Detection of genomic polymorphisms among isolates of the intracellular bacterium *Cowdria ruminantium* by random amplified polymorphic DNA and Southern blotting

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Abstract

Sixteen primers were successfully used in a RAPD assay to generate reproducible fingerprints for six isolates of *Cowdria ruminantium*, a tick-transmitted rickettsia of ruminants. Distinction between stocks was possible by using one or at most two primers. Two stocks were very similar although originating from widely distant geographical regions. A genetic distance tree was constructed by analysing 108 fragments in pairwise comparison between stocks. Three amplification fragments probed with *C. ruminantium* genomic DNA determined a restriction fragment length polymorphism which allowed the distinction between stocks except for the two stocks that had similar RAPD patterns. The potential of RAPD to determine the extent of genetic diversity of *C. ruminantium* and to develop probes or PCR primers for diagnostic purposes is discussed.

**Keywords**: *Cowdria ruminantium*; RAPD assay; Restriction fragment length polymorphism; Genetic diversity

1. Introduction

The tick-borne rickettsia *Cowdria ruminantium* is the causative agent of cowdriosis which affects wild and domestic ruminants in sub-Saharan Africa and several Caribbean islands [1]. It is an obligate parasite of endothelial cells with a tropism for brain microvasculature. Up to now, protection of livestock was achieved by control of the vectors [2] or immunisation by injection of infected blood or tick homogenates followed by treatment of the reacting animals with tetracycline [3]. However, immunisation with inactivated bacteria formulated in oil adjuvants was reported recently for goats [4] and sheep [5]. Research is being conducted to identify relevant antigens which can be incorporated into recombinant vaccines. However, besides immunological studies, there is a need to evaluate the extent of the genetic and antigenic diversity of this organism. Indeed, the level of cross-immunity induced by different stocks is variable [6]. However, no efficient method has yet been described to type the isolates. Serology has

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not led to the identification of true serotypes [7]. Few highly conserved genes have been cloned [8-10]. The study of their polymorphism is rather limited [11] and the results are unlikely to correlate with C. ruminantium cross-immunity profiles. PCR technology, using arbitrary primers, has been successfully used for typing strains or isolates of a wide range of microorganisms. It is able to detect polymorphisms widely dispersed in the genome without requiring any prior DNA sequence information [12,13], and it therefore appeared to be suitable for application on Cowdria.

In this study, we describe the use of the RAPD assay to differentiate isolates of C. ruminantium and to examine the extent of its genetic diversity, also with the aim of developing probes for diagnosis in tick vectors and in infected animals.

2. Materials and methods

2.1. C. ruminantium isolates

Six stocks (isolates) of C. ruminantium from widely distant areas were used: one from Guadeloupe in the Caribbean (Gardel stock) [14], one from West Africa (Senegal stock at passage 4 in culture, not attenuated) [15], one from Central Africa (Cameroun stock), two from East Africa (Umpala stock from Mozambique and Lutale stock from Zambia [15])
C. ruminantium stocks

<table>
<thead>
<tr>
<th>Endothelial cells DNA per dot</th>
<th>B</th>
<th>S</th>
<th>G</th>
<th>W</th>
<th>C</th>
<th>U</th>
<th>L</th>
<th>C. ruminantium DNA per dot</th>
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<tr>
<td>12.5 ng</td>
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<td>500 ng</td>
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<td>25 ng</td>
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<td>250 ng</td>
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Fig. 2. Hybridisation of genomic DNA derived from bovine endothelial cells, with itself (B) and with DNA from six stocks of C. Ruminantium. Abbreviations of Cowdria isolates S, G, W, C, U, L as in Fig. 1.

and one from South Africa (Welgevonden stock) [16].

2.2. C. ruminantium cultivation and DNA extraction

C. ruminantium was cultured in bovine umbilical endothelial cells (BUEC) by conventional methods [7]. Elementary bodies (EB) were purified from the culture supernatant by differential centrifugation [4] and resuspended in 350 μl of PBS (130 mM NaCl, 4 mM Na₂HPO₄, 1.5 mM KH₂PO₄) to which was added 150 μl of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂ and 125 μg of DNase in order to remove contaminating host cell DNA [17]. After 90 min of incubation at 37°C, the reaction was stopped by adding 25 mM EDTA. The EB were washed three times in water and lysed by an overnight incubation at 55°C in a solution of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA, 1.5% SDS, and 250 μg ml⁻¹ of proteinase K. Bacterial DNA was then extracted with phenol-chloroform, precipitated with cold ethanol and redissolved in water. To determine the level of contamination with cell DNA, 2 dilutions of bovine DNA (12.5 and 25 ng) and of each preparation of Cowdria DNA (500 and 250 ng) were spotted on a nylon membrane using a slot blot filtration manifold (Hoefer Scientific Instruments) and hybridised with 50 ng ml⁻¹ of bovine DNA labelled with digoxigenin as described below.

2.3. PCR amplification

Thirty-six decamer arbitrary primers, and primers AB128 (20 nucleotides) and AB129 (25 nucleotides) used for diagnosis [18] were used to randomly am-
plify C. ruminantium genomic DNA. The amplification reaction was performed in a volume of 25 µl containing 1.25 µl 20×polymerase buffer, 1.5 mM MgCl₂, 200 µM of each dNTP (New England Biolabs), 0.5 U Tth DNA polymerase (Epicentre Technology), 0.2 µM of primer and 25 ng of template DNA. Following an initial denaturation of 6 min at 94°C, the PCR was carried out for 45 cycles in a Perkin Elmer Thermal Cycler (Norwalk, CT) programmed for 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, followed by a final chain elongation for 10 min at 72°C. Amplified products were electrophoresed in 1.5% agarose gels containing 0.5 µl ml⁻¹ of ethidium bromide. Gels were photographed under UV light. A minimum of two assays was performed for each primer on two different DNA batches of each C. ruminantium isolate. Genomic DNA of BUEC was used as a negative control.

2.4. Probe preparation, Southern blotting and hybridisation

Genomic DNA (1.5 µg) of the six stocks of C. ruminantium and BUEC DNA (3 µg) were digested by Rsal. The restriction products were electrophoresed in a 0.8% agarose gel and blotted onto nylon N⁺ membranes (Boehringer) by capillary transfer [19]. Amplified DNA fragments of interest separated in low melting point agarose gels were excised and purified using a WIZARD PCR Prep DNA purification kit (Promega). They were labelled with digoxigenin using a commercial kit (Boehringer) and used as probes. Membranes were prehybridised overnight at 68°C in 5×SSC containing 1% (w/v) blocking reagent (Boehringer), 0.1% (w/v) N-lauroylsarcosine, 0.5% (w/v) SDS, and 100 µg ml⁻¹ of denatured salmon sperm DNA. Hybridisation was performed overnight at 68°C with 30 ng ml⁻¹ of probes in prehybridisation buffer. The membranes were then washed successively in 2×SSC/0.5% SDS at room temperature, and in 0.1×SSC/0.5% SDS at 68°C. Hybridised probes were detected by luminescence.

2.5. Calculation of similarity coefficients

Pairwise comparisons of amplified DNA fragments generated by different primers were made between isolates. Similarity coefficients between each pair of isolates were calculated as described by Sokal and Michener [20]. A pairwise distance was calculated and used to construct a dendrogram using the unweighted pair-group method of arithmetic averages (UPGMA) of the SAS program (SAS Institute Inc., Cary, NC).
3. Results

3.1. Differentiation of isolates

Among 38 arbitrary primers with G+C contents varying from 50% to 80%, 18 gave an informative array of fragments. Sixteen of these, 14 decamers and primers AB128 and AB129, gave polymorphic patterns and were subsequently used for the study. The number of bands revealed by each primer varied from 1 to 13 (Fig. 1). The patterns obtained with different DNA preparations of the same stock were always identical and no major DNA fragment common to BUEC and *C. ruminantium* was amplified. By comparing the intensity of hybridisation signals on dots and considering the ratios of homologous and heterologous DNA, the contamination of *Cowdria* DNA preparations by host cell DNA appeared to be far lower than 1% (Fig. 2). Welgevonden and Cameroun stocks differed by only one fragment on the fingerprint generated by primer 31 (Fig. 1), but each isolate was distinguishable by using one or, at most, two primers.

3.2. Genetic distances between isolates

A total of 108 RAPD products generated by the 16 primers were analysed. Minor bands whose intensity varied between amplifications were excluded from the analysis. The resulting dendrogram is shown in Fig. 3. The Senegal stock appears to be most distant from all other stocks. In contrast, Welgevonden and Cameroun isolates were very closely related. Umpala and Lutale were grouped together but did not appear to be closely related. Gardel was located between Senegal and the clusters formed by the other four stocks.

3.3. Southern blotting and hybridisations

To identify shared and stock-specific amplification products, three amplification fragments were used as probes in Southern blots to *RsaI*-digested genomic DNA of the six isolates of *C. ruminantium*.

A DNA fragment of 1.2 kb amplified from the six stocks was purified from the reaction with primer 28 (Fig. 1). The 1.2 kb fragment from the Senegal PCR reaction product and a mixture of the 1.2 kb fragments of the six stocks were used as probes. After hybridisation, the Southern blots showed the same pattern with both probes (Fig. 4A). One monomorphic fragment of 3 kb common to all six stocks and a polymorphic region were revealed. Genomic polymorphic fragments of 0.64 and 1.1 kb were detected for Senegal and Umpala respectively, while the same 0.76 kb DNA fragment characterised Gardel, Welgevonden and Cameroun stocks. Except for the 3 kb fragment, the probe did not hybridise to Lutale genomic DNA.

A second probe was derived from a fragment of 0.56 kb that appeared to be specific for the Senegal stock on the pattern obtained with primer 28 (Fig. 1). The Southern blot pattern was similar to that obtained with the first probe except that the 3 kb fragment could not be revealed (result not shown).

A third probe of 1.9 kb was derived from a fragment obtained with primer 35 which appeared to be specific for the Senegal stock (Fig. 1). As shown in Fig. 4B, this fragment hybridised to the DNA of all stocks except Lutale. Two DNA fragments of 0.24 kb and 0.45 kb were common to the five positive stocks. Welgevonden, Cameroun and Umpala stocks showed the same pattern consisting of five bands: the band of 0.24 kb, a doublet of 0.45 kb and 0.52 kb, and a second doublet of 0.94 kb and 1.1 kb. Two DNA fragments of 0.59 kb and 0.78 kb were specific for the Gardel and Senegal isolates respectively.

The restriction fragment length polymorphism (RFLP) profiles obtained on Southern blots made it possible to differentiate *C. ruminantium* isolates. Gardel, Welgevonden and Cameroun stocks were similar on the blot probed with the 1.2 kb fragment (Fig. 4A). However, Gardel could be differentiated from these two stocks when the blot was probed with the 1.9 kb fragment (Fig. 4B). On the other hand, Welgevonden, Cameroun and Umpala were similar with the 1.9 kb probe but Umpala exhibited a different profile with the 1.2 kb probe. As in the RAPD analysis, Welgevonden and Cameroun isolates were identical.

4. Discussion

In this study, the RAPD technique was applied
successfully to generate DNA fingerprints which were polymorphic between isolates of the intracellular bacterium *C. ruminantium* (Fig. 1). The technique was reproducible since similar patterns were consistently obtained between independent DNA preparations. Contamination with host cell DNA was very limited (Fig. 2). Moreover, although the cultivation of *C. ruminantium* was always done in the same cell line, other assays have shown that the reproducibility of the pattern was not influenced by the origin of the cells (cattle or goat). Finally, the probes developed from purified RAPD products did not hybridise with host cell DNA used as control on Southern blots (Fig. 4), demonstrating that the fragments were specific for the bacterial genome.

A significant diversity in PCR-amplified fragments was demonstrated between isolates of *C. ruminantium* to such a degree that only 24% of the major bands were common among the six stocks. Despite the diversity observed, the Welgevonden and Cameroon stocks, although originating from very distant regions, were very similar. Differentiation between the other stocks studied could be achieved by using one or the combination of two RAPD fingerprints. Besides a simple differentiation between stocks, analysis of RAPD fingerprints can be used to conduct genetic studies and illustrate the relatedness of isolates [21]. A basic assumption in such studies is that bands of similar size are homologous. This can lead to misinterpretations which were minimised by using 16 primers allowing the amplification of a sufficient number of DNA fragments for statistical analysis [22]. The construction of a genetic distance tree based on similarity coefficients illustrates the clustering of the *C. ruminantium* isolates (Fig. 3). Whether the degree of genetic diversity observed in the genus *Cowdria* is correlated to its antigenic variability and especially cross-immunity remains to be investigated. However, although limited, the cross-protection data reported between the isolates used in the present study are concordant with our genetic tree. The Senegal and Welgevonden stocks, which are the most distant in the genetic tree, have been shown to have very limited cross-immunity by Jongejan et al. [6,23]. The same authors [23] also reported that 4/5, 3/5 and 2/5 goats immunised with the Senegal stock were protected against a challenge with Umpala, Lutale and Gardel stocks, respectively. These stocks are intermediate in our dendrogram between Senegal and the Welgevonden-Cameroun cluster. The antigenic relatedness of the Cameroon and the Welgevonden stocks expected from the genetic study has been confirmed by the full cross-protection obtained in four goats immunised by infection and treatment. The Cameroon stock also proved to be pathogenic to mice as is the Welgevonden stock.

The analysis by RAPD was completed by a Southern blot analysis to test for homogeneity of amplified fragments and to look for further polymorphisms in order to develop molecular tools that could be used directly on ticks or infected animals. The 1.2 kb fragment which was shared by the six stocks and therefore appeared to be a good candidate for a genus-specific probe proved to be a mixture of at least two fragments, one which hybridised with a 3 kb *RsaI* fragment (Fig. 4A), and one which revealed a restriction fragment length polymorphism. This demonstrates the possibility of misinterpretation by making the assumption that RAPD fragments of the same size are homologous. In contrast, the 0.56 kb and 1.9 kb probes, which were supposed to be specific for the Senegal stock, hybridised with the other stocks with different fragments of the genome. They are therefore not good candidates for stock-specific probes. A combination of the RFLP profiles obtained on Southern blots with the 1.2 kb and the 1.9 kb probes was sufficient to differentiate *C. ruminantium* isolates except Welgevonden and Cameroun, which appeared identical.

In conclusion, RAPD is a suitable method for typing *C. ruminantium* isolates. It is also of value to determine the extent of the genetic diversity of this microorganism. In addition, amplified fragments can be selected to develop probes. We identified a 1.2 kb fragment that is a good genus-specific *Cowdria* probe, but were unsuccessful in finding a stock-specific probe. To circumvent this problem, sequencing of polymorphic RAPD fragments is being conducted to try to develop genus and stock-specific PCR primers for diagnostic purposes.

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References