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Differentiation of Reinfection from Relapse in Recurrent Lyme Disease

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ABSTRACT

BACKGROUND

Erythema migrans is the most common manifestation of Lyme disease. Recurrences are not uncommon, and although they are usually attributed to reinfection rather than relapse of the original infection, this remains somewhat controversial. We used molecular typing of *Borrelia burgdorferi* isolates obtained from patients with culture-confirmed episodes of erythema migrans to distinguish between relapse and reinfection.

METHODS

We determined the genotype of the gene encoding outer-surface protein C (*ospC*) of *B. burgdorferi* strains detected in cultures of skin or blood specimens obtained from patients with consecutive episodes of erythema migrans. After polymerase-chain-reaction amplification, *ospC* genotyping was performed by means of reverse line-blot analysis or DNA sequencing of the nearly full-length gene. Most strains were further analyzed by determining the genotype according to the 16S–23S ribosomal RNA intergenic spacer type, multilocus sequence typing, or both. Patients received standard courses of antibiotics for erythema migrans.

RESULTS

B. burgdorferi isolates obtained from 17 patients who received a diagnosis of erythema migrans between 1991 and 2011 and who had 22 paired episodes of this lesion (initial and second episodes) were available for testing. The *ospC* genotype was found to be different at each initial and second episode. Apparently identical genotypes were identified on more than one occasion in only one patient, at the first and third episodes, 5 years apart, but different genotypes were identified at the second and fourth episodes.

CONCLUSIONS

None of the 22 paired consecutive episodes of erythema migrans were associated with the same strain of *B. burgdorferi* on culture. Our data show that repeat episodes of erythema migrans in appropriately treated patients were due to reinfection and not relapse. (Funded by the National Institutes of Health and the William and Sylvia Silberstein Foundation.)

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Erythema migrans, the most common clinical manifestation of Lyme disease, is due to cutaneous infection with *Borrelia burgdorferi*.[1] Erythema migrans can disappear and then relapse in untreated patients,[2] or it may transiently improve and then worsen in those who receive antibiotics that lack activity against this bacterium.[3] One or more recurrences of this skin lesion may also develop in patients after appropriate antibiotic treatment; recurrence was observed in approximately 15% of patients who were followed for 5 years in one study conducted in the United States.[4]

Available clinical and epidemiologic data suggest that most recurrences after recommended courses of antibiotic therapy are the result of a new tick-transmitted infection with *B. burgdorferi*, rather than relapse of the original infection.[5,6] Microbiologic evidence that recurrent episodes of Lyme disease are due to reinfection rather than relapse has been provided in anecdotal reports involving two patients in whom genotypically different strains of *B. burgdorferi* were detected in cultures of skin-biopsy specimens obtained during each of the separate episodes of erythema migrans.[7,8] Nonetheless, some persons have attributed recurrent episodes of erythema migrans to relapses in patients treated with recommended courses of antibiotic therapy; they cited experiments in animals that showed persistence of *B. burgdorferi* despite antibiotic treatment.[9]

The outer-surface protein C (OspC) of *B. burgdorferi* is expressed in early infection.[10] At least 19 distinct *ospC* genotypes have been shown to be associated with clinical disease in the United States.[11,12] To investigate whether recurrences of erythema migrans are due to reinfection or relapse with the same strain of *B. burgdorferi*, we systematically analyzed the *ospC* genotypes in patients with two or more consecutive, culture-confirmed episodes of erythema migrans. Strains of *B. burgdorferi* were further analyzed by determining the genotype according to the 16S–23S ribosomal RNA (rRNA) intergenic spacer type, multilocus sequence typing, or both.

METHODS

PATIENTS, CLINICAL SPECIMENS, AND CULTURES

All patients were adults with erythema migrans who had enrolled in prospective studies approved by the institutional review board at New York Medical College. The patients provided written informed consent at the Lyme Disease Diagnostic Center of New York Medical College between 1991 and 2011. Specimens of skin and blood were obtained and cultured for *B. burgdorferi* as described previously.[13,14] Patients were treated with standard courses of antibiotics[15] at each episode of erythema migrans, with subsequent resolution of the skin lesion or lesions.

DETERMINATION OF GENOTYPES

B. burgdorferi DNA was isolated from low-passage cultures (1 to 5 passages) with the use of a nucleic acid extraction kit (IsoQuick, Orca Research). A 522-bp region of *ospC* was amplified by means of seminested polymerase chain reaction (PCR) with the use of external primers OC6(+) and OC623(–) and internal primers OC6(+Fluo) (a fluorescein label was added to the 5′ end) and OC602(–).[16,17] Amplicons were then probed with *ospC* type-specific probes by means of reverse line blot.[16,17] Alternatively, *ospC* was amplified by means of PCR as described above or with the primer set *ospC*-N/*ospC*-C,[18] and amplicons were sequenced in both directions (Genewiz). Isolates with ambiguous sequence results were cloned by means of a limiting-dilution technique; sequence analyses were performed on two clones from each isolate.

In addition to determination of the *ospC* genotype, a 941-bp fragment of the 16S–23S rRNA intergenic spacer was amplified by means of PCR with the use of primers PA and P95.[11] Analyses of PCR-based rRNA intergenic spacer types were performed with the use of the restriction enzyme Tru1I (Fermentas).[11]

Some isolates obtained from patients were subjected to multilocus sequence typing analysis.[19] Sequences for eight individual housekeeping genes were assigned allele numbers, and on the basis of allelic profiles for these genes, isolates were assigned a sequence type according to the multilocus-sequence-typing database.

COMPARISON OF *OSPC* GENOTYPES IN CONSECUTIVE EPISODES OF ERYTHEMA MIGRANS

We identified patients with recurrent erythema migrans and cultures of skin-biopsy specimens, blood cultures, or both that grew *B. burgdorferi* on more than one occasion. Of these patients, we selected for inclusion all patients with *ospC* genotypes that were identified from two or more consecutive episodes of erythema migrans.

The *ospC* genotypes were compared in each pair of consecutive episodes of erythema migrans. We focused on paired consecutive, rather than nonconsecutive, episodes of erythema migrans, because this would logically provide the greatest likelihood of recognizing a microbiologic relapse of infection.

STATISTICAL ANALYSIS

Under the assumptions that the rate of coinfection may be underestimated if it is based on culture results and that coinfection with *n* strains (indicating overall number) of *B. burgdorferi* was initially present in every patient in our study, we calculated the probability that the genotype isolated during the second episode in a paired episode would always differ from the genotype determined during the first episode. It was further assumed, in the event of a relapse of infection, that any of the originally present genotypes were equally likely to be cultured during the second episode (i.e., 1/n of the time). Thus, the probability that, in a putative relapse of infection, a genotype determined during the second episode would differ from that isolated during the first episode for all paired episodes would be $(1-[1/n])^x$, where *x,* an exponent, is the number of paired episodes of erythema migrans.

RESULTS

Twenty-four paired consecutive episodes of erythema migrans were identified in which there was a positive culture of skin or blood for *B. burgdorferi* from both episodes of the pair. Two pairs were excluded from this analysis because borrelial isolates were not available on which to perform *ospC* genotyping. In the remaining 22 paired episodes in 17 patients, *ospC* genotypes were determined and compared (Table 1). Three patients had 2 paired episodes (3 consecutive episodes each) of erythema migrans, and one patient had 3 paired episodes (4 consecutive episodes in total).

The patients, all of whom were believed to have acquired the infections in the Lower Hudson Valley region of New York State, included nine men and eight women, with a median age of 47 years (range, 27 to 80 at the time of the first episode of erythema migrans). In paired episodes of erythema migrans, the second episode occurred from 1 to 15 years (median, 4) after the first episode. The repeat episodes occurred during April, May, June, July, or August (Fig. 1). In 6 of 22 paired episodes (27%), the patient recalled a tick bite at the site of the recurrent erythema migrans within 30 days before this skin lesion developed.

None of the skin or blood cultures grew more than one *ospC* genotype of *B. burgdorferi.* However, in two paired episodes (episodes 1 and 8), the genotype of the skin isolate differed from the genotype of the blood isolate that was obtained at the same time (Table 1).

The same *ospC* genotype was not identified during both episodes of erythema migrans in any of the 22 paired consecutive episodes in any patient (Table 1). Determination of the 16S–23S rRNA intergenic spacer type, multilocus sequence typing genotype, or both of at least one isolate of *B. burgdorferi* obtained from each episode in all 22 paired episodes confirmed that the strains were genotypically distinct. In one patient who had 3 paired episodes (episodes 10 through 12) (Table 1), the same genotype was isolated at the first and third episodes of erythema migrans (on his right ankle and right buttock, respectively); these episodes occurred 5 years apart (Table 2 and Fig. 2).

If it were assumed that every patient had coinfection with two different genotypes at the first episode of erythema migrans, antibiotic failure as the explanation for the findings would be unlikely, since the probability that a genotype that differed from the original isolate would be detected by chance in each of the 22 paired consecutive episodes of erythema migrans was calculated to be 0.0000002. Indeed, the probability of the detection of a different genotype by chance alone is less than 0.01, even if every patient were initially coinfected with five different genotypes.

Our study results cannot be attributed to an inadvertent focus on patients with less severe infection. During the 22 paired episodes of erythema migrans, systemic symptoms such as fever, arthralgias, headache, or fatigue were present during the first episode of the pair in 18 of 22 episodes (82%). In addition, evidence of disseminated infection was present in 13 of 22 episodes (59%) on the basis of a positive blood culture in 11 of 22 episodes (50%), multiple skin lesions in 8 of 22 episodes (36%), or both a positive blood culture and multiple skin lesions in 6 of 22 episodes (27%).

Furthermore, our findings do not support the hypothesis that relapses in antibiotic-treated pa-

Table 1. Genotypes in 22 Paired Consecutive Episodes of Erythema Migrans in 17 Patients with Positive Cultures for *Borrelia burgdorferi*.*

Paired Consecutive Episode	Initial Episode						Second Episode						Duration between Episodes
	Skin Culture			Blood Culture			Skin Culture			Blood Culture			
	ospC†	*RST*	*MLST*	*ospC*†	*RST*	*MLST*	*ospC*†	*RST*	*MLST*	*ospC*†	*RST*	*MLST*	yr
1	N	2	New type‡	NA§	1		H	2	ST4	H	2		7
2¶	K	2					M	3	ST12				10
3¶	M	3	ST12							A‖		ST1	4
4	Negative	Negative	Negative	K‖	2		B‖	1		Negative	Negative	Negative	3
5	G	3					A	1		A	1		5
6	K	2		K	2		I	3		I	3		2
7	I	3		I	3		Negative	Negative	Negative	K	2		5
8	B‖	1		J‖	3	ST34	E‖	3	ST19	A	1		3
9	K‖	2	ST3	K‖	2		Negative	Negative	Negative	B	1		3
10**	K	2	ST3	Negative	Negative	Negative	G‖	3	ST14	Negative	Negative	Negative	4
11**	G	3	ST14	Negative	Negative	Negative	K	2	ST3	Negative	Negative	Negative	1
12**	K	2	ST3	Negative	Negative	Negative	H	2	ST4	Negative	Negative	Negative	1
13††	Negative	Negative	Negative	K	2		I	3	ST16				1
14††	I	3	ST16				E3‖	3	ST40	Negative	Negative	Negative	7
15‡‡	A	1		A	1		N‖	2	ST9	Negative	Negative	Negative	1
16‡‡	N	2	ST9	Negative	Negative	Negative	K	2	ST3	K	2		2
17	M‖	3	ST12	Negative	Negative	Negative	N	2		N	2		4
18	G	3		Negative	Negative	Negative	K	2		Negative	Negative	Negative	4
19	A	1		A‖	1	ST1	B‖	1	ST7	Negative	Negative	Negative	2
20	K‖		ST3	Negative	Negative	Negative	T‖	3	ST37	Negative	Negative	Negative	15
21	B‖	1	ST7	B	1	ST7				A‖	1	ST1	5
22	A‖	1		A	1					K‖	2	ST3	7

* A blank cell indicates that the test was not performed. MLST denotes multilocus sequence typing, NA not available, *ospC* outer-surface protein C, RST 16S–23S ribosomal RNA intergenic spacer type, and ST sequence type.
† Genotyping was performed by means of reverse line-blot analysis unless otherwise specified.
‡ This isolate had a new uvrA allele that does not appear in the multilocus-sequence-typing database; the multilocus ST designation is pending.
§ No sample was available for *ospC* genotyping; however, this isolate was RST1, which has been invariably associated with either *ospC* genotype A or B in our geographic area (the Lower Hudson Valley of New York State).[20]
¶ Three consecutive episodes of erythema migrans occurred in one patient (episodes 2 through 3).
‖ The *ospC* genotype was determined by means of sequencing.
** Four consecutive episodes of erythema migrans occurred in one patient (episodes 10 through 12).
†† Three consecutive episodes of erythema migrans occurred in one patient (episodes 13 and 14).
‡‡ Three consecutive episodes of erythema migrans occurred in one patient (episodes 15 and 16).

tients would be more likely to be culture-negative because antibiotic treatment might alter the phenotype of *B. burgdorferi* to make it uncultivable in vitro.[9] Besides the 24 paired episodes in which both episodes were culture-positive, we also identified 14 additional paired episodes of erythema migrans in which just the first episode was culture-positive. Of the total group of 38 paired episodes with a positive culture at the first episode of erythema migrans, the likelihood of a positive culture at the second episode was 63% (24 of 38 episodes), including a positive skin culture in 19 of 33 episodes (58%) and a positive blood culture in 11 of 30 episodes (37%) in patients in whom these tests were performed.

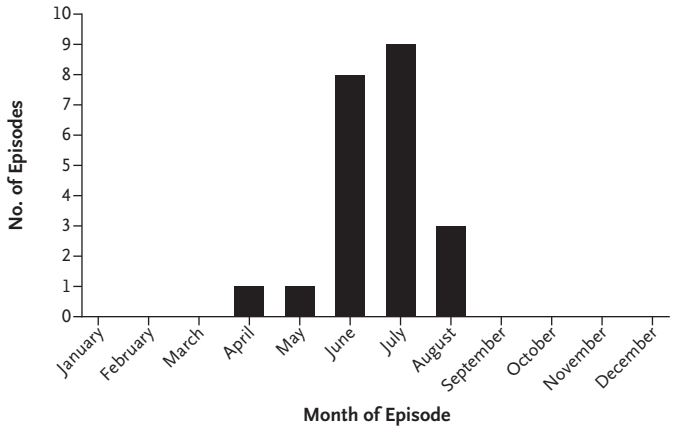

Figure 1. Episodes of Erythema Migrans, According to Month.
The second episodes in 22 paired consecutive episodes of erythema migrans are shown.

DISCUSSION

To determine whether recurrent erythema migrans is associated with a new infection or with relapse of an incompletely treated previous infection, we examined the *ospC* genotypes of *B. burgdorferi* isolates obtained from patients with paired consecutive episodes of erythema migrans. None of the 22 paired consecutive episodes shared the same *ospC* genotype. Furthermore, infection with a different genotype of *B. burgdorferi* was confirmed in all 22 of these paired episodes by means of a separate genotyping method. The probability that this observation was related to chance sampling of different strains that persisted from the original episode after antibiotic treatment was estimated to be less than 0.01, assuming both an initial coinfection rate of 100% and infection with five different genotypes. On the basis of the application of PCR directly to skin-biopsy samples of erythema migrans skin lesions, however, the rate of coinfection has been consistently observed to be less than 50% (range, 1 to 43).[24-26] Furthermore, *Ixodes scapularis* ticks are typically coinfected with fewer than three genotypes, according to direct PCR analysis.[27-30] Thus, our findings suggest that recurrences of erythema migrans after standard courses of antibiotic therapy are reinfections rather than relapses, and they provide further evidence of the success of antibiotics in eradication of *B. burgdorferi* from the skin of patients with erythema migrans in the United States.[31,32]

Clinical and epidemiologic evidence, however, also suggested that our patients had reinfections. Virtually all recurrences occurred 1 year or more apart. In 20 of the 22 paired episodes of erythema migrans (91%), the recurrence was observed during June through August, paralleling the activity of nymphal *I. scapularis* ticks, the vector responsible for more than 90% of cases of Lyme disease in the United States.[33] It is unlikely that a relapsed infection would have such a seasonal distribution when there is a delay of 1 year or more between episodes of erythema migrans. In a previous article describing patients with erythema migrans who had a relapse after ineffective therapy with cephalexin, relapses were observed within days to weeks.[3]

In the 22 paired episodes, 12 different *ospC* genotypes caused infection, including 8 different genotypes at the initial episode of erythema migrans, and 11 different *ospC* genotypes at the second episode (Table 1). Only one patient (in

Table 2. Characteristics of a Patient with Four Consecutive Episodes of Erythema Migrans.

Date	Location of Single Erythema Migrans Lesion	Presence of Punctum*	Genotype in Skin		
			ospC	RST	MLST
8/14/97	Right ankle	Not recorded	K	2	ST3
7/5/01	Left popliteal fossa	Not recorded	G	3	ST14
6/20/02	Right buttock	Yes	K	2	ST3
6/9/03	Right groin	Not recorded	H	2	ST4

* A punctum is a small, usually erythematous central area that is often raised from the surrounding skin and is suggestive of a preceding bite by an arthropod.[21-23]

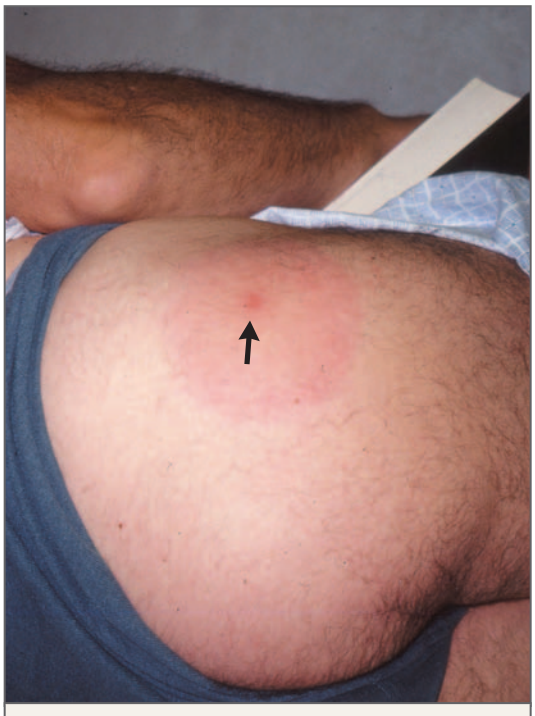

Figure 2. A Single Erythema Migrans Lesion (Paired Consecutive Episode 11).

The central punctum (arrow) is shown. This was the patient's third episode of erythema migrans.

episodes 10 through 12) had infection with the same *ospC* genotype on more than one occasion (at nonconsecutive episodes) (Tables 1 and 2). Although relapse cannot be completely ruled out in this patient, it appears doubtful for a number of reasons. The 2 episodes of erythema migrans caused by the same *ospC* genotype were both solitary erythema migrans skin lesions located at different body sites (the ankle and buttock, respectively), and these episodes were 5 years apart. Furthermore, a punctum was observed at the center of the lesion in the recurrent episode of erythema migrans. A punctum is a small, usually erythematous central area that is often raised from the surrounding skin and is suggestive of a recent preceding bite by an arthropod (Fig. 2).[21-23] Moreover, it is unlikely that insensitivity of our culture techniques is the reason for the failure to identify *ospC* genotype K during the other 2 episodes of erythema migrans in this patient. This *ospC* genotype was the most commonly identified genotype in our study, a finding that is consistent with previous results in patients with erythema migrans in whom the *ospC* genotype K was the most common[34,35] or the second most common[26] genotype isolated. This observed prevalence of *ospC* genotype K may relate to the frequency of this genotype in ticks,[36] the tendency of this *ospC* genotype to cause infection in humans, or both.[11]

We cannot attribute our study results to an inadvertent focus on patients with less severe illness or those with clinically localized infection, who hypothetically might be more likely to have a response to antibiotic therapy. In the 22 paired episodes of erythema migrans, systemic symptoms were present during the first episode of the pair in 18 of 22 episodes (82%), and evidence of disseminated infection (i.e., multiple skin lesions or a positive blood culture) was present in 13 of 22 episodes (59%).

Moreover, our findings do not provide support for the hypothesis (derived from certain studies involving animals) that relapses in patients who received antibiotics would be more likely to be culture-negative because antibiotic treatment might change the phenotype of *B. burgdorferi*.[9] In addition to the 24 paired episodes in which both episodes were culture-positive, we also identified 14 paired episodes of erythema migrans in which just the first episode was culture-positive. In the 38 episodes of erythema migrans with a positive culture at the first episode, the likelihood of a positive skin culture at the second episode was 58% and the likelihood of a positive blood culture was 37%. These rates of positive cultures during the second episode of erythema migrans are similar to our previously published data that showed positive skin cultures in 24 of 47 specimens (51%) and positive blood cultures in 21 of 47 specimens (45%) obtained from patients with erythema migrans.[37]

Our study had insufficient statistical power to address whether type-specific immunity to *ospC* genotypes might exist in humans. There is precedent, however, for the development of type-specific immunity under experimental conditions in mice that receive an OspC vaccine.[38]

A limitation of our study is that isolates of *B. burgdorferi* were unavailable for *ospC* genotyping from 2 of the 24 culture-positive paired episodes of erythema migrans that were identified. Thus, we cannot rule out the possibility of relapse

with the same genotype in the second episode. This would seem unlikely, however, since the clinical histories were suggestive of reinfections. Recurrent erythema migrans lesions developed in both patients in July, after 1 and 2 years, respectively, at body sites that differed from those of the initial lesion.

In summary, different *ospC* genotypes of *B. burgdorferi* were associated with each of 22 paired consecutive episodes of erythema migrans in patients with culture-confirmed infection. These data, in conjunction with available clinical and epidemiologic evidence, show that repeat episodes of erythema migrans in appropriately treated patients were reinfections and not relapses.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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