JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE 80(3-4):57–59 JULY-OCTOBER 2005

DETECTION OF *BORRELIA BURGDORFERI* SEQUENCES IN A BIOPSY FROM A TENNESSEE PATIENT

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ABSTRACT—*Borrelia burgdorferi* is the causative agent of Lyme disease and is transmitted by the black-legged tick, *Ixodes scapularis*. Clinical diagnosis of Lyme disease is often made on the basis of a characteristic Erythema Migrans (EM) rash. Lyme disease cases have been reported in Tennessee since 1991 when Lyme became a nationally notifiable disease. However, to date, there have been no reports of *B. burgdorferi* isolation in culture or detection of genetic sequences characteristic of *B. burgdorferi* in Tennessee. A syndrome related to Lyme disease, southern tick associated rash illness (STARI), has been described and also manifests an EM rash. The putative agent of STARI, which is not a reportable illness, is *Borrelia lonestari*. The presence of *B. lonestari* has been documented in the state. A skin biopsy was taken from an EM rash from a patient in East Tennessee. This study was undertaken to determine if *Borreliae* were present in the biopsy. Following nucleic acid extraction and amplification, a sequence for the *Borrelia* flagellin gene was obtained. There was 98–99% sequence identity between the biopsy and other reported *B. burgdorferi* flagellin gene sequences. This represents the first detection of genetic sequences of *B. burgdorferi* in Tennessee.

Lyme disease is the most common vector borne disease in the United States and is endemic in the northeastern United States. The causative agent is *Borrelia burgdorferi*, which was isolated in culture in 1982 (Burgdorfer et al., 1982). *Borrelia burgdorferi* spirochetes are transmitted by *Ixodes scapularis* ticks (black-legged tick, deer tick) throughout the central and eastern United States. The hallmark symptom of Lyme disease is a characteristic "bull's-eye" rash, Erythema Migrans (EM), which occurs in nearly 80% of patients one to two weeks after *I. scapularis* feeding (CDC, 1997). Erythema Migrans rashes also have been documented at feeding sites of *Amblyomma americanum*, the Lone Star tick (Schulze et al., 1984; Masters et al., 1998; James et al., 2001). Microscopic evaluation of these tissues revealed spirochetes but this organism was not able to be cultured on medium that supported growth of *B. burgdorferi*. Genetic evaluation of this organism revealed a *Borrelia* distinct from *B. burgdorferi*, which has been provisionally named *Borrelia lonestari* (Barbour et al., 1996). *Borrelia lonestari* infection is believed to be responsible for a Lyme-like disease that has been designated southern tick-associated rash illness (STARI) (Burkot et al., 2001). Patient serologic testing fails to adequately distinguish between *B. burgdorferi* and *B. lonestari* as organisms responsible for EM rashes (Kirkland et al., 1997; Masters et al., 1998).

Cases of Lyme disease, putatively attributed to *B. burgdorferi*, have been reported from nearly all states (CDC, 2004). The majority of cases in the United States occur in northeastern states. Lyme disease has been reported in Tennessee each year since Lyme disease became reportable. However, culture of *B. burgdorferi* or detection of genetic sequences has not been documented in the state. *Borrelia lonestari* sequences have been reported from Tennessee (Stegall-Faulk et al., 2003; Stromdahl et al., 2003) and the majority of *B. lonestari* detections have occurred in southeastern states (Burkot et al., 2001; Moore et al., 2003; Varela et al., 2004).

In 2003, a skin biopsy was taken from the margin of an EM rash from a patient residing in East Tennessee. This study was undertaken to evaluate this biopsy for the presence of *Borreliae*, and, if *Borrelia* nucleic acid was found, to determine if sequences were characteristic of *B. burgdorferi* or *B. lonestari*.

MATERIALS AND METHODS

Biopsy—In April 2003, a patient presented at a clinic in East Tennessee with an EM rash. The patient resided in Greene County, Tennessee. A 2 mm skin biopsy was taken from the margin of the EM rash and retained for evaluation. No tick was available for study.

Extraction—Potential *Borrelia* nucleic acid was extracted from the biopsy using a ZR Genomic DNA II kit according to manufacturer instructions except digestion was allowed to proceed for 4 h at 37°C (Zymo Research, Orange, California).

DNA Amplification—The biopsy extract was subjected to two rounds of amplification. The first set of primers, derived from a conserved region of the *Borrelia* flagellin gene, was designed to amplify a fragment of 499 bp for *B. burgdorferi* or 476 bp for *B. lonestari*. The primer sequences were: forward, 5′ CAAAAATTAATACACCAGCAT; reverse, 5′ GCAATCA-TAGCCATTGCAGA (Integrated DNA Technologies, Coralville, Iowa). Following the first amplification, nested PCR was done using internal conserved sequences of the flagellin gene to verify *Borrelia* DNA was present. Expected fragment sizes were 319 bp for *B. burgdorferi* or 298 bp for *B. lonestari*. Nested primer sequences were: forward, 5′ CTAATGTTG-CAAATCTTTT; reverse, 5′ GCATCTTTAATTTGAG-

CATA (Integrated DNA Technologies). Other reagents for PCR included *Taq* polymerase, 10× buffer and dNTPs (Promega, Madison, Wisconsin). Negative dH_2O controls were included in all reactions. PCR products were visualized on a 1.25% agarose gel (NuSieve, FMC BioProducts, Rockland, Maine) stained with ethidium bromide.

Cloning and Sequencing—The PCR amplicon was column cleaned (Quantum Prep, BioRad, Hercules, California). The clean product was ligated into pGEM-T (Promega) and transformed into *Escherichia coli* JM109 (Promega). Transformants were visually inspected for beta-galactosidase activity and plasmids were obtained by standard alkaline lysis. Bi-directional sequencing was done on an Open Gene Automated Sequencer using a Dye Primer Cycle Sequencing Kit (Visible Genetics, Toronto, Ontario, Canada) according to manufacturer instructions. Forward primer M13Cy5.0 was provided in the kit. The reverse primer was Sp6Cy5.0, sequence: 5′ ATTTAGGTGACACTATAGAATAC (Integrated DNA Technologies).

RESULTS

Nested amplification of the biopsy resulted in successful recovery of *Borrelia* DNA. As seen in Fig. 1, an electrophoretic gel containing the PCR product, the nested amplified biopsy extract appears in lane 4. When compared with lane 3 (*B. burgdorferi* positive control) and lane 5 (*B. lonestari* positive control), the amplified biopsy product migrates with the *B. burgdorferi* control while the *B. lonestari* control is slightly lower on the gel (expected fragment size 298 bp).

To ensure that the biopsy contained DNA from *B. burgdorferi*, the nested PCR product was sequenced. The GenBank accession number assigned to this sequence is AY964065. Sequence identity was determined by a nucleotide BLAST search against the GenBank DNA database. The *B. burgdorferi* sequence from the biopsy demonstrated the greatest homology (99%) with *B. burgdorferi* strain B31, differing at only 2 of 319 nucleotides. Sequence identity with all *B. burgdorferi* flagellin sequences in GenBank was 98–99%.

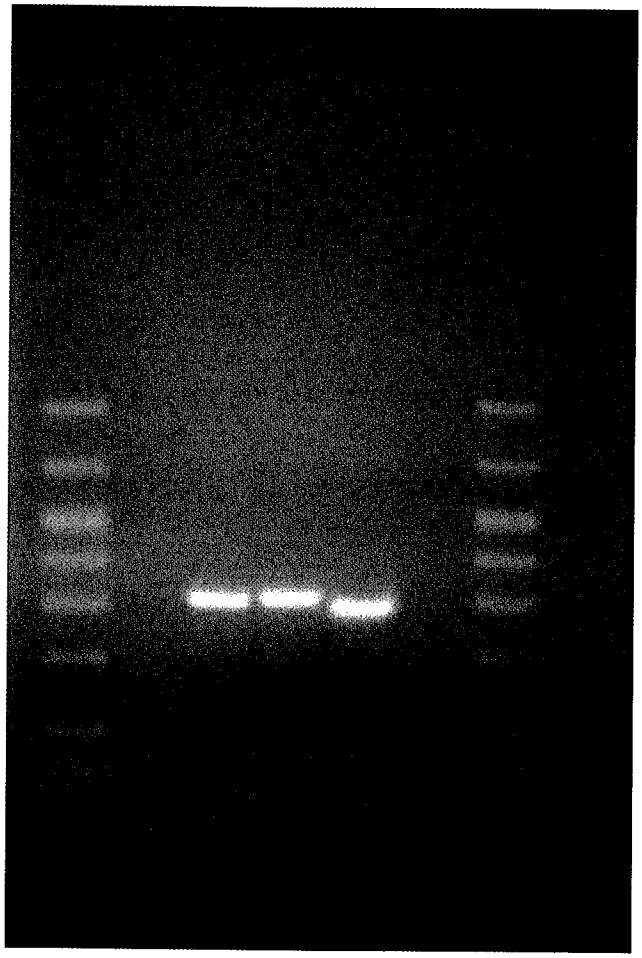

FIG. 1. Electrophoretic Agarose Gel of Amplified Biopsy Sample. Lanes 1 and 7 contain DNA standard ladder BioMarker Low (fragment sizes: 1000, 700, 525, 500, 400, 300, 200, 100, 50 bp; BioVentures, Murfreesboro, Tennessee); Lanes 2, 6, 8 are blank; Lane 3 is a *Borrelia burgdorferi* positive control (319 bp); Lane 4 is the biopsy; Lane 5 is a *Borrelia lonestari* positive control (298 bp).

DISCUSSION

This investigation determined that an EM biopsy contained DNA characteristic of *B. burgdorferi*. This is the first report of a *B. burgdorferi* sequence in Tennessee. Previous investigations have only found DNA from *B. lonestari* (Stegall-Faulk et al., 2003; Stromdahl et al., 2003).

It may be that some cases reported as Lyme disease based solely on the presence of the EM rash may actually represent STARI. Clinical diagnosis of Lyme disease usually does not involve PCR analysis of skin biopsies, although this has been reported as the most sensitive diagnostic method (Nowakowske et al., 2001). Others in the southeastern United States have investigated biopsies of EM sites for evidence of *B. burgdorferi* but, in most cases, culture results were negative (Kirkland et al., 1997; Felz et al., 1999). It is possible that in those reports, *B. lonestari* was present but not able to be cultured or detected. In regard to those studies, it is particularly intriguing that the patients presenting with EM were residents of Georgia, North Carolina and South Carolina. These states, bordering Tennessee, represent a region of the country where *B. lonestari* is most commonly found.

Another explanation for the rarity in detecting *B. burgdorferi* in Tennessee may have to do with the vector presence. While *I. scapularis* is established throughout most of the eastern portion of the United States, there are pockets where *I. scapularis* is not found, including much of Tennessee (Dennis et al., 1998). If the vector of *B. burgdorferi* were scarce, so, too, its pathogen would be expected to be rare. The vector of *B. lonestari*, *A. americanum*, is widely distributed throughout the southeastern United States (Childs and Paddock, 2003). A tick collection study conducted during 2002–2003 in middle Tennessee recovered 8199 ticks. *Amblyomma americanum* represented over 91% of collected ticks; no *I. scapularis* were found (Ludyjan-Ybarra, 2004). Documentation of *B. burgdorferi* in Tennessee by this study may lead to more accurate diagnoses of Lyme disease or STARI in the state.

ACKNOWLEDGMENTS

This work was supported, in part, by grant #0227754 from the National Science Foundation.

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