparum malaria show promise for uniform large-scale monitoring of falciparum malaria in epidemiological studies, but further improvements in the technologies are necessary.

Adequately abundant and species-specific families of repetitive DNA have not been described for most hemotropic species of the phylum Apicomplexa. However, specific synthetic DNA sequences have been defined for rRNA of all four *Plasmodium* species of humans (Lal et al., 1989; Waters & McCutchan, 1989), and it is possible that enzyme-linked synthetic DNA could be used for *in situ* or dot-blot detection of this abundant class of oligonucleotides. However, the range of hybridization temperatures which achieve specificity for these rRNA-targeted synthetic DNA's may be narrower than that for the 21-base pair repetitive family of *P. falciparum* (GLM, unpublished observations). Although progress has been made with regard to RNA fixation onto filters (A.A. Lal, personal communication), successful nonisotopic adaptations of rRNA-targeted synthetic DNA have not, to our knowledge, been documented.

PCR clearly offers the maximum theoretical sensitivity for the direct detection of infectious organisms; the detection of single targets has

been demonstrated (Li et al., 1988). Alternative amplification methods such as with Q Beta polymerase (Lizardi, 1988) are under earlier stages of development and may remain problematic. Detection of falciparum malaria using PCR from loci of defined genes has previously been demonstrated (Jaureguiberry et al., 1990). Although the polymerase is sensitive to heparin and heme, protocols are available which remove such interference (Walsch et al., 1991; Kain & Lanar, 1991). If samples are sufficiently dilute, even simple procedures are feasible. As for dot-blot assays, the selection of abundant targets such as repetitive DNA or rRNA will enhance the effective sensitivity (McLaughlin et al., 1991a). Definition of multiple amplimers will probably be necessary to optimally achieve multiple purposes, e.g. both sensitive detection and strain identification.

Hybridization at reduced stringencies during the PCR assay can allow differential amplification of DNA of taxonomically related protozoan species, e.g. the *Naegleria* complex (McLaughlin et al., 1991a). An advantage of using PCR with polymorphic repetitive targets is that multiple bands are observed which can be analyzed much like restriction fragment length polymorphisms (Huizinga & Mc-Laughlin, 1990) for the genetic typing of given isolates or species (Tautz, 1989). Our initial PCR analysis of the moderately repetitive ITS locus of *Babesia* and *Plasmodium* species yielded banding patterns which may be useful for the PCR fingerprinting of Apicomplexa species. The identity of the amplified DNA bands remains to be demonstrated. The additional bands observed, especially the complex pattern observed for *Babesia* species, could reflect polymorphism at this moderately repetitive locus or simultaneous infections with multiple clones. Because of sequence divergence in these genera, secondary target loci of the parasites are probably also being amplified. Some of these bands may also be due to contaminating host DNA, but for the purpose of comparing strains or species, it is most important that a recognizable pattern for a given pathogen species or strain is obtained. The presented patterns have remained recognizable, but with slight differences in satellite band intensities and/or mobilities, in three independent amplifications, using several sources of infected or cultured blood at different parasitemias. However, although two bands remained conserved, satellite bands differed for two *B. canis* samples with 10-fold different parasitemias, and it remains to be seen whether recognizable patterns can be scored in properly blinded studies for all *Plasmodium* and *Babesia* species and strains. Others have recognized that under nonstringent conditions, even arbitrary primer pairs or single synthetic DNA sequences will generate recognizable patterns of amplified DNA which define given genomes (Williams et al., 1990, Welsch & McClelland, 1990). The phenomenon is in our hands less reproducible when detection of different parasitemia levels is used, or when different sample processing procedures are used. The presence of contaminating DNA from hosts or different pathogen species, may make this type of analysis more problematic for clinical specimens. PCR fingerprinting may prove useful to analyze *Babesia* and *Plasmodium* infections without the need to purify parasites or their DNA, but it seems likely that samples with similar parasitemias should be processed in parallel using rationally selected primers. For protozoan pathogens, we propose to call the rapid analysis of amplified DNA banding patterns to identify particular isolates, strain typing at reduced stringency, or STARS. Analysis of the ITS locus or similar loci may allow rapid genetic typing of new isolates of *Babesia* and *Plasmodium* species and strains without the need for difficult culture or expensive passage in animals.

We have used primers which are expected to amplify at least some ribosomal DNA sequences, whose analysis may aid in taxonomic studies of many *Babesia* and *Plasmodium* species (Olsen, 1988). Among bacteria, some ITS loci are believed to involved with regulation of rRNA gene expression (Bacot & Reeves, 1991). It is known that rRNA genes are differentially expressed in arthropod and vertebrate life cycle stages of *Plasmodium* (Gunderson et al., 1987). Analysis of amplified products may also indicate whether distinct rRNA families are also found in *Babesia*, and may help us understand rDNA gene regulation.

The described type of PCR analysis should be useful in some settings for fingerprinting given isolates or clones (Tibayrenc et al., 1990, 1991) of parasites, that is, for "microepidemology" or case report studies to identify strains with particular phenotypes such as virulence or drug resistance. Amplification of DNA from chloroquine-sensitive and chloroquine-resistant falciparum malaria infected blood using the described amplimers, indeed yielded different banding patterns (McLaughlin & Hassan-King, unpublished observations), but this single observation awaits confirmation, as does the presented conserved and differing bands between low and high *B. canis* parasitemias. Sequence analysis of the genus-specific bands may allow the identification of more

specific amplimers which will enhance the potential utility of this approach. For large-scale diagnosis or "macroepidemiology" studies, improvements in PCR-related methodologies are necessary (Persing, 1991). Different loci, e.g., more highly repetitive DNA (McLaughlin et al., 1991a) and/or (r)RNA with reverse transcriptase (Howe et al., 1992), will probably be necessary to achieve maximal sensitivity using simple sample processing methods.

In summary, the current utility of each of these approaches is limited with regard to disease diagnosis, partly because detailed sequence data are not available, and partly because each has not been well-optimized. Even if each were fully developed and optimized, no single approach or technique is expected to supplant all others. Serodiagnosis is inexpensive, rapid, and its detection of both current and previous infections is advantageous in areas of low disease prevalence, thus, serodiagnosis using multiepitope antigens may find applications if related technologies improve. *In situ* detection maintains parasite morphologies and might provide additional specificity relative to histochemical stains in microscopic studies, but standard microscopy or the quantitative buffy coat (QBC) analysis methodology (Levine & Wardlaw, 1988) will probably remain more convenient. The direct detection of abundant parasite antigens or oligonucleotides in dot-blot formats may allow inexpensive, large-scale and uniform monitoring of current parasitemias, but inherent limitations in sensitivity will remain. PCR amplification offers maximal potential sensitivity of detection, and cost and time considerations are improving (Persing, 1991). PCR is the most promising of the new molecular approaches to disease detection. Using strategies which avoid recognized problems of the technique, the reliable diagnosis of malaria and babesiosis in uniform, automated formats seems feasible. Definition of additional amplimers will be required before PCR-based approaches can be properly evaluated for *Babesia* and *Plasmodium* detection.

In the past decade, established microscopic methods have remained superior to molecular methods for most purposes (Lanar et al., 1989). It remains to be seen whether one or more molecular-based strategies will prove useful for epidemiological research or for routine diagnosis. It is encouraging that molecular research related to improving diagnostic and strain typing techniques can also help to contribute to fundamental understandings of the immunology, taxonomy, ecology, and genetics of *Babesia* and *Plasmodium* species.

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