

The genome of *Theileria parva*: some structural properties

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The DNA of the protozoan *Theileria parva*, the causal agent of the bovine East Coast Fever, has been prepared at least 99% pure from the intra-erythrocytic form of the parasite. Its buoyant density was found to be 1.696 g/cm³ and its calculated G + C content was 36.7%. Fragmentation of *T. parva* by the restriction enzyme *EcoRI* provides some evidence of the presence of repetitive DNA sequences.

Protozoa	<i>Theileria parva</i>	<i>Theileriosis</i>	East Coast Fever
	DNA purification	Repetitive DNA	

1. INTRODUCTION

The protozoan *Theileria parva*, the causal agent of East Coast Fever, a tick-transmitted disease, kills annually several hundred thousand cattle in East and Central Africa. The disease is characterized by two intracellular forms of the parasite, a schizont stage within cells of lymphoid origin and a piroplasm stage within erythrocytes. In a first step, the parasite which is transmitted by the tick *Rhipicephalus appendiculatus* invades host lymphocytes, inducing a rapid proliferation followed by a widespread lymphocytolysis. The red blood cells are then invaded. The intra-erythrocytic form of the parasite is infectious for the tick.

The identification of the antigens of *T. parva* and the use of DNA recombinant technology could supply a novel approach towards development of a vaccine. One of the steps in this approach requires the study of the genomic DNA of the parasite. We have prepared in a pure form the parasite DNA from intra-erythrocytic piroplasms, after having separated the erythrocytes of the parasitized blood from the contaminating bovine white cells.

2. MATERIALS AND METHODS

2.1. Cell separation

Parasitized blood was drawn into heparinized

tubes from a heifer, 17 days after the subcutaneous injection of a tick stabilate containing sporozoites of *T. parva* (Onderstepoort strain). At this time, over 60% of the erythrocytes were found to carry one piroplasm and some carried two or even 3. The blood was diluted (v/v) with phosphate-buffered saline (pH 7.4) and the buffy coat thoroughly removed by eliminating the supernatant and the upper fourth part of the pellet after each of 3 successive centrifugations. The last pellet was treated with distilled water. Lysis of the red blood cells was verified by optical microscopy. The salt concentration was then restored by adding phosphate-buffered saline (10×). The remaining nucleated cell, some nuclei and a few unlysed erythrocytes were spun down (100 × g, 15 min, at 4°C). The resulting supernatant was then centrifuged at 17 000 × g for 30 min at 4°C. To rule out the presence of any remaining nuclei or nucleated cells, the pellet was resuspended in phosphate-buffered saline and the suspension centrifuged again (100 × g, 10 min, 4°C). The homogeneity of the suspension was checked microscopically and the piroplasms were collected by a last centrifugation (17 000 × g, 30 min, 4°C).

2.2. Preparation of DNA

The pellet containing parasites was utilized to prepare DNA as in [1].

2.3. Analytical density gradient centrifugation in CsCl

DNA solutions in 0.005 M NaCl, 0.01 M Tris (pH 7.8) were brought to a density of about 1.700 g/cm³ by adding solid CsCl (Suprapur, Merck, Darmstadt). Centrifugations to equilibrium were carried out at 25°C using a 6-hole titanium An.G rotor, 12 mm double-sector Kel.F cells and a Spinco model E ultracentrifuge equipped with a monochromator, a photoelectric scanner and a multiplexer using phage 2C DNA ($\rho = 1.742$ g/cm³) as a reference [2].

2.4. Restriction enzymes and restriction conditions

The restriction enzyme *Eco*RI was home produced at the Institute Jacques Monod. The digestion of the DNA preparations was performed at 37°C for 1 h in a buffer mixture (11 × buffer = 0.13 M MgCl₂, 1.10 M NaCl, 0.44 M Tris, pH 7.4). The DNA restriction fragments were separated by electrophoresis on 0.8% agarose gel, using λ DNA digested by *Hind*III and *Hind*III + *Eco*RI, as a molecular mass marker.

2.5. Determination of the extent of the contamination by the host DNA

The DNA fragments were transferred from the agarose gel to the nitrocellulose (BA 85 Schleicher et Schull) as in [3]. A ³²P-labeled nick-translated thymus calf probe was prepared as in [4] and assayed for hybridization with the blotted piroplasm DNA fragments. With the aim of revealing even DNA sequences with incomplete homology, the washing following the hybridization was carried out under weak stringent conditions: the last washing was carried in a 1 × SSC solution at 65°C (20 × SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0). The following relationship was utilized to calculate the T_m :

$$T_m = 69.3 + 0.41 \cdot (G + C)\% \quad (5)$$

For *T. parva* this value is 84°C. Usually, washing should be carried out at $t^\circ = T_m - 12^\circ\text{C}$.

3. RESULTS

Electrophoresis of the undigested DNA of *Theileria* indicated that its molecular mass amounted to at least 32 MDa, the molecular mass of the phage λ (fig.2a, track 1) (see section 4).

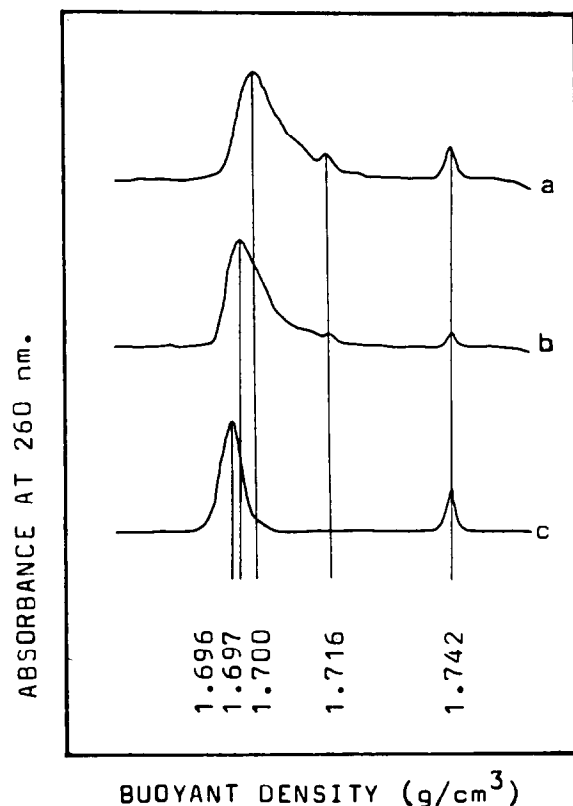


Fig.1. Microdensimeter tracing of bovine (a), bovine + parasite (b) and purified parasite DNA (c) (see sections 2 and 4) centrifuged to equilibrium in CsCl. The peak on the right corresponds to the reference phage 2C DNA ($\rho = 1.742$ g/ml). Centrifugation was performed at 43 000 rpm for 24 h.

Analytical density gradient centrifugation of the DNA of *T. parva* showed a single peak (fig.1). The density of the DNA was found to be 1.696 g/cm³. Using the equation in [6], the (G + C) content was calculated to be 36.7%.

Electrophoresis of the DNA of *Theileria* digested by *Eco*RI resulted in a restriction pattern composed of numerous bands indicative of the likely presence of repetitive sequences (fig.2a, track 5). No fluorescence was detected at the level of the major bovine satellite, which is conspicuous on the tracks relative to digested bovine DNA.

The digested DNA of the piroplasm does not hybridize with a labeled bovine probe (fig.2b, track 6). The implications of these findings with respect to the purity of the prepared sample are discussed below.

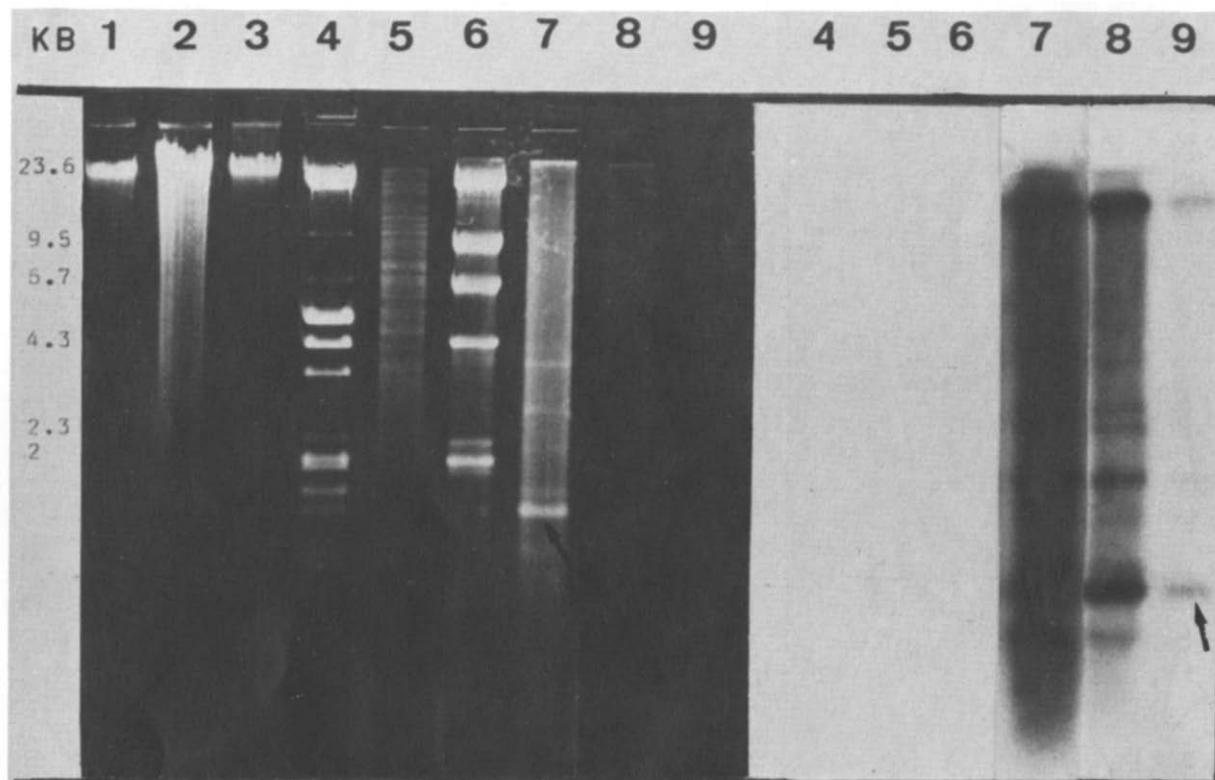


Fig.2. (a) UV fluorescence pattern after electrophoresis (carried on 0.8% agarose in the presence of ethidium bromide in the buffer) of undigested DNAs used as references (tracks 1–3) and the same DNAs after digestion by restriction enzymes (tracks 4–9) (see section 2). Track 1, undigested DNA; track 2, undigested calf thymus DNA; track 3, undigested piroplasm DNA; track 4, λ DNA restricted by *Hind*III + *Eco*RI (Molecular mass marker); track 5, 1 μ g piroplasm DNA restricted by *Eco*RI; track 6, λ DNA restricted by *Eco*RI (molecular mass marker); tracks 7,8,9, 1, 0.1 and 0.001 μ g respectively, of thymus calf DNA digested by *Eco*RI. The arrow in track 7 points to the band corresponding to the bovine major satellite. No fluorescence can be detected at this level in track 5, suggesting the absence of significant contamination. However, this figure shows the presence of repetitive sequences in the piroplasm DNA. (b) Hybridization pattern of digested bovine and parasite DNA. The restriction fragments from the agarose gel (a, tracks 4–9) were transferred to nitrocellulose by the Southern blot method and hybridized with a 32 P-labeled calf thymus DNA probe as described in section 2. Tracks 4 and 6, digested λ DNA (references); track 5, 1 μ g restricted piroplasm DNA. No detectable hybridization after 12 h exposure; tracks 7,8,9 1, 0.1 and 0.001 μ g, respectively, of restricted bovine DNA. The band corresponding to the bovine major satellite DNA (arrow) is conspicuous even in track 9 (0.001 μ g). No hybridization was detectable in the case of the piroplasm digested DNA, even after 14 days exposure.

4. DISCUSSION

The preparation of DNA of *Theileria* with the aim of constructing a genomic library requires a thorough purification.

A preliminary trial involved the removal of the buffy coat and centrifugation on a Ficoll-Paque cushion. Although the smears had shown the presence of only a few remaining leucocytes, analytical centrifugation revealed significant contami-

nation of the DNA of *Theileria* by bovine DNA (fig.1b) (a bovine leucocyte contains about 100-times more DNA than a piroplasm).

As the two components could not be separated by centrifugation because of the close proximity of the density values (fig.1), we achieved separation by a purification procedure performed at the cellular level. Each step was thoroughly checked by microscopical examination and the purity of the resulting DNA could be evaluated by the presence

in bovine DNA of a satellite comprising highly repetitive sequences. This major satellite, of $\phi = 1.715$, whose density peak falls far apart from that of the DNA of *Theileria*, amounts to 5% of the total bovine genome [7]. As shown in fig.2b this would have enabled us to detect even minute contamination. Track 9, relative to the run of 1 ng digested bovine DNA, was assayed for hybridization with a labeled bovine probe. A 12 h exposure was sufficient to reveal light hybridization at the level of the major satellite, while in the track corresponding to 1 μ g *Theileria* DNA, no radioactivity whatsoever could be detected at this location, even after 14 days' exposure. (A negative result, obtained after 12 h exposure only would have ruled out any contamination above 1%.) This confirms and improves on the results obtained with density gradient centrifugation (where the absence of the slightest trace of bovine satellite precludes any contamination greater than 5%) and with electrophoresis (where no UV fluorescence is detected at the level of the major bovine satellite band).

It would be desirable to assess the DNA size of the parasite, but we have not been able to realize the necessary experiments. Effectively, the amount of material at our disposal was too small to realize kinetic complexity investigations.

The fact that the blood had been frozen did not allow any red blood cell count and thus evaluation of the amount of DNA in a single piroplasm.

With respect to the existence of a smear in track 5 of fig.2a (digested *Theileria* DNA) due to the non-repetitive DNA, we can assume that the size of *Theileria* DNA is greater than that of DNA, but the fact that the bands observed in the same track are due to the presence of repetitive sequences excludes the possibility of calculating the DNA size by summing up the lengths of DNA in the visible bands.

The restriction pattern of *T. parva* is reproducible and the presence of multiple bands cannot be explained by possible incomplete enzymatic digestion: phage λ and bovine DNA, treated in the same way, were completely digested (fig.2a). Indeed, repetitive DNA has been also detected in the

case of *Plasmodium berghei* [9] and *P. falciparum* [10]. The presence of repetitive sequences in the haploid genome of a parasite might contribute to the protection of the organism against the consequences of mutations. *Theileria* DNA replicates actively in its intralymphocytic form and the schizonts and piroplasms of *Theileria* are probably haploid, the diploid phase of the organism being found during the tick part of the cycle [11]. In the case of *T. parva*, where a relatively large amount of DNA from two different stages of development could be prepared, it would be of interest to determine whether stage differences could be found in the nature and location of the DNA repetitive sequences.

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