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DNA PROBES FOR THE DETECTION OF *ANAPLASMA CENTRALE* AND *ANAPLASMA MARGINALE*

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ABSTRACT

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Anaplasmosis can be diagnosed either by immunological techniques or by direct microscopic examination of blood smears. Both methods are time-consuming and labour intensive. The use of DNA probes in an hybridization assay may simplify the diagnosis of anaplasmosis in cattle and sheep. A genomic DNA library of *Anaplasma centrale* was constructed in an expression vector and screened to detect clones containing *A. centrale* DNA. Four probes which hybridized to *A. centrale* and *Anaplasma marginale* DNA were isolated. One of these (AC-1) hybridized only to *A. centrale* DNA, whereas AC-2, AC-3 and AC-4 could detect DNA from both *A. centrale* and *A. marginale*. Probes AC-1 and AC-2 could detect 127 ng and 8 ng DNA respectively, while AC-3 and AC-4 detected 64 ng *A. centrale* DNA.

INTRODUCTION

Anaplasmosis is a tick-borne disease of cattle endemic throughout tropical and sub-tropical regions. The causative agents are 2 rickettsia-like organisms, *Anaplasma marginale* and *Anaplasma centrale* (Ristic, 1968). After infection, intraerythrocytic *Anaplasma* are seen within 3–6 weeks. A high parasitaemia occurs during the following 4–9 days, followed by a chronic phase (Ristic, 1977). Recovered animals remain persistently infected, a small number having recurrent disease (Ristic, 1977).

Diagnosis of anaplasmosis, using light microscopy of stained blood, is a reliable, although tedious procedure, not suitable for a large number of samples. Serological diagnosis of *Anaplasma* infections are not wholly reliable and may yield false positive results (Gonzalez, Long & Todorovic, 1978). The available methods are therefore not suited for the rapid detection of the presence of the parasites in large herds on a routine basis.

Splenectomized, susceptible recipients may be used to detect low parasitaemias, but this procedure is too costly for routine use. There is therefore a need to develop improved diagnostic procedures for routine use which will also be useful in surveillance and epidemiological studies of these organisms.

DNA hybridization has been used diagnostically for the identification of bacteria (Forster, McInnis, Skingle & Symons, 1985; Hill, Madden, McCardell, Shah, Jagow, Payne & Boutin, 1983), protozoans (Barker, Suebsaeng, Rooney, Alecrim, Dourado & Wirth, 1986; Gonzalez, Prediger, Huecas, Nogueira & Lizardi, 1984) and a number of viruses (Bornkamm, Desgranges & Grissman, 1983). This technique is sensitive enough to discriminate between closely related species and therefore very suitable for the diagnosis and identification of infectious agents. The sensitivity of the technique depends on the composition of the probe. Repetitive DNA probes may be as sensitive as total genomic probes because closely related species contain different repeated sequences (Jelinek & Schmid, 1982; Peacock, Lohe, Gerlach, Dennis & Appels, 1977). Repetitive DNA probes have been used to detect very small amounts of *Trypanosoma* (Gonzalez *et al.*, 1984) and *Plasmodium* (Barker *et al.*, 1986) DNA.

In this paper we report the isolation of specific DNA probes for *A. centrale* and *A. marginale*.

MATERIALS AND METHODS

DNA isolation: Infected erythrocytes containing *A. centrale* were separated from host white cells by centrifugation and buffy coat removal, followed by passage through a Whatman cellulose column (Richards & Williams, 1972); Ambrosio, Potgieter & Nel, 1986). These erythrocytes were lysed by incubation in 10 % SDS in 10 mM Tris-HC1 pH 7,5, and 0,1 M EDTA. Lysates were digested with Proteinase K (100 μg mℓ^{-1}) for 60 min at 37 °C.

A. centrale DNA was then extracted with phenol, phenol-chloroform and chloroform. After ethanol precipitation, DNA was resuspended in TE (10 mM Tris-HC1 pH 7,5, 1 mM EDTA), purified further by equilibrium centrifugation in cesium chloride (Maniatis, Fritsch & Sambrook, 1982) and dialysed. *A. marginale* DNA was extracted as described above. The DNA concentration was determined by measuring the absorbance at 260 nm.

Library construction and screening: A. centrale DNA was digested with *Eco*R1[1] under conditions which gave an average fragment length of 6 kilobase (kb). Digested parasite and vector (lambda *gt*11)[2] DNA (1μg each) were ligated and packaged *in vitro* (Huynh, Young & Davis, 1985).

Recombinant phages were identified by their ability to form colourless plaques when plates on a *lac*$^-$ host in the presence of XGal. Recombinant phages were harvested as plaques, pooled and stored at 4 °C.

The library was screened with antibody probes for antigen produced by specific recombinant clones, as described by Huynh *et al.* (1985). Positive clones were identified by using peroxidase conjugated second antibody (Adams, Smith & Kuhlenschmidt, 1986), and detected by using 4-chloro-1-naphtol as substrate. Primary antibody was from an hyperimmune animal and was diluted 1:6 in 50 % horse serum before use.

Fusion proteins were produced according to the method of Huyn *et al.* (1985) and identified by western blot analysis, using monoclonal anti-β-galactosidase antibodies[2], followed by goat anti-mouse IgG conjugate[3].

DNA restriction and DNA/DNA hybridization: Restriction enzymes *Pst*1, *Eco*R1, *Bam*H1 and *Hin*d111[4] and *Bgl*11[1], were used at 37 °C in a buffer, as recommended by the manufacturer. *Eco*R1 star activity[5] was obtained by incubating the restriction reaction with an excess of enzyme. Digested DNA was electrophoresed through a 0,6 % agarose gel and transferred to a nylon

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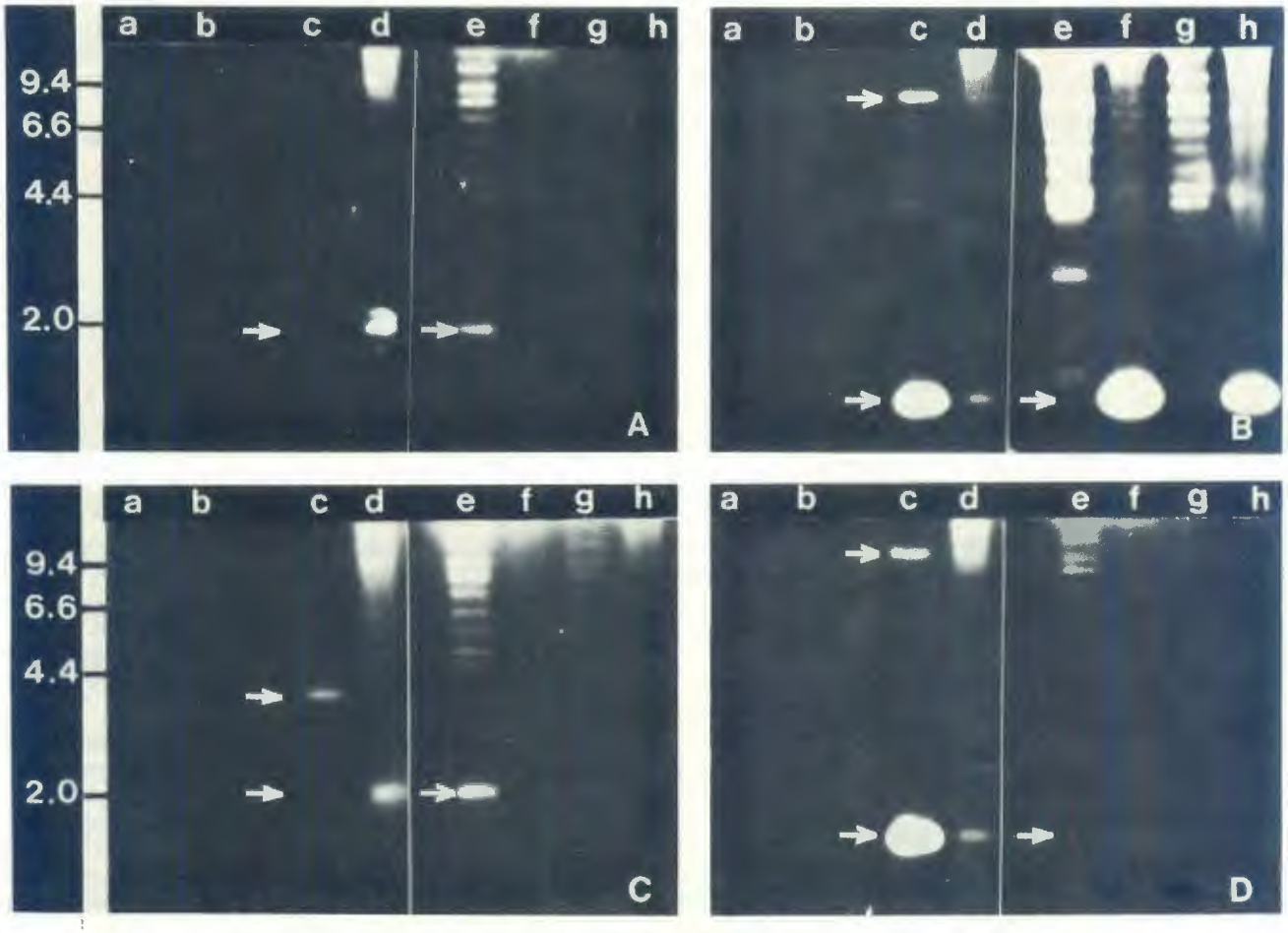

FIG. 1 Southern hybridization of the *A. centrale* probes to DNA from various sources. Genomic DNA was digested with *Eco*R1, size fractionated on 0,6 % agarose gels, transferred to nylon and hybridized with the appropriate probe as described in Materials and Methods.

Panel A: The probe is AC-1 and the tracks contain: a, Bovine DNA + *Eco*R1; b, Sheep DNA + *Eco*R1; c, *A. marginale* DNA + *Eco*R1; d, *A. centrale* DNA + *Eco*R1; e–h, probes AC-1 to AC-4 digested with *Eco*R1.

Panels B, C & D: The probes are AC-2, AC-3 and AC-4 respectively, and the tracks are as in panel A. The sizes of the DNA bands are in kilobase pairs

membrane (Southern, 1975). The blots were hybridized with ^{32}P-dCTP-labelled DNA (specific activity 10^8 dpm μg^{-1}) from different sources (see RESULTS).

Hybridization was performed overnight at 65 °C in a solution containing 6× SSC (1×: 0,15 M NaCl, 0,015 M Na_3-citrate, pH 7,0), 5× Denhardt's solution (Maniatis *et al.*, 1982), 0,5 % SDS and 100 μg mℓ^{-1} denatured herring sperm DNA. Post-hybridization washes were performed in 2 changes of 2× SSC, followed by 2 changes of 0,1× SSC, both containing 0,1 % SDS. The final 0,1× SSC wash was for 15 min at 65 °C.

Nylon membranes were stripped and re-used by washing in 0,4 M NaOH for 120 min, followed by a wash in a buffer containing 0,1× SSC, 10 mM Tris pH 7,5, 0,1 % SDS.

RESULTS

The DNA used in this study was isolated from red blood cells infected with *A. centrale*. The removal of 99 % of the white blood cells, and the DNA extraction and purification procedures are described under Materials and Methods.

A. centrale DNA purified on CsCl/EtBr gradients migrated as a single band on agarose gels. The extracted DNA consisted of linear molecules of between 50 kb and 100 kb. *A. centrale* DNA was digested with *Eco*R1 to generate fragments of between 5 and 7 kb suitable for cloning into bacteriophage λ*gt*11. Following ligation, the DNA was packaged *in vitro* and plated to form isolated plaques on indicator bacteria. A titre of 10^8 pfu/mℓ was obtained for the library.

After screening the library with serum from an hyperimmune bovine, recombinant clones were replated on a lysogenic *Escherichia coli* host strain and tested for production of fusion proteins. These proteins were characterized by western blotting, using monoclonal anti-β-galactosidase antibodies (Huynh *et al.*, 1985). Fusion proteins ranging from 131 kd to 140 kd were identified. Bacteriophage DNA from 4 fusion protein producing plaques was extracted (Maniatis *et al.*, 1982) and tested for its ability to detect *Anaplasma* DNA from bovine blood. The DNA from each of the plaques (termed AC-1, AC-2, AC-3 and AC-4) was radioactively labelled by nick-translation (Rigby, Dieckmann, Rhodes & Berg, 1977) and hybridized to DNA from *A. centrale* and *A. marginale*. This DNA was also hybridized to *Eco*R1, *Bam*H1, *Hin*d111, *Bgl*11 and *Pst*1 digests of *A. centrale* and *A. marginale*.

The AC-1 probe hybridized to a 1,6 kb *A. centrale* DNA band (Fig. 1a, track d). No hybridization was obtained with this probe on *A. marginale* DNA (Fig. 1a, track c).

Hybridization to both *A. centrale* and *A. marginale* was obtained with AC-2, AC-3 and AC-4. Probe AC-2 hybridized to 2 bands of 900 bp and 9 kb on *A. marginale* (Fig. 1b, track c), and to a 900 bp band on *A. centrale*DNA (Fig. 1b, track d). Probe AC-3 hybridized

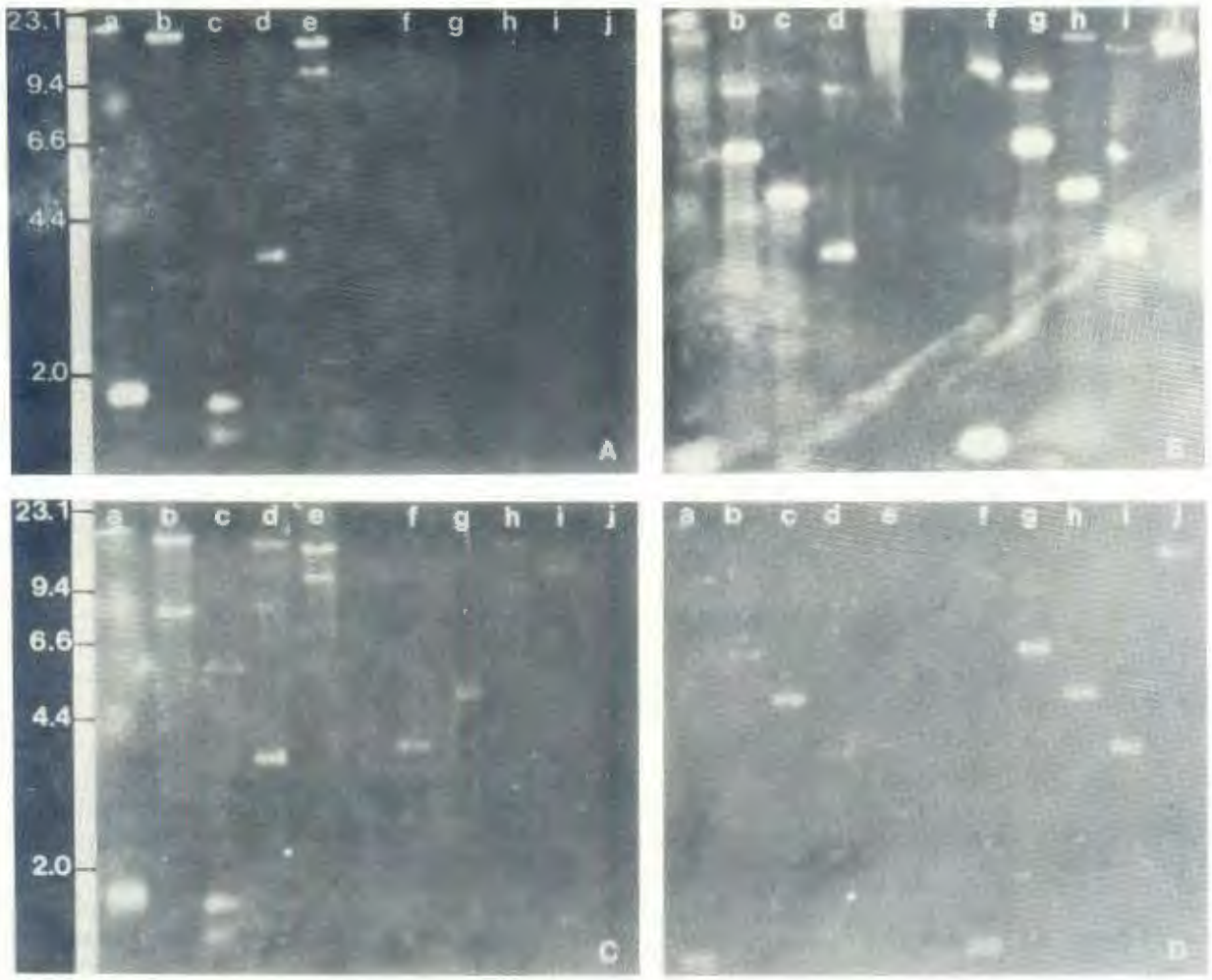

FIG. 2 Southern hybridization of the *A. centrale* probes to various restriction endonuclease digests of *A. centrale* and *A. marginale*.
Panel A: The probe is AC-1 and the tracks contain: a–e, *A. centrale* DNA digested with *Eco*R1, *Hin*d111, *Pst*1, *Bgl*11 and *Bam*H1 respectively; f–j, *A. marginale* DNA digested with *Eco*R1, *Hin*d111, *Pst*1, *Bgl*11 and *Bam*H1 respectively.
Panels B, C & D: The probes are AC-2, AC-3 and AC-4 respectively, and the tracks are as in panel A. DNA sizes are in kilobase pairs

to a 4,3 kb *A. marginale* band as well as to a 1,6 kb *A. centrale* DNA band (Fig. 1c, tracks c & d). Probe AC-4 detected an 800 bp *A. centrale* DNA band as well as an 800 bp and 9 kb *A. marginale* DNA band (Fig. 1d, tracks d & c). Hybridization to the same 1,6 kb band on *A. centrale* DNA was obtained with AC-1 and AC-3. However, AC-1 did not hybridize to *A. marginale* DNA, while AC-3 detected DNA from both species (Fig. 1a & c, tracks c & d).

The possibility of cross-hybridization of these probes to bovine and sheep DNA was investigated. Sheep and bovine DNA was isolated from the white blood cells of uninfected animals as described in Materials and Methods (DNA isolation). The blood was not column-purified. This DNA was blotted onto nylon membranes and hybridized with ^{32}P-labelled AC-1, AC-2, AC-3, and AC-4, respectively. No cross-hybridization was obtained with any of the 4 probes (Fig. 1a-d, tracks a & b).

Tracks e, f, g & h in Fig. 1 contain *Eco*R1-digested probe AC-1–AC-4 DNA (λ*gt*11/*A. centrale* recombinant). Hybridization of probes AC-1, AC-2, AC-3 & AC-4 to these digests shows the *A. centrale* part of the recombinant (arrowed) hybridizing to itself. Probes AC-1 and AC-3, as well as AC-2 and AC-4, cross-hybridize with each other (Fig. 1a & c, tracks e & g; Fig. 1b & d, tracks f & h). No cross-hybridization was obtained between AC-1 and AC-2/AC-4 nor between AC-3 and AC-2/AC-4. The other bands are λ*gt*11 DNA resulting from the star activity of *Eco*R1.

To determine the specificity of these probes we examined the hybridization patterns obtained using AC-1, AC-2, AC-3 & AC-4 on *A. centrale* and *A. marginale* DNA digested with various restriction endonucleases. Fig. 2 shows hybridization of these probes to the different DNA digests. With probes AC-2 and AC-4 almost identical hybridization patterns were obtained, suggesting that these 2 probes are similar (Fig. 2b & d). AC-3 detected more bands on *A. centrale* than on *A. marginale* DNA (Fig. 2c). The band sizes obtained in this experiment are summarized in Table 1.

The multiple hybridization bands obtained with the digested DNA (Fig. 2) suggests that these probes hybridize to similar sequences which occur throughout the *Anaplasma* genome.

When probed with lambda DNA, no cross-hybridization to any of the DNA's tested (bovine, sheep and *Anaplasma*) was obtained (not shown). To determine the sensitivity of these probes, serial dilutions of *A. centrale* DNA (80 μg to 4,8 pg) were Southern transferred to a nylon membrane and probed with labelled AC-1, AC-2, AC-3 & AC-4. The AC-1 and AC-2 probes detected 127 ng and 8 ng DNA respectively. Probes AC-3 and AC-4 detected 64 ng *A. centrale* DNA.

TABLE 1 Fragment sizes detected by probes AC-1 to AC-4

DNA[a]	Probe	Restriction enzymes				
		*Eco*R1	*Hind*111	*Pst*1	*Bgl*11	*Bam*H1
A. centrale	AC-1	1,6	15,1 6,0 3,2	8,9 1,6 1,3	8,5 3,6	14,1 12,9
	AC-2	12,9 0,9	8,5 6,0	4,9	16,4 8,9 3,6	—
	AC-3	1,6	15,1 8,1	6,0 1,6 1,4	15,1 8,5 3,7	14,1 11,5
	AC-4	0,9	8,9 6,3	4,9	3,7	—
A. marginale	AC-1	—	—	—	—	—
	AC-2	10,7 1,0	9,4 6,5	15,1 5,1	14,1 6,5 4,0	14,1
	AC-3	4,0	5,3	15,9 10,0	12,0	—
	AC-4	10,7 1,0	9,4 6,6	5,3	6,5	14,1

[a] Sizes are in kilobase pairs

DISCUSSION

Prior to this study, there were no reports on the use of cloned DNA sequences as hybridization probes for *Anaplasma*. The library we obtained provides a readily accessible source of *A. centrale* DNA. Its value depends on the degree to which it represents the parasite genome. It is for this reason that care was taken to ensure that most of the bovine DNA in the samples was eliminated prior to cloning. The cellulose column method has been found to remove up to 99 % of the bovine white blood cells (Ambrosio *et al.*, 1986). In the present experiments, we focussed on obtaining species-specific DNA probes for *A. centrale*. DNA probes have been isolated for a number of parasites (Moseley, Echeverria, Seriwatana, Tirapat, Chaicumpa, Sakuldaipeara & Falkow, 1982; Hill *et al.*, 1983; Barker *et al.*, 1986) and eventually many of these will be used routinely in diagnostic tests (Owens & Diener, 1981; Wirth & Pratt, 1982).

Four DNA probes were isolated from the *A. centrale* library. The AC-1 probe is specific for *A. centrale* DNA and can detect 127 ng DNA. The hybridization pattern obtained with this probe on enzyme digests of *A. centrale* DNA suggests that this probe contains a unique sequence specific for *A. centrale*.

Probes AC-2, AC-3 and AC-4 hybridized to both *A. centrale* and *A. marginale* DNA. These probes hybridized to *A. marginale* DNA fragments of different sizes. According to the hybridization patterns obtained with probes AC-2 and AC-4 on digests of *A. centrale* and *A. marginale* DNA, it is likely that these 2 probes are similar (Fig. 2).

The usefulness of the probes described in this paper in identifying *Anaplasma* in field trials is currently being evaluated. These probes could be used not only to test blood from field samples, but also to follow experimentally induced parasitaemias in cattle and possibly sheep.

One of the commonest methods for detecting and identifying *Anaplasma* is microscopic examination of blood from an infected host. Similarities between *A. centrale* and *A. marginale* make identification difficult. Definitive identification is dependent on a number of time-consuming tests not suited for the routine screening of a large number of samples. Repetitive DNA can be detected by standard hybridization techniques with a sensitivity only slightly lower than that for genomic DNA. This has allowed for the detection of as little as 25 pg of *P. falciparum* DNA, corresponding to 50 $\mu\ell$ of 0,001 % parasitaemic blood (Wirth & Pratt, 1982). Libraries containing 195 basepair sequences from *Trypanasoma cruzi* were used to detect as few as 30 parasites (Gonzalez *et al.*, 1984). We intend to identify short sequences suitable for highly sensitive synthetic probes as has been done for *Plasmodium falciparum* (Franzen, Shabo, Perlman, Wigzell, Westin, Aslund, Persson & Petterson, 1984) and to develop a methodology suitable for the rapid identification of *Anaplasma* in the field.

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