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Use of repetitive DNA elements to define genetic relationships among *Anaplasma marginale* isolates

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Abstract

Anaplasma marginale genomic DNA was tested for the presence of repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC)-like sequences in order to evaluate the genetic diversity of multiple *A. marginale* isolates. *A. marginale* isolates were obtained from cattle of six different states of Brazil, from the US and an *Anaplasma centrale* strain was obtained from Uruguay. Patterns obtained from *A. marginale* isolates varied from 14 to 17 fragments by REP-polymerase chain reaction (PCR) and 6 to 14 fragments by ERIC-PCR. All *A. marginale* isolates presented a 0.75-kb fragment by REP and two common fragments (0.38 and 1.0 kb) by ERIC-PCR. These two fragments were not detectable in *A. centrale*. Both methods produced similar patterns (80%) among *A. marginale* isolates obtained from the same region, although some isolates within regions shared less similarity. Isolates from Parana and Pernambuco, were differentiated by these methods. The study demonstrates the presence of ERIC and REP-like elements in *A. marginale* isolates and shows that *A. marginale* isolates and strains can be differentiated by these methods. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Anaplasma marginale, Genetic relationships, ERIC-PCR, REP-PCR

1. Introduction

Anaplasma marginale is an intraerythrocytic rickettsia that causes bovine anaplasmosis and is transmitted by infected ticks or by contaminated fomites [1]. High-level rickettsemia results in anemia, abortion, weight loss and, in some cases, death during the acute phase [2]. Despite its importance, little is known about the biology of this rickettsia at the molecular level. *A. marginale* isolates exhibit morphological differences [3], variability within their surface proteins [4,5] and have a circular genome of between 1200 and 1260 kb [6]. Further characterization of *A. marginale* isolates has importance in the development of diagnostics, epidemiological studies and for vaccine development.

Most genetic information concerning *A. marginale* relates to the DNA sequences encoding six major outer membrane proteins designated major surface proteins (MSPs) MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4 and MSP-5 [4,7–10]. Some of these genes encode proteins which are polymorphic and are encoded by multi-gene families [7–9].

Several genetic typing methods have been used to differentiate bacterial strains. Repetitive extragenic palindromic (REP) elements [11], and enterobacterial repetitive intergenic consensus (ERIC) sequences [12] are dispersed throughout the prokaryotic genome. Polymerase chain reaction (PCR) studies of eubacteria revealed that inter-REP or inter-ERIC distances and patterns are specific for bacterial species and strains within species [13].

The objective of this study was to investigate the utility of REP- and ERIC-PCR in *A. marginale* in determining the similarity among *A. marginale* strains and isolates from different Brazilian regions.

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2. Materials and methods

2.1. *Anaplasma spp.* isolates and strains

A. marginale isolates were obtained from cattle in different regions/states of Brazil. These regions/states are Central-West/Mato Grosso do Sul (MS-CG), Northeast/Pernambuco (PE-M, PE-A and PE-S), Southeast/São Paulo (SP), Southeast/Minas Gerais (AUFV1) [14], South/Rio Grande do Sul (RS) and South/Paraná (PR-LS1 and PR-HV). The Florida strain has been described previously [15]. The *Anaplasma centrale* strain [16] was obtained from the Centro de Investigaciones Veterinarias 'Miguel C. Rubino', Uruguay.

The isolates were purified following experimental infection of splenectomized cattle, negative for *A. marginale* by enzyme-linked immunosorbent assay. Blood samples were collected by venipuncture in acid citrate, and washed four times in phosphate-buffered saline (PBS) to remove plasma and buffy-coat. Erythrocytes were resuspended in PBS to a final concentration of 10^9 erythrocytes ml^{-1}, and were then frozen at −20°C until use. Purified DNA from leucocytes of negative cattle for *A. marginale* was used as a negative control for DNA amplification.

2.2. DNA purification

DNA purification of *A. marginale* and control DNA was carried out according to supplier recommendations (Puregene, Gentra Systems, Minneapolis, MN, USA), with some modifications. Briefly, 300 μl of each sample was thawed in a water bath, added to 1000 μl of erythrocyte lysis solution in a 1.5 ml Eppendorf tube and incubated for 10 min. The samples were then centrifuged at 13 000×*g* for 2 min. The supernatant was removed and the pellet was incubated with 300 μl of cell lysis solution containing proteinase K (200 μg ml^{-1}) for 1 h at 37°C. The samples were then cooled and 200 μl of protein precipitation solution added. After centrifugation at 13 000×*g* for 3 min the DNA was extracted with isopropanol, precipitated with ethanol and 100 μl of DNA hydration solution was added. DNA was quantified by comparison with λ DNA (Gibco BRL) on 1% agarose gel stained with ethidium bromide and visualized by UV. DNA was stored at 4°C until use.

2.3. PCR for detection *A. marginale*

Nested PCR was performed according to Torioni de Echaide et al. [10] to confirm the presence of *A. marginale*. The primers, obtained from the DNA sequence of the *msp5* gene of Florida *A. marginale* (MSP5-GenBank M3392), were external forward 5′-CATAGCCTCCCCC-TCTTTC-3′; external reverse 5′-TCCTCGCCTTGCCCC-TCAGA-3′ and internal forward 5′-TACACGTGCCCT-ACCGACTTA-3′. PCR was performed in a final volume of 25 μl as per instructions (PCR master kit Boehringer Mannheim) with a Perkin Elmer thermocycler for 5 min at 95°C, 35 cycles at 95°C for 1 min, 65°C for 2 min and 72°C for 1 min with a final extension at 72°C for 10 min followed by cooling to 4°C. A 100-bp ladder (Gibco BRL) was co-electrophoresed to serve as molecular size standards. A DNA band of 345-bp size was visualized with ultraviolet light and photographed. High-purity water and DNA from leucocytes were used as negative controls.

2.4. ERIC- and REP-PCR

REP-PCR was performed with the primers REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-IC-GICTTATCIGGCCTAC-3′), and ERIC-PCR with the primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCA-C-3′); ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGC-G-3′) at a concentration of 50 pmol, as previously described [13], using a thermal cycler (Gene Amp PCR System 9700/Perkin Elmer). Amplification reactions were performed in a final volume of 25 μl with 25 ng *A. marginale* DNA, 0.2 mM deoxynucleoside triphosphate, 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ and 1.25 U of Taq DNA polymerase (Gibco BRL, Life Technology). The ERIC- and REP-PCR reactions were incubated for 5 min at 95°C, followed by 40 cycles at 94°C for 1 min, 52°C for 1.5 min for ERIC and 45°C for 1 min for REP, and 72°C for 8 min, and a final extension at 72°C for 16 min. High-purity water and DNA from leucocytes were used as negative controls. DNA from *Escherichia coli* C600 was used as positive control, and *A. centrale* DNA was used to test for differences between the species. Aliquots of the products REP- and ERIC-PCR (12 μl) were resolved in 1.5% agarose gel (Type II, Sigma) containing 0.5× Tris–borate–EDTA buffer (0.045 M; 0.001 M EDTA pH 8.0) after electrophoresis at 3.2 V cm^{-1} (80 V/4.5 h) (Pharmacia Biotech EPS 600). The gel was stained with ethidium bromide and photographed on a UV-transluminator.

2.5. Analysis of ERIC- and REP-PCR patterns

The sizes of the bands shown by electrophoresis of REP- and ERIC-PCR products were determined by direct comparison with a 100-bp ladder (Life Technologies, Gibco BRL). The gels were analyzed by visual inspection considering all visible bands. Variations in intensity and shape of bands among isolates were not considered a difference. The ERIC- and REP-PCR fingerprints were converted to binary matrix (1, presence; 0, absence of bands). Similarities were determined by a simple matching coefficient, and clustering correlation coefficients were calculated by the unweighted pair group method with arithmetic (UPGMA) averages generating the phenograms by SAHN (sequential agglomerative hierarchical and nested) of NTSYS-PC version 1.7 (Applied Biostatistics) [17].

3. Results and discussion

The optimal amplification conditions for *A. marginale* DNA with REP and ERIC primers with respect to number, intensity and size of bands, were at annealing temperatures of 45 and 52°C, respectively. The optimal DNA concentration was 25 ng. REP- and ERIC-PCR patterns of each isolate on three different days were the same and all comparisons of isolates were performed on the same gel.

Anaplasma DNA generated distinct bands ranging in size from 0.1 to 2.0 kb (Figs. 1A and 2A). Bovine DNA from leucocytes and water did not amplify. Patterns of *A. marginale* isolates varied from 14 to 17 bands by REP-PCR and from 6 to 14 bands by ERIC-PCR. The *A. centrale* strain presented 13 bands by both methods (Figs. 1A and 2A). There were bands common for all *A. marginale* strains on REP- (0.75 kb) and ERIC-PCR (0.38 and 1.0 kb), which were not seen after amplification of *A. centrale* and are identified by arrows (Figs. 1A and 2A).

These results suggest the presence of ERIC and REP-like sequences in *A. marginale* isolates, since high-stringency conditions of amplification were used which reduces experimental variation and increases the reproducibility of these techniques. When ERIC-PCR is applied to non-enterobacterial targets, it may be used at 52°C to be a highly reproducible and sensitive method for specific DNA

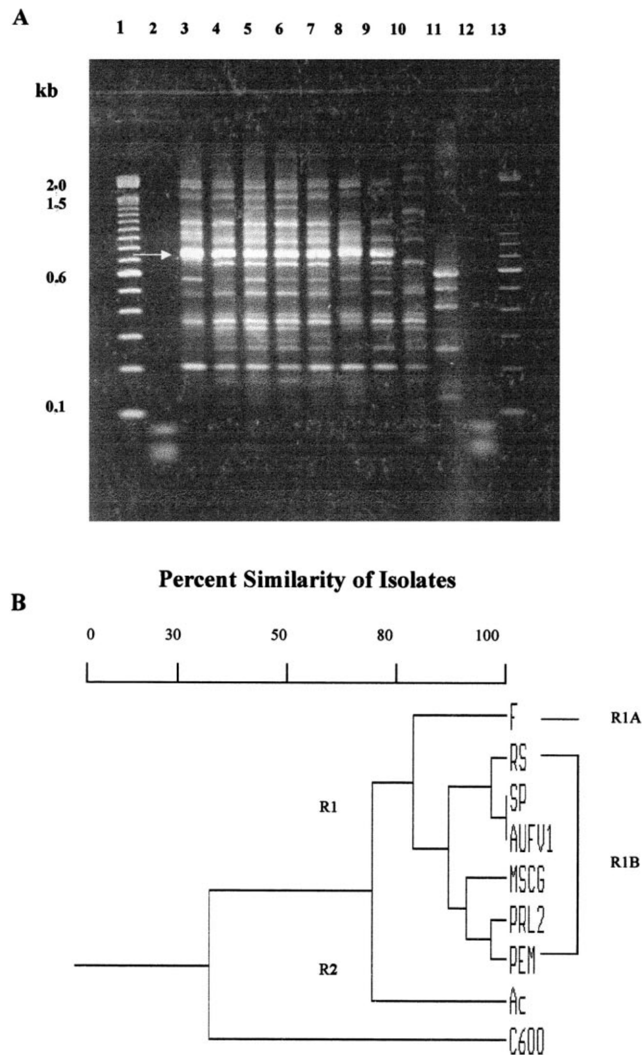

Fig. 1. REP-PCR fingerprint patterns of genomic DNA from *A. marginale* isolates. (A) Lanes: 1 and 13, molecular size markers 100-bp ladder; 2, negative control of amplification (H_2O); 3–9, *A. marginale* isolates: Florida, RS-SP-J, AUFV1, MS-CG, PR-L2, PE-M: 10, *A. centrale* strain; 11, *E. coli* C600; 12, negative control of amplification of bovine leucocyte DNA. Sizes (in kb) are indicated on the left and the arrow identifies the 0.75-kb band. (B) Phenogram (clustered using UPGMA) showing the relationship between the analyzed bacterial strains based on Simple Matching similarity coefficients calculated from REP-PCR analysis data. The bar at the top indicates the similarity index.

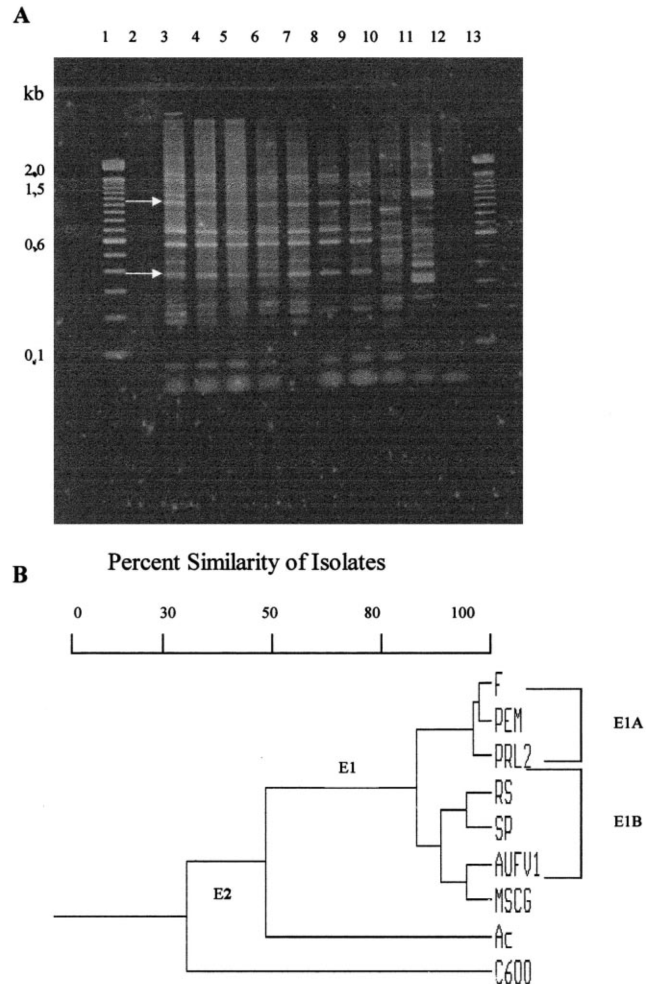

Fig. 2. ERIC-PCR fingerprint patterns of genomic DNA from *A. marginale* isolates. (A) Lanes: 1 and 13, molecular size markers 100-bp ladder; 2, negative control of amplification (H_2O); 3–9, *A. marginale* isolates: Florida, RS, SP-J, AUFV1, MS-CG, PR-L2, PE-M; 10, *A. centrale* strain; 11, *E. coli* C600; 12, negative control of amplification of bovine leucocyte DNA. Sizes (in kb) are indicated on the left and the arrows identify the 0.38- and 1.0-kb bands. (B) Phenogram (clustered using UPGMA) showing the relationship between the analyzed bacterial strains based on Simple Matching similarity coefficients calculated from ERIC-PCR analysis data. The bar at the top indicates the percent of similarity.

amplification and fingerprinting. Gillings and Holley [18] and Versalovic et al. [13] used 40°C for REP-PCR in eubacteria. In *Listeria* sp. strains, these elements were found even with lower annealing temperatures, 40°C for REP and 50°C for ERIC [19].

REP and ERIC sequences are highly conserved within the eubacterial kingdom [13]. These sequences have been used as PCR primers to characterize many bacterial strains including *Actinobacillus* [20], *Bordetella* [21], *Bartonella* [22], *Listeria* [19] and *Mycobacterium* [23].

The phenograms generated by the UPGMA method in REP- and ERIC-PCR are shown in Figs. 1B and 2B. REP- and ERIC-PCR showed seven and eight different profiles, respectively, from seven *A. marginale* isolates and one *A. centrale* strain. The isolates segregated into two clusters with similarities greater than 80% by REP- and ERIC-PCR. Cluster R1 obtained by REP-PCR was divided into two subclusters R1A and R1B with 82% of similarity. R1A contained only the Florida strain and R1B contained all other isolates analyzed (Fig. 1B). The cluster E1 obtained by ERIC-PCR was divided into two subclusters, E1A and E1B with 85% similarity. E1A contained the Florida strain, PE-M and PR-L2 isolates, and E1B contained RS, SP-J, AUFV1 and MS-CG (Fig. 2B). Cluster number 2 from REP-PCR (R2) and from ERIC-PCR (E2) contained *A. centrale*, with 70% and 50% similarity with *A. marginale* isolates (Fig. 2A,B).

The degree of similarity between *E. coli* and *Anaplasma* isolates and strains was approximately 35%, by both methods. Phylogenetic analysis of 16S ribosomal DNA from *A. marginale* showed that *A. marginale* is related to the genera *Rickettsia* and *Ehrlichia*, which are approximately 0.03 of evolutionary distances from *E. coli* [24].

Both methods showed a high degree of similarity (80%) among *A. marginale* isolates that belonged to the same cluster, although some isolates were subclustered differently. The ERIC-PCR detected differences between the SP and AUFV1 strains, although those strains presented 100% of similarity by REP-PCR, showing that ERIC-PCR was more discriminatory than REP-PCR. The patterns created by REP-PCR were more complex than those generated by ERIC-PCR, since REP-PCR produced a greater number of bands (14–17 bands).

Others found similar results with a range of different bacterial species. Appuhamy et al. [20] reported that ERIC-PCR was more discriminatory than REP-PCR for the differentiation of strains of *Actinobacillus seminis*; among 24 isolates they found five REP types and nine ERIC types. Sander et al. [22] found that the fingerprints created by REP-PCR (17–19 bands) were more complex than the patterns generated by ERIC-PCR (7–11 bands), but that ERIC-PCR and pulsed-field gel electrophoresis (PFGE) provided the highest discriminatory potential for subtyping *Bartonella henselae* strains.

The degree of similarity between *A. marginale* isolates and *A. centrale* was lower than that among the *A. marginale* isolates themselves, by both methods. The Florida strain was the only one allocated to sub-cluster R1A, whereas two isolates from Brazil from distant regions (PE-M and PR-L2) were allocated to the same sub-cluster (E1A), showing that these isolates are closely related, although not identical.

Conversely, this work also showed that isolates from the same region can be genetically different. By means of REP-PCR, two *A. marginale* isolates from Paraná State (PRL1 and PRL2) showed only 85% similarity, whereas, two isolates from Pernambuco State (PE2 and PE3) showed 95% similarity, but demonstrated only 88% similarity to a third isolate (PE1) from the same state (data not shown).

There are few studies about genetic relatedness among *A. marginale* isolates. The restriction endonuclease patterns produced from five different isolates (Florida, Louisiana, Oklahoma, St. Croix and Virginia) with the restriction enzymes *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I and *Pst*I, were not identical [25]. Restriction endonuclease patterns of Virginia, Florida and South Idaho strains by PFGE were similar, but not identical [6].

Our results suggest the presence of REP and ERIC-like elements in the genome of *A. marginale* (members of the Anaplasmataceae family) are useful in determining the genetic relatedness among *A. marginale* isolates and strains.

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