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Specificities and Sensitivities of Four Monoclonal Antibodies for Typing of *Borrelia burgdorferi* Sensu Lato Isolates

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***Borrelia burgdorferi*, the agent of Lyme borreliosis, is genetically more heterogeneous than previously thought. In Europe five genospecies have been described from the original *B. burgdorferi* sensu lato (sl): *B. burgdorferi* sensu stricto (ss), *B. garinii, B. afzelii, B. lusitaniae*, and *B. valaisiana*. In the United States, *B. burgdorferi* ss as well as *B. bissettii* in California and *B. andersonii* on the East Coast were differentiated. In Asia, *B. japonica* has been identified along, with *B. garinii, B. afzelii*, and *B. valaisiana*. In order to evaluate sensitivity and specificity of four species-specific monoclonal antibodies, we analyzed 210 *B. burgdorferi* sl isolates belonging to eight genospecies by immunoblot and confirmed genospecies by restriction fragment length polymorphism (RFLP) of *rrf* (5S)-*rrl* (23S) intergenic spacer amplicon. Monoclonal antibody H3TS had 100% sensitivity for 55 *B. burgdorferi ss* isolates but showed reactivity with all four isolates belonging to *B. bissetii*. Monoclonal antibody I 17.3 showed 100% specificity and sensitivity for 45 *B. afzelii* isolates. Monoclonal antibody D6 was 100% specific for *B. garinii* but missed 1 of 64 isolates (98.5% sensitivity). Monoclonal antibody A116k was 100% specific for *B. valaisiana* but was unreactive with 4 of 24 isolates (83.5% sensitivity). Genetic analysis correlated well with results of reactivity and confirmed efficacy of the phenotypic typing of these antibodies. Some isolates showed atypical RFLP. Therefore, both phenotypic and genotypic analyses are needed to characterize new *Borrelia* isolates.**

Borrelia burgdorferi, the agent of Lyme borreliosis, has been found to be genetically more heterogeneous than previously thought. In Europe, five genospecies have been described from the original *B. burgdorferi*, now called *B. burgdorferi* sensu lato: *B. burgdorferi* sensu stricto, *B. garinii, B. afzelii, B. lusitaniae*, and *B. valaisiana* (3, 9, 19, 32). Barbour et al. (4) and Wilske et al. (35) first provided early evidence of the heterogeneity among European isolates of *B. burgdorferi*, whereas U.S. isolates appeared to be a homogeneous group except for a few variants observed in California (6, 25) and on the East Coast from *Ixodes dentatus* (*B. andersonii*). In Asia, some new species have been differentiated as *B. japonica* and *B. turdae*. *B. garinii, B. afzelii*, and *B. valaisiana* also appear to be endemic species (11; T. Masuzawa, Y. Imai, and Y. Yanagihara, Abstr. VIII. Int. Conf. Lyme Borreliosis Other Tick-Borne Dis., 1999, abstr. O5, p. 5).

The European borreliae apparently have distinct reservoir hosts. *Apodemus* mice and *Clethrionomys glareolus* voles appear to be the main reservoirs for *B. afzelii* (14, 16). Similarly, birds of the genus *Turdus* are reservoirs for *B. garinii* and *B. valaisiana* (15), and the red squirrel *Sciurus vulgaris* might be a reservoir for *B. burgdorferi* sensu stricto and *B. afzelii* (13). Although not absolute, associations of particular clinical manifestations in humans with distinct species of *B. burgdorferi* sensu lato have been documented. Acrodermatitis chronica atrophicans is clearly associated with infection due to *B. afzelii* (9, 10). Patients with Lyme arthritis are more often infected with *B. burgdorferi* sensu stricto (18) or show higher serological reactions with this particular *Borrelia* species, and patients with neuroborreliosis are more frequently infected with *B. garinii* (8) or present serological reactions in accordance with this association (1, 2, 24, 29). We have previously reported serological evidence for a pathogenic potential of *B. valaisiana* in humans (29). Sera from three patients with neuroborreliosis and from one patient with Lyme arthritis showed higher reactivity with this *Borrelia* species.

Genetic analysis based on 16S rDNA, restriction fragment length polymorphism (RFLP), arbitrarily primed PCR, and other methods for phylogenetic study of bacterial population, such as multilocus enzyme electrophoresis, all confirmed the subdivision of *B. burgdorferi* sensu lato into different species worldwide.

The serotyping method developed by Wilske et al. (34) and classification based on protein profiles provided similar data. Monoclonal antibodies specific to some of these species have been described (3, 9, 22), and a new monoclonal antibody to *B. valaisiana* has been produced in our laboratory. In the present study, we evaluated the specificity and sensitivity of four species-specific monoclonal antibodies based on the analysis of 210 isolates of *B. burgdorferi* sensu lato.

MATERIALS AND METHODS

Culture of *Borrelia* isolates. All isolates (Table 1) were cultured in BSK II medium at 34°C, and spirochetes were harvested during the late log phase by centrifugation at 10,000 × *g* for 10 min. The pellet was washed twice in phosphate-buffered saline with 5 mM $MgCl_2$ and finally resuspended in distilled water. Protein concentration was adjusted to 1 mg/ml. The preparation was frozen at −20°C until use.

T. Balmelli, G. Baranton, A. G. Barbour, S. Bergström, A. van den Bogaard, W. Burgdorfer, S. J. Cutler, A. J. van Dam, L. Gern, A. Gylfe, I. Heinzer, P. F. Humair, K.-J. Hwang, T. Masuzawa, S. Nuncio, D. Postic, V. Preac-Mursic, S. Rijpkema, V. Sambri, J. Schmidli, T. Schwan, J. Wilhelm, M. M. Wittenbrink, and B. Wilske kindly provided us with various isolates.

Phenotypic typing of *B. burgdorferi* sensu lato. Electrophoresis and immunoblots were performed as previously described (23). Briefly, a suspension of

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TABLE 1. *B. burgdorferi* sensu lato isolates evaluated in this study[a]

Strain	Country of isolation	Biological origin	RFLP pattern	Source
B. afzelii				
934U	Korea	*Apodemus agrarius*	D	K.-J. Hwang
A26S	The Netherlands	Human (skin)	D	A. J. van Dam
A38S	The Netherlands	Human (skin)	D	A. J. van Dam
A39S	The Netherlands	Human (skin)	D	A. J. van Dam
A40S	The Netherlands	Human (skin)	D	A. J. van Dam
A42S	The Netherlands	Human (skin)	D	A. J. van Dam
A45aS	The Netherlands	Human (skin)	D	A. J. van Dam
A51T	The Netherlands	*I. ricinus*	D	A. J. van Dam
A58T	The Netherlands	*I. ricinus*	D	A. J. van Dam
A59T	The Netherlands	*I. ricinus*	D	A. J. van Dam
A76S	The Netherlands	Human (skin)	D	A. J. van Dam
A100S	The Netherlands	Human (skin)	D	A. J. van Dam
ACA1	Sweden	Human (skin)	D	S. Bergström
BO23	Germany	Human (skin)	D	B. Wilske and V. Preac-Mursic
DK3	Denmark	Human (skin)	D	T. Balmelli
DK8	Denmark	Human (skin)	D	T. Balmelli
F1	Sweden	*I. ricinus*	D	S. Bergström
IP3	CIS	*I. persulcatus*	D	S. Bergström
Iper	Japan	*I. persulcatus*	D	D. Postic
M7	China	*I. persulcatus*	D	D. Postic
M55	The Netherlands	*I. ricinus*	D	A. van den Bogaard
NE28	Switzerland	*C. glareolus*	D	P. F. Humair
NE29	Switzerland	*C. glareolus*	D	P. F. Humair
NE30	Switzerland	*C. glareolus*	D	P. F. Humair
NE35	Switzerland	*Apodemus flavicollis*	nd	P. F. Humair
NE36	Switzerland	*C. glareolus*	D	P. F. Humair
NE39	Switzerland	*C. glareolus*	D	P. F. Humair
NE42	Switzerland	*C. glareolus*	D	P. F. Humair
NE43	Switzerland	*C. glareolus*	D	P. F. Humair
NE44	Switzerland	*C. glareolus*	D	P. F. Humair
NE45	Switzerland	*C. glareolus*	D	P. F. Humair
NE53	Switzerland	*I. ricinus*	D	P. F. Humair
P/Sto	Germany	Human (skin)	D	B. Wilske and V. Preac-Mursic
PGau	Germany	Human (skin)	D	B. Wilske and V. Preac-Mursic
PKo 2-85	Germany	Human (skin)	D	B. Wilske and V. Preac-Mursic
Pspe	Germany	Human (CSF)	D	B. Wilske and V. Preac-Mursic
Pwud I	Germany	Human (skin)	D	B. Wilske and V. Preac-Mursic
SMS1	Sweden	*Apodemus flavicollis*	D	D. Postic
SO2	United Kingdom	*I. ricinus*	D	S. J. Cutler
UM01	Sweden	Human (skin)	D	S. Bergström
VS18	Switzerland	*I. ricinus*	D	OL
VS25R-Or	Switzerland	*Apodemus flavicollis*	D	OL
VS42	Switzerland	*I. ricinus*	D	OL
VS42R-R	Switzerland	*Apodemus sylvaticus*	D	OL
VS461	Switzerland	*I. ricinus*	D	OL
B. andersonii				
19952	United States	*I. dentatus*	L	G. Baranton
21123	United States	*I. dentatus*	L	G. Baranton
B. bissettii				
25015	United States	*I. scapularis*	K	G. Baranton
CA-128	United States	*I. neotomae*	J	D. Postic
CA-55	United States	*I. neotomae*	J	D. Postic
DN127	United States	*I. pacificus*	I	G. Baranton
B. burgdorferi sensu stricto				
297	United States	Human (CSF)	A	D. Postic
13062	Yugoslavia	*I. ricinus*	A	J. Wilhelm
13063	Yugoslavia	*I. ricinus*	A	J. Wilhelm
20006	France	*I. ricinus*	A	G. Baranton
A44S	The Netherlands	Human (skin)	A1	A. J. van Dam
ATCC35211	Switzerland	*I. ricinus*	A	A. Barbour
B31	United States	*I. scapularis*	A	W. Burgdorfer
BE1	Switzerland	Human (synovial fluid)	A	J. Schmidli
CA-2-87	United States	*I. pacificus*	A1	D. Postic
CA-5	United States	*I. pacificus*	A	D. Postic
Charlie Tick	United States	*I. scapularis*	A1	G. Baranton
Geho	Germany	Human (skin)	A	B. Wilske and V. Preac-Mursic
HUM3336	United States	*I. pacificus*	A	D. Postic
IP1	France	Human (CSF)	A	G. Baranton
IP2	France	Human (CSF)	A	G. Baranton
IP3	France	Human (CSF)	A	G. Baranton
IRS	Switzerland	*I. ricinus*	A	A. Barbour
IXD	United States	*I. scapularis*	A	W. Burgdorfer
M14	The Netherlands	*I. ricinus*	A	A. van den Bogaard
MAC3EMCNY86	United States	Human (skin)	A	W. Burgdorfer

Continued on following page

TABLE 1—*Continued*

Strain	Country of isolation	Biological origin	RFLP pattern	Source
NE48	Switzerland	*I. ricinus*	A	P. F. Humair
NE49	Switzerland	*I. ricinus*	A2	L. Gern
NE50	Switzerland	*I. ricinus*	A1	P. F. Humair
NE56	Switzerland	*I. ricinus*	A	P. F. Humair
PBre	Germany	Human (skin)	A	B. Wilske and V. Preac-Mursic
PKA	Germany	Human (skin)	A	B. Wilske and V. Preac-Mursic
SON328	United States	*I. pacificus*	A	D. Postic
VS2	United States	*I. scapularis*	A	OL
VS14	Switzerland	*I. ricinus*	A	OL
VS44	Switzerland	*I. ricinus*	A	OL
VS73	Switzerland	*I. ricinus*	A1	OL
VS82	Switzerland	*I. ricinus*	A1	OL
VS106	Switzerland	*I. ricinus*	A1	OL
VS108	Switzerland	*I. ricinus*	A1	OL
VS109	Switzerland	*I. ricinus*	A	OL
VS115	Switzerland	*I. ricinus*	A1	OL
VS123	Switzerland	*I. ricinus*	A	OL
VS130	Switzerland	*I. ricinus*	A	OL
VS134	Switzerland	*I. ricinus*	A1	OL
VS137	Switzerland	*I. ricinus*	A1	OL
VS139	Switzerland	*I. ricinus*	A1	OL
VS146	Switzerland	*I. ricinus*	A1	OL
VS149	Switzerland	*I. ricinus*	A1	OL
VS161	Switzerland	*I. ricinus*	A1	OL
VS206	Switzerland	*I. ricinus*	A	OL
VS215	Switzerland	*I. ricinus*	A	OL
VS219	Switzerland	*I. ricinus*	A	OL
VS293	Switzerland	*I. ricinus*	A	OL
VS393	Switzerland	*I. ricinus*	A	OL
VS396	Switzerland	*I. ricinus*	A	OL
VS405	Switzerland	*I. ricinus*	A	OL
VS423	Switzerland	*I. ricinus*	A	OL
VS619	Switzerland	*I. ricinus*	A1	OL
VS623	Switzerland	*I. ricinus*	A1	OL
VS753	Switzerland	*I. ricinus*	A	OL
B. garinii				
387	Germany	Human (CSF)	B	G. Baranton
20047	France	*I. ricinus*	B	G. Baranton
935T	Korea	*I. persulcatus*	B1	K.-J. Hwang
A19S	The Netherlands	Human (skin)	B2	A. J. van Dam
A77C	The Netherlands	Human (CSF)	B	A. J. van Dam
AR-1	The Netherlands	*I. ricinus*	B	S. Rijpkema
BB153	France	*I. ricinus*	B	D. Postic
BITS	Italy	*I. ricinus*	B	V. Sambri
FAR01	Denmark	*I. uriae*	B	A. Gylfe
FAR02	Denmark	*I. uriae*	B	A. Gylfe
FIS01	Iceland	*I. uriae*	B	A. Gylfe
G25	Sweden	*I. ricinus*	B	D. Postic
HP3	Japan	*I. persulcatus*	B	D. Postic
Ip89	CIS	*I. persulcatus*	C	G. Baranton
Ip90	CIS	*I. persulcatus*	B	S. Bergström
IUB18	Sweden	*I. uriae*	B1	A. Gylfe
M50	The Netherlands	*I. ricinus*	B	A. van den Bogaard
M63	The Netherlands	*I. ricinus*	B	A. van den Bogaard
N34	Germany	*I. ricinus*	B	G. Baranton
NBS16	Sweden	*I. ricinus*	B1	S. Bergström
NBS23a	Sweden	*I. ricinus*	B	S. Bergström
NE2	Switzerland	*I. ricinus*	nd	L. Gern
NE11H	Switzerland	*I. ricinus*	B	L. Gern
NE47	Switzerland	*I. ricinus*	B	P. F. Humair
NE51	Switzerland	*I. ricinus*	B	P. F. Humair
NE52	Switzerland	*I. ricinus*	B	P. F. Humair
NE58	Switzerland	*I. ricinus*	B	L. Gern
NE60	Switzerland	*I. ricinus*	B	L. Gern
NE83	Switzerland	*I. ricinus*	B	L. Gern
NE84	Switzerland	*I. ricinus*	B	L. Gern
NT29	Japan	*I. persulcatus*	C	G. Baranton
P/Bi	Germany	Human (CSF)	B	B. Wilske and V. Preac-Mursic
P/Br	Germany	Human (CSF)	B	B. Wilske and V. Preac-Mursic
PD89	China	Human (blood)	B	D. Postic
Pwud II	Germany	*I. ricinus*	B	B. Wilske and V. Preac-Mursic
SIKA1	Japan	*I. ovatus*	B	D. Postic
SIKA2	Japan	*I. persulcatus*	B	D. Postic
SO1	United Kingdom	*I. ricinus*	B	S. J. Cutler
T25	Germany	*I. ricinus*	B	D. Postic
TN	Germany	*I. ricinus*	B	D. Postic

Continued on following page

TABLE 1—*Continued*

Strain	Country of isolation	Biological origin	RFLP pattern	Source
VS3	Switzerland	*I. ricinus*	B	OL
VS100	Switzerland	*I. ricinus*	B	OL
VS102	Switzerland	*I. ricinus*	B	OL
VS156	Switzerland	*I. ricinus*	B	OL
VS185	Switzerland	*I. ricinus*	B	OL
VS244	Switzerland	*I. ricinus*	B	OL
VS277	Switzerland	*I. ricinus*	B	OL
VS286	Switzerland	*I. ricinus*	B	OL
VS290	Switzerland	*I. ricinus*	B	OL
VS307	Switzerland	*I. ricinus*	B	OL
VS416	Switzerland	*I. ricinus*	B	OL
VS421	Switzerland	*I. ricinus*	B	OL
VS464	Switzerland	*I. ricinus*	B	OL
VS468	Switzerland	*I. ricinus*	B	OL
VS488	Switzerland	*I. ricinus*	B1	OL
VS492	Switzerland	*I. ricinus*	B1	OL
VS518	Switzerland	*I. ricinus*	B	OL
VS600	Switzerland	*I. ricinus*	B	OL
VS641	Switzerland	*I. ricinus*	B	OL
VS704	Switzerland	*I. ricinus*	B	OL
VS711	Switzerland	*I. ricinus*	B1	OL
VSBM	Switzerland	Human (CSF)	B	OL
VSBP	Switzerland	Human (CSF)	B	OL
VSDA	Switzerland	Human (CSF)	B1	OL
B. japonica				
COW611a	Japan	*I. ovatus*	E	G. Baranton
COW611c	Japan	*I. ovatus*	E	G. Baranton
F63B	Japan	*I. ovatus*	E	G. Baranton
Fi340	Japan	*I. ovatus*	E	T. Masuzawa
FiAE2	Japan	*Apodemus speciosus*	E	T. Masuzawa
FiEE2	Japan	*Eothenomys smithi*	E	T. Masuzawa
FsAE4	Japan	*Apodemus argenteus*	E	T. Masuzawa
HO14	Japan	*I. ovatus*	E	D. Postic
IKA2	Japan	*I. ovatus*	—	D. Postic
O612	Japan	*I. ovatus*	E	G. Baranton
B. lusitaniae				
BR41	Czech Republic	*I. ricinus*	G	D. Postic
IR345	Belorussia	*I. ricinus*	G	D. Postic
POTIB1	Portugal	*I. ricinus*	G	S. Nuncio
POTIB2	Portugal	*I. ricinus*	G	S. Nuncio
POTIB3	Portugal	*I. ricinus*	H	S. Nuncio
B. valaisiana				
AG1	Switzerland	*I. ricinus*	F	I. Heinzer
AR-2	The Netherlands	*I. ricinus*	F	S. Rijpkema
F10.8.94	Germany	*I. ricinus*	F	M. M. Wittenbrink
Frank	Germany	*I. ricinus*	F	M. M. Wittenbrink
M7	The Netherlands	*I. ricinus*	F	A. P. van Dam
M19	The Netherlands	*I. ricinus*	F	A. van den Bogaard
M52	The Netherlands	*I. ricinus*	F	A. P. van Dam
M53	The Netherlands	*I. ricinus*	F	A. P. van Dam
M57	The Netherlands	*I. ricinus*	F	A. van den Bogaard
NE168	Switzerland	*I. ricinus*	F	P. F. Humair
NE218	Switzerland	*Turdus merula*	F	P. F. Humair
NE223	Switzerland	*Turdus merula*	F	P. F. Humair
NE224	Switzerland	*Turdus merula*	F	P. F. Humair
NE225	Switzerland	*Turdus merula*	F	P. F. Humair
NE226	Switzerland	*I. ricinus*	F	P. F. Humair
NE229	Switzerland	*Turdus merula*	F	P. F. Humair
NE230	Switzerland	*Turdus merula*	F	P. F. Humair
NE231	Switzerland	*Turdus merula*	F	P. F. Humair
NE248	Switzerland	*I. ricinus*	F	P. F. Humair
NE253	Switzerland	*Turdus merula*	F	P. F. Humair
UK	United Kingdom	*I. ricinus*	F	S. J. Cutler
VS116	Switzerland	*I. ricinus*	F	OL
VS732	Switzerland	*I. ricinus*	F	OL
Z6.11.93	Germany	*I. ricinus*	F	M. M. Wittenbrink
H11	Italy	Human (blood)	B	V. Sambri
B. anserina	United States	*Ornithodorus anserina*	nd	T. Schwan
B. coriaceae	United States	*Ornithodorus coriaceae*	nd	T. Schwan
B. hermsii	United States	*Ornithodorus hermsii*	nd	T. Schwan
B. parkeri	United States	*Ornithodorus parkeri*	nd	T. Schwan
B. turicata	United States	*Ornithodorus turicata*	nd	T. Schwan

[a] Abbreviations: CIS, Commonwealth of Independent States; OL, our laboratory; nd, not determined. —, no specific amplification.

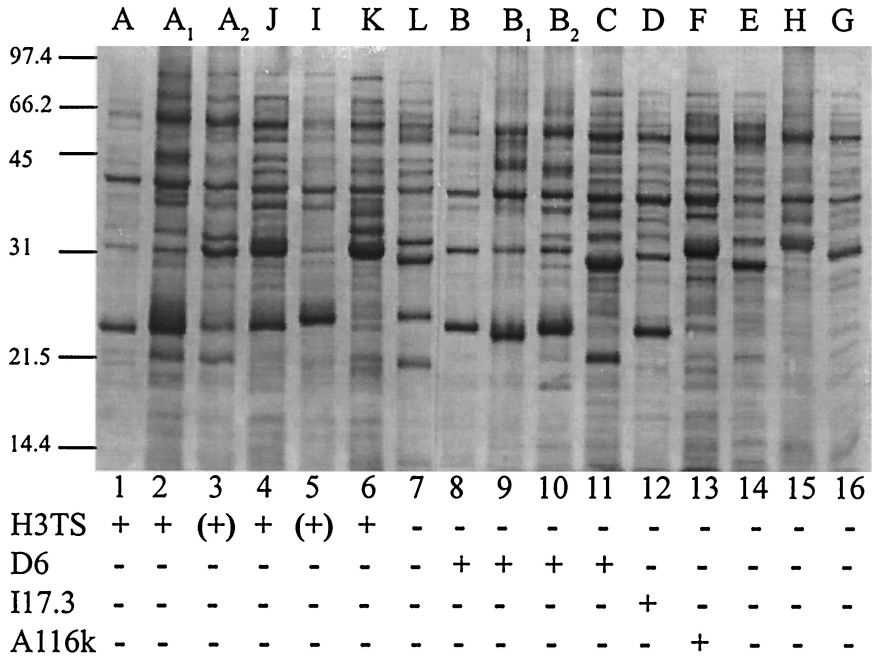

FIG. 1. (Top) Protein profiles of *B. burgdorferi* sensu lato isolates after polyacrylamide gel electrophoresis and Western blot on polyvinylidene difluoride membrane stained with Coomassie blue. Representative isolates of each *Borrelia* genospecies are ordered according to their RFLP pattern. Sizes are shown in kilodaltons. Lanes 1 to 3, *B. burgdorferi* sensu stricto (VS215, VS619, and NE49). Lanes 4 to 6, *B. bissettii* (CA128, DN127, and 25015). Lane 7, *B. andersonii* (21123). Lanes 8 to 11, *B. garinii* (VS102, VSDA, AS19s, and NT29). Lane 12, *B. afzelii* (VS461). Lane 13, *B. valaisiana* (VS116). Lane 14, *B. japonica* (FiAE2). Lanes 15 and 16, *B. lusitaniae* (PotiB3 and IR345). (Bottom) Reactivity with monoclonal antibodies. (+), weak reactivity.

washed borreliae (protein concentration, 1 mg/ml) was dissolved (1:1) in sample buffer with 0.6% sodium dodecyl sulfate (final concentration) and 50 mM dithiothreitol as a reducing agent. The samples were boiled for 5 min before undergoing electrophoresis (constant voltage, 170 V) on a polyacrylamide gel at 12.5% for the separating gel. Standards (Bio-Rad low-range protein molecular weight standards) were used as a reference for the calculation of relative molecular masses. After electrophoresis, proteins were transferred by Western blot to polyvinylidene difluoride (Immobilon; Millipore, Bedford, Mass.) membranes.

After transfer, the membrane was stained with Coomassie blue. The membrane was then cut at the level of OspA and OspB as well as below the 14.4-kDa marker, and these two pieces were destained in a bath of pure methanol for a few seconds. They were saturated with 5% gelatin in a Tris-NaCl buffer (pH 7.5) for 1 h at 37°C and washed three times for 5 min each in a Tris-Tween 20 (0.05%) buffer containing 0.1% gelatin. The pieces containing OspA and OspB were incubated for 2 h at room temperature with monoclonal antibodies H3TS (Symbicon, Stockholm, Sweden) or A116k (K. Ryffel, unpublished data) and I17.3 (kindly provided by G. Baranton) (9) diluted 1:500, 1:1,000 and 1:500,000, respectively, in the same buffer with 1% gelatin. The piece below 14.4 kDa was incubated as described above with monoclonal antibody D6 (22) diluted 1:100. After washing, monoclonal antibodies fixed specifically on the antigens were demonstrated by a second goat anti-mouse immunoglobulin for H3TS, A116k, and I17.3 monoclonal antibodies or goat anti-mouse immunoglobulin M (μ-chain specific) for D6 monoclonal antibody conjugated to alkaline phosphatase, followed by three washes and the addition of 5-bromo-4-chloro-3-indolyl *p*-toluidine phosphate and *p*-nitroblue tetrazolium chloride substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.).

At least one isolate each of *B. burgdorferi* sensu stricto, *B. garinii, B. afzelii*, and *B. valaisiana* were run in each blot as positive controls for reactivity with the monoclonal antibodies.

Genotypic typing. The method described by Postic (25) was used for typing, using the restriction pattern of amplicons in the *rrf* (5S)-*rrl* (23S) intergenic spacer region.

In short, 50 μl of reaction mixture containing 5 μl of bacterial thermolysate (95°C for 10 min) with 2 U of Extra-Pol II DNA polymerase (Eurobio, Les Ulis, France) and 200 μmole of deoxynucleoside triphosphate mix were subjected to 40 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) in a Perkin-Elmer GeneAmp PCR System 9600.

Several negative controls were included in each run as well as reference strains. Amplified products were electrophoresed on 2% agarose gels stained with ethidium bromide at 0.5 μg/ml.

A total of 210 amplicons were digested overnight at 37°C with restriction endonucleases *Mse*I (2.5 U/10 μl of PCR product), and if needed (43 amplicons) with *Dra*I (10 U/10 μl of PCR product) (Gibco-BRL, Life Technologies, Paisley, United Kingdom). The digested products were electrophoresed on 16% acrylamide-bisacrylamide (19:1) (Bio-Rad, Hercules, Calif.) for 3 h at 100 V.

To resolve particular RFLP, amplicons were purified using a Qiaquick PCR purification kit (Qiagen, Hilden, Germany). DNA sequencing was performed on a Li-Cor 4000 automated sequencer, using IRD800-labeled primers (Lincoln, Neb.) and Thermo-sequenase (Nycomed, Amersham, United Kingdom).

RESULTS

Protein profiles based on OspA and OspB allowed us to easily recognize three of the five European groups. Two of these showed both OspA and OspB with distinct electrophoretic mobility (Fig. 1). *B. burgdorferi* sensu stricto had an OspA of 31 kDa and an OspB of 34 kDa, and *B. afzelii* had an OspA of 32 kDa and an OspB of 35 kDa. The other groups presented a pattern with an OspA without an apparent OspB, also with distinct electrophoretic mobility, *B. garinii* of 32.5 kDa and *B. valaisiana* of 33 to 33.5 kDa. *B. lusitaniae* showed an OspA profile very similar to that of *B. valaisiana*, which did not provide sufficient characteristics to differentiate them. We used these distinct protein patterns to predict the reactivity of the isolates with species-specific monoclonal antibodies.

H3TS was confirmed to react specifically with OspA of all *B. burgdorferi* sensu stricto isolates expressing an OspA of 31 kDa and an OspB of 34 kDa. All 55 isolates originally classified as *B. burgdorferi* sensu stricto were reactive with H3TS. In addition, four *B. bissettii* evaluated in this study, two isolates

TABLE 2. Sensitivity and specificity of four monoclonal antibodies to 210 *B. burgdorferi* sensu lato isolates and five relapsing-fever *Borrelia* species

Antibody[a]	No. of isolates unreactive/no. tested (% specificity)	No. of isolates detected/total (% sensitivity)	Species
H3TS	156/160 (97.5)	55/55 (100)	*B. burgdorferi* sensu stricto
D6	151/151 (100)	63/64 (98.5)	*B. garinii*
I17.3	170/170 (100)	45/45 (100)	*B. afzelii*
A116k	87/87 (100)	20/24 (83.5)	*B. valaisiana*

[a] H3TS showed cross-reactivity with four isolates of *B. bissettii* and weak reactivity with isolate NE49.

from *Ixodes neotomae* (CA128 and CA55), one from *Ixodes scapularis* (25015), and one from *Ixodes pacificus* (DN 127) were also reactive (Table 2). This last isolate reacted very weakly. Isolate NE49, whose OspA and OspB profiles are typical of *B. burgdorferi* sensu stricto, showed at most very weak reactivity with H3TS. The 156 other isolates were not reactive with this monoclonal antibody.

Among the 64 isolates classified as *B. garinii* with respect to their protein profile (OspA of 32.5 kDa), only strain 935T isolated from a Korean *Ixodes persulcatus* did not react with monoclonal antibody D6. We observed that a few isolates (NT29, IP89, and A19S) presented a protein reactive with monoclonal antibody D6 of lower molecular mass (<12 kDa) than the other *B. garinii* isolates (Table 2). Monoclonal antibody D6 did not react with any of 151 isolates belonging to other species. Similarly, only the 45 isolates of *B. afzelii* were all specifically recognized by monoclonal antibody I17.3. These isolates typically had an OspA of 32 kDa and an OspB of 35 kDa (Table 2). No other of 170 isolates were reactive with this monoclonal antibody.

The specificity and sensitivity of monoclonal antibody A116k were evaluated with 111 *Borrelia* isolates, including all the *B. valaisiana* isolates available (Table 2). This monoclonal antibody appears to be specific but was not reactive with 4 of the 24 *B. valaisiana* isolates.

The four monoclonal antibodies tested did not show any reaction with *B. anserina, B. coriaceae, B. hermsii, B. parkeri, B. turicata, B. japonica, B. lusitaniae*, or *B. andersonii*.

Monoclonal antibodies D6, I17.3, and A116k showed 100% specificity, whereas H3TS had a specificity of 92.5% (12 of 13 evaluated species) or 98% with respect to all isolates examined (211 of 215). Sensitivity of 100% was observed with monoclonal antibodies H3TS (55 of 55) and I17.3 (45 of 45), 98.5% (63 of 64) with monoclonal antibody D6, and 83.5% (20 of 24) with monoclonal antibody A116k (Table 2).

One isolate (H11) was received as a mixture of two *Borrelia* species (*B. burgdorferi* sensu stricto and *B. garinii*). In the first passages, H11 was confirmed to be *B. burgdorferi* sensu stricto, and after two to four additional passages in our BSK II medium, a mixed population of *B. garinii* and *B. burgdorferi* sensu stricto was observed. In further passages, only the *B. garinii* population of spirochetes persisted. Similar results were obtained from several aliquots of an original culture that we have received. We could clearly observe the appearance of the mixed populations with the Osp profiles on the Coomassie blue staining as well as with the reactivity with monoclonal antibodies H3TS and D6.

Genotypic typing. Confirmation of the phenotypic determination was made at the genetic level. Amplicons generated by PCR in the *rrf* (5S)-*rrl* (23S) intergenic spacer region had sizes of about 250 bp (226 to 266 bp). The isolate IKA2 generated a nonspecific fragment of about 700 bp.

Several restriction patterns were observed for *B. burgdorferi* sensu stricto, *B. garinii*, and *B. lusitaniae*, including some hitherto undescribed patterns (Fig. 2 and Table 3).

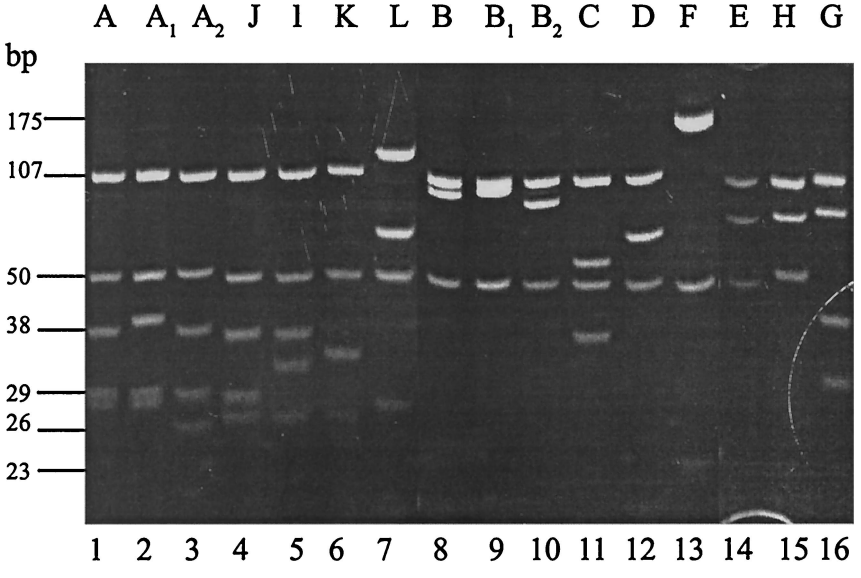

FIG. 2. *Mse*I restriction patterns of *rrf-rrl* intergenic spacer amplicons of *B. burgdorferi* sensu lato. Representative isolates of each *Borrelia* genospecies are ordered according to their RFLP pattern. Lanes 1 to 3, *B. burgdorferi* sensu stricto (VS219, VS619, and NE49). Lanes 4 to 6, *B. bissettii* (CA128, DN127, and 25015). Lane 7, *B. andersonii* (21123). Lanes 8 to 11: *B. garinii* (VS618, VSDA, AS19s, and IP89). Lane 12, *B. afzelii* (VS18). Lane 13, *B. valaisiana* (AG1). Lane 14, *B. japonica* (FiAE2). Lanes 15 and 16, *B. lusitaniae* (PotiB3 and IR345).

TABLE 3. *Mse*I and *Dra*I restriction fragments of *rrf-rrl* intergenic spacer amplicons[a]

Pattern	Sizes (bp) of fragments produced by *Mse*I	No. of isolates	Sizes (bp) of fragments produced by *Dra*I	No. of isolates
A	107, 52, 38, 29, 28	37	144, 53, 29, 28	2
A1	107, 52, 40, 29, <28	17[b]	146, 53, 29, 28	10
A2	107, 52, 38, 29, 26	1[b]	144, 80, 29	1
B	107, 95, 50	54	204, 49	4
B1	107, 98, 50	7[b]	204, 49	7
B2	107, (93–94), 50	1	204, 49	1
C	107, 57, 50, 38	2	nd	
D	107, 68, 50, 20	45	nd	
E	107, 78, 50	9	235	3
F	175, 50, (23), (7)	24	206, 49	1
G	107, 81, 39, 29	4	nd	
H	107, 79, 52, (16)	1	nd	
I	107, 52, 38, 33, 27	1	144, 53, 33, 27	1
J	107, 52, 38, 29, 27	2	144, 53, 29, 27	2
K	107, 52, 34, 27, (17), (12), (4)	1	174, 53, 27	1
L	120, 67, 52, 28	2	nd	

[a] *Dra*I fragments were defined by Postic et al. (25–27). Parentheses indicate that the fragment was not always detected in gels or of the estimated size. nd, not done.

[b] Sequence from this study and Péter et al. (24).

The majority of *B. burgdorferi* sensu stricto isolates (37 of 55, 67%) showed the typical *Mse*I pattern A, whereas 17 isolates (31%), including 13 isolates from our region (Valais, Switzerland), revealed a pattern referred to as A1 (Table 3). The isolates belonging to pattern A1 possessed a particular fragment of 40 bp instead of the 38-bp fragment. One isolate, NE49, presented a unique pattern, with a fragment of 26 bp instead of 28 bp. After *Dra*I digestion, 11 isolates with the A or A1 RFLP exhibited identical patterns, but NE49 (A2) showed three fragments of 144, 80, and 29 bp instead of four fragments of 144, 53, 29, and 28 bp.

Among the 64 *B. garinii* isolates analyzed, 54 (84%) had RFLP pattern B, identical to reference strain 20047 after digestion with *Mse*I. Two isolates showed pattern C, and seven other isolates had a particular pattern called B1, with a fragment of 98 bp instead of the expected 95-bp fragment. In one isolate from the Netherlands, A19S, a fragment of 93 to 94 bp was observed (not sequenced). This pattern was called B2. *Dra*I digestion of 12 amplicons with RFLP patterns B, B1, and B2 resulted in the same RFLP, with fragments of 204 and 49 bp, respectively.

All 45 *B. afzelii* isolates analyzed showed pattern D after digestion of amplicons with *Mse*I, although the 20-bp fragment was rarely observed in our gels. The 24 *B. valaisiana* amplicons (pattern F) consistentely showed the typical fragment of 175 bp after *Mse*I digestion, along with the other fragments, though the 7-bp fragment was never detected. *B. andersonii* isolates (pattern L) were also easily identified by their 120-bp fragment, characteristic of this *Borrelia* species, after *Mse*I digestion. The *B. lusitaniae* isolates presented two patterns, named G and H. Since the expected 16-bp fragment was not visible on our gel, pattern H was almost indistinguishable from pattern E described for *B. japonica*. The four *B. bissettii* fell within three different patterns named I, J, and K, with pattern J being close to pattern A of *B. burgdorferi* sensu stricto.

All the isolates classified by phenotypic characteristics were confirmed by genetic analysis. By RFLP analysis, we were also able to show the presence of two *Borrelia* species (*B. burgdorferi* sensu stricto and *B. garinii*) in the H11 isolate, as detected by phenotypic analysis as well.

DISCUSSION

Comparison of both phenotypic and genotypic analyses revealed an excellent agreement for isolates belonging to *B. burgdorferi* sensu stricto, *B. garinii, B. afzelii*, and *B. valaisiana*, each specifically recognized by species-specific monoclonal antibodies. Monoclonal antibody H3TS had an estimated sensitivity of 100% to *B. burgdorferi* sensu stricto, but was also reactive with some *B. bissettii* isolates. Monoclonal antibody I17.3 recognized all and only the *B. afzelii* isolates, revealing both a sensitivity and a specificity of 100%. Monoclonal antibody D6 was reactive to all *B. garinii* isolates except one isolate from Korea. Specificity was 100%, and estimated sensitivity was 98.5% (63 of 64).

The specificity of monoclonal antibody A116k was 100%, and the sensitivity was about 83.5%. The OspA to which this monoclonal antibody is reactive appears to be quite heterogeneous in *B. valaisiana* isolates, showing different electrophoretic mobilities, as reported previously (32). Based on both the electrophoretic mobility of the OspA and OspB (22) and the reactivity of monoclonal antibodies H3TS, I17.3, D6, and A116k, the phenotypic characterization of new European *Borrelia* isolates is efficient. At the regional level, we were able to define the *Borrelia* species merely from the electrophoretic profile of OspA and OspB. For example, of 50 local isolates, 48 were classified correctly, and two isolates belonging to *B. valaisiana* would not have been differentiated from *B. lusitaniae* isolates. We realize that our observation still needs confirmation by specific methods with monoclonal antibodies and further genetic analysis.

The genetic analysis by RFLP with *Mse*I, as described by Postic et al. (25, 26), is an excellent and powerful typing method for *B. burgdorferi* sensu lato isolates. Isolate NE49 was readily identified as a new subgroup in *B. burgdorferi* sensu stricto. This observation was confirmed by Postic et al. (27). All the other RFLP groups were previously described by Postic et al. (25). However, about 10% of all isolates needed a second step of digestion with the enzyme *Dra*I. Some RFLP patterns were not easily resolved, above all when a difference of ±2 bp was observed on one fragment (i.e., groups A, A1, A2, and J or groups E and H). In order to interpret this, the sequence of such amplicons will have to be determined. One additional problem arose with short restriction fragments (<20 bp). These fragments were usually not visible in our gels and thus were not informative. Only full sequencing of the amplicons allowed us to determine the original size of such small fragments.

Among all the isolates investigated, we noticed that different *B. burgdorferi* sensu lato isolates have identical names, such as M7, one isolated from *Ixodes ricinus* in the Netherlands (*B. valaisiana*), and one isolated from *I. persulcatus* in China (*B. afzelii*), or IP3, isolated from human cerebrospinal fluid (CSF) in France (*B. burgdorferi* sensu stricto) and one isolated from *I. persulcatus* in the Commonwealth of Independent States (*B. afzelii*). One *B. garinii* referred to in this study as isolate M50 (isolated from *I. ricinus* in the Netherlands) was typed as *B. valaisiana* in a previous report (32). Similarly, isolate A76S

was clearly identified as *B. afzelii* in this study but was previously described as *B. garinii* (31). We do not know whether these isolates were not initially pure and if further passages in culture medium in the two laboratories have selected two different isolates, or if these isolates were incorrectly labeled. In our hands, these isolates were never found to be a mixture of two *Borrelia* species.

Other genetic methods often used, such as DNA-DNA hybridization (3), pulsed-field electrophoresis (5), multilocus enzyme electrophoresis (7), arbitrarily primed PCR (33), specific typing of 16S rDNA (20), and species-specific hybridization (28), allow us to type *B. burgdorferi* sensu lato isolates. All these methods have confirmed the presence of these different species among *B. burgdorferi* sensu lato, with some particular geographical distribution (30). There is no doubt that the description of these different *Borrelia* species allowed us to better understand the complex natural history of Lyme borreliosis in Europe. For example, different reservoir hosts were described for some *Borrelia* species (13–15). Similarly, several reports have suggested an association of particular clinical symptoms in human with some *Borrelia* species (1, 2, 10, 24, 29). The typing of *B. burgdorferi* sensu lato isolates is essential to clarify this specific point and consequently to understand the physiopathology of each *Borrelia* species (12, 17, 21).

Our results have shown that some *B. burgdorferi* sensu lato isolates cannot be easily typed by genetic methods and need cumbersome techniques. In this respect, monoclonal antibodies may greatly help to type closely related isolates at one particular point of the genome. Therefore, phenotypic and genotypic methods appear to be complementary. Phenotypic methods, particularly monoclonal antibodies, are helpful epidemiological tools that may be essential for laboratories which lack facility in genetic methods.

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