Cross-Reactivity to *Borrelia burgdorferi* Proteins in Serum Samples from Residents of a Tropical Country Nonendemic for Lyme Disease

Thomas R. Burkot, Martin E. Schriefer, and Sandra A. Larsen

Reports of Lyme disease from areas where the disease is not endemic have increased. Eighty-six human serum samples from Papua New Guinea (nonendemic for Lyme disease) were examined for the presence of IgG antibodies that recognize *Borrelia burgdorferi* antigens, using the currently recommended two-tiered system of analysis (sensitive ELISA with Western blot). The percentage of positive tests dropped from 50% to 10% when individual negative controls were included in the two-tiered analysis. Positive serum samples failed to inhibit the growth of *B. burgdorferi* in culture and did not yield positive reactions in the fluorescent treponemal antibody-absorption test. These characteristics, together with atypical Western blot antigen recognition patterns and the absence of known vectors, provide evidence that seropositive results for these persons are not the result of exposure to *B. burgdorferi*. Individual negative controls may minimize false-positive results for serologic tests for Lyme disease, and these tests must be interpreted in the context of clinical and epidemiologic data.

Lyme disease spirochetes have been isolated from North America, Europe, and Asia, including Japan [1]. However, even in the absence of known enzootic cycles of any of the causative agents, the disease has also been increasingly reported from South America, sub-Saharan Africa, and Australia [1]. These reports are based on clinical observations, serologic analyses, or both. The problem of antigenic cross-reactivities among bacteria coupled with the protean nature of Lyme disease symptoms make overdiagnosis of the disease possible [2].

The Centers for Disease Control and Prevention case definition of Lyme disease in the United States requires physician-diagnosed erythema migrans or evidence of late Lyme disease manifestations (e.g., carditis, neurologic disease, arthritis) and a confirmatory serologic test result [3]. At present, the recommended 2-tiered standardized serologic test for Lyme disease caused by *Borrelia burgdorferi* uses a sensitive ELISA followed by Western blot to test equivocal and positive ELISA results [1, 4].

In this study, serum samples from Papua New Guinea (PNG), a tropical country where Lyme disease is not endemic, were subjected to stringent evaluation using a recommended 2-tiered Lyme disease serologic methodology and interpretive criteria [1, 4, 5] or an ELISA alone [6].

**Materials and Methods**

*Human sera.* Serum samples were obtained by venipuncture from subjects in Buksak village, Madang Province, PNG, separated from cells, and stored at -70°C until analyzed [7].

*Antibody ELISAs.* Serum samples were evaluated by ELISA for IgG reactivity to *B. burgdorferi* antigens. Two *B. burgdorferi* antigen preparations were used: a whole cell sonicate of low-passage strain B31 (WCS) [6] and an enriched flagellin preparation (Fla; Kirkegaard & Perry, Gaithersburg, MD) [4]. Individual sera were tested in duplicate at a 1:500 dilution against WCS, Fla, and uncoated wells.

In addition to using the uncoated wells as individual negative controls, 6 serum samples from persons living in an area of the...
United States not endemic for *B. burgdorferi* and who had no history of *B. burgdorferi* exposure were assayed on each plate [4]. Absorbance values were analyzed using two approaches. In the first, serum was considered to be positive if the absorbance generated in the antigen-coated wells was greater than the mean + 3 SD of the negative US sera [4]. A negative response was defined as one that was less than the mean + 1 SD. Absorbances between the positive and negative cutoff values were considered equivocal.

In the second analysis, the average absorbance difference between the antigen-coated and uncoated wells for a serum sample was compared with the absorbance difference for antigen-coated and uncoated wells for the negative US serum. Positive and negative cutoff values were as described above. Serum samples scored as positive or equivocal in the ELISA were further analyzed by Western blot.

**Western blots.** Western blots (MarDx Diagnostics, Carlsbad, CA) were done according to the manufacturer’s recommendations. Positive and negative control sera (MarDx Diagnostics) were simultaneously run on the same sets of nitrocellulose strips with serum samples at a 1:100 dilution. Western blots were scored as positive or negative using the criteria of Dressler et al. [5]: An IgG blot was considered positive if 5 of 10 antigens (18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa) were recognized.

**Other serologic tests.** Serum samples scored as positive by the ELISA—Western blot criteria were evaluated for the presence of antibodies to *Treponema pallidum* [8] by use of microhemagglutination assay, rapid plasma reagin test, fluorescent treponemal antibody-absorption (FTA-Abs) test, and Western blot.

**Growth inhibition assays.** In vitro *B. burgdorferi* growth inhibition assays were done in 96-well tissue culture plates as previously described [9], using log-phase low-passage strain B31 or high-passage strain HB19 spirochetes at 10^6/well. Serum samples were added at serial dilutions from 1:4 to 1:256 to wells containing BSK II medium supplemented with 20% guinea pig serum (as a complement source). Plates were maintained for 72 h at 34°C in a candle jar before being scored for spirochete growth. Growth was quantitated by microscopic examination (×400) and with an outer surface protein (Osp) A antigen-capture ELISA [10]. Purified serum from a rabbit, hyperimmunized by repeated needle inoculation of *B. burgdorferi*, served as a positive control.

**Results**

**ELISA.** PNG serum samples were tested by ELISA for reactivity to *B. burgdorferi* WCS and Fla. When total absorbances of PNG sera were compared directly with those of controls, 98% and 90% of 86 serum samples were positive or equivocal against WCS and Fla, respectively (table 1). When absorbances in nonantigen-coated wells were subtracted from absorbances in antigen-coated wells prior to comparison of PNG and control serum reactivity, the positivity rate declined to 63% and 23% for WCS and Fla, respectively.

**Western blot analysis.** By the criteria of Dressler et al. [5], 48 of 84 (57%) of the PNG sera tested were positive for IgG in Western blots (figure 1). The number of *B. burgdorferi* antigens recognized ranged from 2 to 17, with a median and mean of 10 antigens and a mode of 6 antigens. The 10 diagnostically important *B. burgdorferi* antigens [5] with molecular masses of 41, 93, 66, 58, 39, 45, 28, 30, 18, and 21 kDa (OspC) were recognized by 82%, 81%, 74%, 49%, 41%, 30%, 14%, 13%, 4%, and 0% of PNG sera, respectively. OspA and OspB were recognized by 6% and 75% of PNG serum samples, respectively.

**Table 1.** Analyses of serum samples from Papua New Guinea for antibodies recognizing *B. burgdorferi* antigens by ELISA: effect of using individual negative controls.

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>Without individual controls</th>
<th>With individual controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WCS</td>
<td>Fla</td>
</tr>
<tr>
<td>Positive</td>
<td>64 (74)</td>
<td>39 (45)</td>
</tr>
<tr>
<td>Equivocal</td>
<td>21 (24)</td>
<td>39 (45)</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (1)</td>
<td>8 (9)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%). WCS, whole cell sonicate of low-passage strain B31 of *B. burgdorferi* [6]; Fla, flagellin-enriched extract of *B. burgdorferi* [4].

Discussion

It has been reported that a large proportion of persons with any one of a number of clinically diagnosed illnesses, including infectious diseases, generate positive serologic test results for Lyme disease [2]. There have also been reports of Lyme disease from areas lacking ecologic or epidemiologic evidence, including Australia, South America, and South Africa [1]. Neither competent vectors nor known causative agents of Lyme disease have been isolated from these areas. These reports are based on either serologic reactivity in an ELISA or immunofluorescent assay,
PNG, a country nonendemic for Lyme disease. Serum samples from persons with confirmed Lyme disease and from persons from the two-tiered test. Antigens revealed significant differences between sera from persons with serologic "confirmation." or clinical criteria, or on a combination of clinical observations with serologic "confirmation."

In Australia, Russell [11] was unable to provide evidence by either polymerase chain reaction or darkfield microscopy of any known Lyme disease pathogens from >12,000 Australian ticks collected in reported Lyme disease-endemic areas. Laboratory studies also failed to identify any competent vectors [11]. Furthermore, Western blot analyses of 78 sera from suspected Lyme disease patients, including patients diagnosed with late-stage disease, failed to yield any positive results by the criteria of Dressler et al. [5], leading Russell [11] to question the existence of Lyme disease in Australia.

In the current study, 50% of the serum samples of residents of a PNG village were found to be positive when the currently recommended approach for serologic testing for Lyme disease in the United States (ELISA and Western blot [1, 4]) was used. However, elevated immunoglobulin levels in residents of tropical regions and cross-reactive antibodies generated against other pathogens are likely sources of false reactivity in ELISAs and Western blots. To minimize the problem of cross-reactive antibodies, individual negative controls are commonly incorporated into tests for serum from tropical areas for antigen recognition. When individual controls were incorporated with the Fla ELISA for Lyme disease in testing serum from PNG, a significant drop, from 50% to 10%, was seen in positive results from the two-tiered test.

Examination of the pattern of IgG recognition of different antigens revealed significant differences between sera from persons with confirmed Lyme disease and that from persons from PNG, a country nonendemic for Lyme disease. Serum samples from infected populations generally respond in similar proportions to OspA and OspB because their genes are in the same operon. In early infections, 8% of patients developed IgG antibodies to OspB, while 11% had IgG antibodies to OspA [12]. In late infections, 32% and 28% responded to OspB and OspA, respectively, in one study [13], while 14% and 13% developed IgG antibodies to OspB and OspA, respectively, in another study [14].

In contrast, IgGs in 75% of PNG sera recognized OspB, while only 6% recognized OspA. Of the 8 persons who met the combined ELISA and Western blot criteria to be considered serologically positive for Lyme disease, 6 recognized OspB, but none reacted to OspA. Furthermore, the expanded IgG response in persons with late Lyme disease characteristically recognizes additional B. burgdorferi antigens. 92%, 79%, 71%, and 41% of sera from late Lyme disease patients react to the 58-, 45-, 30-, and 21-kDa (OspC) antigens [5], respectively. These molecules were recognized by only 49%, 30%, 13%, and 0% of the PNG sera, respectively.

Further evidence against Lyme disease infections causing the IgG reactivity in PNG sera is the inability of any of the 8 seropositive sera to inhibit the growth of B. burgdorferi in vitro. A previous study reported that 69% of serum from Lyme disease patients inhibited the growth of B. burgdorferi in culture [9].

The lack of a positive reaction to the syphilis tests by the subjects with serologic tests positive for Lyme disease in PNG also argues against their exposure to B. burgdorferi. In previous studies, as many as 43% of confirmed Lyme disease patients had positive FTA-Abs tests [15].

The results from this study highlight the caution that must be exercised when applying serologic tests developed for use in temperate areas to tropical areas, where individuals often have elevated immunoglobulin levels. The prevalence of positive ELISA results was extremely high until more stringent controls were incorporated into the tests. In the absence of both clinical data and isolates of Lyme disease spirochetes, serologic results must be cautiously interpreted, particularly with samples from areas lacking supporting epidemiologic evidence of Lyme disease.

Four lines of evidence argue against B. burgdorferi being present in PNG. First, all known vectors of Lyme disease are absent. Second, while Western blot analysis of PNG sera using B. burgdorferi as antigen recognized a high mean number of antigens, the antigen recognition pattern, particularly for OspA, OspB, OspC, and the 58-, 45-, and 30-kDa antigens was very different from that seen in patients with confirmed Lyme disease. Last, in contrast to sera from confirmed Lyme disease patients, PNG serum samples positive in the Lyme disease assays did not inhibit B. burgdorferi growth in culture and failed to cross-react in the syphilis tests, characteristics of true Lyme disease cases. While the possibility of a previously undescribed pathogen being responsible for the clinical observations of Lyme disease in areas not known to have either known
vectors or enzootic cycles cannot be excluded, the observations reported here are unlikely to have resulted from infection with *B. burgdorferi*.

This paper addresses several issues associated with false positivity and therefore specificity of current Lyme disease serology. While the value of using individual negative controls to minimize false positives is demonstrated, the affect of this approach on true positive serologies and test sensitivity remains to be ascertained. In addition, it is imperative that clinical and epidemiologic supporting data be considered in conjunction with serologic analyses when defining an area as endemic for Lyme disease.

**Acknowledgments**

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**References**