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Transhemispheric Exchange of Lyme Disease Spirochetes by Seabirds

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Lyme disease is a zoonosis transmitted by ticks and caused by the spirochete *Borrelia burgdorferi* sensu lato. Epidemiological and ecological investigations to date have focused on the terrestrial forms of Lyme disease. Here we show a significant role for seabirds in a global transmission cycle by demonstrating the presence of Lyme disease *Borrelia* spirochetes in *Ixodes uriae* ticks from several seabird colonies in both the Southern and Northern Hemispheres. *Borrelia* DNA was isolated from *I. uriae* ticks and from cultured spirochetes. Sequence analysis of a conserved region of the flagellin (*fla*) gene revealed that the DNA obtained was from *B. garinii* regardless of the geographical origin of the sample. Identical *fla* gene fragments in ticks obtained from different hemispheres indicate a transhemispheric exchange of Lyme disease spirochetes. A marine ecological niche and a marine epidemiological route for Lyme disease borreliae are proposed.

The genus *Borrelia* contains several major human and animal pathogens. Among these are the relapsing fever *Borrelia* species, the Lyme disease *Borrelia* species, and the etiologic agent of avian spirochetosis, *Borrelia anserina*. Lyme disease can be caused by three different genomic species, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (4, 9). Another species, *B. japonica*, was recently described, but its relevance as a human pathogen remains unclear (17). The *Borrelia* flagellin has been studied extensively with the aim of developing diagnostic tools for Lyme disease, through both immunological detection and PCR amplification methodology. Moreover, molecular studies of the *fla* gene and its product, flagellin, have been used to perform phylogenetic studies of borreliae and to classify the different Lyme disease-causing species (26–28).

The *Borrelia* spirochetes have complicated transmission cycles involving ticks, mammals, and birds (1, 20, 21). In their enzootic cycles, Lyme disease spirochetes are transmitted mainly by ticks of the *Ixodes ricinus-I. persulcatus* complex (subgenus *Ixodes*). The terrestrial cycles involving small mammals as the main reservoir have been studied in the greatest detail.

Seabirds are the approximately 300 avian species that feed mainly at sea (14). To avoid predators, these birds often breed on isolated islands and peninsulas (18). This important feature leads to the aggregation of hundreds of thousands, and sometimes millions, of individuals during the breeding season. This crowding habit makes the seabirds and their offspring vulnerable to endo- and ectoparasites (10, 29). The seabird-associated tick *I. uriae* (subgenus *Ceratixodes*) has a large and unique bi- and circumpolar distribution. Infestation by *I. uriae* of more than 50 species of seabirds colonizing regions of high latitudes in both hemispheres has been reported (11, 23, 25).

Recently, *B. garinii* was isolated from the seabird tick *I. uriae* from a mammal-free island in the Baltic Sea (25). It was also shown that seabirds such as razorbills (*Alca torda*) were infected with Lyme disease borreliae and that *I. uriae* was the vector of infection. The protein profile of the spirochete and the sequences of its *ospA* and *fla* genes were identical to those of a spirochete isolated from *I. ricinus* on a nearby island (8). Of particular interest was the finding of a case of human neuroborreliosis on the latter island with a specific immune response against that particular borrelial strain (8).

To determine whether Lyme disease spirochetes are common in ticks infesting seabirds, 523 *I. uriae* ticks were collected from different locations in the Northern and Southern Hemispheres. In the present study, we examined the ticks by phase-contrast microscopy, an immunofluorescence assay, and DNA amplification by the PCR for evidence of the presence of Lyme disease *Borrelia* infection. To further characterize the *Borrelia* DNA found in the ticks, flagellin gene sequencing was performed. Our result suggests that seabirds may be reservoirs of Lyme disease spirochetes and important for their global spreading.

MATERIALS AND METHODS

Tick collection. The following nine locations were investigated by seabird-ringing groups who collected ticks either from seabird nestlings or from the ground within the seabird colonies (Fig. 1). (i) Campbell Island, New Zealand, holds 30,000 pairs of black-browed albatrosses (*Diomedea melanophris*) and 5,000 pairs of grey-headed albatrosses (*D. chrysostoma*). The terrestrial mammals present are feral sheep, cattle, feral cats, and brown rats (*Rattus norvegicus*) (14a). (ii) The Crozet Islands hold 34 species of breeding seabirds. The most abundant (each numbering millions of pairs) are macaroni penguins (*Eudyptes chrosolophus*), Salvin's prions (*Pachyptila salvini*), and South Georgia diving petrels (*Pelecanoides georgicus*). The population of king penguins (*Aptenodytes patagonicus*) is estimated at a half a million pairs. Feral cats and black rats (*R. rattus*) are present on the islands. (iii) West Point, Falkland Islands, holds 12,100 pairs of black-browed albatrosses. Brown rats and a few feral sheep are present in the colony area (31a). (iv) Egg and St. Lazaria Islands, Alaska, hold 100,000 pairs of tufted puffins (*Lunda cirrhata*) and fork-tailed storm petrels (*Oceanodroma furcata*). No terrestrial mammals are present (25b). (v) Gannet Island, Labrador, Newfoundland, Canada, holds 33,000 pairs of puffins (*Fratercula arctica*), 20,000 pairs of guillemots (*Uria aalge*), and 5,800 pairs of razorbills. No terrestrial mammals are present (23a). (vi) Flatey Island, located off the northwestern part of Iceland, holds 450 pairs of black guillemots (*Cepphus grylle*). No terrestrial mammals are present (25a). (vii) Nólsoy, Faeroe Islands, holds 10,000 pairs of puffins (*F. arctica*). House mice (*Mus musculus*) and feral sheep are present adjacent to the colony area (15a). (viii) Cape Sizun, Brittany, France,

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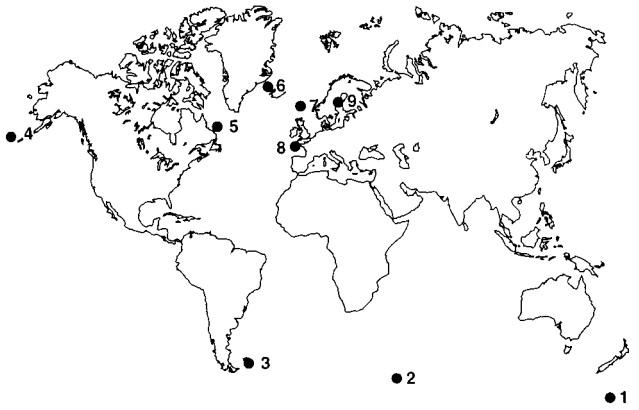

FIG. 1. Locations of seabird colonies where *I. uriae* ticks were collected. 1, Campbell Island, New Zealand; 2, Crozet Islands; 3, West Point, Falkland Islands; 4, Egg and St. Lazaria Islands, Alaska; 5, Gannet Island, Canada; 6, Flatey Island, Iceland; 7, Nólsoy, Faeroe Islands; 8, Cape Sizun, France; 9, Bonden Island, Sweden.

holds 950 pairs of kittiwakes (*Rissa tridactyla*), 300 pairs of herring gulls (*Larus argentatus*), and 30 pairs of guillemots (*U. aalge*). Red foxes (*Vulpes vulpes*) and weasels (*Mustela nivalis*) can be found in association with the bird colonies (8a). (ix) Bonden, situated in the northern part of the Baltic Sea, Sweden, holds 4,000 pairs of razorbills, 800 pairs of guillemots, and 500 pairs of black guillemots. No terrestrial mammals are present (25).

Tick identification and spirochete detection. After collection, all ticks were kept in plastic vials. The ticks were examined with a binocular dissecting microscope (6 to 50× lens objective) and their species, life cycle stage, and status of engorgement were recorded (13). Live ticks were cleaned with 70% ethanol prior to dissection. Part of the tick body contents was excised aseptically on a glass slide, placed into a drop of sterile phosphate-buffered saline (PBS), and examined by phase-contrast microscopy at a magnification of ×400. In smears where spirochetes could be seen, attempts to culture the spirochetes in BSK-II medium were made (5). The cultures were incubated at 34°C and checked weekly for at least six weeks.

Immunofluorescence assay. Smears on slides were air dried and fixed in acetone. After being dried, the smears were incubated with a covering drop of PBS containing either the genus-specific monoclonal antibody H9724 (1:10 dilution) (kindly provided by Alan G. Barbour), which is directed against the flagellin of the borreliae (6), or the monoclonal antibody 84C (1:10 dilution) (kindly provided by Denée Thomas), which recognizes outer surface protein B (OspB) of most strains of Lyme disease borreliae (30). After incubation at 37°C for 30 min in a moist chamber, the slides were gently washed with PBS prior to incubation with goat anti-mouse immunoglobulin G–fluorescein isothiocyanate (Becton Dickinson, San Jose, Calif.) diluted in PBS (1:5). After additional incubation for 30 min in a moist chamber, the slides were washed with sterile water and then with PBS. Examination for bound fluorescent antibodies was performed with a fluorescence microscope at a magnification of ×600.

DNA extraction and PCR amplification. One half of each of the collected ticks was used for DNA isolation. The tick sample was boiled in 100 μl of autoclaved water for 3 min. After the sample was boiled, the supernatant was carefully removed and transferred to a 1.5-ml Eppendorf tube. The procedure thereafter followed the method of Ishizawa et al. (15). Briefly, 300 μl of a solution containing 6 M sodium iodide (NaI), 13 mM EDTA, 0.5% sodium *N*-lauroylsarcosine, 10 μg glycogen, and 26 mM Tris-HCl, pH 8, was added to the supernatant, and the mixture was incubated at 60°C for 30 min. An equal amount of isopropanol was added to the tube to precipitate the DNA. After being vigorously shaken, the samples were incubated at room temperature for 30 min. The pellets were centrifuged at 10,000 × *g* for 15 min. The samples were washed twice with 70% ethanol, vacuum dried, and dissolved in 50 μl of sterile water. Five microliters of this solution was used as a template for DNA amplification. Two pairs each of oligonucleotide primers specific for the *ospA* gene and the flagellin gene of *B. burgdorferi* B31 were used for DNA amplification. The *ospA* primers, located at positions 44 to 70 (3′-TGGATAAGGGTTATTTATAAAAAAGTA-5′) and at positions 204 to 225 (3′-CGTTGTCATCTGTTCGAACTCG-5′), direct the synthesis of a 185-bp fragment (16). From a sequence of the flagellin gene of *B. burgdorferi* B31, oligonucleotides fla F (3′-AACGTTGTAATC GACGTATTTATA-5′) and 7C (3′-TGTTGATGTCAACTACGATTAT-5′), at nucleotide positions 563 to 586 and 721 to 742, respectively, were used as primers (24). They direct the synthesis of a 156-bp fragment from a species-variable region of the flagellin gene. The DNA was amplified in a 50-μl reaction vial for a total of 35 cycles in an automated thermal incubator (Perkin-Elmer Cetus, Norwalk, Conn.). Denaturation was performed at 94°C for 30 s, annealing was performed at 55°C for 1 min, and extension was performed at 72°C for 1 min. The amplification cycles were concluded with an extension reaction at 72°C for 7 min. Amplified DNA was electrophoresed through a 1.5% agarose gel and visualized by ethidium bromide staining. In all DNA amplification experiments, *Cryptococcus neoformans* DNA was used as a negative control and *B. burgdorferi* DNA from strain B31 was used as a positive control. All pre- and post-PCR reactions were performed in different areas in order to minimize cross-contamination. All PCR reagents were pipetted with aerosol-resistant tips (Molecular Bio-Products, Inc., San Diego, Calif.) and tested for PCR reactivity without the addition of DNA.

Flagellin gene sequencing. The PCR-amplified fragments of the flagellin gene were isolated and ligated into pT7Blue T-Vector (Novagen, Madison, Wis.) according to the instructions of the manufacturer. Competent *Escherichia coli* DH5α was transformed with the recombinant plasmids containing the *fla* gene by standard methods (3). Both strands of positive clones containing the PCR-amplified *fla* gene fragment were sequenced by primer-directed sequencing by the dideoxy chain termination method with α-^{35}S-dATP (T7 sequencing kit; Pharmacia, Uppsala, Sweden).

RESULTS

All ticks were identified as *I. uriae* (13). All three active developmental stages (larvae, nymphs, and adults) were represented, but there was a predominance of nymphs and adults. The idiosomas of all live ticks were examined by phase-contrast microscopy. None of the ticks from the Crozet Islands or the Falkland Islands were alive, so they were not subjected to microscopy. In 43 of 385 live ticks, spirochete-like organisms were observed (Table 1). Spirochetes were successfully cultivated from three ticks from the Faeroe Islands, one tick from Iceland, and two ticks from Sweden. Smears of ticks for which DNA amplification was successful were examined by immunofluorescence with the *Borrelia* genus- and *B. burgdorferi* sensu lato-specific monoclonal antibodies H9724 and 84C, respectively. In 1 of 18 live ticks from New Zealand, spirochetes were seen after staining with H9724. The spirochetes from the ticks collected from Alaska, the Faeroe Islands, Iceland, and Sweden reacted with both antibodies, indicating that they belong to *B. burgdorferi* sensu lato.

To further characterize the spirochetes, total DNA from one half of each of the 523 ticks was extracted and PCR amplified with *fla*- and *ospA*-specific primers. Amplification with the flagellin gene primers was successful for 113 ticks. With the *ospA*-specific primers, 115 ticks gave the expected PCR products (Table 1). The amplified flagellin gene fragments from all positive ticks from Campbell Island, the Crozet Islands, and randomly selected samples from other localities were subjected to DNA sequencing. The *fla* fragments showed a high degree of mutual sequence homology regardless of the source (Fig. 2). Altogether, six different sequences were found. They were all more closely related to the *fla* gene of *B. garinii* than to that of any other Lyme disease *Borrelia* species.

To study the heterogeneity among spirochetes at a particular location, the 23 *fla* PCR products from ticks obtained from the Crozet Islands were sequenced. Four different sequence patterns were recognized (Fig. 2). Three of these sequences (sequences 1, 3, and 4 [Fig. 2]) were also found in ticks collected from different localities in the Northern Hemisphere, i.e., Iceland, the Faeroe Islands, and Alaska.

DISCUSSION

Infestation of seabirds with *I. uriae* infected by *B. garinii* has previously been demonstrated only in Sweden (25). To further analyze the global distribution of seabird ticks infected with spirochetes, 523 ticks were collected from nine different locations in both the Northern and Southern Hemispheres (Fig. 1).

The present data extend the geographic range of *B. garinii* in *I. uriae*. As *I. uriae* has been reported to feed on more than 50

TABLE 1. Detection of spirochetes and Lyme disease spirochete DNA in *I. uriae* ticks infesting seabirds

Location	Source of ticks	No. of ticks[a]			
		Examined	With spirochetes detected by microscopy[b]	With *Borrelia* DNA detected by PCR	
				fla gene	*ospA* gene
Campbell Island, New Zealand	Black-browed albatrosses	41 (1 N, 40 A)	1[c] (1 A)	3 (3 A)	3 (3 A)
Crozet Islands	King penguins	46 (5 N, 41 A)	Not done[d]	23 (1 N, 22 A)	33 (2 N, 31 A)
West Point, Falkland Islands	Black-browed albatrosses	57 (49 N, 8 A)	Not done[d]	0	0
Egg and St. Lazaria islands, Alaska	Tufted puffins	8 (8 A)	0	0	0
	Fork-tailed storm petrels	24 (1 N, 23 A)	2[e] (2 A)	11 (11 A)	11 (11 A)
Gannet Island, Canada	Guillemot nests[f]	4 (4 A)	0	0	0
Flatey Island, Iceland	Black guillemots	60 (1 N, 59 A)	26 (26 A)	29 (29 A)	20 (20 A)
Nólsoy, Faeroe Islands	Puffins	120 (1 L, 31 N, 88 A)	11 (1 L, 5 N, 5 A)	38 (1 L, 12 N, 25 A)	36 (1 L, 12 N, 23 A)
Cape Sizun, France	Kittiwakes	23 (11 N, 12 A)	0	0	0
Bonden Island, Sweden	Razorbills	88 (12 N, 76 A)	3 (3 A)	6 (2 N, 4 A)	8 (2 N, 6 A)
	Guillemots	52 (12 N, 40 A)	0	3 (3 A)	4 (4 A)

[a] L, larvae; N, nymphs; A, adults.
[b] Spirochetes were detected in smears from live ticks by phase-contrast microscopy and/or by immunofluorescence assays with monoclonal antibodies H9724 (directed against flagellin) and 84C (directed against OspB) (6, 30).
[c] Only live ticks (18 of 41) were examined.
[d] No ticks were alive.
[e] Only live ticks (12 of 24) were examined.
[f] Ticks were collected in and around the birds' nests.

species of seabirds, it is likely that *B. garinii* is widely distributed in seabird colonies at high latitudes in both hemispheres.

The presence of *B. garinii* was demonstrated by culturing with ticks from the Faeroe Islands; Bonden, Sweden; and Iceland and by immunofluorescence with both a genus-specific antiflagellin monoclonal antibody and a Lyme disease *Borrelia*-specific monoclonal antibody against a conserved epitope of the OspB protein (6, 30). Further analyses were performed by amplification of the *fla* and *ospA* genes of the spirochetes in samples from several mutually distant localities in both the Northern and Southern Hemispheres.

The terrestrial cycles of transmission of Lyme disease are complex and involve a number of species of the *I. ricinus-I. persulcatus* complex that parasitize mammals, birds, and reptiles (19). The isolation of Lyme disease *Borrelia* spirochetes from birds and from larvae that feed on birds in the United States and Japan suggests that birds are competent reservoirs of these borreliae (2, 20, 22, 33). Our finding of *Borrelia* spirochetes in parasitizing seabird ticks in the absence of other hosts suggested that seabirds may be competent reservoirs and amplifying hosts. This hypothesis is strengthened by the observation that in five of the seabird colonies where ticks were collected, no mammals that could maintain the spirochetes in an enzootic cycle are present. In these colonies, seabirds are the only possible vertebrate reservoirs. In addition, we detected *B. garinii* in the footweb of a razorbill nestling, thus strengthening the notion of an important role of seabirds as a reservoir for Lyme disease borreliae (25). This postulated bird-tick cycle would be less complex than the previously described terrestrial enzootic *Borrelia* cycles. In four of the locations where *I. uriae* ticks were collected, mammals could be found in association with the seabird colonies. Even though there are reports that *I. uriae* may occasionally feed on mammals, including humans, it is not clear whether this tick is a competent vector for transfer of borreliae to mammals (23).

Interestingly, heterogeneity was found among samples from the same region as well as among samples from distant geographic regions. This finding, together with the demonstration of identical sequences in samples obtained from the Northern and Southern Hemispheres, supports the case for a transhemispheric exchange of the bacteria. Although our results suggest a spread of *B. garinii* by migratory seabirds, it has not been conclusively demonstrated that this spread is necessarily dependent on the transport of infected ticks. Another possibility is the presence of spirochetes in migrating birds. The relatively low (and, for Lyme disease borreliae, suitable) body temperature of 38°C in certain seabirds such as procellariids (shearwaters, storm petrels, and albatrosses) (32), compared with the body temperature of terrestrial birds, 40°C, suggests that seabirds may be competent hosts for *Borrelia* spirochetes.

In the Northern and Southern Hemispheres, several species of seabirds are possible candidates for this transhemispheric exchange. Many species of procellariids are highly mobile and undertake long, complex migrations of several thousand kilometers. Great (*Puffinus gravis*), sooty (*P. griseus*) and short-tailed (*P. tenuirostris*) shearwaters are three very common seabird species that, after breeding on subantarctic islands, make a transequatorial migration to northern parts of the Atlantic and Pacific Oceans. Migration in the opposite direction is exhibited by Manx shearwaters (*P. puffinus*), which have a wide distribution in the North Atlantic and winter in subantarctic waters of South America and South Africa (14). Thus, it is probable that certain seabirds are important for the dispersal of infected ticks and are also important for the multiplication and survival of *Borrelia* spirochetes.

Apparently, *B. garinii* is strongly associated with *I. uriae* ticks. Although Lyme disease is not common at the high latitudes where these ticks are prevalent seabird parasites, all prerequisites for their spread from seabirds to other birds and mammals, including humans, may be fulfilled. It is likely that *Borrelia* spirochetes can be transmitted between birds and mammals by ticks, especially *I. uriae*, feeding on the animals. Of particular interest is the finding of suspected cases of Lyme disease in Australia and South Africa, although no Lyme disease-causing spirochete has been isolated from these regions yet (7, 31). Most of the findings in Australia are based on

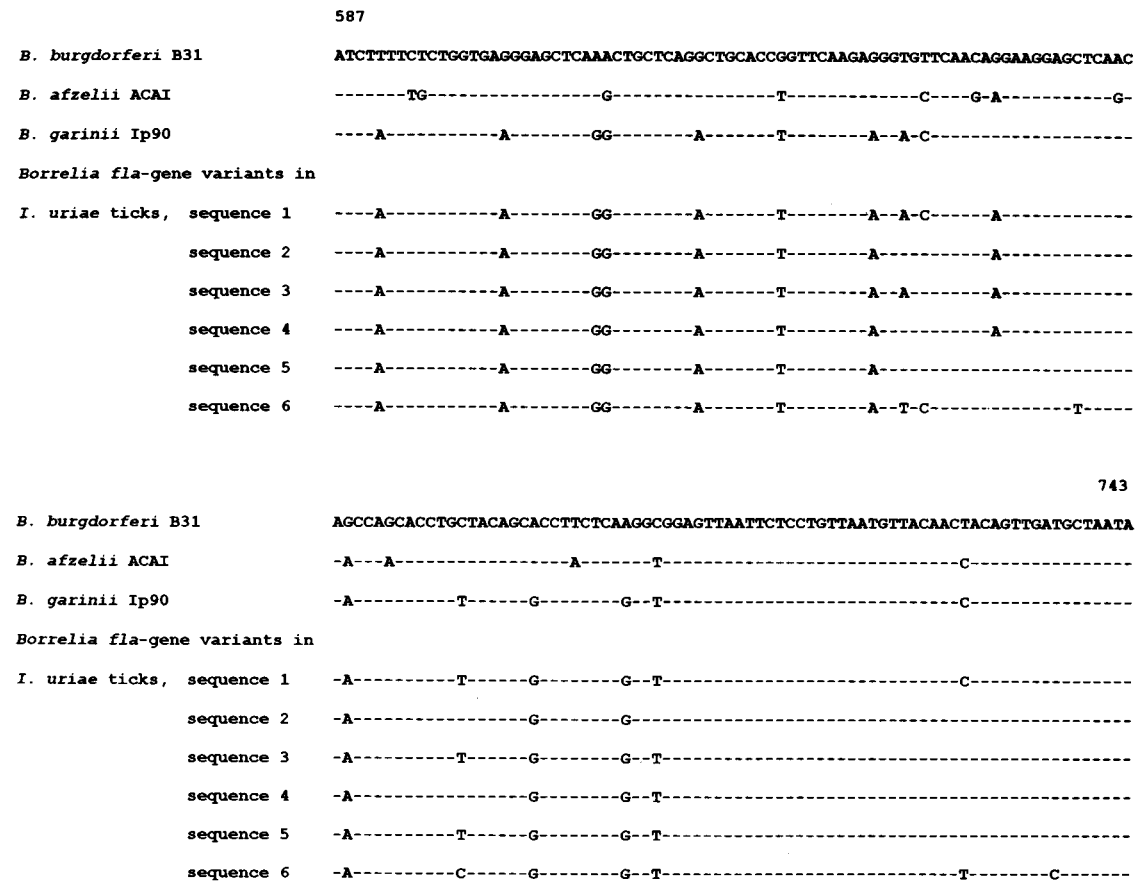

FIG. 2. Nucleotide sequences of 156-bp internal *fla* gene fragments from *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, and *I. uriae* ticks. Sequence 1 is representative of 3 ticks from Campbell Island, 3 ticks from Egg Island, and 11 ticks from the Crozet Islands; sequence 2 is representative of 6 ticks from the Crozet Islands; sequence 3 is representative of 4 ticks from the Crozet Islands and 2 ticks from Nólsoy; sequence 4 is representative of 5 ticks from Flatey Island, 3 ticks from Nólsoy, and 2 ticks from the Crozet Islands; sequence 5 is representative of 8 ticks from Egg Island; and sequence 6 is representative of 5 ticks from Bonden Island.

serological data and clinical cases with symptoms typical of Lyme disease. Our finding of *Borrelia* DNA in *I. uriae* ticks obtained from the Crozet Islands and Campbell Island suggests that Lyme disease enzootic foci are present in that part of the world. However, in that region the exchange between the marine enzootic cycle of *B. garinii* and a theoretical terrestrial cycle may be rare. In general, the seabirds and consequently the *I. uriae* ticks in the southern oceans are restricted to the open sea, remote oceanic islands, and peninsulas. They are only accidentally brought, by extreme winds, to the inner mainland.

Routine exploitation of colonial seabirds is important at high latitudes in both hemispheres (12). Persons involved in such harvests, who are frequently members of indigenous groups in isolated locations lacking medical facilities, may be exposed to Lyme disease. Serological surveys of these populations are needed in order to assess this risk. The importance of birds, and in particular of seabirds, in the global distribution of Lyme disease must be considered one of the major factors in the worldwide dispersal of pathogens causing this disease.

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