Laboratory confirmation of Lyme disease

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TG SCHWAN, WJ SIMPSON, PA ROSA. Laboratory confirmation of Lyme Disease. Can J Infect Dis 1991;2(2):64-69. Lyme disease can be confirmed in the laboratory by isolation or detection of its causative agent, a tick-borne spirochete *Borrelia burgdorferi*, or by a diagnostic change in the titre of antibodies specific to the agent. *B burgdorferi* can be isolated and cultivated in Barbour-Stoenner-Kelly II medium. It can be detected by light microscopy in tissue sections or, rarely, in blood smears using various staining methods. There is interest in the development of alternative detection methods, including identification of specific antigens of *B burgdorferi* in the urine of suspected cases and demonstration of the presence of species-specific DNA using polymerase chain reaction assays. Currently, serological tests (indirect immunofluorescence assay, enzyme-linked immunosorbent assay and Western immunoblot) are the most practical and available methods for confirming Lyme disease. The quest to improve the specificity and sensitivity of serological tests – for example, through the use of specific recombinant antigens – continues.

Key Words: Borrelia burgdorferi, *Enzyme-linked immunosorbent assay, Indirect immunofluorescence assay, Laboratory confirmation, Western immunoblot*

Examens de laboratoire et confirmation de la maladie de Lyme

RESUME: La maladie de Lyme peut être confirmée en laboratoire grâce à l'isolement ou à la détection de l'agent étiologique, le spirochète *Borrelia Burgdorferi*, ou par des titres significatfs d'anticorps spécifiques antispirochètes. *B burgdorferi* peut être isolé et cultivé en milieu de culture B-S-K II. Il peut être détecté dans des coupes de tissus sous microscopie classique ou, rarement, sur des lames sanguines soumises à diverses méthodes de coloration. On s'intéresse à la mise au point d'autres méthodes possibles de détection – identification des antigènes spécifiques de *B burgdorferi* dans l'urine des sujets chez qui on soupçonne la maladie et mise en évidence d'ADN spécifique d'espèces grâce à la réaction en chaîne à la polymérase. Présentement, les tests sérologiques (étude en immunofluorescence indirecte, méthode immunoenzymatique, et Western immunoblot) sont les plus pratiques et les plus accessibles pour confirmer la maladie de Lyme. La recherche se poursuit donc pour améliorer la spécificité et la sensibilité des tests sérologiques – par l'utilisation de l'antigène flagelline, notamment.

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LYME DISEASE CAN BE CONFIRMED IN THE LABORATORY by the isolation or detection of its causative agent, the tick-borne spirochete *Borrelia burgdorferi*, or by a diagnostic change in the titre of antibodies specific to the agent when a patient's sequential sera are compared. In the absence of an acute serum, a single convalescent serum with an antibody titre above a specified level may be acceptable for serological confirmation. However, the vast majority of the human population of Canada and the United States lives outside the hyperendemic areas for Lyme disease. Throughout these nonendemic or less endemic areas, human cases of Lyme disease are being reported. In the absence of isolation or detection of the spirochete, these cases are 'confirmed' by serological tests with problems in sensitivity, specificity and standardization. Improvements in laboratory tests, therefore, will both confirm cases of Lyme disease and aid the identification of patients not infected with the spirochete.

The demands placed on clinical laboratories to confirm or rule out Lyme disease in such a large and diverse environment as Canada, will likely be great. The following is a brief review of some of the methods currently used in the laboratory to confirm Lyme disease, as well as a discussion of problems associated with these methods.

ISOLATION OF THE SPIROCHETE

B burgdorferi can be isolated and cultivated in a complex, undefined liquid culture medium (1), although the efficacy of the medium for growing the spirochete during its primary isolation from infected mammalian or tick tissues is unknown. The breakthrough for the in vitro cultivation of borreliae came in 1971, when Kelly (2) described a liquid medium that successfully maintained the relapsing fever spirochete *Borrelia hermsii* for 80 continuous subpassages, after which the spirochetes were still infectious for laboratory mice. In 1974, Stoenner (3) modified the method for preparing the Kelly medium and later fortified it by adding CMRL (GIBCO Laboratories) tissue culture media and yeastolate (DIFCO Laboratories) (4). It was in this fortified Kelly medium (as it was then called) that, in November 1981, Barbour isolated Lyme disease spirochetes from the infected midguts of *Ixodes dammini* ticks that had been given to him by Burgdorfer (5). Barbour et al (6) subsequently added neopeptone and HEPES, removed the yeastolate, and changed the name to 'BSK medium', apparently standing for Barbour-Stoenner-Kelly medium. BSK (or BSK I) became BSK II with the removal of glutamine and the addition again of yeastolate. BSK II is the medium currently used by most workers to isolate and maintain *B burgdorferi* in the laboratory (1). The spirochete is cultured most successfully when the medium has a neutral pH and is incubated in a microaerophilic environment at 30 to 37°C. The culture medium is monitored for spirochetes by darkfield light microscopy for four to six weeks, although cultures may be positive in less than a week (7) or may require many months if the medium lacks gelatin and rabbit serum and is incubated at lower temperatures (8).

Once isolated in the medium, the spirochetes may be identified as *B burgdorferi* by reactivity with species-specific monoclonal antibodies (9), although hybridization with specific DNA probes (10,11) or amplification of specific DNA using the polymerase chain reaction (12,13) can be done as discussed below.

Many laboratories may be hesitant to commit the resources and technical assistance required to attempt the isolation of *B burgdorferi*. There is no substitute, however, for isolation of the spirochete when one is attempting to identify infected human patients, domestic and wild mammals, or ticks, or when one is establishing an area as being endemic for Lyme disease. The spirochete can be isolated most frequently from the skin, blood and cerebrospinal fluid, although success rates are only moderate to poor (8,14,15). In spite of these poor rates, clinicians and diagnostic laboratories are encouraged to attempt to isolate the spirochete when patients in nonendemic areas present with what appears to be typical acute Lyme disease.

DETECTION OF THE SPIROCHETE BY STAINING

B burgdorferi, like other spirochetes, can be detected by light microscopy in tissue sections or, rarely, in blood smears using various staining methods. The Warthin-Starry silver stain has been used most often (16), even though spirochetes stained with silver cannot be identified as *B burgdorferi* by this property alone. Immunological stains using fluoresceinated polyclonal antiserum to *B burgdorferi* have also been used to detect spirochetes, but again, detection does not identify the spirochete because of antigens shared by other species of borrelia. Indirect staining with fluoresceinated monoclonal antibodies specific for *B burgdorferi*, eg, H5332 (9), has the potential both to detect and to identify spirochetes as *B burgdorferi* in clinical samples (17).

Given the apparent scarcity of *B burgdorferi* in mammalian tissues, the time required, and the expense involved in detecting spirochetes in stained sections of human tissues, it is unlikely that histological sectioning and staining for the Lyme spirochete will have any practical applica-

tion in clinical laboratories for routine confirmation of cases.

DETECTION OF *B BURGDORFERI* ANTIGENS

Given the difficulties of cultivating or detecting spirochetes by staining, considerable interest has been directed at developing alternatives for the detection of spirochetes. One approach has been to identify specific antigens of *B burgdorferi* in the urine of patients suspected of having Lyme disease. Several groups have already shown that Lyme spirochetes may be present in the urine of naturally infected white-footed mice, *Peromyscus leucopus* (18), and that the urinary bladders of both experimentally and naturally infected rodents often produce spirochetes when the organs are triturated and inoculated into BSK II medium (7,19). Antigens of *B burgdorferi*, including outer surface proteins A and B and the flagellar protein, have been detected in the urine of both laboratory mice infected with *B burgdorferi* and humans with Lyme disease (20). Urine containing these antigens was filtered through a membrane that was then incubated with one of three specific monoclonal antibodies in a dot blot immune assay.

Although the initial results were encouraging and one urine-based antigen test was marketed commercially for a brief time, no such test for confirming Lyme disease in the laboratory is currently available.

DETECTION OF *B BURGDORFERI* USING DNA PROBES AND THE POLYMERASE CHAIN REACTION

In the past few years, another strategy developed at Rocky Mountain Laboratories to detect *B burgdorferi* has been to demonstrate the presence of DNA specific to this spirochete. First, hybridization probes were developed to identify specifically both purified DNA of *B burgdorferi* and whole spirochetes (10,11,21). Although the probes specifically identified Lyme spirochetes, the sensitivity of some probes was unsatisfactory because detection required a minimum of 10,000 spirochetes. Subsequently, assays have been developed using the polymerase chain reaction. These assays are specific for *B burgdorferi*, capable of detecting as few as one to five spirochetes, and able to type spirochetes from North America and Eurasia into two groups (12,13).

Other workers have subsequently developed further polymerase chain reaction assays amplifying other regions of the spirochete's genome to identify *B burgdorferi* DNA (22,23) and to detect the spirochete in fresh ticks (24), ticks preserved in alcohol (25), or the urine of human patients with Lyme disease (26).

Polymerase chain reaction for the laboratory confirmation of Lyme disease is not yet commercially available.

LABORATORY CONFIRMATION BY SEROLOGICAL TESTS

Immunofluorescent assay: At the present time, serological tests are the most practical and available methods for confirming Lyme disease in the clinical laboratory. The history of Lyme serology dates back fewer than 10 years to late 1981, when the newly isolated spirochetes were used in an indirect immunofluorescence assay (IFA) with convalescent sera of Lyme patients from the northeastern United States (5). Since then, the arena of Lyme serology has grown rapidly, with numerous tests described in the scientific literature, and new test kits available in an increasingly competitive commercial market. Already numerous published reviews and editorials describe some of these tests, their potential problems, and their lack of standardization (27-33).

The IFA, as mentioned above, was the first serological test used to confirm Lyme disease and is still used in many laboratories (34). In this test, Lyme spirochetes are first fixed onto a glass microscope slide and then incubated with human serum at one or several dilutions. To detect human anti-*B burgdorferi* antibodies bound to the spirochetes, a second antibody raised against human immunoglobulin in some other species of mammal, and labelled with fluorescein isothiocyanate, is then incubated on the slide. The binding of this second antibody is visualized with a fluorescence microscope and indirectly demonstrates the presence of *B burgdorferi* antibodies (34).

While this test is easy to do, and truly high titre sera will fluoresce beautifully, there are problems with the test's specificity, reproducibility and usefulness when large numbers of samples need to be tested, as well as with its lack of quantification and subjective interpretation. Due to the presence of nonspecific antibodies in some normal sera, most workers require that a positive sample be reactive when diluted to 1:256 or more, although not everyone adheres to this threshold for a positive result. Unfortunately, a titre of 1:256 or greater may occur if the patient has been exposed to other species of spirochetes, or to even more distantly related bacteria that share antigens with the Lyme spirochete (35). Therefore, a patient who has not been exposed to *B burgdorferi* but who has had tick-borne relapsing fever, syphilis, leptospirosis or periodontal disease, may have antibodies that react positively in a Lyme test (35,36).

Determining the endpoint or titre is also somewhat subjective, as each individual reading the

test may have his or her own cutoff based on the number of spirochetes fluorescing and the intensity of fluorescence.

Enzyme-linked immunosorbent assay: The enzyme-linked immunosorbent assay (ELISA) or enzyme immune assay is probably the most widely used serological test for confirming Lyme disease in laboratories in which large numbers of samples are routinely tested (34,37,38).

This type of assay has advantages over the IFA. It allows workers to test many samples quickly, and uses a spectrophotometric determination that is quantifiable and subject to statistical analysis.

The assay in its simplest form uses a 96-well plastic microtitre plate coated with either intact spirochetes or a suspension of spirochetal antigens produced by disruption of the bacteria by sonication. Human sera are then incubated in the wells, allowing anti-*B burgdorferi* antibodies to bind to the antigens adsorbed to the surface of the wells. Following removal of the sera, an antibody to human immunoglobulins (polyvalent or specific for IgM or IgG [37,39]), which has been chemically bound to one of several enzymes, is incubated in each well. Finally, a chemical substrate is added which results in a colour change by the action of the enzyme bound indirectly to anti-*B burgdorferi* antibodies. Very positive sera produce enough colour to allow the result to be determined with the unaided eye; however, the test is usually quantified with a spectrophotometer.

While this method is fast, efficient for testing many samples, and quantifiable with less subjective error, it has the same weaknesses of specificity as the IFA (35,36), and the ELISA is even less standardized than the IFA. As mentioned above, sera from patients exposed to certain other pathogenic bacteria may react falsely. Also, there is considerable variation in how the ELISA is done and in how results are reported.

Western immunoblot: The problem of false positive results when using the IFA or the ELISA, especially for patients with atypical clinical manifestations living in areas currently considered to be nonendemic, has necessitated a potentially more specific serological test. The Western immunoblot technique, considered by some investigators to be the 'gold standard', could potentially increase the specificity of Lyme serology. In this assay, Lyme spirochetes are lysed by chemical treatment and heat, and the various molecular components separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (40). After the components have been separated in the gel, they are transferred by electroblotting onto a membrane, on which they bind with specific anti-*B burgdorferi* antibodies in the serum incubated with the membrane. Antibodies to the different antigens are then visualized by one of several techniques using either an enzyme-substrate mediated colour change similar to that used for the ELISA, or autoradiography.

Done properly, a negative serum can be identified with a high degree of confidence. When the assay begins to produce bands resulting from antibodies binding to one or more of the antigens, the question of what is really positive becomes a concern. Considerable variation in the reagents and methods used subject this procedure to the same types of interlaboratory variation that exist for the ELISA.

Other diagnostic methods: The potential lack of specificity of many of the tests described above has led the authors and others to search for components of the bacterium that can be used as diagnostic antigens which will improve the specificity and sensitivity of serological tests.

Two approaches to obtaining specific antigens have been either to lyse the spirochetes and then selectively purify the target antigen (38,41-47), or to use recombinant DNA techniques to clone and express a specific spirochetal antigen in a foreign bacterial cell such as *Escherichia coli* (48-51). One of the antigens receiving the most attention has been the 41 kilodalton protein, flagellin, because it is thought that flagellin elicits the earliest antibodies produced during infection (41,42). Interest in this antigen has continued in spite of its partial amino acid sequence similarity with flagellin of other pathogenic spirochetes (42), which could result in false positive tests.

Recently workers at Rocky Mountain Laboratories cloned and expressed, in *E coli*, a 39 kilodalton species-specific antigen of *B burgdorferi* that reacted with all sera from human cases of Lyme disease that were positive (greater or equal to 1:256) by an IFA (50). Most of these sera, however, did not react with flagellin, raising the possibility that anti-P39 antibodies have been mistaken for antibodies to the closely migrating flagellin.

The authors' recent experimental work with mice (*P leucopus* and *Mus musculus*), corroborates their earlier study with humans. When 40 mice were infected with *B burgdorferi*, all 40 produced anti-P39 antibodies, whereas only seven (18%) produced antiflagellin antibodies (51). Additional mice, infected by the bite of *I dammini* ticks, produced anti-P39 antibodies only two to four days after the ticks had detached (51). Commercial test kits using the P39 antigen are currently being developed by several companies, and may be available in the near future.

CONCLUSIONS

Many aspects of serological testing of Lyme disease have not been discussed in this article, such as the influence of in vitro cultivation (52), temperature (53) and strain variation (54) on the reactivity of *B burgdorferi*, the effect of antibiotic treatment on the host's antibody response (14), the possible problem of seronegative Lyme disease and the contribution of antigen-antibody complexes (55), the use of T cell proliferative assays to identify true seronegative Lyme patients (56), IgM versus IgG assays to recognize early antibody production and early detection of cases (37,39), and more. Even with all the work described during the past 10 years, serological testing for Lyme disease is still in need of improvement and standardization. Within the next year, the use of recombinant antigens for antibody tests, and polymerase chain reaction to detect the spirochete may become available and help improve laboratory confirmation of Lyme disease. For the present, the IFA and ELISA are the most available and practical methods. Again, however, the importance of attempting to isolate and culture *B burgdorferi* from humans and other animals suspected of having Lyme disease must be emphasized. This isolation and culturing will establish a more accurate picture of the distribution of the Lyme spirochete in North America, and possibly identify other closely related, but as yet unknown, spirochetes that could be confusing the epidemiological picture of this important tick-borne pathogen.

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