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A mutagenic PCR identifies isolates of *Borrelia garinii* responsible for Lyme borreliosis

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Abstract

Borrelia garinii is one of the three major *Borreliae* responsible for Lyme borreliosis in Europe. We have characterized a protein of *B. garinii* (VS102) and a genomic fragment from the gene encoding this protein was cloned. The DNA sequence of the fragment showed high homology with a known gene of *B. burgdorferi* sensu stricto. The protein encoded by this gene in *B. burgdorferi* sensu stricto is a phosphocarrier protein (histidine-containing protein). A mutation T to G polymorphism at codon 57 was found to be specific to *B. garinii*. A PCR-based approach that allows the rapid detection of this mutation made it possible to specifically discriminate *B. garinii* from other *B. burgdorferi* genospecies with high sensitivity and specificity. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Borrelia garinii*; Lyme borreliosis; Mutagenic polymerase chain reaction; Phosphocarrier protein HPR

1. Introduction

Borrelia garinii, along with *B. burgdorferi* sensu stricto and *B. afzelii*, is one of the three major *Borreliae* responsible for Lyme borreliosis [1]. It is associated with the majority of neuroborreliosis cases in Europe [2–4]. *B. garinii* appears to be a very heterogeneous group which includes several serogroups [5] exhibiting divergences at the genetic level [1,6]. In Europe, *B. garinii* is the most common *Borrelia* species [7,8]. Its geographical distribution appears to be restricted to Europe and Asia, although seabirds, as a potential reservoir, may spread *B. garinii* between both hemispheres via a specific vector, *Ixodes uriae* [9]. It has been demonstrated that birds of the genus *Turdus* are reservoirs for *B. garinii* and *B. valaisiana*, based on direct isolation of these *Borrelia* species and xenodiagnoses [10].

After two-dimensional gel electrophoresis, followed by Western blot, two co-migrating proteins of about 11 kDa

were identified on Immobilon membrane stained with amido black. One of these proteins showed a species-specific polymorphism, which we characterized at the DNA level. Our data suggest that this single nucleotide variation can be used as a molecular tool for the identification of *B. garinii* isolates.

2. Materials and methods

2.1. *Borrelia* isolates and other unrelated bacteria

All *Borrelia* isolates were grown in BSK II medium [11] at 34°C and were harvested during exponential-phase growth, washed twice in phosphate-buffered saline (pH 7.4) ([12], appendix B12) containing 5 mM MgCl₂. Pellets were resuspended in distilled water and frozen at –80°C until use. The 100 Eurasian and four American *Borrelia* isolates are listed in Table 1. They belong to six genospecies and originated from ticks ($n = 69$), rodents ($n = 8$), humans ($n = 25$) and birds ($n = 2$). All the isolates used were typed by restriction fragment length polymorphism (RFLP) of *rrf* (5S)–*rrl* (23S) intergenic spacer amplicons [6].

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Table 1
Origins of *B. burgdorferi* sensu lato and of other bacterial strains used in this study

Isolate	Biological origin	Geographical origin	Isolate	Biological origin	Geographical origin
<i>B. afzelii</i>			FAR02	<i>I. uriae</i>	Denmark
934U	<i>Apodemus agrarius</i>	Korea	FIS01	<i>I. uriae</i>	Iceland
A100S	Human (skin)	The Netherlands	G25	<i>I. ricinus</i>	Sweden
A26S	Human (skin)	The Netherlands	HP3	<i>I. persulcatus</i>	Japan
A39S	Human (skin)	The Netherlands	Ip89 ^a	<i>I. persulcatus</i>	CIS
A42S	Human (skin)	The Netherlands	Ip90	<i>I. persulcatus</i>	CIS
A45aS	Human (skin)	The Netherlands	M50	<i>I. ricinus</i>	The Netherlands
A51T	<i>Ixodes ricinus</i>	The Netherlands	M63 ^a	<i>I. ricinus</i>	The Netherlands
A58T	<i>I. ricinus</i>	The Netherlands	N34	<i>I. ricinus</i>	Germany
A76S	Human (skin)	The Netherlands	NBS16 ^a	<i>I. ricinus</i>	Sweden
ACA1	Human (skin)	Sweden	NE2	<i>I. ricinus</i>	Switzerland
DK3	Human (skin)	Denmark	NE83	<i>I. ricinus</i>	Switzerland
DK8	Human (skin)	Denmark	NT29 ^a	<i>I. persulcatus</i>	Japan
F1	<i>I. ricinus</i>	Sweden	P/Br	Human (CSF)	Germany
IP3 (Iper3)	<i>Ixodes persulcatus</i>	CIS	PD89	Human (blood)	China
Iper	<i>I. persulcatus</i>	Japan	T25	<i>I. ricinus</i>	Germany
M55	<i>I. ricinus</i>	The Netherlands	TN	<i>I. ricinus</i>	Germany
M7	<i>I. persulcatus</i>	China	VS3 ^a	<i>I. ricinus</i>	Switzerland
NE36	<i>Clethrion glareolus</i>	Switzerland	VS102 ^a	<i>I. ricinus</i>	Switzerland
NE39	<i>C. glareolus</i>	Switzerland	VS156	<i>I. ricinus</i>	Switzerland
Pwud I	Human (skin)	Germany	VS244	<i>I. ricinus</i>	Switzerland
SMS1	<i>Apodemus flavicollis</i>	Sweden	VSBM	Human (CSF)	Switzerland
UM01	Human (skin)	Sweden	VSBP	Human (CSF)	Switzerland
VS25R-Or	<i>A. flavicollis</i>	Switzerland	VSDA	Human (CSF)	Switzerland
VS42R-R	<i>Apodemus sylvaticus</i>	Switzerland	<i>B. japonica</i>		
VS461 ^a	<i>I. ricinus</i>	Switzerland	COW611c	<i>Ixodes ovatus</i>	Japan
<i>B. burgdorferi</i> sensu stricto			FI340	<i>I. ovatus</i>	Japan
A44S	Human (skin)	The Netherlands	FiAE2	<i>A. speciosus</i>	Japan
B31 ^a	<i>Ixodes dammini</i>	USA	FiEE2	<i>Eothenomys smithi</i>	Japan
CA-5	<i>Ixodes pacificus</i>	USA	HO14	<i>I. ovatus</i>	Japan
Charlie Tick	<i>I. dammini</i>	USA	IKA2	<i>I. ovatus</i>	Japan
Geho	Human (skin)	Germany	<i>B. lusitaniae</i>		
IP1	Human (CSF)	France	BR41	<i>I. ricinus</i>	Czech Republic
IP2	Human (CSF)	France	IR345	<i>I. ricinus</i>	Byelorussia
IP3	Human (CSF)	France	POTIB1	<i>I. ricinus</i>	Portugal
IRS	<i>I. ricinus</i>	Switzerland	POTIB2	<i>I. ricinus</i>	Portugal
M14	<i>I. ricinus</i>	The Netherlands	POTIB3	<i>I. ricinus</i>	Portugal
NE48	<i>I. ricinus</i>	Switzerland	<i>B. valaisiana</i>		
NE50	<i>I. ricinus</i>	Switzerland	AG1	<i>I. ricinus</i>	Switzerland
NE56	<i>I. ricinus</i>	Switzerland	AR-2	<i>I. ricinus</i>	The Netherlands
BE1 (PIG)	Human (synovial fluid)	Switzerland	F10.8.94	<i>I. ricinus</i>	Germany
VS2	<i>I. dammini</i>	USA	Frank	<i>I. ricinus</i>	Germany
VS44	<i>I. ricinus</i>	Switzerland	M19	<i>I. ricinus</i>	The Netherlands
VS73	<i>I. ricinus</i>	Switzerland	M52	<i>I. ricinus</i>	The Netherlands
VS82	<i>I. ricinus</i>	Switzerland	M53	<i>I. ricinus</i>	The Netherlands
VS106	<i>I. ricinus</i>	Switzerland	M57	<i>I. ricinus</i>	The Netherlands
VS108	<i>I. ricinus</i>	Switzerland	NE168	<i>I. ricinus</i>	Switzerland
VS115	<i>I. ricinus</i>	Switzerland	NE218	<i>Turdus merula</i>	Switzerland
VS134	<i>I. ricinus</i>	Switzerland	NE223	<i>T. merula</i>	Switzerland
VS146	<i>I. ricinus</i>	Switzerland	VS116	<i>I. ricinus</i>	Switzerland
VS161	<i>I. ricinus</i>	Switzerland	Other bacterial strains tested		
VS219	<i>I. ricinus</i>	Switzerland	<i>Staphylococcus aureus</i>		
<i>B. garinii</i>			<i>Staphylococcus epidermidis</i>		
387	Human (CSF)	Germany	<i>Escherichia coli</i>		
935T ^a	<i>I. persulcatus</i>	Korea	<i>Salmonella</i> sp.		
A19S ^a	Human (skin)	The Netherlands	<i>Shigella sonnei</i>		
A77C	Human (CSF)	The Netherlands	<i>Klebsiella pneumoniae</i>		
AR-1	<i>I. ricinus</i>	The Netherlands	<i>Pseudomonas aeruginosa</i>		
BITS	<i>I. ricinus</i>	Italy			
FAR01	<i>Ixodes uriae</i>	Denmark			

^aSequenced isolates.

Seven other unrelated bacterial strains, shown in Table 1, were tested and used as a control of PCR specificity.

2.2. Protein separation

Proteins of the isolate VS102 (*B. garinii*) were separated by two-dimensional gel electrophoresis. In the first dimension immobilized pH gradient strips (Pharmacia-LKB, Uppsala, Sweden), with a gradient from pH 3 to 10, were used. The second dimension was performed on polyacrylamide gel (gradient from 9 to 16%). After transfer to Immobilon P (polyvinylidene difluoride (PVDF) membrane, Millipore, Bedford, MA, USA) the membrane was stained with amido black. The protein was sequenced with an automated protein microsequencer from the PVDF membrane (SWISS-2DSERVICE, sequencing service, Geneva, Switzerland). Molecular techniques were carried out (see Section 3) as described in Maniatis et al. [12].

2.3. Mutagenic PCR

A bacterial suspension (20 or 50 μ l, protein concentration 1 mg ml⁻¹) was heated for 5 min at 95°C for lysis. The PCR reaction was carried out in a 50- μ l reaction mixture containing 2 μ l of bacterial lysate (Taq: 2.5 U, nucleotides 200 μ M). The upstream primer was 5'-ATG-GTAAAAAAGAAGC-3' and the downstream primer was 5'-CCTCACCTCAGCACATATCAAAGCTTTT-TACCAG-3'. A Biometra T-Gradient (Biometra, Göttingen, Germany) was used and cycling conditions were as follows: 95°C for 15 min followed by 29 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min) and a final elongation at 72°C for 5 min. The PCR product (10 μ l) was electrophoresed through a 3% agarose gel (Agarose STG, Eurobio, Les Ulis, France) in 1 \times TBE ([12], appendix B23), for 20 min at 100 V.

2.4. Digestion with PvuII

A total of 40 μ l PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). 15 μ l of purified PCR products were incubated with 2 U of PvuII (Gibco BRL, Life Technologies, Basel, Switzerland) for 1 h at 37°C, with an additional 2 U of enzyme for another 2 h of incubation. Products of digestion (8 μ l) were analyzed through a 3% agarose gel (Agarose STG Eurobio) in 1 \times TBE for 30 min at 90 V.

2.5. Sequencing PCR

Sequencing of the mutagenic PCR fragment was performed with upstream primer 5'-TGTAACGACGGC-CAGTATGGTAAAAAAGAAGC-3' and downstream primer 5'-AAATCCTTCTAAAATTCA-3' used to amplify DNA. The amplification reactions were optimized and carried out in Biometra T-Gradient. The cycling condi-

tions were: 95°C for 15 min followed by 5 cycles of (95°C for 1 min, 50°C for 1 min, 72°C for 1 min) and 30 cycles of (95°C for 1 min, 43°C for 1 min, 72°C for 1 min, 72°C for 1 min).

2.6. Nucleotide sequence accession numbers

The full-length sequence of the gene encoding the phosphocarrier protein (histidine-containing protein) of *B. garinii* (VS102 isolate) has been assigned the GenBank accession number AF291154 and partial sequences of the same gene in *B. garinii* isolates: VS3 (AF291150), 935T (AF291151), NT29 (AF291152), NBS16 (AF291153), IP89 (AF293454), A19S (AF293455), M63 (AF293456) and in *B. afzelii* VS461 reference strain (AF291155).

3. Results and discussion

Following two-dimensional gel electrophoresis and transfer onto Immobilon membrane, the membranes were stained with amido black. A cluster of two spots was observed at 11 kDa. The larger spot with an estimated *pI* of 5.7 was sequenced. The N-terminal sequence of the first 14 amino acids was determined and subsequently confirmed from a second preparation. From this sequence we designed a degenerate oligonucleotide probe which was used on Southern blots of several restriction digests (*EcoRI*, *EcoRV*, *BglII*, *PstI* and *HindIII*) of genomic DNA from *B. garinii*. An *EcoRI* and a *HindIII* fragment each showed strong homology to the probe. Both fragments were cloned in plasmids and sequenced.

Results from DNA sequencing indicated a very high homology with a known sequence of *B. burgdorferi* sensu stricto (TIGR database) [13] at positions 569 283–569 544, referred to as BB557 HPR (histidine-containing protein). This protein is a phosphocarrier protein (phosphoenolpyruvate:phosphotransferase system) well conserved in bacteria (e.g. *E. coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*), and involved in fructose uptake. Using primers tailed with the M13–21 sequence, we amplified the relevant gene segment from several *Borrelia* isolates. This segment's sequence was then determined in five *B. garinii*, one *B. burgdorferi* sensu stricto and one *B. afzelii* isolates. Comparative analysis of all seven sequences showed 95% identity between these *Borrelia* isolates (Fig. 1). All five *B. garinii* isolates had an identical substitution at nucleotide 169, where a G was found instead of a T as in the other two species. This substitution leads to an amino acid change at position 57, where in *B. garinii* an alanine replaces the serine residue, typically found in the other isolates. Because of the lack of a restriction site at positions 167–170, we designed a mutagenic PCR system as reported in Fig. 1, in order to screen a larger number of *Borrelia* isolates. The downstream primer contained a base substitution creating a restriction site recognized by

eral *Borrelia* isolates. We thank Alexia Couturier-Maret for excellent technical assistance and Jean-Claude Piffaretti for critical review of the manuscript and helpful suggestions. This work was supported by the Swiss National Foundation for Scientific Research (Grant 3200-052739) and the Foundation for research and development of the Institut Central des Hôpitaux Valaisans. The automated DNA sequencer was provided by the E. Boninchi Foundation to Pierre Hutter.

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