Borrelia burgdorferi Genotype Predicts the Capacity for Hematogenous Dissemination during Early Lyme Disease

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Background. Lyme disease, the most common tickborne disease in the United States, is caused exclusively by *Borrelia burgdorferi* sensu stricto in North America. The present study evaluated the genotypes of >400 clinical isolates of *B. burgdorferi* recovered from patients from suburban New York City with early Lyme disease associated with erythema migrans; it is the largest number of borrelial strains from North America ever to be investigated.

Methods. Genotyping was performed by restriction fragment–length polymorphism polymerase chain reaction analysis of the 16S–23S ribosomal RNA spacer and reverse line blot analysis of the outer surface protein C gene *(ospC)*. For some isolates, DNA sequence analysis was also performed.

Results. The findings showed that the 16S–23S ribosomal spacer and *ospC* are in strong linkage disequilibrium. Most *B. burgdorferi* genotypes characterized by either typing method were capable of infecting and disseminating in patients. However, a distinct subset of just 4 of the 16 *ospC* genotypes identified were responsible for >80% of cases of early disseminated Lyme disease.

Conclusions. This study identified the *B. burgdorferi* genotypes that pose the greatest risk of causing hematogenous dissemination in humans. This information should be considered in the future development of diagnostic assays and vaccine preparations.

Lyme disease is a tickborne zoonosis that occurs globally in the Northern Hemisphere [1, 2]. In North America, Lyme disease appears to be caused exclusively by *Borrelia burgdorferi* sensu stricto, whereas in Europe other species of *Borrelia* predominate [3–6]. A variety of typing systems exist to distinguish genetically distinct strains of *B. burgdorferi* [3]. On the basis of restriction fragment–length polymorphism (RFLP) of the 16S–23S rRNA intergenic spacer, *B. burgdorferi* has been classified into 3 distinct genetic subgroups (herein called genotypes), arbitrarily called ribosomal spacer type (RST) 1, RST2, and RST3 [7, 8]. Greater separation into distinct genotypes is achievable on the basis of the sequence heterogeneity of the outer surface protein C gene *(ospC)* [9, 10]. Genetically distinct genotypes of *B. burgdorferi* appear to differ ecologically and epidemiologically [11–14], suggesting that genotype classification is relevant to understanding the basic biology of the spirochete.

Differential pathogenicity of *B. burgdorferi* on the basis of genotype has been reported in several studies. For example, in an analysis of 104 borrelial strains recovered from the skin of patients with a diagnosis of erythema migrans in Westchester County, New York, it was found that patients with disseminated infection (i.e., positive blood culture and/or multiple erythema migrans skin lesions) were at least 5 times more likely to have been infected with RST1 strains of *B. burgdorferi* than with RST3 strains [15]. A subsequent study of patients from Connecticut and Rhode Island with erythema migrans similarly found that infections believed to have disseminated were 5 times more common among RST1-infected patients compared with those infected with RST3 strains of *B. burgdorferi* [16]. In a study from New York State, dissemination of *B. burgdorferi* to blood or cerebrospinal fluid (CSF) was exclusively associated

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with *ospC* genotypes A, B, I, or K [9]. Subsequent studies from other geographic areas, however, have suggested that hematogenous dissemination is not restricted to just these 4 *ospC* genotypes [16–18].

The present study was undertaken to evaluate the relationship between RST and *ospC* genotypes and to examine the association between specific genotypes and objective evidence of dissemination of the spirochete in patients, using the largest sample of clinical isolates of *B. burgdorferi* studied to date. In addition, the frequency distribution of RST and *ospC* genotypes in the skin or blood of patients with culture-confirmed erythema migrans was compared with that present in local tick populations.

METHODS

Subjects, clinical specimens, and cultures. All human subjects were adults with erythema migrans enrolled in prospective studies at the Lyme Disease Practice of the Westchester Medical Center between 1991 and 2005. This practice serves patients in suburban New York City who live or work in the lower Hudson Valley of New York State. Specimens from skin, whole blood, serum, or plasma were collected and cultured as described elsewhere [15, 19].

Typing of* B. burgdorferi *strains. *B. burgdorferi* DNA was isolated from low-passage (1–5) cultures using a nucleic acid extraction kit (IsoQuick; Orca Research). A 941-bp fragment of the 16S–23S intergenic spacer was amplified by polymerase chain reaction (PCR) using primers PA and P95, as described elsewhere [7]. PCR-based RFLP analyses of the 16S–23S intergenic spacer were performed using the restriction enzyme *Tru*1I (Fermentas) [8, 20]. A 522-bp region of *ospC* was amplified by PCR using external primers OC6(+) and OC623(−) and internal primers OC6(+Fluo) and OC602(−) [11, 21]. Amplicons were then probed with *ospC* type–specific probes by reverse line blot [11, 21]. The *ospC* amplicons that did not hybridize with any *ospC* type–specific probes were reamplified and sequenced in both directions (Genewiz) using either primer set *ospC*-N/*ospC*-C [5] or OC6(+)/OC623(−). Isolates that produced ambiguous sequence results were cloned by limiting dilution, and sequence analyses were performed on 2 clones from each isolate. Some of the isolates in the present study have been reported in the context of other investigations of the pathogenicity of particular genotypes of *B. burgdorferi* [9, 15, 22–24]. The *ospC* typing of *B. burgdorferi* in extracts from infected ticks was done by reverse line blotting, essentially as described above and elsewhere [11, 21].

Statistical analyses. Differences in the frequency distributions of genotypes were assessed using a χ^2 analysis [25]. *P* values were estimated using a Monte Carlo simulation with 100,000 replicates when the marginal values summed to <5, making the χ^2 distribution inappropriate [26]. The Bonferroni correction for multiple tests ($P = .05$/number of tests) was applied where appropriate in determining statistical significance [25]. Analyses were performed in R [27].

RESULTS

Genotyping of* B. burgdorferi *isolates from patients with early Lyme disease. *B. burgdorferi* isolates were obtained from either the skin or blood of 374 adult patients with erythema migrans evaluated at the Lyme Disease Practice of the Westchester Medical Center. RST genotyping was performed on 462 isolates (319 from skin and 143 from blood), and *ospC* genotyping was performed on 432 isolates (298 from skin and 134 from blood). At least 16 skin cultures and at least 5 blood cultures grew more than a single genotype of *B. burgdorferi,* on the basis of RST and/or *ospC* genotyping. Among the 94 patients infected with only a single genotype in skin and for whom a blood culture was positive and the isolates typed, the same genotype of *B. burgdorferi* grew from blood in 82 (87.2%). The blood culture yielded a different genotype for the other 12 patients; for 9 patients a single genotype was obtained from blood and for 3 patients 2 genotypes were obtained, only 1 of which was the same genotype as that recovered from skin. Patients for whom >1 genotype of *B. burgdorferi* was cultured on the basis of a particular genotyping method were excluded from the analyses described below.

Association between specific* B. burgdorferi *genotypes and dissemination. In this article the terms "invasive infection" and "disseminated infection" are used interchangeably. The frequency distribution of RST genotypes of *B. burgdorferi* strains recovered from either blood or skin during 1991–1996 did not significantly differ from those recovered during 1997–2005, when the high-volume plasma culture method [15] was performed routinely (data not shown). Therefore, all analyses reported here are for the entire 15-year time interval.

All 3 RST genotypes and at least 12 *ospC* genotypes were capable of hematogenous dissemination. However, the frequency distribution of RST genotypes among strains of *B. burgdorferi* recovered from skin differed from that among strains recovered from blood (Pearson's χ^2 test, $\chi^2 = 8.8$ and $P = .01$) (table 1). Table 2 shows a similar result based on *ospC* typing (Pearson's χ^2 test, $\chi^2 = 27.4$ and $P = .017$).

The relationship between RST and *ospC* genotypes among 422 isolates of *B. burgdorferi* obtained from clinical specimens of skin or blood was analyzed (table 2). The results indicate that the 16S–23S intergenic spacer region is in strong linkage disequilibrium with the gene encoding OspC. Each RST genotype was associated with 2 or more *ospC* genotypes. RST1 corresponded uniquely to *ospC* genotypes A and B; RST2 only to *ospC* genotypes F, H, K, and N; and RST3 to the remaining 10 *ospC* genotypes found among the strains that were evaluated (C, D, E, G, I, J, M, O, T, and U).

The prevalence among patients with a positive blood culture or multiple erythema migrans skin lesions was evaluated specif-

Table 1. Frequency distribution of ribosomal spacer type (RST) genotypes of *Borrelia burgdorferi* in skin and blood cultures from patients from suburban New York City with erythema migrans evaluated between 1991 and 2005.

RST genotype	No. (%)	
	Skin cultures	Blood cultures
1	86 (28.2)	52 (37.4)
2	133 (43.6)	65 (46.8)
3	86 (28.2)	22 (15.8)
Total	305 (100)	139 (100)

ically for 249 patients with a positive skin culture in whom both blood and skin cultures were performed at the time of presentation. More than half of these patients (51.4%) had evidence of disseminated infection (either a positive blood culture or multiple erythema migrans skin lesions). An association was seen between RST genotype and the presence of disseminated infection (Pearson's χ^2 test, $\chi^2 = 10.6$ and $P = .005$) (table 3). The presence of spirochetemia (a positive blood culture) was also contingent on RST genotype (Pearson's χ^2 test, $\chi^2 = 7.9$ and $P = .02$). In both circumstances, RST1 was overrepresented in blood, and RST3 was underrepresented. Although the frequency of having multiple erythema migrans skin lesions varied across RST genotypes, there was no association between the presence of multiple erythema migrans skin lesions and RST type (Pearson's χ^2 test, $\chi^2 = 0.9$ and $P = .69$). The frequency distribution of *ospC* genotypes among total infections differed from the distribution among disseminated infections (Pearson's χ^2 test, $\chi^2 = 43.7435$ and $P < .001$) and from the distribution among either patients with spirochetemia (Pearson's χ^2 test, $\chi^2 = 37.8098$ and $P < .001$) or patients with multiple erythema migrans skin lesions (Pearson's χ^2 test, $\chi^2 = 28.7819$ and $P = .02$). All differences were statistically significant after correction for 2 posthoc tests ($\alpha = .025$).

Several *ospC* genotypes represented within the RST3 genotype (*ospC* genotypes C, D, J, O, and T) were infrequently recovered from either skin or blood specimens (table 2). When the analysis was restricted to those *ospC* genotypes represented by at least 5 skin isolates of *B. burgdorferi*, it was clear that the most invasive *ospC* genotypes were I, A, H, and B, each of which was associated with disseminated infection in >50% of patients infected in the skin with the same genotype (table 4). Both of the *ospC* genotypes within the RST1 genotype were highly invasive, whereas only genotype H within RST2 was comparably invasive. Among the *ospC* genotypes within the RST3 genotype, only *ospC* genotype I was frequently associated with dissemination, and no RST3-associated *ospC* genotypes were cultivated from blood more than once, except for genotypes I and C. Indeed, cutaneous infection due to *ospC* genotypes D, O, T, and U was never associated with either spirochetemia or the presence of multiple erythema migrans skin lesions. Although *ospC* genotype I was the single most commonly isolated genotype in the RST3 genotype, it comprised only 24.4% of the 82 RST3 strains recovered from skin.

B. burgdorferi *genotype frequency in ticks versus patients with early Lyme disease.* *Ixodes scapularis* nymphs transmit *B. burgdorferi* to the majority of patients with Lyme disease in the northeastern United States. Therefore, we attempted to compare the frequency distribution of RST and *ospC* genotypes of *B. burgdorferi* among patients with that found among questing *I. scapularis* nymphs collected from the same region (lower Hudson Valley, New York) during an overlapping time period (2002–2004) [11, 28]. Because the frequency distribution of RST and *ospC* genotypes did not differ significantly among nymphs between years (Pearson's χ^2 test, $\chi^2 = 30.1$ and $P = .45$), tick data from 2002–2004 were pooled. The *ospC* genotype in these ticks was determined on the basis of direct PCR testing of the ticks; the RST genotype was not determined directly, but, given the strong association between the RST locus and *ospC* alleles demonstrated above and in other publications [29–31], the RST genotype was inferred from the *ospC* genotype. Overall, the frequency distribution observed among ticks differed significantly from

Table 2. Frequency distribution of *ospC* genotypes of *Borrelia burgdorferi* in skin and blood cultures from patients from suburban New York City with erythema migrans evaluated between 1991 and 2005.

RST, *ospC* genotype	No. (%)	
	Skin cultures	Blood cultures
1		
A	46 (15.9)	31 (23.5)
B	37 (12.8)	19 (14.4)
2		
F	9 (3.1)	2 (1.5)
H	13 (4.5)	7 (5.3)
K	86 (29.7)	44 (33.3)
N	17 (5.9)	7 (5.3)
3		
C	2 (0.7)	2 (1.5)
D	4 (1.4)	0 (0)
E	14 (4.8)	1 (0.8)
G	14 (4.8)	1 (0.8)
I	20 (6.9)	16 (12.1)
J	3 (1.0)	1 (0.8)
M	11 (3.8)	1 (0.8)
O	1 (0.3)	0 (0)
T	2 (0.7)	0 (0)
U	11 (3.8)	0 (0)
Total	290 (100)	132 (100)

NOTE. RST, ribosomal spacer type.

Table 3. Frequency of disseminated infection (positive blood culture and/or multiple erythema migrans [EM] skin lesions) by ribosomal spacer type (RST) genotype among patients from suburban New York City with EM whose skin culture was positive for *Borrelia burgdorferi*.

RST genotype	Total no.	No. (%)		
		Blood culture positive	Multiple EM	Blood culture positive and/or multiple EM
1	70	40 (57.1)	20 (28.6)	46 (65.7)
2	113	45 (39.8)	26 (23.0)	57 (50.4)
3	66	23 (34.8)	15 (22.7)	25 (37.9)
Total	249	108 (43.4)	61 (24.5)	128 (51.4)

that found in either skin or blood for both RST (Pearson's χ^2 test for ticks vs. blood, $\chi^2 = 32.3$ and $P < .001$; Pearson's χ^2 test for ticks vs. skin, $\chi^2 = 17.4$ and $P < .001$) and *ospC* genotype (Pearson's χ^2 test for ticks vs. blood, $\chi^2 = 93.7$ and $P < .001$; Pearson's χ^2 test for ticks vs. skin, $\chi^2 = 66.0$ and $P < .001$) (table 5). RST1 strains increased in frequency from 22.8% among infected ticks to 28.6% of skin isolates and 37.9% of blood isolates. RST2 strains were found in 33.7% of infected ticks, compared with 43.1% of skin isolates and 45.5% of blood isolates. The frequency of RST3 strains decreased steadily from 43.5% among infected ticks to 28.3% of skin isolates and 16.7% of blood isolates. Five *ospC* genotypes (A, B, C, I, and K) showed a progressive increase in frequency from ticks to skin to blood, whereas 7 *ospC* genotypes (D, E, F, G, J, M, and T) showed a progressive decrease; 4 showed no consistent pattern (table 5).

Table 4. Frequency of disseminated infection (positive blood culture and/or multiple erythema migrans [EM] skin lesions) by *ospC* genotype among patients from suburban New York City whose skin culture was positive for *Borrelia burgdorferi*.

RST, *ospC* genotype	Total no.	No. (%)		
		Blood culture positive	Multiple EM	Blood culture positive and/or multiple EM[a]
1				
A	38	21 (55.3)	15 (39.5)	27 (71.1)
B	26	14 (53.8)	5 (19.2)	14 (53.8)
2				
F	7	1 (14.3)	0 (0)	1 (14.3)
H	11	6 (54.5)	4 (36.4)	6 (54.5)
K	72	26 (36.1)	14 (19.4)	32 (44.4)
N	10	3 (30.0)	3 (30.0)	4 (40.0)
3				
C	2	2 (100)	0 (0)	2 (100)
D	3	1 (33.3)	0 (0)	1 (33.3)
E	8	1 (12.5)	1 (12.5)	1 (12.5)
G	9	2 (22.2)	1 (11.1)	2 (22.2)
I	13	10 (76.9)	8 (61.5)	11 (84.6)
J	1	0 (0)	0 (0)	0 (0)
M	7	1 (14.3)	1 (14.3)	1 (14.3)
O	1	0 (0)	0 (0)	0 (0)
T	2	0 (0)	0 (0)	0 (0)
U	11	0 (0)	0 (0)	0 (0)
Total	221	88 (39.8)	52 (23.5)	102 (46.2)

NOTE. RST, ribosomal spacer type.

[a] Patients for whom a blood culture was not performed or whose blood culture was contaminated were excluded.

Table 5. Comparison of the frequency distribution of different genotypes of *Borrelia burgdorferi* among infected nymphal stage *Ixodes scapularis* ticks with that found in the skin or blood of infected patients.

RST, *ospC* genotype	No. (%)		
	Nymphal ticks[a]	Skin cultures	Blood cultures
1			
A	59 (13.1)	46 (15.9)	31 (23.5)
B	44 (9.8)	37 (12.8)	19 (14.4)
2			
F	31 (6.9)	9 (3.1)	2 (1.5)
H	24 (5.3)	13 (4.5)	7 (5.3)
K	88 (19.5)	86 (29.7)	44 (33.3)
N	9 (2.0)	17 (5.9)	7 (5.3)
3			
C	0 (0)	2 (0.7)	2 (1.5)
D	40 (8.9)	4 (1.4)	0 (0)
E	30 (6.7)	14 (4.8)	1 (0.8)
G	25 (5.5)	14 (4.8)	1 (0.8)
I	15 (3.3)	20 (6.9)	16 (12.1)
J	9 (2.0)	3 (1.0)	1 (0.8)
M	39 (8.6)	11 (3.8)	1 (0.8)
O	1 (0.2)	1 (0.3)	0 (0)
T	23 (5.1)	2 (0.7)	0 (0)
U	14 (3.1)	11 (3.8)	0 (0)
Total	451 (100)	290 (100)	132 (100)

NOTE. RST, ribosomal spacer type.

[a] Nymphs collected from suburban New York City in Dutchess County, New York (lower Hudson Valley), in 2002–2004, as reported elsewhere [11, 28].

DISCUSSION

This investigation involved an analysis of the largest collection of clinical isolates of *B. burgdorferi* from the United States ever studied. *B. burgdorferi* can be subdivided into 3 distinct genotypes on the basis of an RFLP typing system of the 16S–23S rRNA intergenic spacer region and into at least 16 different *ospC* genotypes. Each of the *ospC* genotypes is in strong linkage disequilibrium with a specific RST genotype. For example, all of the 130 strains of *ospC* genotype K that were analyzed fell into the RST2 genotype, and all of the 77 strains of *ospC* genotype A were typed as RST1. These findings confirm and extend the findings of another study with fewer specimens [16] and support those of other investigators who demonstrated that isolates of *B. burgdorferi* are mostly clonal [32, 33].

The findings of the present study demonstrate that certain genotypes of *B. burgdorferi* are more invasive than others and clarify the role of each of the typing systems evaluated in identifying highly invasive strains. A significant association was observed between RST genotype and disseminated infection, with RST3 infections being the least invasive and RST1 infections the most (table 3). However, one of the *ospC* genotypes comprising the RST3 genotype, genotype I, was highly invasive. This *ospC* genotype was associated with the highest prevalence of both spirochetemia (76.9%) and multiple erythema migrans skin lesions (61.5%) compared with any of the other relatively common *ospC* genotypes, irrespective of RST genotype (table 4). Thus, of the 2 typing systems evaluated here, the *ospC* genotyping system was the more informative for defining the risk of disseminated *B. burgdorferi* infection in humans.

Note that the 16S–23S rRNA intergenic spacer is a noncoding sequence that should not contribute to pathogenicity but rather must be linked to pathogenicity-determining loci. In contrast, OspC is required for migration of spirochetes from the tick midgut to the salivary glands and/or for establishing initial infection in a mammal [34, 35]. However, given the observed linkage disequilibrium between the 16S–23S rRNA intergenic spacer and *ospC* (the former located on the chromosome and the latter on a circular plasmid), it is possible that *ospC* is also in linkage with other loci that are required for dissemination.

An earlier study that included fewer clinical isolates concluded that only *ospC* genotypes A, B, I, and K are invasive [9]. The present study demonstrates that, in fact, most *ospC* genotypes can cause spirochetemia on occasion, but the most invasive *ospC* genotypes in humans, in descending order, were I, A, H, and B. However, because genotype K infections were more common than infections due to other genotypes, this genotype was the one most frequently associated with disseminated infection in patients (tables 4 and 6); conversely, genotype H infections were generally infrequent and therefore were responsible for dissemination in a much smaller number of cases (6 cases of disseminated infection for genotype H vs. 32 for genotype K). Genotype H is comparatively rare among both ticks and primary skin infections (table 5). Therefore, in our geographic area, the 4 *ospC* genotypes A, B, I, and K comprise >80% of the cases of culture-confirmed early Lyme disease associated with spirochetal dissemination, although they comprise only 45.7% of the borrelial strains found in representative nymphal tick populations from the lower Hudson Valley. A limitation of our study is

Table 6. Hierarchical analysis of invasive *Borrelia burgdorferi* infection among patients from suburban New York City with erythema migrans.

ospC genotype	RST genotype	Patients with proven dissemination,[a] %
K	2	31.4
A	1	26.5
B	1	13.7
I	3	10.8

NOTE. Findings were calculated from the data shown in table 4. RST, ribosomal spacer type.

[a] Proven on the basis of positive blood culture and/or multiple erythema migrans skin lesions.

that, for several *ospC* genotypes, the number of infected patients was too small for us to evaluate invasive capability confidently. Nevertheless, our findings have potential implications for inclusion of particular *ospC* antigen(s) in diagnostic assays that incorporate this antigen [36] and for identifying highly clinically relevant *Borrelia* strains to evaluate the efficacy of experimental Lyme disease vaccines.

In agreement with other studies [9, 24], our data also suggest that there is differential pathogenicity for most strains of *B. burgdorferi,* not only between skin and blood but also between *I. scapularis* nymphs and skin. Of the common *ospC* genotypes, most within the RST3 genotype (except for I and U) and 2 of the 4 *ospC* genotypes within RST2 (F and H) were underrepresented in skin compared with their frequency in regional ticks, suggesting that these are the least pathogenic strains of *B. burgdorferi* for humans. These results should be viewed cautiously, however, because the data on the frequency of *B. burgdorferi* genotypes among the field-collected ticks considered in our analysis may not be representative of the actual ticks parasitizing humans in our region during the entire duration of this study. In addition, identification of infections in ticks was based on PCR of tick material, whereas identification of infections in patients was based on PCR of cultured isolates of *B. burgdorferi*. In our experience, cultures of skin biopsy specimens are negative in ~45% of patients with a clinical diagnosis of erythema migrans who can be shown to have *B. burgdorferi* infection on the basis of PCR and/or serologic evidence [37]. The strains of *B. burgdorferi* that appear to be underrepresented in skin relative to ticks may have been responsible for infection in such culture-negative cases. In addition, patients with coinfection in skin were excluded from this study and in general may be underappreciated by culture; therefore, failure to recognize or consider coinfection could be another factor contributing to the apparent underrepresentation of certain strains of *B. burgdorferi* in skin relative to ticks. Strains of *B. burgdorferi* that grow least well in culture would be systematically underrepresented in studies based on culture [8]. Alternatively, it is theoretically possible that *B. burgdorferi* strains that appear to be underrepresented in skin may have disseminated without causing erythema migrans. In certain European studies, it has been observed that strains of *Borrelia garinii* are underrepresented in cultures of erythema migrans relative to the frequency with which this species can be demonstrated in regional ticks but overrepresented in cases of neuroborreliosis associated with a positive CSF culture [38].

In summary, our findings establish that the 16S–23S rRNA intergenic spacer region and *ospC* are in strong linkage disequilibrium owing to the clonal nature of *B. burgdorferi* clinical isolates. Additionally, the study further strengthens the concept that a distinct subset of *B. burgdorferi* genotypes is responsible for the vast majority of cases of early disseminated Lyme disease in suburban New York City. Thus, although most *B. burgdorferi* genotypes are capable of producing human infection, several factors appear to contribute to the ability of specific *B. burgdorferi* genotypes to cause disseminated infection. These include the prevalence of the specific genotype among the local nymphal tick population, the transmissibility of a particular genotype from tick to human skin, and the ability of that genotype to disseminate from the initial site of skin infection to blood. It should be noted that the present study focused on hematogenous dissemination. Little is currently known regarding the relationship between *B. burgdorferi* genotype and invasion of sites such as CSF or joints. Classifying strains of *B. burgdorferi* into 1 of 3 RST genotypes or 1 of 16 *ospC* genotypes is useful for identifying those strains that pose the greatest risk of hematogenous dissemination. Furthermore, these findings should be considered in the future development of diagnostic assays and vaccine preparations.

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