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Simple Method to Differentiate among *Rickettsia* Species

Gaspar Peniche-Lara^a, Jorge Zavala-Velazquez^a, Karla Dzul-Rosado^b, D.H. Walker^c, and Jorge Zavala-Castro^b

^aDepartment of Parasitic and Infectious Diseases, Faculty of Medicine, University of Yucatán, Yucatán, Mexico

^bDepartment of Emergent and Re-Emergent Diseases, Centro de Investigaciones Regionales 'Dr. Hideyo Noguchi', Universidad Autónoma de Yucatán, Yucatán, Mexico

^cDepartment of Pathology, University of Texas Medical Branch, Galveston, Tex., USA

Abstract

In this work we present a new option to identify 11 rickettsial species that cause human rickettsioses, with some advantages over the previous methods described. Using rickettsial isolates from 11 *Rickettsia* species as a sample, we used the polymerase chain reaction to amplify a 990- to 1,000-bp DNA fragment from the *ompB* gene, common for the 11 *Rickettsia* species analyzed in this study, which were digested with *AluI* restriction enzyme to obtain different digestion patterns. This restriction pattern can be visualized using a polyacrylamide gel electrophoresis technique. Using this method we could differentiate between the 11 *Rickettsia* species analyzed regardless of the group to which the *Rickettsia* belonged. We developed a simple method to identify 11 *Rickettsia* species which cause human rickettsioses using polymerase chain reaction and restriction fragment length polymorphism techniques with the advantage that it only needs one amplicon and only one restriction enzyme to obtain the restriction pattern. The identification of the species infecting vectors, reservoirs, and humans is essential to establish the ecological and behavioral ecosystem involved in its maintenance and transmission in nature in the specific region where the pathogen is circulating. This method is very helpful to identify *Rickettsia* species in a short time.

Keywords

Rickettsia; Restriction fragment length polymorphism; *Rickettsia* infections; Polymerase chain reaction

Introduction

Rickettsioses are diseases caused by obligate intracellular bacteria belonging to the genus *Rickettsia*. Actually, the genus comprises the typhus group (TG) rickettsiae (*Rickettsia prowazekii*, *R. typhi*), the spotted fever group (SFG) rickettsiae (*R. rickettsii*, *R. sibirica*, *R. conorii*), the transitional group (TGR) rickettsiae (*R. felis*, *R. akari*), and the ancestral group (AG) rickettsiae (*R. bellii*, *R. canada*). The first three groups include *Rickettsia* species that

cause human rickettsioses and the fourth group does not contain any organism that is well documented to cause human rickettsiosis [1].

Currently there are several diagnostic tools to differentiate among *Rickettsia* strains including: *Rickettsia* isolation, immunological detection of antibodies including immunofluorescence assay, which needs multiple microimmunofluorescence assay titers of the sera against different species to identify the rickettsial species, and molecular testing including polymerase chain reaction (PCR) (very sensitive and specific if applied to infected tissue), sequencing (very specific but requiring specific equipment, time-consuming and in some cases expensive) and restriction fragment length polymorphism (RFLP) (which is considered a reasonable approach to routine identification of multiple isolates); most of these methods are focused on SFG rickettsioses [2, 3]. Development of methods to identify *Rickettsia* isolates using RFLP analysis began in 1991 when Regnery et al. [4] developed a method for identification of SFG rickettsiae using PCR and RFLP on two amplified genes: citrate synthase (*gltA*), and the 190-kDa antigen (*ompA*). Then, in 1994, Ereemeeva et al. [5] improved the RFLP method developed by Regnery et al. to identify *Rickettsia* species targeting *gltA*, outer-membrane protein A (*ompA*) and the outer-membrane protein B (*ompB*). To date, many researchers use this method to identify the *Rickettsia* spp. as belonging to the SFG [6] or identifying the *Rickettsia* species with PCR/RFLP technique using a 17-kDa fragment as a target. However, with this method in the TG, only *R. typhi* could be differentiated from other *Rickettsia* species using an RFLP of the 17-kDa gene amplicon. Many researchers have developed other PCR/RFLP methods to differentiate specific *Rickettsia* species using 17-kDa, *ompB* and *sca4* gene as a target for the restriction [7, 8] or to analyze the relations between *Rickettsia* isolates from the same species [9]. Previously described PCR/RFLP methods can identify some *Rickettsia* species or require more than one amplicon or more than one restriction enzyme to obtain different restriction patterns for each *Rickettsia* species, thus requiring more time to obtain the result.

The identification of *Rickettsia* species in human patients, reservoirs and vectors has an enormous importance for establishing the dynamics of the transmission cycle depending on the *Rickettsia* species, in order to perform actions for preventing the infection in humans and animals, and to establish the epidemiological spectrum and prevalence of the rickettsial diseases.

In this work, we propose a two-step method for rickettsial identification using a single restriction enzyme in only one PCR product, making this identification easier, cheaper, and quicker. Here we demonstrate that our method is useful to identify 11 *Rickettsia* species belonging to three of the four groups making this less expensive to do as a routine method for *Rickettsia* species identification.

Results

Rickettsial Genome Analysis

We analyzed 11 rickettsial complete genome sequences in the search of a gene fragment useful for the protocol. *rrs*, *omp1*, *tolc*, *sca1*, *sca2*, *sca3*, *sca4*, and *sca5* genes were the ones analyzed and which are common for all *Rickettsia* species.

pDRAW32 software allowed us to identify the gene, fragment of the gene and the restriction enzyme that could create a unique restriction pattern for each *Rickettsia* species analyzed. The analysis showed that the best option was *ompB* gene, because it was the only one that had a restriction site for an enzyme that produces specific and individual patterns. With the software, we found that a 990–999 fragment from *ompB* gene was the target for the *AluI* restriction enzyme. We chose this *ompB* fragment because the restriction patterns obtained from *AluI* enzyme are longer than the fragment obtained from the PCR/RFLP method previously reported [4, 5, 7].

We designed new primers for this method from the *R. prowazekii ompB* gene that allowed the amplification of the ~990- to ~1,000-bp DNA amplicon from the 11 *Rickettsia* species studied. The *AluI* DNA digestion produced distinct individual patterns for all the strains composed of several bands ranging from 2 to 590 bp that coincided with the profiles determined by the pDRAW32 software and differentiated them from each other. However, due the complexity of the lower bands in the pattern and the difficulty to separate them in the 30% polyacrylamide gel, we only considered the upper bands from 139 to 590 bp to analyze the RFLPs because they still had a unique pattern for the strains (fig. 1).

Typhus Group *Rickettsia* Identification

R. prowazekii and *R. typhi* showed three and four migration bands, respectively, and both RFLP patterns are easily differentiated from each other (fig. 1, lanes 6 and 10). In order to explore any change in the RFLP pattern depending on different strains, we compared the RFLP patterns of three *R. prowazekii* strains (Madrid E, Breinl, and GvV-250) with different genotyping previously reported [10], and found that the RFLP was identical for the three strains, confirming the utility of our analysis to identify the species (fig. 2).

Transitional Group *Rickettsia* Identification

The RFLP pattern of *R. felis* showed a condensed band of ≈ 318 bp that corresponds to the two upper bands in the pDRAW32 software analysis (324 and 297 bp) (fig. 1, lane 4), which did not appear as separate bands in the 30% polyacrylamide gel. However, the pattern formed by the remaining two bands is different from the other 10 strains analyzed, including the pattern of the other member of the TGR, *R. akari* (fig. 1, lane 2), and *R. typhi* which does not belong to the TGR but usually is confused clinically and molecularly with *R. felis*. The same phenomenon occurred with the lower bands of *R. akari* (162 and 159 bp), which appear to migrate as a single band (fig. 1).

Spotted Fever Group *Rickettsia* Identification

Seven species of the SFG were analyzed: *R. rickettsii*, *R. africae*, *R. honei*, *R. japonica*, *R. australis*, *R. conorii* Indian tick typhus strain, and *R. sibirica*. The individual pattern from each allowed the species identification without difficulty (fig. 1). The most similar patterns were the patterns of *R. felis* with *R. africae*, and *R. rickettsii* with the *R. conorii* Indian tick typhus strains. However, slight differences in the migration of the upper bands made the patterns distinctive for all the SFG rickettsiae analyzed.

Verification

In all the samples tested by the PCR/RFLP method, the restriction patterns correspond to the *Rickettsia* species identified by sequencing in the previous studies (table 2). Human cases of rickettsioses caused by *R. felis*, *R. rickettsii*, *R. akari* and *R. typhi* reported in Yucatan State were corroborated by this method (fig. 3).

Discussion

ompB plays an important role in the bacterial pathogenesis, particularly in host cell attachment and entry [11, 12]. Functionally, the *ompB* gene has been well conserved in rickettsial evolution, and has been used as a taxonomic, phylogenetic, and diagnostic tool [10, 13–14]. The identity of the *ompB* sequence among species belonging to the same group is high, and on average the SFG *Rickettsia* have 96%, the TG 92%, and the TRG 91% identity. In contrast, *ompB* shows differences in the identity of the sequence between groups, such as the TG which has only 81% identity in the *ompB* sequence with respect to the SFG, and 82% with the TRG.

The utility of the method identifying strains from the same species was corroborated using three strains of *R. prowazekii*, and the results demonstrate the efficacy of the test identifying the same pattern no matter the differences in their genotype (fig. 2) [10], and to differentiate clinically related rickettsioses such as murine typhus and fleaborne spotted fever (fig. 1) [15, 16].

The atypical migration of some bands could be the result of conformational alterations as a result of differences in the sequence of those fragments between rickettsial strains. However, the patterns produced allow the differentiation among the 11 strains.

Validation of the method using samples from diagnosed patients, reservoirs, and vectors was successfully done. The results have shown a perfect match between the *ompB* RFLP pattern and the infective species diagnosed by sequence analysis of the amplified products using *gltA*, *htrA*, and *ompB* in other reported studies in our laboratory.

We propose to use this method as an easy technique to diagnose not only rickettsial diseases but also to diagnose specific *Rickettsia* species infections in countries where more than one *Rickettsia* species are circulating in the population like Yucatan State where four different *Rickettsia* species cause rickettsial diseases: *R. felis* [17], *R. rickettsii* [18], *R. typhi* [19] and *R. akari* [20].

Our method provides an easy and accurate tool to identify *Rickettsia* species without the requirement of sequencing the PCR products or using several primers. The method makes more accessible the species identification in infected humans, animals or vectors, and it would be more plausible to determine the epidemiological spectrum and prevalence of the rickettsial diseases in developing countries in order to suggest strategies for prevention and control of the transmission cycle.

Experimental Procedures

Rickettsial Genome Analysis

We obtained the complete genome sequences of the 11 *Rickettsia* species analyzed in the study from the GenBank. The rickettsial species and their sequence accession numbers from each *Rickettsia* are listed in table 1. Using BioEdit sequence alignment software v.7.0.5.3 (Tom Hall), we compared the complete genome sequence of the 11 *Rickettsia* species (table 1) in order to identify genes which are common for all *Rickettsia* species with the lowest percentage of identity. With this analysis we determined that *ompB* was the best target for the study.

ompB Gene Analysis

pDRAW32 DNA analysis software (AcaClone software) was used to find the lowest number of restriction enzymes for the RFLP analysis in order to differentiate *Rickettsia* species. Although the *ompB* sequences of *R. sibirica*, *R. honei*, *R. australis*, and Indian tick typhus *Rickettsia* reported in GenBank were not complete, the electrophoretic pattern obtained with *Alu I* RFLP from the 11 species analyzed showed a unique restriction pattern for all species, and closely matched those obtained by the electrophoretic analysis (fig. 1).

Rickettsial Strains and DNA Purification

The strains used in this study were provided by the Rickettsial and Ehrlichial Diseases Research Laboratory, University of Texas Medical Branch, and are listed in table 1. Genomic DNA was extracted using QIAamp DNA kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions, and the PCR amplicons were purified using Qiagen Gel Extraction Kit (Qiagen) according to the manufacturer's instructions with a final elution volume of 10 µl.

PCR and RFLP

PCR amplification was performed with primers designed for this study based on the sequence of *R. prowazekii ompB* (GenBank accession No. AF161079): Rp.330(2) Fw 5'-ATGGCTTCAAAAACCAAATTTTCTAA-3' and Rp.330(2) Rv 5'-AGCTCTACCTGCTCCATTATCTGTACC-3', which amplify a 990- to 1,000-bp PCR product depending on the species. We decided to obtain a long amplicon because it would be easy to see long restriction patterns by RFLP analysis. Each PCR was carried out in a Techne Genius FGEN02TP thermal cycler (Teche, Stone, Staffs., UK). The reactions were performed with 150 ng of rickettsial DNA in a 50-µl reaction mixture containing 25 pmol in each primer, 200 µM (each) dATP, dCTP, dGTP and dTTP (Invitrogen, Carlsbad, Calif., USA), 1 U platinum Taq DNA polymerase (Invitrogen); 5 µl of 10. PCR buffer; 1.5 µl of 50 mM magnesium chloride. The conditions used for amplification were: initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 60 s at 60 °C, and extension for 1 min 30 s at 72 °C, and a final holding reaction for 7 min at 72 °C. PCR products obtained were resolved by agarose gel electrophoresis and ethidium bromide as a stain. PCR products were visualized using a UVP light source.

For RFLP, 5 µl of amplified products were digested using *AluI* restriction endonuclease (Invitrogen) for 1 h at 37 ° C, and the restriction products were separated in a 12% polyacrylamide gel that was run at 100 V for 3 h, stained with ethidium bromide, and visualized with an UV light source (UVP).

Method Verification

To verify the utility of the method in clinical specimens, we compare the restriction patterns obtained from the PCR amplicons performed from DNA samples of the reference strains of *R. felis*, *R. typhi*, *R. rickettsii*, and *R. akari* listed in table 1, with the restriction patterns obtained from the PCR performed using DNA samples of previously reported rickettsioses that were diagnosed by PCR sequence and reported to the GenBank (table 1). The restriction patterns were identical and indirectly demonstrate the utility of our species identification method in clinical and biological samples (table 2).

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Fig. 1.

Comparison between restriction pattern obtained from pDRAW32 software (VR) and restriction pattern obtained from 12% acrylamide gel electrophoresis. Slight differences between virtual restriction and real restriction (RR) are observed possibly due to acrylamide concentration or electrophoresis running time. RM: 1-kb DNA ladder (Invitrogen); VM: 1-kb DNA ladder (Invitrogen); 1: VR *R. sibirica*; 2: RR *R. sibirica*; 3: VR *R. felis*; 4: RR *R. felis*; 5: VR *R. prowazekii*; 6: RR *R. prowazekii*; 7: VR *R. rickettsii*; 8: RR *R. rickettsii*; 9: VR *R. typhi*; 10: RR *R. typhi*; 11: VR *R. australis*; 12: RR *R. australis*; 13: VR *R. africae*; 14: RR *R. africae*; 15: VR *R. conorii*. Dotted line divides the bands used for the RFLP pattern (upper bands).

Fig. 2.
RFLP obtained from amplicons of three *R. prowazekii* strains. 1: Breinl strain; 2: GvV strain; 3: Madrid strain.

Fig. 3.
12% acrylamide gel electrophoresis of restriction patterns obtained from *Rickettsia* species blood samples in Yucatan State. M: 100-bp molecular weight maker; 1: *R. akari*; 2: *R. felis*; 3: *R. rickettsii*; 4: *R. typhi*.

Table 1

Species and strains included in the study

Species	Strain	Geographic origin	Source	GenBank Accession No.
<i>R. felis</i>	URRWXC2	USA	<i>Ctenocephalides felis</i>	NC_007109
<i>R. rickettsii</i>	Sheila Smith	USA	Human	NC_009882
<i>R. akari</i>	Kaplan	USA	Human	AF123707
<i>R. typhi</i>	Wilmington	USA	Rat	NC_006142
<i>R. prowazekii</i>	GvV	USA	Flying squirrel	AF211821
	Madrid	Spain	Human	AJ235273
	<i>Breidl</i>	Poland	Human	AF161079
<i>R. australis</i>	Phillips	Australia	Human	AF123709
<i>R. conorii</i>	Indian tick typhus	India	<i>Rhipicephalus sanguineus</i>	AF123726
<i>R. africae</i>	ESF-5	Ethiopia	<i>Amblyomma variegatum</i>	AF123706
<i>R. japonica</i>	YM	Japan	Human	AF123713
<i>R. honei</i>	TT118	Thailand	<i>Ixodes</i> sp.	AF123724
<i>R. sibirica</i>	246	Russia	<i>Dermacentor nuttali</i>	AF123722

Table 2

Comparison of previously reported *Rickettsia* species from different samples by sequencing with PCR/RFLP technique developed using the same sample stored or collected

Sample	<i>Rickettsia</i> species sequence identification	PCR/RFLP restriction pattern obtained	Samples analyzed, n	Reference
Blood	<i>R. typhi</i>	<i>R. typhi</i>	1	Zavala-Castro et al. [19]
	<i>R. felis</i>	<i>R. felis</i>	2	Zavala-Castro et al. [17]
	<i>R. akari</i>	<i>R. akari</i>	2	Zavala-Castro et al. [20]
<i>D. marsupialis</i> tissue			1	GenBank Accession No. GU447233.1
Spleen	<i>R. felis</i>	<i>R. felis</i>	1	
Kidney	<i>R. felis</i>	<i>R. felis</i>	1	
Brain	<i>R. felis</i>	<i>R. felis</i>	1	
Flea				GenBank Accession No. JX198506
<i>C. felis</i>	<i>R. felis</i>	<i>R. felis</i>	9	