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Business Manager

For correspondence
Karen Vanderhoof-Forschner
Lyme Disease Foundation
One Financial Plaza
Hartford, CT 06103-2610
Telephone: 860-525-2000
Fax: 860-525-8425
e-mail: lymefnd@aol.com
Internet: www.lyme.org

Publisher

For submissions and subscriptions
SLACK Incorporated
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Thorofare, NJ 08086
609-848-1000

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Attachment of Human and Rhesus *Serpulina pilosicoli* to Cultured Cells and Comparison with a Chick Infection Model

Nagaraja Muniappa, BVSc, MS, PhD; Mohan R. Ramanathan*, BVSc, MS; Ross P. Tarara†, DVM, PhD; Ralph B. Westerman‡, PhD; Michelle R. Mathiesen*; and Gerald E. Duhamel*, DMV, PhD*

ABSTRACT

Intimate attachment of spirochetes to the apical membrane of colonic enterocytes is pathognomonic of colonic spirochetosis, a disease of human beings and animals caused by *Serpulina pilosicoli*. The aims of this study were to develop a cultured cell model of *S pilosicoli* attachment and to compare the spirochete-cell interaction with the chick infection model of colonic spirochetosis.

Three rhesus monkey *Serpulina pilosicoli* isolates were compared to a reference human isolate for attachment to six eukaryotic cell lines and to the cecal epithelium of chicks using light and electron microscopy. The attachment of *S pilosicoli* to H407 cells was examined further by fluorescent actin staining and radioimmunoassay.

Attachment of *S pilosicoli* was highest with the human embryonic intestinal epithelial H407 cells, less with CaCo-2 and HEp-2 cells, minimal with FaDu and HeLa cells, and absent with HT-29 cells. Attachment of spirochetes was diffuse, non-polar, time-dependent, and did not involve actin rearrangement. By contrast, chick cecal enterocytes colonized by the spirochetes had cap-like structures overlying areas of rarefaction of the terminal web microfilaments and accumulation of membrane-bound cytoplasmic vesicles.

Attachment of human and rhesus *S pilosicoli* to cultured eukaryotic cells correlated with the chick infection model, but each model displayed unique morphological features that were representative of the natural disease.

Key words: intestinal spirochetes, *Serpulina pilosicoli*, spirochete attachment, spirochete adherence, spirochetosis

INTRODUCTION

A newly recognized pathogenic intestinal spirochete, *Serpulina pilosicoli*[1-3] is the etiologic agent of a disease variously referred to as intestinal spirochetosis,[3-11] colonic spirochetosis,[12-14] colorectal spirochetosis,[15,16] or rectal spirochetosis.[17] Colonic spirochetosis (CS) is preferred because it more accurately describes the location of the intestinal lesions in human beings,[4-8,15-21] nonhuman primates,[9,11,20-24] dogs,[9,10] swine,[12,13,25-28] guinea pigs,[9] opossums,[9] and birds[29] with the disease. Another intestinal spirochete different from *S pilosicoli*, and called *Brachyspira aalborgi*, also has been found in human beings with CS in Denmark, but the epidemiological significance of this spirochete is unknown.[30] More recently, *B aalborgi* alone or together with *S pilosicoli* and some unclassified flagellated bacteria have been seen in CS of colony-raised rhesus monkeys.[24]

Koch's postulates for *S pilosicoli* have been fulfilled with gnotobiotic swine[26] and conventional swine.[13,25,27,28] Swine challenge-exposed with human and porcine *S pilosicoli* develop diarrhea and reduced growth with focal to diffuse attachment of spirochetes to the cecal and colonic enterocytes. Similarly, crop inoculation of one-day-old chicks with human, porcine, and canine *S pilosicoli* results in attachment of the spirochetes to the cecal enterocytes accompanied with focal erosions and sometimes local invasion of the gut wall.[14,29,31,32] Because the morphologic changes in the colon of human beings with

*From the *Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln; the †California Regional Primate Research Center, University of California-Davis; and the ‡College of Veterinary Medicine, Kansas State University, Manhattan.*

Address correspondence to Gerald E. Duhamel, Veterinary Basic Science Bldg., Department of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0905.

CS are similar to those found in animals and because inoculation of swine and chicks with human *S pilosicoli* produces CS, animal models are considered relevant to studies of the pathogenetic mechanisms involved in human CS.[27,29,32]

Non fimbriae-mediated intimate attachment of bacteria to the plasma membrane of intestinal epithelial cells is an initial and essential step in the pathogenesis of many diseases of the alimentary tract. One such intimate attachment is characterized by attaching and effacing (AE) lesions at the point of contact between the bacterium and the host cell plasma membrane.[33-35] This is seen with enterocolitis caused by attaching and effacing *Escherichia coli* (AEEC),[33-35] certain strains of enteropathogenic *Yersinia pseudotuberculosis*,[36] *Citrobacter freundii*, the cause of transmissible murine colonic hyperplasia,[37] and strains of *Hafnia alvei* isolated from children with diarrhea.[38] Morphologically, AE lesions are characterized by 1) an irregular surface epithelium with focal epithelial cell degeneration and sloughing, 2) effacement of apical microvilli, 3) elevation of the host plasma membrane forming "cups" or "pedestals" at the base of individually attached bacteria, and 4) electron dense fibrillar modifications in the terminal web area beneath the attached bacteria that corresponds to F-actin rearrangement.[33-36] Genetically, the development of AE lesions requires the expression of conserved virulence genes located within the locus of enterocyte effacement present in AEEC, *C freundii*, and *H alvei*.[38]

The intimate attachment seen with *Helicobacter pylori* type I strains appears different from the AE lesion. Although attachment of *H pylori* to gastric cells in patients with type B gastritis and gastric and duodenal ulcers causes effacement of microvilli and a slight elevation of the host plasma membrane, the molecular basis of *H pylori* interaction is fundamentally different from that of AE bacterial enteric pathogens.[39-42] Neither the cytoplasmic electron-dense deposits or F-actin rearrangement beneath the point of bacterial attachment, nor the *eae* gene, a virulence gene located within the locus of enterocyte effacement, are present in *H pylori*.[40] And although uptake of bacteria by host cells is seen with *H pylori* and AE bacteria, only *H pylori* is seen penetrating between epithelial cells and disrupting intercellular junctions.[39,41]

Previous morphological studies using the chick infection model suggest that the mechanism of mucosal association with human, canine, and porcine *S pilosicoli* is different from that described previously for AE gastroenteric bacterial pathogens.[32] *Serpulina hyodysenteriae*, the cause of swine dysentery, attaches to primary and continuous cell lines, and the mechanism is suggestive of an adhesion-receptor interaction involving sialic acid.[43,44] Because *S pilosicoli* has a broader host range than *S hyodysenteriae*, and because attachment of *S pilosicoli* to the colonic epithelium is a key early event in CS, it is important to define the basis of host-parasite interactions at the molecular level. The aims of the present study were to develop a cultured cell model of *S pilosicoli* attachment, and to compare the morphology of the in vitro spirochete-cell interaction with the chick model of CS. Attachment of *S pilosicoli* to cultured eukaryotic cells correlated with the chick infection model, but each model displayed unique morphological characteristics of the natural disease. The *S pilosicoli* eukaryotic cell interaction more closely resembled *H pylori* than other non fimbriae-mediated intimate bacterial attachments characterized by AE lesions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Serpulina innocens isolate B256 (American Type Culture Collection [ATCC]# 29796), human *S pilosicoli* isolate SP16 (ATCC# 49776), and the AE *Escherichia coli* strain 2430-78 (CW Maddox, Pennsylvania State University, PA) were used in this study. Three spirochete strains, designated MMU27669, MMU26986, and MMU26717, were isolated from the colons of colony-raised rhesus macaques (*Macaca mulatta*) at the California Regional Primate Research Center. Each strain was identified by specific amplification of purified chromosomal DNA with a *S pilosicoli* but not with a *B aalborgi* 16S rRNA gene-based PCR assays as previously described.[24] Isolate MMU26717 was from a one year and ten month old female that had been euthanatized, whereas isolates MMU26986 and MMU27669 were from female monkeys, aged one year and nine months, and nine months, respectively, that were found dead. Each monkey was born in a different outdoor cage between 1992 and 1993, and rhesus MMU26717 and MMU26986 had been hospitalized respectively for one month and several months, because of chronic diarrhea, wasting, and dehydration. Concurrent infections with *Shigella flexneri* and *Yersinia pseudotuberculosis* were present in rhesus MMU26717 and MMU27669. Pure cultures of spirochetes were propagated in pre-reduced anaerobically-sterilized trypticase soy broth as previously described.[2,14]

Spirochete attachment to cultured cells

Cell lines. The human intestinal epithelial cell lines: embryonic small intestine (H407; ATCC# CCL6), colonic adenocarcinomas (CaCo-2; ATCC# HTB37 and HT-29; ATCC# HTB38), and the human nonintestinal epithelial cell lines: laryngeal carcinoma (HEp-2; ATCC# CCL23), pharyngeal carcinoma (FaDu; ATCC# HTB43), and cervical carcinoma (HeLa; ATCC# CCL2) were examined for spirochetal attachment. The cells were grown in 5%

CO_2 in air at 37°C in Dulbecco's Modified Eagle Medium (MEM; Gibco BRL, Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum.

Light microscopy. The attachment of spirochetes to cultured cells was examined using a previously described method.[44] Epithelial cells in 4-well chamber slides (Nunc, Inc., Naperville, IL) were incubated at 37°C and 5% CO_2 in air for 2 hours, washed with MEM, and inoculated with either sterile medium or medium containing approximately 1×10^8 spirochetes. After incubation at 37°C and 5% CO_2 in air for 2 hours, the wells were washed three times with phosphate buffered saline (PBS; pH 7.2), fixed with methanol for 2 minutes, and stained with the Giemsa's stain. Attachment was estimated subjectively by light microscopic examination at 400 × magnification in at least two separate experiments. Results were expressed as (-) = no attachment; (+/-) = < 20% of the cells with < 10 spirochetes/cell; (+) = < 20% of the cells with > 20 spirochetes/cell; (++) = > 80% of the cells with < 20 spirochetes/cell; (+++) = > 80% of the cells with > 20 spirochetes/cell.

Fluorescent actin staining. Control H407 cells and cells with attached human *S pilosicoli* were examined for actin filament rearrangement in two separate experiments using a previously described method.[35] The cells were fixed with 3% (vol/vol) formaldehyde for 20 minutes at room temperature, washed with PBS, and treated with 0.1% (vol/vol) Triton X-100 (Sigma, St. Louis, MO) in PBS for 4 minutes at room temperature. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-phalloidin (5 µg/mL in PBS; Sigma) for 20 minutes at room temperature, washed with PBS, and examined under ultraviolet light using a fluorescence microscope (Nikon Microphot, Nikon Inc., Garden City, NY). The actin rearrangement positive *E coli* strain 2430-78 and sterile medium were used as positive and negative controls, respectively.

Radioimmunoassay. Approximately 1×10^6 H407 cells were seeded onto wells of 24-well cell culture plates (Costar, Cambridge, MA) and incubated at 37°C and 5% CO_2 in air for 2 hours. After washing with MEM, individual wells were inoculated with either sterile medium or medium containing *S pilosicoli*, and incubated for 15 minutes, 30 minutes, 1, 2, 4, and 6 hours. After washing with PBS and blocking with 10% (wt/vol) non fat dry milk in PBS, the mouse monoclonal antibody 10G3, produced against the porcine *S pilosicoli* isolate B1555a,[14,45] was added to each well and incubated for 1 hour at 37°C and 5% CO_2 in air. After washing with PBS, an appropriate dilution of [^{125}I]-labelled sheep antimouse IgG $F(ab')_2$ fragments (New England Nuclear, Boston, MA) was added to each well (80,000 cpm/well) and incubated for 1 hour at 37°C and 5% CO_2 in air. After washing with PBS, the bound radioactivity was eluted with 10% (wt/vol) sodium dodecyl sulfate solution and counted in a gamma counter (ICN Micromedic Systems, Huntsville, AL). Results were expressed as mean radioactivity ± standard deviation of the mean for three separate experiments.

Electron microscopy. Approximately 1×10^8 H407 cells were seeded onto 12 mm culture plate inserts (Millicell-H™, Millipore Products Division, Bedford, MA) for 2 hours before inoculation with either sterile medium or medium containing 5×10^8 human *S pilosicoli*. Replicate cultures were incubated at 37°C and 5% CO_2 in air for 4, 12, and 24 hours for scanning electron microscopy and for 2, 4, and 6 hours for transmsission electron microscopy. At the end of the incubation period, the inserts were washed with medium and fixed with 3% (vol/vol) glutaraldehyde in 0.1 mol/L Sorenson's phosphate buffer (SPB; pH 7.2). After washing with SPB, the inserts were post-fixed with 2% (wt/vol) osmium tetroxide in SPB for 30 minutes, washed with SPB and dehydrated in a series of ethanol (50, 70, 95, 100, 100, and 100%). Each insert was divided in half and one half was processed for scanning electron microscopy by transferring to a carbon dioxide critical-point drying apparatus (Samdri pvt-3, Tousimis Research Corp., Rockville, MD). After drying, the specimen was mounted onto an aluminum stub with copper tape, edged with silver paste and lightly coated with carbon by vacuum evaporation (Denton Vacuum DV-502, Cherry Hill, NJ). The specimen was sputter-coated with gold palladium and examined in a scanning electron microscope (AMRAY 100-A, Bedford, MA) at 20 kV. The other half of the insert was processed for transmission electron microscopy by washing with propylene oxide and incubation in a 1:1 propylene oxide araldite embedding resin overnight. The next day, the specimen was soaked in fresh araldite for 6 hours and heated in molds at 65°C overnight to allow polymerisation. Ultrathin sections were stained with 2% (wt/vol) uranyl acetate and lead citrate and examined with a transmission electron microscope (Philips 201, Eindhoven, The Netherlands). The attachment indices at each time point were determined by counting the number of spirochetes in contact with the plasma membrane of 10 epithelial cells. Similarly, indices of intracellular uptake were determined by counting the number of spirochetes either free within the cytoplasm or inside cytoplasmic vacuoles in 10 epithelial cells at each time point. The mean number of spirochetes ± standard error of the mean in contact with the plasma membrane or inside H407 cells were calculated.

Spirochete attachment to chick cecal epithelium

One-day-old conventional California White X White Leghorn chicks (Ross Hill Hatcheries, Lincoln, NE) or HyLine Leghorn chicks (Hy-Vac, Adel, IA) were ran-

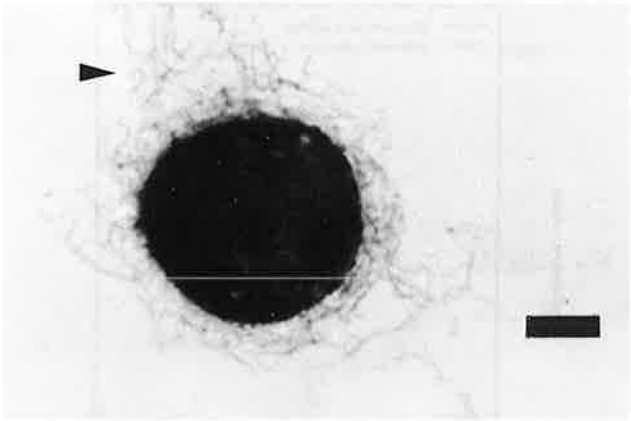

Figure 1. Light microscopic photomicrograph of a human embryonic small intestinal epithelial H407 cell 2 hours after inoculation with human Serpulina pilosicoli. *Many spirochetes are attached on the surface of the cell (arrowhead). Giemsa. Bar = 10 μm.*

Table 1. Kinetics of human* Serpulina pilosicoli *isolate SP16 surface attachment and intracellular uptake by human embryonic small intestinal H407 cells as determined by transmission electron microscopy.

Indices	Time (hours)		
	2	4	6
Surface Attachment	13.7 ± 10.5†	23.3 ± 11.7	31.3 ± 14.6
Intracellular Uptake			
Cytoplasm	0.0 ± 0.0	0.4 ± 0.5	0.1 ± 0.3
Vacuole	0.3 ± 0.7	1.2 ± 2.2	1.2 ± 1.5

†Surface attachment is expressed as mean number of spirochetes in apposition with the plasma membrane of 10 epithelial cells ± standard error of the mean. Intracellular uptake is expressed as mean number of spirochetes either free in the cytoplasm or inside cytoplasmic vacuoles of 10 epithelial cells ± standard error of the mean.

domly allocated to groups of three to four birds and housed and cared for according to approved guidelines of the University of Nebraska-Lincoln Institutional Animal Care and Use Committee as previously described.[32] Chicks were inoculated on the day of hatching by crop gavage with 0.5 mL volumes of either sterile medium or medium containing approximately 2×10^8 human or rhesus *S pilosicoli*. On day 21 postinoculation, the chicks were humanely killed and the ceca were processed for bacteriologic and pathologic examinations as previously described.[14,32] Attachment of spirochetes to the cecal epithelium was assessed by light and electron microscopic examinations as previously described.[32]

RESULTS

Spirochete attachment to cultured cells

Attachment of *S pilosicoli* to epithelial cells was highest (+++) with H407 cells; greater than 20 spirochetes were diffusely attached on the surface of greater than 80% of the cells (Figure 1). The attachment of spirochetes was less (++) with CaCo-2 and HEp-2 cells, minimal (+) with FaDu and HeLa cells, and absent (-) with HT-29 cells. *Serpulina innocens* did not attach to H407, CaCo-2, HT-29, and HEp-2 cells. Attachment of the rhesus *S pilosicoli* was similar to that of the human *S pilosicoli* and did not involve accumulation or rearrangement of actin at the point of contact between the spirochete and the plasma membrane of H407 cells. Conversely, H407 cells incubat-

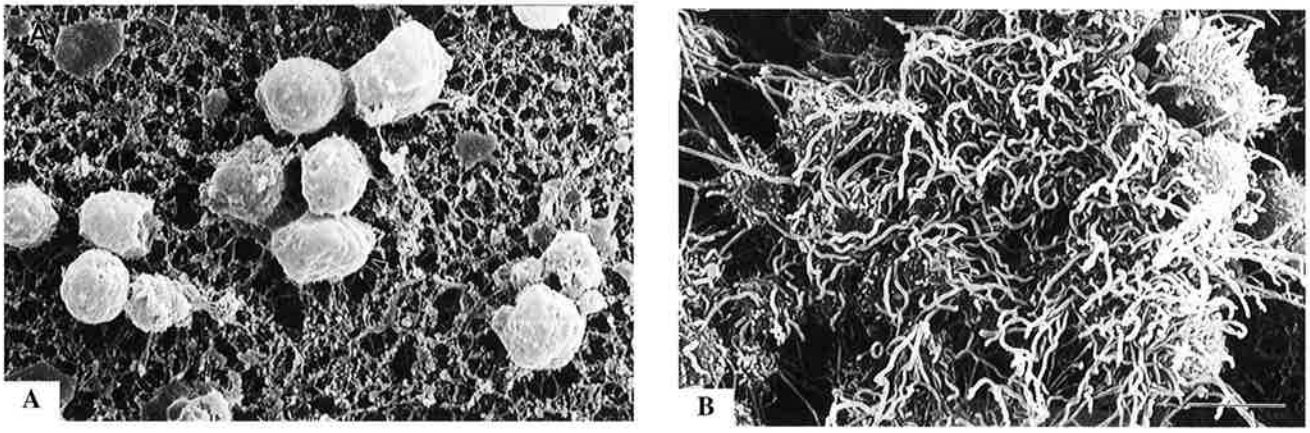

Figure 2. Scanning electron photomicrographs of human embryonic small intestinal epithelial H407 cells 12 hours after incubation with either sterile medium ***(A)*** *or human* Serpulina pilosicoli ***(B)****. Note cells with normal morphology (arrowhead) attached to the solid support (A), and diffuse attachment of spirochetes (arrowheads) in all different orientations on the surface of a group of cells (B). Bar = 10 μm.*

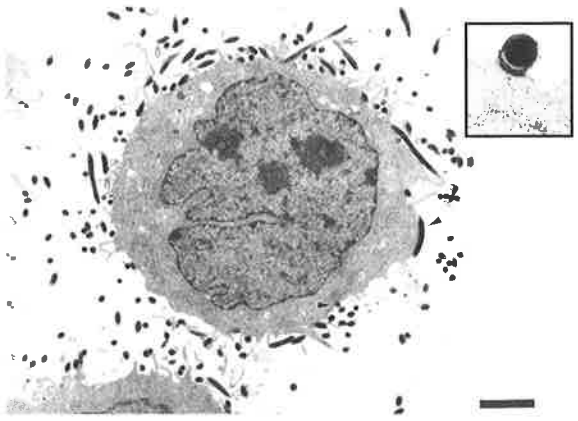

Figure 3. Transmission electron photomicrograph of a human embryonic small intestinal epithelial H407 cell 6 hours after incubation with human Serpulina pilosicoli. *Spirochetes are closely apposed to the plasma membrane (**arrowheads**). Insert: Higher magnification of an area of spirochete attachment shows absence of electron-dense fibrillar accumulation in the cytoplasm beneath the point of contact with the spirochete. Uranyl acetate and lead citrate. Bar = 2.5 μm.*

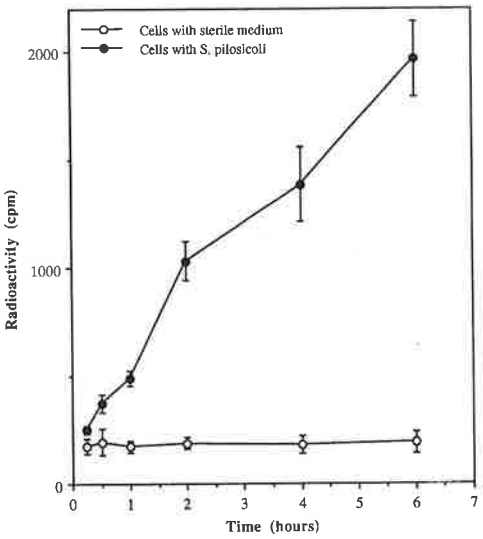

Figure 4. Kinetics of human Serpulina pilosicoli *attachment to human embryonic small intestinal epithelial H407 cells as determined by radioimmunoassay. Results are expressed as mean radioactivity ± standard deviation of the mean for three separate experiments.*

ed with the AE *E coli* showed typical actin accumulation at the site of bacterial attachment. Scanning electron microscopy confirmed the diffuse attachment of *S pilosicoli* to H407 cells seen by light microscopy. The numbers of spirochetes on the surface of H407 cells increased between 4 and 12 hours (Figure 2), and by 24 hours large clumps of spirochetes were present on the surface of most cells. By transmission electron microscopy, intimate points of contact were present between the sides of the spirochete outer membrane and the plasma membrane of H407 cells (Figure 3). The plasma membrane of H407 cells at the point of apposition with the spirochetes did not form adhesion pedestals nor was any accumulation of electron-dense fibrillar material seen in the cytoplasm immediately beneath adherent spirochetes. There was a linear relationship between the index of spirochete attachment and the duration of incubation with H407 cells using a direct counting method (Table 1) and radioimmunoassay (Figure 4). Intracellular uptake of spirochetes characterized by spirochetes either free in the cytoplasm or within membrane-bound vacuoles plateau after 2 hours (Table 1).

Spirochete attachment to chick cecal epithelium

At necropsy, the cecal contents of control chicks and chicks inoculated with *S pilosicoli* were yellowish brown, foamy and semi–solid. By light microscopy, there were no significant lesions in the ceca of chicks inoculated with sterile medium; the cecal mucosa was lined by tall columnar enterocytes interspersed with goblet cells, and small numbers of lymphocytes were present in the lamina propria (Figure 5A). Chicks inoculated with the rhesus spirochetes showed focal to diffuse basophilic thickening of the brush border (Figures 5B and 5C). The brush border thickening stained dark brown or black by the Warthin-Starry stain and was restricted to the superficial enterocytes located between crypt lumens. Ultrastructurally, control chicks had regularly spaced microvilli and tonofibrils in the apical cytoplasm of cecal enterocytes (Figure 6A). By contrast, the ceca of chicks inoculated with the rhesus spirochetes had polar attachment of spirochetes to the apical membrane of enterocytes in a picket-fence fashion (Figures 6B and 6D). Attachment of spirochetes commonly occurred at the intercellular junctions between adjacent enterocytes (Figures 6B and 6C) and often ended abruptly with neighboring cells (Figure 6D). At the point of contact with spirochetes, the cells showed partial to complete effacement of microvilli and disruption of the terminal web microfilaments (Figures 6B, 6C, and 6D). Additionally, the apical plasma membrane of colonized enterocytes often was raised above adjacent cells, resulting in the formation of a cap-like structure (Figure 6D). The cytoplasm underneath the terminal web of colonized enterocytes had variable numbers of membrane bound vesicles with electron-lucent contents (Figure 6C). Similar vesicles were less prominent in adjacent enterocytes without spirochetal attachment. The tip of the attached end of the spirochetes in contact with the plasma membrane of enterocytes had electron-lucent protrusions and variable numbers of outer surface ring-like structures similar to ciliary necklaces (Figure 7). Invasion of spirochetes beyond the epithelial cell layer was not seen. There was a good correlation between spirochetal attachment and bacteriologic cultures of the cecal mucosa and cecal contents (Table 2).

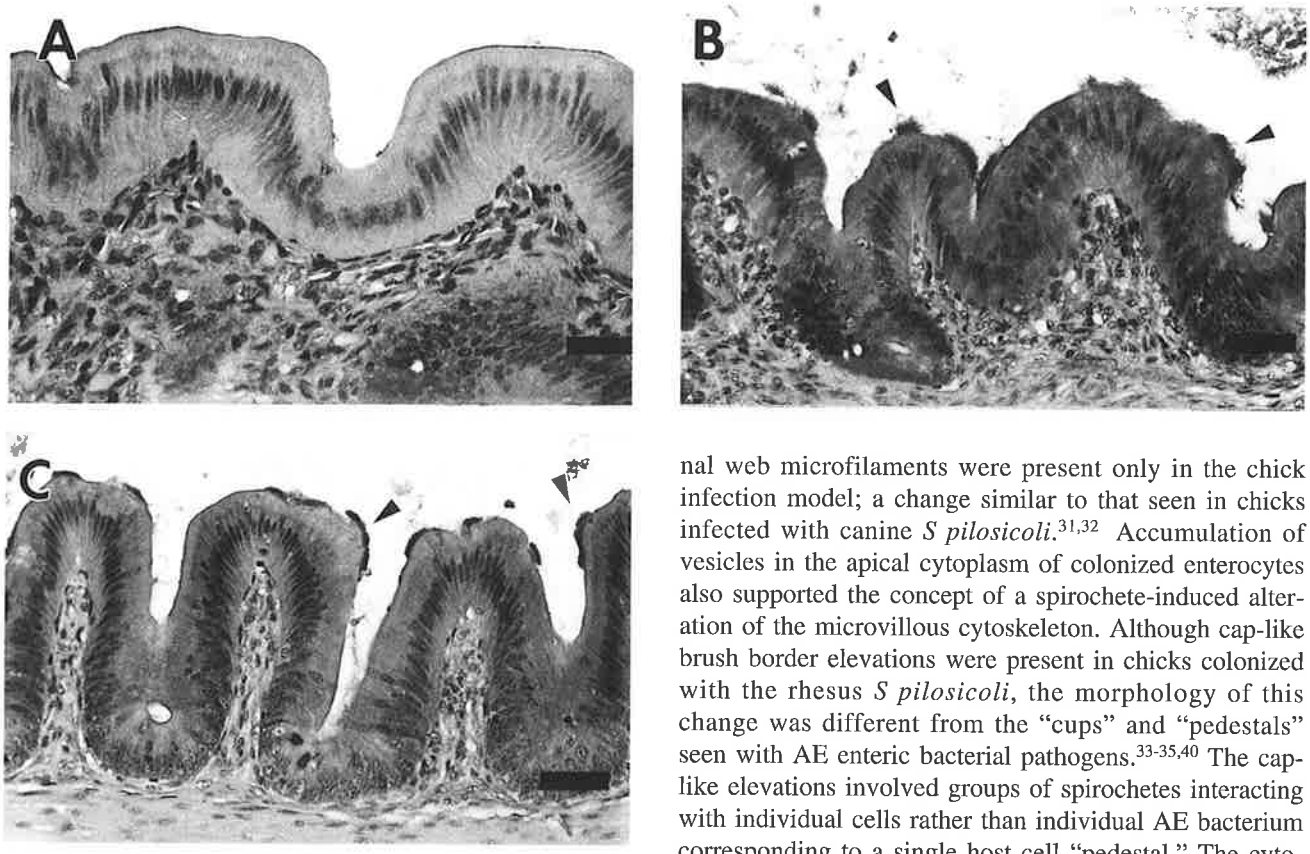

*Figure 5. Light microscopic photographs of cecal mucosa of chicks 21 days after crop inoculation with either sterile medium (**A**), human* Serpulina pilosicoli *(**B**), or rhesus* Serpulina pilosicoli *isolate MMU26717 (**C**). Note absence of bacteria on the surface of enterocytes of a control chick (A) compared with spirochetes (arrowheads) closely apposed to the brush border of the surface enterocytes of infected chicks (B and C). HE stain. Bar = 40 μm.*

DISCUSSION

Recent technological advances in genetic-based identification methods for intestinal spirochetes have spurred renewed interest into the role of spirochetes in colonic diseases of human beings and animals. The newly identified intestinal spirochete, *Serpulina pilosicoli* is now recognized as a cause of diarrhea and wasting in a broad range of hosts from human beings and non-human primates to swine, dogs, and birds. We found that the human and all three rhesus *S pilosicoli* strains had attaching phenotypes when examined using the H407 cell assay and the chick infection model. However, each model displayed unique morphological features that were representative of the natural disease.

Light microscopic and ultrastructural examinations of challenge-exposed chicks revealed attachment of human and rhesus *S pilosicoli* to cecal enterocytes in a pattern similar to the eukaryotic cell assay. However, effacement of the microvillous structure and rarefaction of the terminal web microfilaments were present only in the chick infection model; a change similar to that seen in chicks infected with canine *S pilosicoli*.[31,32] Accumulation of vesicles in the apical cytoplasm of colonized enterocytes also supported the concept of a spirochete-induced alteration of the microvillous cytoskeleton. Although cap-like brush border elevations were present in chicks colonized with the rhesus *S pilosicoli*, the morphology of this change was different from the "cups" and "pedestals" seen with AE enteric bacterial pathogens.[33-35,40] The cap-like elevations involved groups of spirochetes interacting with individual cells rather than individual AE bacterium corresponding to a single host cell "pedestal." The cytological alterations present in colonized cecal enterocytes of chicks stongly suggest that *S pilosicoli* establishes a parasitic relationship with the host. This is in agreement with data indicating interference of host absorption of specific amino sugars by spirochetes attached to the rectal mucosa of human subjects with naturally-occurring CS.[21]

In the chick infection model, the attachment of spirochetes was restricted to surface enterocytes and ceased abruptly at the junctions between enterocytes. This pattern of attachment may be indicative of expression of a specific receptor by more mature enterocytes located near the extrusion zone of cecal crypt units. The findings that *S pilosicoli* attached only to certain eukaryotic cell lines in vitro, and that the commensal intestinal spirochete *S innocens* had a negative attachment phenotype further confirmed the specificity of the interaction, and implied that attachment to eukaryotic cell may be a characteristic of pathogenic intestinal spirochetes.[42,43]

Ultrastructural examination of *S pilosicoli* interaction with cecal enterocytes confirmed the findings with the H407 cell attachment assay and provided further morphologic evidence indicating that the attachment is intimate and involves the outer membrane of the spirochete. These observations were similar to *H pylori* and *H mustelae* interaction with HEp-2 cells.[40,42] The absence of cytoplasmic electron-dense fibrillar material beneath the point

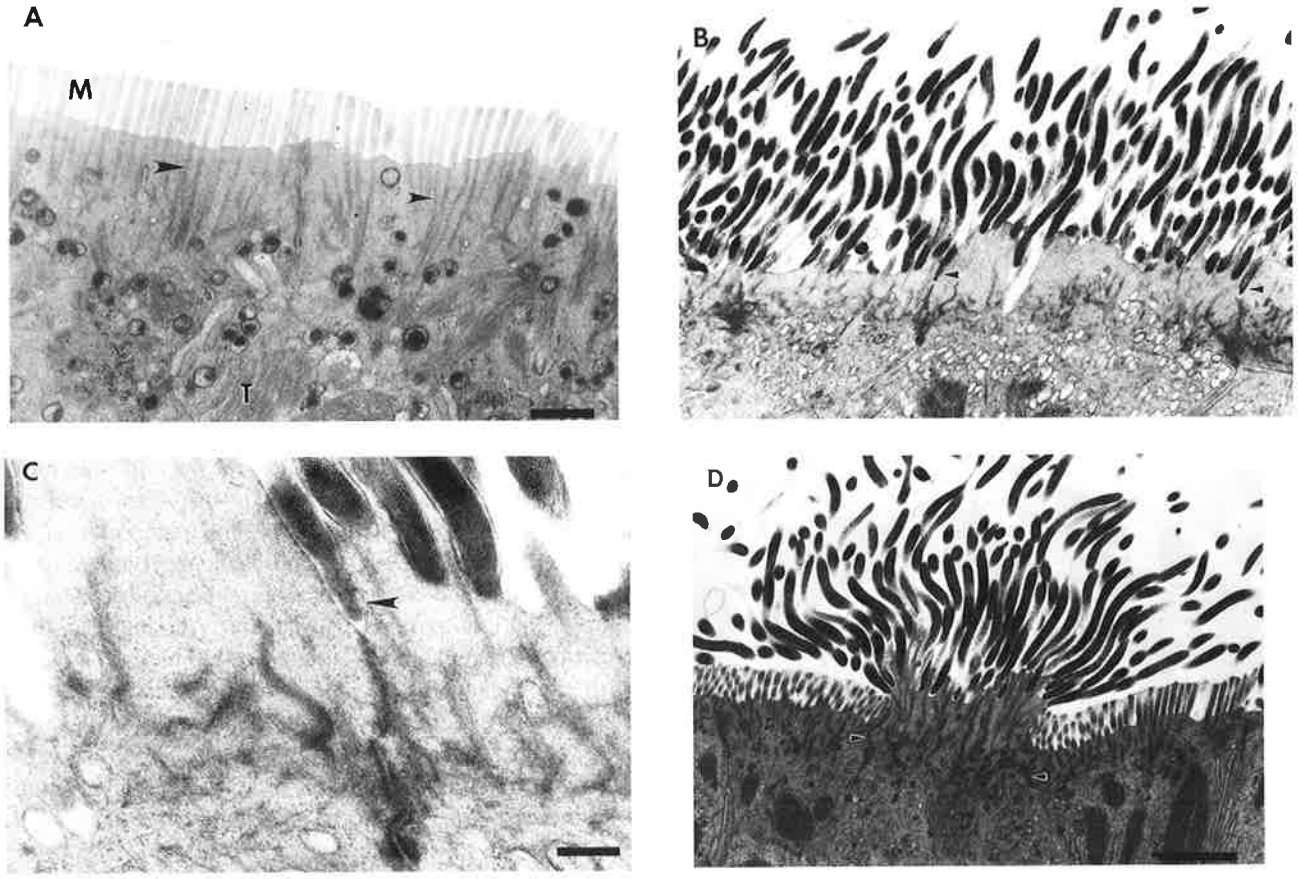

*Figure 6. Transmission electron photomicrographs of cecal enterocytes of chicks 21 days after crop inoculation with either sterile medium (**A**), rhesus* Serpulina pilosicoli *isolate MMU26717 (**B and C**), or isolate MMU26986 (**D**). Note regularly spaced microvilli (M) with microfilamenis (arrowheads) and tonofiblils (T) extending in the underlying terminal web area in enterocytes of a control chick (A). Polar attachment of spirochetes to cecal enterocytes causing effacement of microvilli and rarefaction of terminal web microfilaments with cytoplasmic vesicles near the terminal web of colonized enterocytes in an infected chick (B). Higher magnification of B shows spirochetes (arrowheads) wedged at the intercellular junctions between adjacent enterocytes (C; Bar = 0.25 μm). Spirochetes attached along adjacent enterocytes formed a cap-like structure with abrupt cessation of attachment (arrowheads) at the intercellular junctions with normal enterocytes (D). Note the presence of cytoplasmic vesicles near the terminal web of colonized enterocytes. Bar = 2 μm.*

of cell membrane attachment *in vitro* and *in vivo*, together with the lack of actin rearrangement at the spirochete-cell interface *in vitro* suggested that the mechanism of attachment of *S pilosicoli* is similar to *H pylori*.[40] Sequential ultrastructural examinations and labelling studies with the H407 cell assay further showed that *S pilosicoli* readily attached to epithelial cells in a time-dependent fashion. By contrast with the chick infection model in which invasion was not seen, the H407 cell assay revealed intracellular uptake of spirochetes through an intracisternal pathway with few spirochetes also free in the cell cytoplasm. This is in agreement with observations made in human beings,[4,7,16,18,19,20] non-human primates,[20] dogs,[9,10] swine,[12] and opossums[9] with the natural disease.

Surface rings similar to those seen with canine *S pilosicoli* at their point of contact with cecal enterocytes of challenge-exposed chicks,[32] were seen with the human and rhesus *S pilosicoli*. These structures were similar to intramembranous particles seen by freeze-fracture electron microscopy of spirochetes at the site of attachment to colonic enterocytes of rhesus monkeys with naturally-acquired CS.[21] Although similar structures were not seen in the H407 cell attachment assay, their presence could not be ruled out since the time frames for examination post inoculation were different; hours for the cell assay versus days for the chick infection model. Nevertheless, because these structures are seen only in the portion of the spirochete in close apposition with the eukaryotic plasma membrane, it is likely that they represent specialized organelles involved in either the attachment process or the transport of nutrients across the spirochetal membrane.

The attachment of AE *E coli* in a chick infection model is accompanied by focal epithelial cell degeneration and necrosis, but diarrhea and gross cecal changes have not

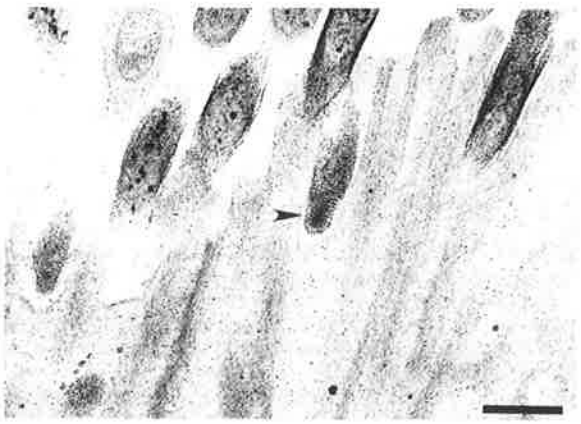

Figure 7. Transmission electron photomicrograph of a cecal enterocyte of a chick 21 days after crop inoculation with the rhesus Serpulina pilosicoli *isolate MMU26986. The tip of a spirochete inserted into an invagination of the plasma membrane of an enterocyte has ring structures suggestive of ciliary necklaces (arrowhead). Bar = 0.25 μm.*

been reported.[34] This is not unlike what is seen in chicks colonized with most strains of *S pilosicoli*,[14,32] but certain strains of *S pilosicoli* appear more virulent, and invade the cecal mucosa,[14,29] or cause massive loss of surface area and diarrhea in the chick infection model.[31] It is likely that the pathogenic potential of *S pilosicoli* varies among strains, with some strains capable of invading beyond the intestinal epithelial barrier,[4-7,9,10,12,18-20] as well as in extra-intestinal sites.[46] In fact, the human and rhesus *S pilosicoli*, like certain canine and porcine strains, attach at the intercellular junctions of cecal enterocytes in a pattern similar to *H pylori* in the gastric antral mucosa.[39,41] A subtilisin-like outer membrane-associated serine protease identified in intestinal spirochetes, including the human and rhesus *S pilosicoli* strains examined in the present study, may indirectly contribute to disease production by dissociation of intercellular bridges.[47]

From the available information obtained using animal infection models, it is hypothesized that following association with the cecal and colonic mucus gel, *S pilosicoli* attaches to the apical surface of enterocytes in a picket-fence fashion.[13,25,27] Clinical signs of diarrhea are attributable to displacement and later effacement of microvilli by attached spirochetes and reduction in the absorptive surface area of the large intestine. However, diarrhea is not always seen with CS and some unknown factors may be necessary in order to compromise the reserve absorptive capacity of the colon and induction of diarrhea. With time *S pilosicoli* is no longer seen on the surface of the colonic epithelium, but persistent infection results in chronic inflammation and altered colonic function.[25,27,28]

Table 2. Bacteriologic and light microscopic examinations of ceca of chicks 21 days after crop inoculation with either control medium or medium containing human or rhesus* Serpulina pilosicoli *isolates.†

Inoculum	Origin	Isolation (%)	Attachment (%)
Sterile TSB		0/9 (0.0)	0/9 (0.0)
SP16	Human	6/7 (85.7)	4/7 (57.1)
MMU26717	Rhesus	5/6 (83.3)	5/6 (83.3)
MMU26986	Rhesus	6/6 (100.0)	5/6 (83.3)
MMU27669	Rhesus	4/10 (40.0)	3/10 (30.0)

†Results are expressed as number of chicks positive for spirochetal isolation or attachment/total number of chicks challenged, in three separate experiments.

Attachment of *S pilosicoli* to the apical membrane of enterocytes is pathognomonic of CS, but the mechanism and significance of this interaction are poorly understood. This is the first demonstration of *S pilosicoli* attachment to cultured cells and correlation with the chick infection model. On the basis of morphologic parameters and the lack of actin rearrangement, we concluded that the mechanism of epithelial cell attachment by *S pilosicoli* involved a bacterial ligand (adhesion)-host cell receptor interaction that more closely resembled *H pylori* than that of AE enteric bacterial pathogens. Although several animal models of spirochete attachment are available, the identification of permissive and non-permissive cell lines will provide a useful tool for basic studies aimed at identifying the molecular basis of *S pilosicoli* attachment to eukaryotic cells. The high correlation between attachment to H407 cells and the chick infection model will allow rapid screening of field isolates for attachment phenotype without the need for animal experimentations.

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Evidence for in utero Transmission of *Borrelia burgdorferi* from Naturally Infected Cows

Mira M. Leibstein, BS, Mazhar I. Khan, DVM, PhD, and Sandra L. Bushmich, MS, DVM

ABSTRACT

Natural infection of dairy cattle with *Borrelia burgdorferi* has been described with clinical signs that include lameness, swollen joints, and skin rash. Serologic diagnosis of *B burgdorferi* is difficult because of cross-reactive antibodies and occurence of subclinical infection in cattle. Definitive diagnosis of *B burgdorferi* infection in cattle requires the isolation or detection of *B burgdorferi* from body fluids or tissues. The effects of maternal infection with *B burgdorferi* on the bovine fetus are not known. The primary objective of this study was to determine if in utero transmission of *B burgdorferi* occurs in naturally infected dairy cattle. Blood specimens were obtained immediately after parturition from adult cows naturally exposed to *B burgdorferi* and their calves (n=15) for *B burgdorferi* serology (indirect fluorescent antibody (IFA) and immunoblot) and antigen detection with *B burgdorferi* Osp A specific polymerase chain reaction (PCR) primers. Other samples collected at parturition for *B burgdorferi* culture or PCR included placenta, uterine fluid, and colostrum. Thirteen of 14 cows tested had antibodies ≥ 3 *B burgdorferi* specific proteins and 4 of 14 calves tested had antibody response to 1 or 2 *B burgdorferi* specific proteins by immunoblot. Five of 15 adult cows were spirochetemic at parturition; 4 of the calves from these cows were also spirochetemic at birth (PCR). Spirochetes were cultured from the placentas in 2 of 10 cows and from the uterine fluid in 1 of 8 cows. *B burgdorferi* DNA was detected in the colostrum in 4 of 12 cows. Three of 15 calves were stillborn; *B burgdorferi* DNA was detected by PCR in 3 of 3 and spirochetes cultured from 2 of 3 stillborn calves. Fetal tissues from which *B burgdorferi* DNA was detected include blood, spleen, bladder, kidney, synovial fluid and tissue, heart, cerebrum, and aqueous humor. *B burgdorferi* was cultured from the spleen of one stillborn calf and the kidney of another. Detection of *B burgdorferi* DNA from the tissues of stillborn calves, as well as spirochetemia in neonatal live-born and stillborn calves, gives evidence for in utero transmission of *B burgdorferi* in naturally infected dairy cattle.

Key words: *Borrelia burgdorferi*, Lyme disease, cattle, pregnancy, colostrum, fetal infection

INTRODUCTION

Lyme borreliosis, a multisystemic bacterial infection caused by *B burgdorferi,* is endemic throughout the northeastern United States, including Connecticut. This often debilitating disease is commonly transmitted via the bite of infected *Ixodes* species (spp.) ticks.[1] Lyme borreliosis has been reported in both humans and domestic animals, including dogs and cats,[2-5] horses,[6-11] and dairy cattle.[6,10,12-16] Clinical signs in domestic animals include mild to severe musculoskeletal manifestations, including single or shifting limb lameness, arthritis, and swollen joints, with or without fever.[2,4,9,12,15] However, subclinical infection with *B burgdorferi* is frequently seen in domestic animals.[6,17-18]

Natural infection with *B burgdorferi* has been described in dairy cattle, often occurring in first-calf heifers as they enter full milk production.[10] Acute cases of *B burgdorferi* infection in cattle may result in fever, stiffness, lameness, swollen joints, erythematous skin rash and decreased milk production.[13-14,19] Arthritis and laminitis have also been reported in cattle.[12] *B burgdor-*

From the Department of Pathobiology at the University of Connecticut, Storrs, Connecticut.

Address correspondence to Sandra L. Bushmich, MS, DVM, Department of Pathobiology, U-89, The University of Connecticut, 61 North Eagleville Road, Storrs, CT 06269-3089

feri has been isolated via culture from lung, liver, blood, synovial fluid, colostrum, and urine of infected cattle.[6,12,20] Chronic weight loss, laminitis, and reproductive problems have been suspected of being caused by *B burgdorferi* in some herds.[15,19,21] Experimental infection of neonatal dairy calves with *B burgdorferi* has been described,[22] resulting in erythematous rash at the site of inoculation, local lymphadenopathy, and disseminated infection, with live *B burgdorferi* shed in the urine.

Diagnosis of Lyme borreliosis in dairy cattle is often hampered by the sparse numbers of the spirochete in tissues and body fluids, difficulty in culturing *B burgdorferi*, and inconsistency of the serological response. The use of serology as a diagnostic tool in cattle has proven especially difficult. Animals with cases of infection confirmed by culture or PCR may develop only low antibody titers to *B burgdorferi*; alternately, cattle with strong serological response may exhibit little or no clinical illness.[14] Currently, the value of utilizing serology in the diagnosis of Lyme borreliosis in cattle is limited; it should be used in conjunction with the isolation or detection of the organism from tissues and/or fluids such as blood or urine.

In utero infection has been reported in humans,[23,24] laboratory mice,[25,26] domestic animals including horses[11,27] and dogs.[28] In utero infection was suspected when *B burgdorferi* was cultured from the blood of a neonatal calf, and when an aborted calf had antibodies to *B burgdorferi,*[6] since maternal antibodies do not cross the placenta in cattle.[29] Other studies in mice[30] and rats[31] have failed to demonstrate transplacental transmission of *B burgdorferi.* A definite link between in utero infection with *B burgdorferi* and adverse pregnancy outcome remains controversial[32] and warrants further investigation.

MATERIALS AND METHODS

Experimental plan

The objective of this study was to demonstrate if in utero transmission of *B burgdorferi* occurs in naturally infected dairy cattle. The cattle studied were first-calf dairy heifers and their offspring (n=15) from a Connecticut herd with a documented history of natural *B burgdorferi* infection.[14] The heifers had been pastured, through much of their gestation, in a field known to harbor infected *I scapularis* ticks. The heifers and their neonatal offspring were evaluated for evidence of infection with *B burgdorferi* immediately after parturition, before calves were given colostrum.

Blood specimens were obtained from adult cows and their calves after parturition for *B burgdorferi* serology (IFA, immunoblot) and antigen detection (PCR) with *B burgdorferi* OspA specific primers.[33] Additional samples collected from the cows at parturition included placenta, uterine fluid, and colostrum for culture in BSK media and/or PCR analysis. Each adult cow was evaluated at this time for signs of clinical illness related to *B burgdorferi* infection, including joint swelling, limb edema, skin rash and evidence of lameness.

The following organs and fluids were collected from stillborn calves for culture or PCR analysis: blood, urine, CSF, brain tissue, lung tissue, aqueous humor, synovial fluid and tissue, testes, heart, kidneys, liver, bladder, and spleen.

Indirect fluorescent antibody

Spirochetes were prepared for indirect fluorescent antibody (IFA) from approximately 40 to 60 mL of active, low passage *B burgdorferi* N40 (gift of Dr. Stephen Barthold) in BSK agarose-free media produced in our laboratory. *B burgdorferi* cultures were pooled and washed three times in 1× phosphate buffered saline (PBS) with 0.1% merthiolate. The cells were then resuspended in 1×PBS with 0.1% merthiolate and diluted to the desired concentration of a full field of nonoverlapping spirochetes when checked by dark field microscopy at 200×. Diluted spirochetes were then stored frozen or at 4°C for up to three weeks.

Multi-well, hydrophobic acetone resistant slides were then coated with a single layer of the prepared spirochete suspension. Test sera, along with controls, was prepared in two-fold serial dilutions, from 1:32 through 1:1024, transferred to corresponding wells, and incubated in a moist chamber at 37°C for 30 minutes. Slides were then rinsed two times in 1×PBS and once in distilled water. Fluorescein isothyiocynate (FITC) labeled goat anti-bovine IgG (H+L) (Kirkegaard and Perry, Gaithersburg, MD) was then added to each well, and slides were incubated and rinsed as above. Slides were air dried, mounted, and examined at 400× by dark field fluorescent microscopy. Sera from calves experimentally infected with *B burgdorferi* and noninfected control calves were used as low positive and negative controls, respectively. Sera from PCR (+) clinical bovine Lyme disease cases were used as high positive controls. Titers of ≥1:64 were considered positive, based upon previous studies.[22]

SDS-PAGE and immunoblot

Spirochetes were prepared for discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by washing whole, low passage *B burgdorferi* N40 cells three times in 1×PBS with 0.1% merthiolate, and resuspended in 1×PBS. Approximately 300 µg of protein (500 µL) was then mixed with 187 µL 4× SDS

sample buffer (0.25 mol/L Tris-HCL, 40% glycerol, 2% SDS, 20% 2-mercaptoethanol, 0.025% bromophenol blue) and denatured by heating at 100°C for 5 minutes. SDS-PAGE of *B burgdorferi* proteins and molecular weight standards (BioRad, Richmond, CA) was performed as previously described[34] (SE 600 vertical slab gel electrophoresis unit, Hoefer Scientific, San Fransico, CA), using a 4% stacking gel and 11% resolving gel.

Proteins were electrophoresed at 100 mA until the dye-front reached approximately 1 cm from the bottom of the gel. The gel was pre-equilibrated in transfer buffer (25 mmol/L Tris base, 38 mmol/L glycine, 20% methanol, pH 8.3) for 30 minutes, then transferred onto 0.45 µm nylon supported nitrocellulose membranes (BioRad Laboratories, Hercules, CA) at 70V for 2.5 hours.[35]

Nitrocellulose membranes were then soaked in Ponceau stain (Sigma Chemical Company, St. Louis, MO) for approximately 10 minutes to determine if proteins were properly transferred. Membranes were cut into strips and blocked in 2% bovine serum albumin Fraction V (BSA) (Fisher Scientific, Fair Lawn, NJ) and 1% horse serum in Tris-buffered saline, rocking for 1 hour at room temperature. Strips were rinsed three times for 10 minutes in wash buffer (150 mmol/L NaCl, 10 mmol/L Tris, 0.5% Tween 20) and test sera was added at a dilution of 1:100. The strips were incubated for 2 hours and washed as above. Phosphatase labeled conjugate (goat anti-bovine IgG (H+L), 1:500) (Kirkegaard and Perry, Gaithersburg, MD) was added, strips were incubated 1 hour and washed as above. BCIP/NBT phosphatase substrate (Kirkegaard and Perry, Gaithersburg, MD) was added and strips rocked 1-10 minutes until protein bands were visualized. The reaction was stopped with distilled water. A standard curve was generated by plotting the relative mobility (Rf) values of the protein standards against their known molecular weights. Control sera were described under IFA methods. Immunoblots were considered reactive if cattle responded to ≥ 3 *B burgdorferi* specific proteins including the 93/83, 39, 34, 31, 25, and 22 kDa proteins.[36] Immunoblots were equivocal if there was antibody response to 1 or 2 *B burgdorferi* specific proteins, or to any number of genus-specific or other uncharacterized proteins, including the 66, 60, 45, 41, 37, 35, 30, 28/29, 20, and 18 kDa proteins.[36,37]

Culture

Approximately 0.5 mL of liquid or 20 to 25 mg minced fetal tissue were inoculated into 7 mL of sterile Barbour-Stoenner-Kelly (BSK) media containing ciprofloxacin (40 mg/mL) and rifampicin (20 mg/mL). All media was inoculated under a laminar flow hood to maintain sterile conditions. Cultures were incubated at 32°C and examined by dark field microscopy weekly for a minimum of six weeks.

DNA preparation

Blood and tissue samples were extracted in a biohazard hood in a laboratory not used for *B burgdorferi* culture, using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) with minor modifications. Control DNA for PCR reactions were extracted separately from clinical samples, also using the Puregene DNA isolation kit. Positive controls (low passage *B burgdorferi* N40 cells in BSK media), and negative controls (distilled water) were then stored at -20° C until use.

Blood

Twenty-five µL of whole blood were added to 600 µL of cell lysis solution and incubated at 55°C for 60 to 90 minutes. If cell clumps were visible after mixing, the samples were then incubated at room temperature until the solution was homogenous. Protein precipitation solution (200 µL) was added, the samples were vortexed and then spun at 12 000×*g* for 4 minutes. The protein pellet was discarded and the supernatant transferred to 600 µL 100% isopropanol. The samples were inverted 40 times, spun at 14 000×*g* for 4 minutes, and washed with an equal volume of ethanol. The supernatant was discarded and the pellet allowed to air dry for 15 to 30 minutes. The DNA was rehydrated in TE buffer, heated at 65°C for 1 hour and allowed to cool to room temperature. The samples were Rnase treated (3-5µL) for 15 to 60 minutes at room temperature and stored at –20°C.

Tissue

Approximately 20 to 25 mg of previously frozen (-70°C) or fresh tissue was used for extraction. A 1.5 mL microfuge tube containing 600 µL cell lysis solution was chilled on ice. Tissue samples were added and quickly homogenized using ≥ 50 strokes with a microfuge tube pestle. Five µL of proteinase K (20 mg/mL) was added to the lysate and the sample incubated at 55°C overnight or 65°C for 1 to 3 hours. Protein precipitate solution (200 µL) was added, each sample vortexed for 20 seconds, and centrifuged 13 000×*g* for 5 minutes. Supernatant was transferred to 600 µL 100% isopropanol, inverted 40 times, and centrifuged at 14 000×*g* for 5 minutes. The DNA pellet was air dried for 15 to 30 minutes and resuspended in 50 µL TE buffer. The samples were rehydrated at 65°C for 1 hour, Rnase treated (3 µL) for 15 minutes at 37°C, and stored at –20°C until use.

Colostrum

Colostrum (10-15 mL) was extracted as previously described[38] with minor modifications. The samples were defatted two times with 1 mL of chloroform and allowed to settle. The supernatant was centrifuged at 14 000×*g* for

30 minutes. The pellet was dissolved in 1.5 mL 1×PBS, pH 8.0, transferred to a 1.5 mL microfuge tube, and stored at –70°C. For analysis, 500 µL was centrifuged at 14 000×*g* for 20 minutes, the pellet dissolved in 100 µL 1×PBS, and 100 µL of Instagene Matrix (BioRad, Hercules, CA) added. The samples were vortexed vigorously, heated at 100°C for 5 minutes, and centrifuged at 12 000×*g* for 3 minutes. The colostrum samples were then chilled on ice for 15 minutes and stored at –20°C until use.

Polymerase chain reaction

The *B burgdorferi* specific oligonucleotide primers used have been previously described,[33] with sequences as follows:

OspA primer #1 CAATGGATCTGGAGTACTTGAAGGCG

OspA primer #2 GCAGTTAGAGTTCCTTCAAGAACATAGCT

The primers were synthesized by the Biotechnical Services at the University of Connecticut, Storrs, CT, and the DNA content was estimated by measuring the optical density at 260 nmol/L.[39] This primer pair amplifies a 309 bp segment of *B burgdorferi* outer surface protein A (Osp A), without amplifying other *Borrelia* spp. showing significant OspA homology, including *B coriaceae,* a spirochetal pathogen of cattle.[33,40]

Gene products were amplified as previously described,[41] with minor modifications, using the GeneAmp PCR Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT). Each 100 µL reaction volume contained 100 mmol/L Tris HCl (pH 8.3); 500 mmol/L KCl; 200 µmol/L each of dATP, dGTP, dCTP, and dTTP; 1.0 µmol/L of each primer; 2.5 units Amplitaq DNA Polymerase; and 2.5 mmol/L MgCl.

Ten microliters of sample DNA was used as template, overlaid with 50 µL mineral oil. All reactions were run, with positive and negative controls in triplicate, on a Perkin-Elmer Thermocycler 480 (Perkin-Elmer Cetus, Norwalk, CT). Positive and negative controls were aliquoted at the beginning, middle, and end of each run to screen for carry-over contamination. Each amplification run consisted of 35 cycles as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 40 seconds, and extension at 74°C for 55 seconds, followed by a final extension of 5 minutes at 74°C. To ensure the presence of amplified gene products, all positive and negative controls were then run on a 0.8% agarose gel in 1×TBE buffer at 60V for 90 minutes, stained with ethidium bromide (1 µg/mL), and washed in distilled water. The DNA was visualized under UV light; if the positive controls were not clearly visible, and all three negative control lanes clear, the entire run was repeated. The detection level for this procedure in our laboratory is approximately 10 *B burgdorferi* organisms/mL in fluid samples. All PCR products were stored at –20°C.

Slot blot hybridization

Amplified gene products were detected using gel electrophoresis and hybridization.[42] PCR products were electrophoresed on 0.8% agarose gels and denatured (75 mL 1 mol/L NaOH, 75 mL 5 mol/L NaCl, 100 mL dH20) while rocking at room temperature for one hour. Each gel was neutralized in 75 mL 5 mol/L NaCl and 175 mL 1mol/L Tris Cl while rocking at room temperature for 45 minutes. For Southern hybridization, the amplified DNA was transferred to Zeta-Probe membranes (BioRad, Richmond, CA), previously soaked in water and 10×SSC, for 18 to 20 hours, and allowed to air dry before baking at 80°C for two hours. Positive control PCR products were pooled, purified using the QIAquick Spin PCR Purification Kit (QIAGEN Inc., Chatsworth, CA), and labeled with ^{32}P-dATP (3000Ci/mmol/L) using the DECAprime DNA labeling kit (Ambion Inc., Austin, TX). Each membrane was then hybridized with the ^{32}P-labeled PCR products in 1mmol/L EDTA, 0.5mol/L NaH2PO4 (pH 7.2), and 7% SDS at 65°C overnight. The membrane was washed twice in 1mM EDTA, 40 mmol/L NaH2PO4 (pH 7.2), 5% SDS and twice in 1 mmol/L EDTA, 40 mmol/L NaH2PO4 (pH 7.2) and 1% SDS. Each membrane was air dried before exposure to KODAK X-OMAT film (Sigma) film at –70°C, typically for 18 hours (range of 3-24 hours, depending on isotope activity).

RESULTS

Thirteen of the fifteen adult cows had positive *B burgdorferi* antibody titers (range 1:64–1:256); 2 of 15 cows had negative titers of 1:32 (Tables 1, 2). Four of 14 cows tested by immunoblot had antibody response to > 4 nonspecific *B burgdorferi* proteins, and 13 of 14 had response to ≥ 3 *B burgdorferi* specific proteins (Figure 1; Tables 1, 2).

Sera from 5 of 15 neonatal calves, including 2 of 3 stillborn calves, had low positive antibody titers (range: 1:64 -1:128) (Tables 1, 2). Four of 14 calves tested by immunoblot, including 1 of 3 stillborn calves, showed specific antibody response to the 39 kDa protein of *B burgdorferi*, as well as antibody response to between one and seven nonspecific *B burgdorferi* proteins (Figure 1: lanes 7, 13, 17, 23; Tables 1, 2). An additional 2 calves showed antibody response to a single nonspecific *B burgdorferi* protein (Table 1). The remaining 8 of 14 calves, including 2 of 3 stillborn calves, had negative immunoblots (Figure 1; Tables 1, 2). One of three stillborn calves was seronegative to *B burgdorferi* by IFA and immunoblot (Table 2).

Table 1. Brief summary of data for cows naturally infected with **Borrelia burgdorferi** *at parturition and liveborn neonatal calves.*

	Spirochetemia (*Bb* PCR)		IFA Titer		# *Bb* Specific Antibodies		# Nonspecific *Bb* Antibodies	
COW ID	Dam	Calf	Dam	Calf	Dam	Calf	Dam	Calf
UC 359	(-)	(-)	1:32	NEG	3/6	0	7/10	0
UC 360	(-)	(-)	1:64	NEG	3/6	1/6	8/10	1/10
UC 376[1]	(-)	NT	1:128	1:64	3/6	0	8/10	0
UC 382	(-)	NT	1:64	NEG	3/6	0	5/10	1/10
UC 385	(-)	(-)	1:64	1:128	3/6	2/6	8/10	6/10
UC 386	(-)	(-)	1:64	NEG	4/6	0	9/10	1/10
UC 388	(-)	(-)	1:64	NEG	3/6	0	6/10	0
UC 390[3]	(-)	(-)	1:64	1:64	3/6	1/6	6/10	5/10
UC 392[3]	(+)	(-)	1:64	NEG	NT	NT	NT	NT
UC 933	(-)	(-)	1:32	NEG	2/6	0	5/10	0
UC 406	(+)	(+)	1:128	NEG	5/6	0	10/10	0
UC 408[2,3]	(+)	(+)	1:64	NEG	3/6	0	6/10	0

1 = Bb *culture positive placenta and uterine fluid*
2 = Bb *culture positive placenta*
3 = Bb *PCR positive colostrum*
NT = sample not tested

B burgdorferi DNA was demonstrated in whole blood from 5 of 15 adult dairy cattle at the time of parturition when subjected to PCR using *B burgdorferi* OspA specific primers[33] (Figure 2: lanes 10, 21,25, 28, and additional data; Tables 1, 2). The calves of four of these five individuals, including 2 of 3 stillborn calves, were also spirochetemic at birth (Figure 2: lanes 11, 26, 27, 29). *B burgdorferi* DNA was also detected by PCR in the colostrum of 4 of 12 adult dairy cattle, including 3 of 4 spirochetemic cattle, at parturition (Figure 3: lanes 10, 11 14, 15). All cattle studied were healthy, and none showed clinical signs suggestive of *B burgdorferi* infection.

Spirochetes were isolated by culture from 2 of 10 placenta and 1 of 8 uterine fluid samples collected at parturition (Table 1). Both of these cows had *B burgdorferi* specific antibody response at parturition by immunoblot and IFA (Figure 1: lanes 8, 28; Table 1). One of these cows and her calf were spirochetemic (Table 1) at parturition.

Three of 15 calves were stillborn; *B burgdorferi* DNA was detected by PCR in 3 of 3 and spirochetes were cultured from 2 of 3 stillborn calves (Table 3). Two of three stillborn calves, and their dams, were spirochetemic at birth (Figure 2: lanes 10, 11, 28, 29). The other tissues from which *B burgdorferi* DNA was detected in these two calves included spleen, kidney, synovial fluid and tissue, cerebrum, and aqueous humor (Figures 3B, C). *B*

Table 2. Brief summary of data for cows naturally infected with **Borrelia burgdorferi** *at parturition and stillborn calves.*

	Spirochetemic (Bb PCR)		IFA Titer		# *Bb* Specific Antibodies		# Nonspecific *Bb* Antibodies	
COW ID	Dam	Calf	Dam	Calf	Dam	Calf	Dam	Calf
UC 378	(-)	NT	1:64	1:64	4/6	0	9/10	0
UC 380	(+)	(+)	1:64	1:64	3/6	1/6	9/10	1/10
UC 410[1]	(+)	(+)	1:256	NEG	4/6	0	7/10	0

1 = Bb *PCR positive colostrum*
NT = sample not tested

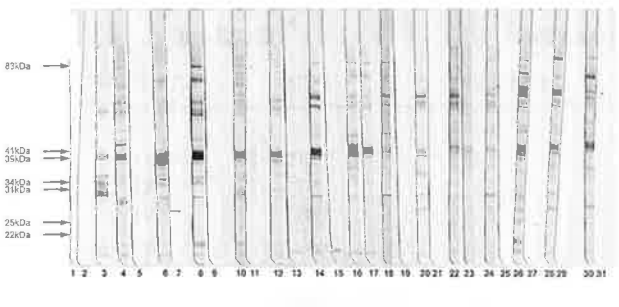

Figure 1. Borrelia burgdorferi *immuoblot of sera from naturally infected adult dairy cattle at parturition and their calves prior to nursing.* Bb *specific proteins are labelled in kDa. Lane 1; Conjugate control; Lane 2: Negative control calf serum; Lane 3: Positive control calf serum; Lanes 4,5: UC 359 and calf; Lanes 6, 7: UC 360 and calf; Lanes 8,9:UC 376 and calf; Lanes 10, 11: UC 378 and stillborn calf; Lanes 12, 13: UC 380 and stillborn calf; Lanes 14, 15: UC 382 and calf; Lanes 16, 17: UC 385 and calf; Lanes 18, 19: UC 386 and calf; Lanes 20, 21: UC 388 and calf; Lanes 22, 23: UC 390 and calf; Lanes 24, 25: UC 933 and calf; Lanes 26, 27: UC 406 and calf; Lanes 28, 29: UC 408 and calf; Lanes 30, 31: UC 410 and stillborn calf.*

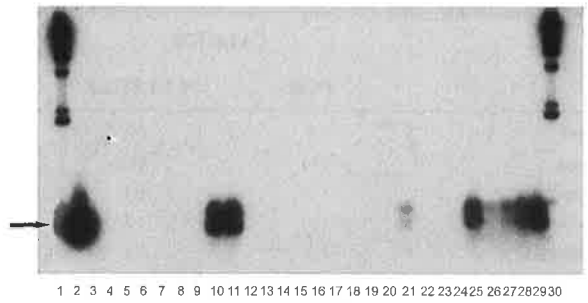

Figure 2. Autoradiograph of ^{32}P labelled PCR products. Whole blood samples were collected at parturition from cattle naturally infected with B burgdorferi *and their calves. Lane 1: Hind III digested lambda DNA (GIBCO); Lane 2: Positive control* B burgdorferi *genomic DNA; Lane 3: Negative control; Lane 10: UC 380 blood; Lane 11: UC 380 stillborn calf blood; Lane 21: UC 392 blood; Lane 25: UC 406 blood; Lane 26: UC 406 calf blood; Lane 27: UC 408 calf blood (UC 408 also positive, not shown here); Lane 28: UC 410 blood; Lane 29 UC 410 stillborn calf blood; Lane 30: Hind III digested lambda DNA.*

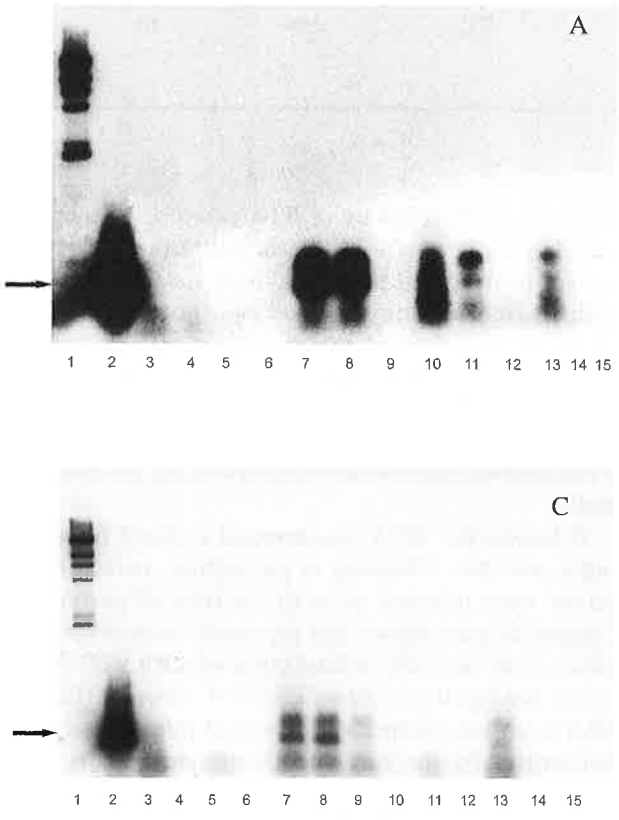

*Figure 3. Autoradiographs of ^{32}P labelled PCR products from tissues of 3 of 3 stillborn calves. (**A**) UC 378 stillborn calf. Lane 1: Hind III digested lambda DNA (GIBCO); Lane 2: Positive control* B Burgdorferi *genomic DNA; Lane 3: Negative control; Lane 7: spleen; Lane 8: left carpus fluid; Lane 10: left hock fluid; Lane 11: bladder; Lane 13: heart. All other samples tested were negative; (**B**) UC 380 stillborn calf. Lane 1: Hind III digested lambda DNA (GIBCO); Lane 2: Positive control* B Burgdorferi *genomic DNA; Lane 3: Negative control; Lane 4: bladder; Lane 9: spleen; Lane 10: left carpus fluid. All other samples tested were negative; (**C**) UC 410 stillborn calf. Lane 1: Hind III digested lambda DNA (GIBCO); Lane 2: Positive control* B Burgdorferi *genomic DNA; Lane 3: Negative control; Lane 7: left carpus fluid; Lane 8: left carpus synovium; Lane 9: cerebrum; Lane 13: aqueous humor. All other samples tested were negative.*

burgdorferi was also isolated by culture from the kidney of one of these calves, and subsequently confirmed by PCR (Table 3).

The dam of one stillborn calf was not spirochetemic (Figure 2: lane 9). The calf's blood was not tested; however, *B burgdorferi* DNA was detected by PCR in the spleen, bladder, synovial fluid, and heart (Figure 3A) and spirochetes were isolated by culture from the spleen of

Table 3. Summary of data from organs and tissues collected from stillborn calves for culture or PCR analysis.

	CALF 378		CALF 380		CALF 410	
	PCR	CULTURE	PCR	CULTURE	PCR	CULTURE
Blood	(-)	(-)	(+)	(-)	(+)	(-)
Spleen	(+)	(+)	(+)	(-)	(-)	(-)
Urine	(-)	(-)	(-)	(-)	NT	NT
Bladder	(+)	(-)	(+)	(-)	NT	NT
L kidney	(-)	(-)	(-)	(-)	(-)	NT
R kidney	(-)	(-)	(-)	(-)	(-)	(+) (PCR+)
L carpus fluid	(+)	(-)	(+)	NT	(+)	(-)
L carpus tissue	NT	NT	(-)	NT	(+)	NT
L hock fluid	(+)	(-)	NT	NT	NT	NT
L hock tissue	(-)	NT	(-)	NT	NT	(-)
L hip-tissue	(-)	NT	NT	NT	NT	(-)
Liver	(-)	NT	NT	NT	NT	NT
Heart	(+)	NT	(-)	NT	NT	NT
Testes	(-)	NT	NT	NT	NT	NT
CSF	(-)	(-)	(-)	NT	(-)	(-)
Brain stem	NT	NT	NT	NT	(-)	(-)
Cerebrum	NT	NT	NT	NT	(+)	NT
Cerebellum	NT	NT	NT	NT	(-)	NT
Lung	NT	NT	NT	NT	(-)	NT
Aqueous humor	NT	NT	NT	NT	(+)	(-)

NT = sample not tested

this stillborn calf (Table 3).

DISCUSSION

This study clearly demonstrates in utero transmission of *B burgdorferi* from naturally infected cows to their calves. Most of the cows tested were seropositive, and one third (5 of 15) were spirochetemic at parturition. Four of these cows gave birth to spirochetemic calves, suggesting a relationship between maternal and neonatal spirochetemia. *B burgdorferi* DNA was also detected in colostrum from adult cattle, and viable *B burgdorferi* was cultured from the placenta or uterine fluid of two cows, indicating disseminated infection with *B burgdorferi* at the time of parturition.

Three of fifteen calves in this study were stillborn at term. Two were spirochetemic (the blood from the third was not tested), and all showed evidence of disseminated *B burgdorferi* infection by the detection of *B burgdorferi* DNA in multiple tissues. Live spirochetes were cultured from the spleen of one stillborn calf and the kidney of another, and subsequently confirmed as *B burgdorferi* by PCR, demonstrating that the *B burgdorferi* was viable, in at least some tissues. Not all PCR positive calf organs were culture positive; this could be due to the presence of nonviable *B burgdorferi* DNA in some tissues, or to the difficulty in direct culture of *B burgdorferi*. Although the sample size is small, 20% term stillbirths in first calf heifers from a well managed, well vaccinated herd is high. Paired serology for common bovine infectious agents causing abortion was performed on sera from the dams of the stillborn fetuses, and reflected vaccine response. However, the lack of controlled experimental infection in this study make it impossible to determine if *B burgdorferi* infection was responsible for the three fetal deaths.

B burgdorferi DNA was detected in blood from adult cattle and their offspring at parturition, indicating the calves were infected prior to the time of parturition. Congenital spirochetosis has previously been observed in calves from clinically normal cows infected with *B coriaceae*, the agent of epizootic bovine abortion (EBA).[43] EBA is an asymptomatic spirochetal infection of cattle transmitted by the soft tick *Ornithodoros coriaceus,* which frequently results in late-term abortion or the birth of weak calves.[44] This disease, and its tick vector, are nonendemic to New England. Another spirochetal agent of abortion in cattle, *Leptospira* spp., often results in third trimester abortion, and maternal clinical signs can vary from mild to severe, with abnormal milk, renal disease and reproductive problems predominating.[45,46] The cattle

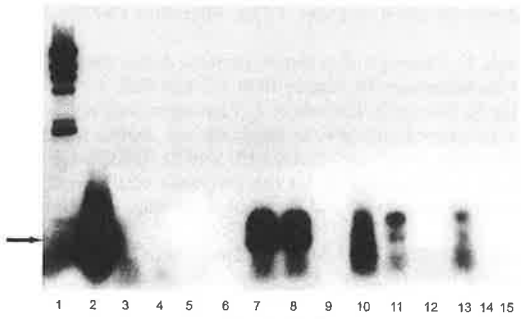

Figure 4. Autoradiograph of ^{32}-P labelled PCR product from colostrum collected from dairy cattle naturally infected with B burgdorferi. *Lane 1: Hind III digested lambda DNA (GIBCO, Gaithersburg, MD); Lane 2: Postive control Bb genomic DNA; Lane 3: Negative control; Lane 10: UC 390 colostrum; Lane 11: UC 392 colostrum; Lane 14: UC 408 colostrum; Lane 15: UC 410 colostrum. All other cattle tested were negative.*

in this study were well vaccinated against several *Leptospira* serovars; paired serology of dams with stillborn calves showed only vaccine response to this agent.

None of the cattle observed at parturition showed clinical signs commonly associated with *B burgdorferi* infection such as lameness, edema or joint swelling. This observation supports previous findings that cattle, as well as other domestic animals, are often subclinically infected with *B burgdorferi*.[14]

Antibody response to *B burgdorferi* infection in cattle is known to be highly variable and cross-reacting antibodies to other *Borrelia* species may cause positive IFA serology; however, at this time there is no evidence to suggest that other *Borrelia* species that commonly infect cattle, including *B coriaceae* or *B theileri*, or their tick vectors, are endemic to this area. In this study, the antibody response to several *B burgdorferi* specific proteins seen in immunoblots of adult cattle suggests long-standing exposure to *B burgdorferi*. However, many of these same adults had quantitatively low antibody titers by IFA. While the antibody response seen in the calves was not strong using either method (IFA/immunoblot), it was specific against *B burgdorferi* proteins by immunoblot. The antibody response observed in the calves must reflect in utero exposure, since maternal-fetal transfer of antibody in cattle only occurs after birth via colostrum.[29] A specific antibody response against *B burgdorferi* proteins has also been seen in neonatal dairy calves when experimentally infected with *B burgdorferi*.[22]

Evidence both supporting and refuting in utero transmission of *B burgdorferi* has been presented in the literature.[6,11,25-32] The disparate results may reflect the many variables that occur in the course of fetal pathogen exposure, including the stage of gestation, placental type, route of infection, maternal response, and pathogenicity of the infectious agent. The findings of this study of natural *B burgdorferi* infection in pregnant dairy heifers support previous observations of both natural and experimental in utero infection with *B burgdorferi* in domestic animals, and give further evidence that transmission of *B burgdorferi* occurs during gestation in naturally infected cattle. Additional studies are needed to investigate maternal and fetal response to *B burgdorferi* infection.

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Borrelia theileri: A Review

Ronald D. Smith, DVM, PhD and Arlin B. Rogers, DVM, MS

INTRODUCTION

Borrelia theileri Laveran 1903[1] is a relatively nonpathogenic member of the genus that is transmitted by several ixodid ticks. It is most commonly reported from cattle, but also parasitizes a number of other domestic and wild vertebrate hosts. Its importance lies in its widespread geographic distribution and possibility of being confused with more pathogenic members of the genus. In this paper we review current knowledge of the biology of this spirochete and suggest directions for future research on this species.

HISTORY AND SYSTEMATICS

Until recently, classification of spirochetes has been based largely on morphological characteristics and host distribution. Live spirochetes were first observed in the blood of South African cattle by Sir Arnold Theiler in 1902, who named the associated disease "spirillosis of cattle."[2] Theiler also reported seeing the organism in a febrile horse and sheep, but was unable to transmit the organism with blood from infected cattle to naive cattle, horses, sheep, goats, dogs, rabbits, or guinea pigs.

In 1903 Laveran studied stained smears of Theiler's spirochetes, which he named *Spirillum theileri*.[1] He reported that spirochetal organisms found in bovine blood smears ranged from 8 to 30 µm in length. In 1904 Theiler described different shapes that the spirochetes might take and stated that "the longest microbes measure from 20 to 30 µm."[2] Subsequent references erroneously concluded that the mean length of *B theileri* was 20 to 30 µm, which led to confusion as to the identity of bovine spirochetes. Thus, when bovine spirochetes were reported from other parts of the world with mean lengths less than 20 µm, it was believed that they were not *B theileri*. Investigators in Argentina reported a bovine spirochete with a mean length of 15 µm and named it *Borrelia najeri* sp. nov.[3] Callow commented: "It would appear that early reviewers did not read past the statement that the largest forms can be 20–30 µm long."[3]

Borrelia theileri is currently classified in the family Spirochaetaceae and the bacterial order Spirochaetales.[4] Other pathogenic spirochetes in the family include *Treponema* and *Leptospira*. *Borrelia theileri* has undergone several taxonomic reclassifications since it was first named *Spirillum theileri*. The 1923 edition of *Bergey's Manual* listed it as *Spironema theileri*. Mulhearn named the organism found in Australia *Spirochaeta theileri*.[3] In 1952 Seddon claimed that the bacterium should be placed in the genus *Treponema*.[3] The name *Borrelia theileri* first appeared in the 1957 edition of *Bergey's Manual of Systematic Bacteriology*. Callow's 1967 Communication[3] helped clear up the discrepancies and the name *Borrelia theileri* is now applied to comparable bovine spirochetes around the world. However, the 1984 edition of *Bergey's Manual* still described *B theileri* as having a mean length of 20–30 µm.[4]

Geographic and host distribution

Since its original description in South African cattle, *B theileri* has been reported from southern Russia, Bulgaria, Australia, Texas, Mexico, and South America.[3,5-9] Naturally-occurring infections are most commonly reported in cattle, but tick-borne infections have been induced in sheep, horses, and deer.[10]

Two other species of Borrelia infect cattle: *B burgdorferi* and *B coriaceae*. Since the geographic ranges of vector ticks and vertebrate hosts for *B theileri*, *B burgdorferi*, and *B coriaceae* overlap, species distinctions are important. A case of *Borrelia burgdorferi* infection, the agent of Lyme disease, was described in a cow from Wisconsin[11] and a statistical relationship between circulating *B burgdorferi*–reactive antibody and lameness in cattle from the upper Midwestern US has also been reported.[12] *Borrelia coriaceae* infection has been associat-

From the Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL.

Address correspondence to Ronald D. Smith, DVM, PhD, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL 61802.

ed with epizootic bovine abortion (also known as Foothill abortion) in California,[13,14] although a causative role remains unproven.[15]

The geographic distribution of the bovine borreliae is determined by the distribution of their tick vectors. *Borrelia theileri* has been reported almost exclusively from tropical latitudes, correlating with the distribution of its tick vectors, *Boophilus microplus*, *B annulatus*, *B decoloratus*, and *Rhipicephalus evertsi*, on the African, Australian, and American continents.[3,7-10,16,17] *Borrelia burgdorferi* and *B coriaceae* are found principally in temperate climates. Borreliosis was reported in ruminants on the Dutch island of Ameland in the North Sea.[18,19] Although it was originally ascribed to *B theileri*, it was later postulated to be an early finding of *B burgdorferi*.[20] None of the tick species known to harbor *B theileri* were present in Ameland. However, the tick *Ixodes ricinus*, a proven vector of Lyme disease in Europe, was present on the island.[21]

As ticks that transmit *B theileri* may also be vectors for bovine babesiosis, the presence of *B theileri* in Giemsa–stained blood smears is often an incidental finding during studies on babesiosis.[7,22]

Pathogenesis

Bovine borreliosis due to *B theileri* is benign in most cases, even in splenectomized calves.[8] Parasitemia is first detectable in Giemsa–stained thin blood smears from 2 to 4 weeks postexposure to infected ticks, but lasts only a few days.[7] During the parasitemic phase infected cattle may exhibit mild temperature elevations, lethargy, reductions in packed cell volume, and possibly hematuria.[2,3,7] Clinical illness usually lasts only a few days and is followed by uneventful recovery. Parasite recrudescences, characterized by spirochetemia without outward signs of illness, may occur.[2,23]

Morphology in bovine and tick hosts

As a group, the borreliae are more pleomorphic than leptospires and treponemes.[24] The borreliae contain loose coils whose wave amplitudes may vary considerably, even within the same organism. Replicating borreliae elongate and then divide by transverse fission to form two daughter cells.[25] Dividing cells are longer, more linear, and thinner than nondividing forms. Borreliae stain well with aniline dyes such as the Romanowsky–type stains used in routine blood smears. Giemsa stain is most commonly used. *Borrelia theileri* is weakly Gram negative, but the bacterial DNA is readily visible using acridine orange staining and ultraviolet illumination.[26] Spirochete morphology and staining properties have been used to distinguish among the pathogenic genera of the family Spirochaetaceae (Table 1).[25,27]

A number of workers have studied *B theileri* morphology in bovine blood, tick hemolymph, and tick tissues using light microscopy, scanning, and transmission electronic microscopy.[3,7,8,23,26] Some of these findings are summarized in Table 2.

Bovine blood forms of *B theileri* (Figure 1) are generally shorter, thicker, and show less evidence of active division than tick tissue or hemolymph forms (Figure 2), but lengths of all forms vary considerably. *Borrelia theileri* in Giemsa–stained bovine blood smears consist of populations of post and predivision forms in approximately a 2:1 ratio.[8] Apparent constrictions midway along the length of many of the longer forms support the contention that they are preparing for division.[8,23] The relative proportions of these two populations will affect estimates of the mean length of bovine and tick populations.

Within a given spirochete the diameter is relatively constant (0.1–0.3 μm depending on stage of division) except for a gradual tapering to a point near the poles. Wave amplitude within and between organisms is too variable to permit meaningful measurements. Wave amplitudes of less than 0.25 μm are common in the long, thin dividing forms (Figure 2). In contrast, the short nondividing forms (Figure 1) may exhibit wave amplitudes up to 1.25 μm in the center, which decrease to less than 0.5 μm near the poles.

The borrelial surface structure consists of an outer surface layer, an outer membrane, and a cytoplasmic membrane. Periplasmic flagella (Figure 3) are located between the outer and cytoplasmic membranes. Synonyms for these structures are axial fibrils, periplasmic fibrils, and endoflagella.[4] Periplasmic flagella originate near the poles of the spirochete and course towards the middle of its length where they overlap. The number of flagella varies between species and also within species. Most borreliae have flagella ranging in number from 16 to 30.[4] The number of flagella originating from each pole of *B burgdorferi* has been shown to vary from 7 to 11.[28] From 5 to 10 flagella have been observed in cross sections of *B theileri*, suggesting that 5 flagella originate from each of the poles and intersect in the middle of the organism (Figure 4). However, organisms may occasionally contain up to 14 to16 flagella.[26] Membrane blebs, or gemmae, can also be seen in negative stains of *B theileri*.

When wet mounts of infected bovine blood, tick tissues, or short term cultures are observed by darkfield or phase contrast microscopy, *B theileri* display translation (linear propulsion) fore and aft, flexion, and rotational motility,[26] similar to that of other borreliae.[27] Free swimming organisms propel themselves linearly or curvilinearly, or may remain stationary while undergoing rapid gyrations. Periods of activity may alternate with periods of quiescence. Spirochetes often have one end embedded in

Table 1. Criteria for morphologic distinction between the pathogenic spirochetes.

Genus	Staining with Aniline Dyes	Wavelength	Coils	Distal Ends
Borrelia	Good	Irregular	Loose	Tapered
Leptospira	Poor	Short	Tight	Hooked
Treponema	Poor	Long	Tight	Blunt

a cell or piece of tissue while the free ends undergo gyrations to varying degrees.

Development in and transmission by ticks

Most studies of *B theileri* in ticks have focused on the one-host cattle tick, *B microplus*. Replication of *B theileri* appears to occur at a higher rate in *B microplus* ticks than in bovine blood, as masses of replicating *B theileri* are often found in association with tick hemocytes (Figure 2).[8,26] Spirochetes have also been observed in close association with tick ovaries (Figures 5 and 6) and in oviposited eggs, but they are relatively rare.[26] Despite extensive invasion and replication in tick tissues, *B theileri* appears to be relatively nonpathogenic for the tick.[7]

Only the nymphal and adult stages of *B microplus* transmit the spirochete to cattle,[3,10] despite the fact that transovarial infection occurs and borreliae can be found in larvae within 72 hours after being placed on calves.[7] It may be that too few spirochetes arrive in the larval salivary glands to effect transmission. Ticks are also capable of transmitting *B theileri* to sheep, horses, and deer under experimental conditions.[3] *Borrelia theileri* can also be transmitted by injecting fresh, whole blood from borrelemic into naive cattle.[3]

In vitro cultivation

Prior to 1992 the only reported attempt to cultivate *Borrelia theileri* in vitro was that of Theiler who wrote that "cultivations on the usual artificial media were repeatedly tried, but always with negative results."[2]

Rogers[26] established short-term primary cultures of *B theileri* using a modification of a medium previously developed to maintain *B recurrentis* (the relapsing fever spirochete) in vitro.[29] The original medium was modified by using high concentrations of bovine rather than rabbit serum. Cultures were established using ovaries from infected *B microplus* ticks and monitored for spirochete viability by phase contrast and darkfield microscopy.

Active *B theileri* were observed in the culture for 6 weeks post-inoculation. Viable organisms were observed in association with tick ovarian tissue (Figure 7). One end of most spirochetes was embedded in tissue while the free end gyrated rapidly. Sometimes clusters of spirochetes were seen in the absence of tick tissue. Free–swimming individual borreliae were occasionally observed. Spirochetes exhibited translational (linear) motility, flexion, and corkscrew rotation. Replication of *B theileri* led to high concentrations of organisms over the first few weeks in vitro. Large clusters containing hundreds to thousands of borreliae were seen in the first few weeks. In subsequent weeks the number of active organisms diminished.

Table 2. Morphologic features of Borrelia theileri *in bovine blood, tick hemolymph, and tissues.*

Morphologic Feature*	Bovine Blood	Tick Hemolymph	Tick Tissue
Length (μm)	10.2-13	14.8-15.4	17.7
	(6-19.5)	(8.3-32.0)	(8-28)
No. Spirals	5.0-5.6	7.2-7.3	—
	(1-8)	4-11	—
Wavelength	1.6	1-5	2
	1-1.9	—	—

**Mean values with ranges in parentheses*

The short-term cultures enabled the extraction of relatively pure suspensions of organisms for use as antigen in immunologic testing. Other media reported to support in vitro growth of one species of *Borrelia*, including *B burgdorferi*, failed to sustain *B theileri*.[26]

IMMUNOLOGIC RELATIONSHIPS WITH OTHER BORRELIAE

The discovery of the borrelial etiology of Lyme disease has prompted studies on the serologic cross–reactivity between spirochetes of different genera and species. Cross–reactivity among members of the genus *Borrelia* sp., *Leptospira* sp., and *Treponema* sp. has been reported.[30,31]

Since *B theileri* can be readily transmitted among cattle and other herbivores without clinical detection, it may complicate the diagnosis of Lyme disease in the bovine, especially if there is serologic cross–reactivity between *B theileri* and *B burgdorferi*. Rogers[26] evaluated the immune response of cattle to *B theileri* infection and the role of serologic cross-reactivity in the interpretation of seroepidemiologic studies. *Borrelia theileri*–infected calves produced antibodies that cross-reacted with *B burgdorferi* and *B coricaeae* in indirect fluorescent antibody (IFA) tests, using whole cells of all three organisms as antigen. Titers were first detectable three weeks after tick-borne exposure, then rose rapidly and persisted at high levels through 10 weeks post-exposure when the

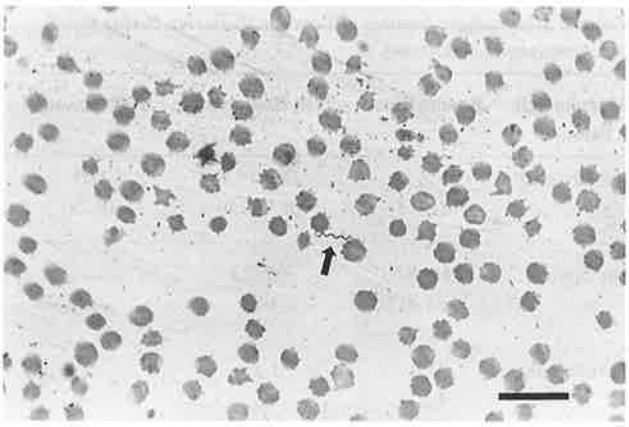

Figure 1. Borrelia theileri *(arrow) in bovine blood smear. Giemsa stain; bar=20 µm.*

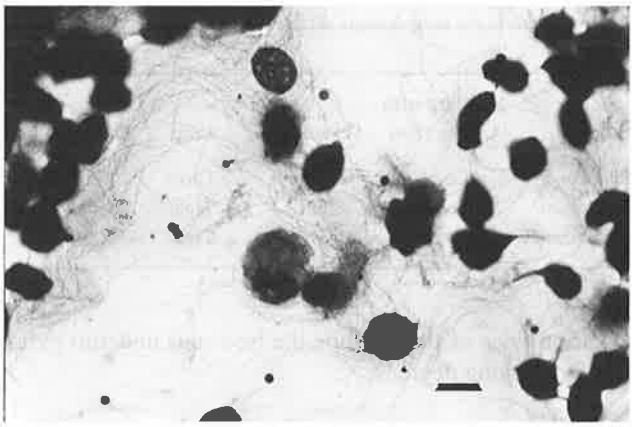

Figure 2. Borrelia theileri *in* Boophilus microplus *hemolymph. Note close association with hemocytes and many long thin forms suggestive of active replication. Giemsa stain; bar=20 µm.*

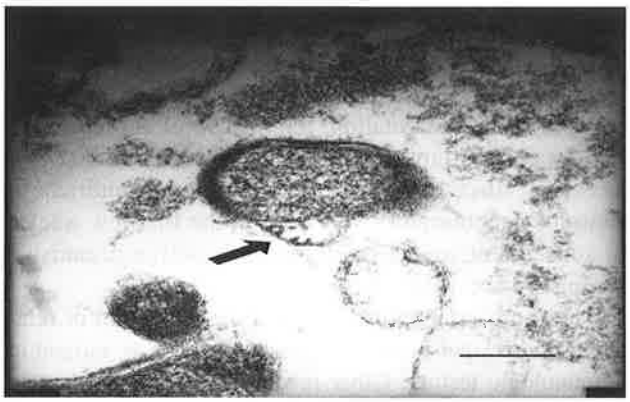

*Figure 3. Transmission electron micrograph of tick (*Boophilus microplus*) origin* Borrelia theileri *with pouching of outer membrane enclosing numerous periplasmic flagella (arrow). Bar=0.1 µm.*

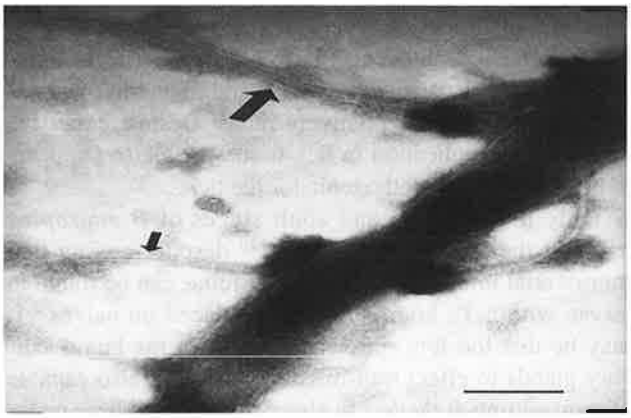

*Figure 4. Negatively-stained transmission electron micrograph of disrupted tick (*Boophilus microplus*) origin* Borrelia theileri *surface with exposure of 5 periplasmic flagella (3 at large arrow, 2 at small arrow), Bar=0.3 µm.*

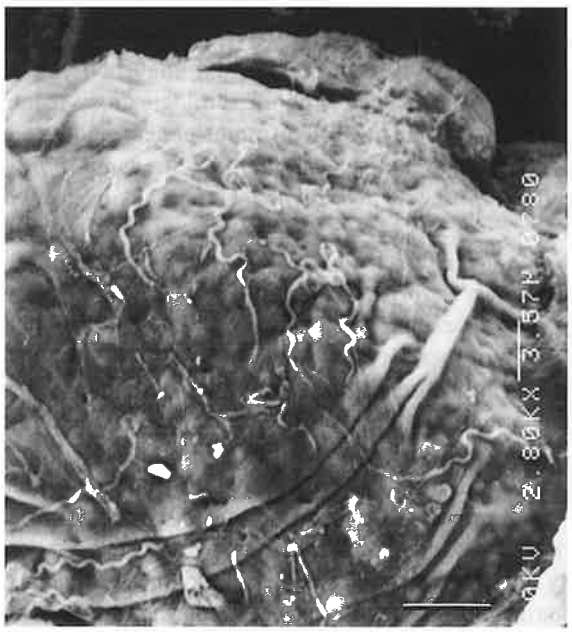

Figure 5. Scanning electron micrograph of Borrelia theileri *on the surface of* Boophilus microplus *oocyte. Bar = 12 µm.*

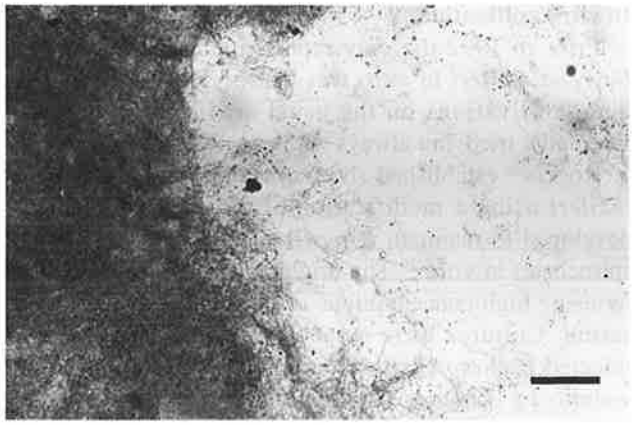

Figure 6. Transmission electron micrograph of Borrelia theileri *(arrow) within the capsule of a* Boophilus microplus *oocyte. Bar = 6 µm.*

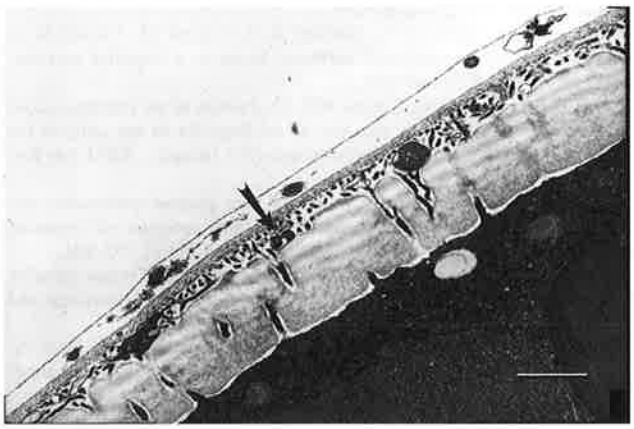

Figure 7. Borrelia theileri *in culture. Note association of spirochete clusters with detritus of inoculated tick ovarian tissue (left). Giemsa stain; bar = 20 μm.*

study was terminated. Antibody titers to *B burgdorferi* and *B coriaceae* were lower than to *B theileri* antigen.

Serologic cross–reactivity among these species is probably due, in part, to a shared, genus-specific 41 kDa flagellar antigen[32] that was detected in IFA testing of whole cell antigen with monoclonal antibody.[26] Ji et al[33] also reported cross–reactions to a purified 41 kDa *B burgdorferi* flagellar antigen in an ELISA test using these same *B theileri*–infected calf sera. As expected, the *B burgdorferi*–specific outer surface protein A (OspA) antigen[34,35] was not present on either *B theileri* or *B coriaceae*.[26]

Genetic relationships between *B theileri* and other borreliae have also been initially explored. Highly conserved fragments of borrelial flagellin and 16S rDNA gene sequences of *B theileri* and other borreliae have been amplified, sequenced, and compared[36] (Telford, SM, Personal Communication, 1997). One of the important findings was that *B theileri* is distinct from a newly described uncultivable borrelia from *Amblyomma americanum* ticks associated with a Lyme disease-like illness in the Chesapeake Bay area of the United States.[36]

DIRECTION FOR FUTURE STUDIES

Although *Borrelia theileri* causes relatively mild disease in cattle, it remains important because of its potential to be confused with the Lyme disease spirochete, *B burgdorferi*, and with the putative agent of epizootic bovine abortion, *B coriaceae*. Recently, uncultivable borreliae have been implicated as a potential cause of Lyme disease-like illness.[36,37] Characterization of potentially zoonotic borreliae, including *B theileri*, is therefore relevant to both human and animal health.

Host, morphologic and serologic criteria may be inadequate for establishing the relationship between *B theileri* and other members of the genus. More specific markers, based on biochemical analysis, immunoblotting, molecular subtyping, and gene amplification are needed. Their development would be greatly facilitated if relatively pure preparations of *B theileri* could be obtained. Successful long-term propagation of the organism would thus appear to be the key to better understanding *B theileri* and its relationship to other members of the genus *Borrelia*.

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Limitations of the OspA Vaccine for Humans: A Review*

Denise M. Foley*, PhD; David R. Blanco†, PhD; Michael A. Lovett†‡, MD, PhD; James N. Miller†, PhD

INTRODUCTION

Presently, the OspA vaccine is the most developed and defined candidate for generating protection against Lyme disease. As has been reported in the literature, significant protection has been achieved for several animal models including mice,[1-9] dogs,[10] rabbits,[11] and monkeys.[12] An important issue for any vaccine is that it not only be effective, but also safe. In this regard, vaccination studies in monkeys[13] and humans[14-16] have indeed shown that the OspA vaccine is safe with only minor reactions being reported in a small percentage of persons. More importantly, both the Pasteur Merieux Connaught and SmithKline Beecham Laboratories have now reported on phase III human vaccine trials where it was demonstrated that OspA provides significant protection against Lyme disease.[17,18]

While these studies indicate that OspA is a very promising vaccine against Lyme disease, there are limitations based upon experimental studies in animals that may have important implications for humans. The purpose of this paper is to discuss the recombinant OspA vaccine and, in particular, raise issues regarding its limitations for humans by reviewing data obtained from published studies. Many investigators have described their rationale for these limitations that tend to support the necessity for utilizing other protective immunogens in concert with OspA in a "cocktail" vaccine. These concerns will be included in the context of this review.

OspA HETEROGENEITY

While it has been observed that OspA varies greatly or is completely absent in European isolates, it was hoped that OspA vaccination would provide protection for vaccinated persons in North America where there is less OspA heterogeneity.[19] Recently, however, a growing concern about diversity among North American isolates has been noted.[20-31] The ability of some OspA serotypes to avoid killing with antibodies raised against other serotypes has been shown.[24,31] In the study by Lovrich et al,[27] the authors concluded that although cross protection occurred against some strains expressing different antigenic types of OspA, vaccination with a single OspA type did not provide complete protection against challenge with all strains. Even more surprising was the finding that the presence of anti-OspA antibodies elicited from some isolates did not result in protection against challenge with the homologous strain.

The current OspA vaccine utilizes a *Borrelia burgdorferi* sensu stricto OspA molecule which, to date, has been found in the majority of the isolates from North America.[19,26] However, one type of North American OspA variant, typified by strain 25015, has been shown to infect mice vaccinated with N40 OspA,[21] a molecule similar to the current OspA vaccinogen. This variant type, isolated from upstate New York, has also been isolated from Illinois,[32] (presented by Maria Picken, 11th Annual Scientific Conference on Lyme Borreliosis, New York, April 25-27, 1998). It is therefore possible that, individuals infected with this variant strain may not be protected with the current vaccine. Furthermore, the probability of discovering other variants against which the vaccine will fail is high given the propensity for the organism to undergo mutational and recombinational events at the OspA locus,[33-38] and the discovery of variant *Borrelia* strains in locations such as California,[25,39] New York,[30,40] Texas,[40] Missouri,[40,41] Illinois,[32] Georgia, and Florida,[42-44] which have yet to be tested in vaccination protocols.

*From the *Department of Biomolecular Science, Chapman University Division of Natural Sciences, Orange, CA; and the †Department of Microbiology and Immunology; and the ‡Division of Infectious Diseases, UCLA School of Medicine, Los Angeles.*

Address correspondence to: Denise M. Foley, PhD, Divison of Natural Science, Chapman University, 333 North Glassell, Orange, CA 92866.

**Due to the lengthy review process, this report was written and revised prior to the recent FDA approval of the vaccine for Lyme disease.*

While the greatest variation of the OspA molecule occurs in European isolates, the increasing evidence of OspA variability in North America, together with the observation that cross protection is not always achieved with OspA vaccination, implies that even a vaccine that includes several serotypes of OspA molecules will not result in complete protection of the vaccinated North America population.

OspA downregulation in the vertebrate and host adaptation

Another key issue to be considered when using OspA as the sole vaccinogen is the widely accepted fact that OspA is not expressed during vertebrate infection. The studies by Schwan et al[45] and de Silva et al[46] demonstrate that OspA is present on *B burgdorferi* before tick feeding but is lost after initiation of the bloodmeal. Furthermore, in the study by Schwan et al, it was demonstrated that OspC, a more heterogeneous molecule than OspA, is unregulated after tick feeding. They suggest this downregulation of OspA and corresponding upregulation of OspC is crucial for the ability of the organism to infect the vertebrate host.

Further evidence that OspA is not expressed in the vertebrate host can be gathered from studies in which animals inoculated with low numbers of *B burgdorferi,* whether it be from needle injection or tick transmission, do not develop antibodies to OspA in spite of developing an antibody response to other *B burgdorferi* antigens.[45,47-51] It has also been observed that many Lyme borreliosis patients either do not produce antibodies that react with OspA or produce relatively low OspA antibody titers.[52-57] In a study by Schutzer et al,[58] 12 of 16 early Lyme disease patients with neurological involvement were found to have cerebrospinal fluid (CSF) and serum IgM directed against OspC and 5 of these 12 also had IgM to OspA that was restricted to the CSF. These data suggest that in certain neurological Lyme disease patients, OspA may be selectively expressed in the central nervous system (CNS) and not in the peripheral blood or skin. When considering the abundance of the OspA protein in ex vivo cultured organisms and the evidence for its downregulation during tick feeding, the absence of a universal serum antibody response to OspA in humans would seem to support the theory that the majority of spirochetes will not initially express this protein in the infected human. If some organisms do revert to expression of OspA once they reach the CNS or other specific site, this may explain why some patients develop a response to this protein. It is assumed that vaccinated persons will destroy these organisms if the appropriate OspA serotype is being expressed. However, the population of organisms within the OspA-vaccinated host, which continue to keep OspA downregulated, would remain unaffected.

The studies of Barthold et al[59] provide additional evidence that OspA is not expressed by *B burgdorferi* while in the host. These data are particularly convincing because they demonstrate that OspA vaccinated mice exposed to organisms taken directly from a vertebrate host via a skin transplant from an infected syngeneic mouse are susceptible to infection. These studies were extended by de Silva et al[60] who demonstrated that mice passively administered immune mouse serum were still susceptible to infection by *B burgdorferi* following homologous challenge with infected mouse skin or by tick bite. All mice became infected despite being administered immune sera over the course of 14 days. These investigators concluded that the organisms may be resistant to immune serum antibodies through a mechanism of "host adaptation" that results in immune evasion. The ability of these organisms to evade host immune defenses is clear from the course of natural infection where the establishment of chronic infection and late debilitating manifestations is a common feature. This is also illustrated by the fact that in spite of high levels of borreliacidal antibodies present in humans during stages of Lyme disease,[61-63] these patients remain infected. The fact that immune sera administered after challenge does not abort the infection, which is in contrast to the ability of this same sera to prevent infection if administered before challenge,[59] is compelling evidence that the organism quickly "adapts" once inside the host. This adaptation allows the organism to persist in the presence of what would otherwise be an effective immune response. Although these authors concluded in the same study that OspA vaccination was completely protective, it is also pertinent to consider the possibility of host adaptation and immune evasion when examining OspA vaccination.

It has been shown that infected ticks feeding upon an OspA-vaccinated host results in the destruction of the majority of spirochetes in the tick. However, it has also been shown that some spirochetes survive within some ticks after a bloodmeal containing OspA antibodies.[6,9,12,46] Presumably, these organisms were not expressing OspA or expressed an OspA variant resistant to the killing antibodies present in the bloodmeal. A relevant question is, what is the disposition of the few spirochetes which do survive in the engorged ticks after feeding upon an OspA-vaccinated host? One would predict that these organisms, for perhaps a significant period of time, do not express OspA in response to the downregulating effects of the bloodmeal. Therefore, do these organisms represent a real or merely a theoretical danger to the individual vaccinated with only OspA? If these organisms gain entry to the vaccinated human host, their ability to quickly adapt and resist the anti-OspA immune response creates a

potentially dangerous scenario. Transmission in presumably very low numbers might establish an undetected asymptomatic infection which later exacerbates as debilitating chronic manifestations of late stage Lyme disease.

THE SIGNIFICANCE OF LATENCY

The issue of the potential development of a latent infection in a previously vaccinated individual or animal has not been vigorously investigated. This is particularly pertinent in view of the recognized capacity for spirochetal pathogens, including *B burgdorferi,* to cause latent infection.[64] Others have recognized this gap in the literature as evidenced by this statement:

> "...it is surprising that so little attention has been paid to the question of asymptomatic infection (as manifested by seroconversion) in experimental test systems of vaccine candidates, given the presumption that latency may occur in human *B burgdorferi* infections." GP Wormser. *Infection* 1996;24:203.

In a published human vaccine trial with OspA plus adjuvant administered to individuals residing in endemic areas of the US, Steere et al[17] reported a vaccine efficacy of 76% (16 confirmed cases) after 3 doses of immunogen. Although no silent seroconverters were found in these vaccinees, 2 persons who received only 2 doses were diagnosed as asymptomatic. A similar study by Sigal et al[18] showed a 92% success rate among individuals who received 3 vaccine doses. However, male subjects $\geq$ 60 years of age displayed an efficacy of only 75% (L. Sigal et al, Infectious Disease Society of America, San Francisco, CA, 1997, abstract). Absence of infection in the latter study was based upon the lack of evidence of clinically apparent disease in the vaccinated population, although no testing for seroconversion was performed in vaccinated persons who were not presenting evidence of clinical disease. It should be noted that in both the Steere et al[17] and Sigal et al[18] studies, a lesser degree of protection (49% and 68%, respectively) was obtained when only 2 vaccine doses were administered. These data take on added significance when considering that 1) primary vaccinated individuals may not complete the required vaccine series over a 1-year period, and 2) a proportion of those not protected may harbor a latent infection. The question remains as to whether some vaccinated individuals, after exposure to *B burgdorferi,* harbor a low level latent infection. The possibility that a low level infection may not stimulate a measurable antibody response but may exacerbate into clinical Lyme disease at a later time, is suggested by several OspA animal vaccine studies.

In OspA-vaccinated rhesus monkeys, the detection of *B burgdorferi* DNA and antigens in tissues was found at a time when overt symptoms of the disease and Western blot reactivity were absent. These findings suggest the presence of organisms in these tissues although an attempt to "activate" this potential latent infection by administering immunosuppressive drugs was not successful.[12] Although many have argued that the detection of DNA does not indicate the presence of living organisms, a study by Malawista et al[65] has shown a very high correlation between the detection of DNA and positive cultures. Similarly, at the earliest times tested after antibiotic treatment of infected mice the ability to amplify DNA disappeared in concordance with the disappearance of cultivatible spirochetes. The failure to activate a potentially latent infection in the vaccinated monkeys does not unequivocally imply its absence. A parallel can be drawn with latent syphilis in which reactivation is known to occur among latent syphilitics despite the fact that the "triggering" mechanism is not known. During latent infection of rabbits, spirochetes are known to persist despite difficulty in reactivating the infection. In a study by McLeod and Magnuson,[66] symptomatic reactivation was achieved in one rabbit when toxic levels of cortisone were administered. A 33% increase in spirochetemia was observed in drastically immunosuppressed rabbits when compared to latent infected controls and 1 of 7 surviving animals from an original group of 30 developed a darkfield positive skin lesion. Similar doses of cortisone had less of an effect on mice. Of further interest is the fact that infection of mice and rabbits with low numbers of *Treponema pallidum* can establish latency without an antibody response[67] (JN Miller, unpublished studies).

Another question to be considered among OspA-vaccinated persons is whether partial immunity, either from a waning resistance or from an incomplete vaccination regimen, results in an altered disease state upon exposure or a "masking" phenomenon in which infection in the absence of characteristic clinical manifestations such as erythema migrans (EM) occurs. In support of this hypothesis, we found that 4 of 11 OspA-vaccinated rabbits became infected upon challenge with *B burgdorferi* strain B31.[11] In contrast to completely susceptible animals where each site typically develops EM, these infected rabbits developed EM at only 8 of 40 challenged sites while all exhibited disseminated infection. The remaining 7 OspA-immunized rabbits did not develop either EM or disseminated infection. Furthermore, in rabbits exhibiting infection-derived immunity, 2 of 11 exhibited atypical infection while the remaining animals showed complete immunity. The two rabbits from this group became dermally infected following intradermal challenge in the absence of the development of an EM rash and disseminated infection. Further evidence for the potential development of a low level infection in vaccinated animals can be found in a study by Telford et al[6] who showed that 1 of 24 OspA-

vaccinated and heterologously challenged mice, although culture negative, exhibited arthritic changes in the joint.

We believe that these observations support the theory that when states of partial immunity exist, altered forms of disease may arise in some vaccinated and exposed individuals which may make diagnosis more difficult. Because the clinical manifestations may be inconsistent with typical disease, a differential diagnosis that includes Lyme disease, might not be considered given that some physicians may conclude that vaccination reduces or eliminates the chances of acquiring the disease. Furthermore, as suggested by these studies, the infection may be subclinical but could emerge at a later time as more difficult to treat late manifestations.

CAN WE PREDICT A FUTURE VACCINE FAILURE?

Information pointing to the prediction of when spirochetes are likely to evade the immune response of the vaccinated host may currently be available. In two separate studies, a correlation was demonstrated between protective antibody and a specific epitope on OspA, defined by the monoclonal antibody LA-2.[68,69] It was shown in the study by Golde et al[68] that the LA-2 antibody titer is a reliable indicator of immune status following immunization with OspA; vaccinated mice and dogs with a low LA-2 antibody response were susceptible to infection upon challenge. Furthermore, in the human vaccine study conducted by Steere et al,[17] it was reported that patients with breakthrough cases of Lyme disease were found to have significantly lower LA-2 antibody levels at two months following the second injection when compared to a group of vaccinated individuals who had not come down with disease. Interestingly, Padilla et al[61] found that although significant levels of borreliacidal antibodies were elicited in vaccinated individuals and hamsters after two doses, the borreliacidal activity quickly diminished within 180 days. The decline in borreliacidal activity is of concern due to the demonstration by Johnson et al[69] that this activity correlates with protection. The information regarding LA-2 may be useful for determining which individuals are susceptible to *Borrelia burgdorferi* sensu stricto strain B31 type isolates, however, high LA-2 antibody titers may have little or no effect upon variant strains. The frequency of exposure to these variants among a vaccinated population remains to be determined. Thus, the central question still remains as to how many vaccinated patients will develop a classic or altered disease state after exposure, and how this will influence the ability to make an accurate diagnosis.

FUTURE DIRECTIONS TOWARD THE DEVELOPMENT OF A MORE EFFICACIOUS VACCINE

For almost as long as recombinant OspA has been tested as a vaccine candidate, many investigators have recognized the need for an improved vaccine. Recognizing the proven and potential limitations of an OspA vaccine, several investigators have suggested the addition of other components to the vaccine. Such a "cocktail" may include one or more additional recombinant proteins including various OspA serotypes as well as other *B burgdorferi* molecules. Several laboratories have suggested the inclusion of decorin binding protein A (Dbp-A)[31,70,71] which apparently is expressed by *B burgdorferi* while in the vertebrate host.[31] Inclusion of this molecule in a vaccine has been proposed as a means of overcoming the potential danger of spirochetes gaining entry into the OspA-vaccinated host and avoiding antibody-mediated destruction due to the absence of expression in the vertebrate. However, variation in the gene sequence for Dbp-*A* has been demonstrated in some strains[70] and antibodies against Dbp-A do not protect against some variant types.[31] Thus this heterogeneity among Lyme disease spirochetes emphasizes the probability that no one component will be universally expressed by *B burgdorferi* strains thereby necessitating the inclusion of several components in order to achieve optimal protection against Lyme disease.

In closing, the following quote from the literature summarizes the beliefs of many in the field regarding the OspA vaccine and applies to other potential vaccinogens that will be considered in the future.

> Although there is compelling evidence that immunization with OspA will provide protection, questions remain regarding the duration of protection from such immunization, the necessity to have a minimum level of neutralizing antibodies at all times for protection, and the relationship of an immune response to OspA and autoimmune features of Lyme borreliosis. (A Sadziene, AG Barbour. Infection 1996;24:195)

In conclusion, although the OspA vaccine is the most promising candidate thus far, there clearly remains a need for a Lyme disease vaccine that stimulates high levels of long lasting protection against all strains of *Borrelia* responsible for Lyme disease.

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INFORMATION FOR CONTRIBUTORS

Journal of Spirochetal and Tick-borne Diseases

Dedicated to science and art in spirochetal and tick-borne diseases

INFORMATION FOR AUTHORS AND EDITORIAL POLICY

The following guidelines are in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" and the International Committee of Medical Journal Editors (the "Vancouver Group") statement, agreed at the January 1993 Meeting.

The *Journal of Spirochetal and Tick-borne Diseases* publishes quarterly reviews and original work studies about any aspect of spirochetal and tick-borne diseases. The primary purpose is to broaden our understanding of spirochetal and tick-borne diseases. Special focus is given to Lyme borreliosis (also known as Lyme disease), as the most prevalent spirochetal and tick-borne disease. The clinical topics may involve all medical disciplines, nursing, and pharmacy, as well as the social, ethical, and biological features of spirochetal and tick-borne diseases.

Reviews

Each issue includes a series of state-of-the-art articles on a topic related to spirochetal and tick-borne diseases. The articles represent invited presentation by authorities in the field on topics related to spirochetal and tick-borne diseases, with an emphasis on Lyme borreliosis.

Submissions to this category should present a comprehensive state-of-the-art analysis and should be accompanied by an abstract of 300 words or less summarizing major points.

Peer Review Articles

Original articles of 5000 words or less may be submitted to the editorial office. Each article should be accompanied by an abstract of 300 words or less describing the findings of the original research. All articles will be peer reviewed within a 3-week period with subsequent notification to the authors within 5 weeks of submission.

Case Reports

Specific clinical case reports describing a unique approach to Lyme disease and other related disorders in the area of diagnosis or treatment may be submitted for review. An abstract of 250 words or less should accompany the text.

Photographic Section

The topical photographic section will be a regular feature. Photographs pertinent to articles presented in the Journal, as well as other photographs related to any aspect of spirochetal or tick-borne diseases, will be considered for the publication. The guidelines for the submission are designated in **Illustrations**.

Conflict of Interest

The Journal asks authors to disclose at the time of submission any financial or other arrangements they may have with a company whose product figures in the submitted manuscript or with a company making a competing product. Such information will be held in confidence while the paper is under review and will not influence the editorial decision, but if the article is accepted for publication, the editors will discuss with the authors the manner in which such information is to be communicated to the reader.

Submission of Manuscript

An original and three copies of the manuscript should be submitted to:

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part of its essential substance has been or will be published or submitted for publication elsewhere before appearing in the Journal.

All manuscripts should be accompanied by a letter of copyright transmittal. This must be signed and dated by all authors. The letter is required before any manuscript can be considered for publication and should contain the following wording:

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Titles and Author's Names

With the manuscript, provide a page giving the title of the article; titles should be concise and descriptive (not declarative). Also include a running head of fewer than 40 letter spaces; the name(s) of the author(s), including the first name(s) and academic degree(s); the name of the department and institution in which the work was done; the institutional affiliation of each author; and the name and address of the author to whom reprint requests should be addressed. Any grant support that requires acknowledgment should be mentioned on this page.

Abstract

Provide on a separate page an abstract of not more than 300 words (original and review articles) or 250 words (case report). This abstract should consist of four paragraphs, labeled Background, Methods, Results, and Conclusion. They should briefly describe the problem being addressed in the study, how the study was performed, the results, and what the authors conclude from the results.

Text

All material should be typed and double-spaced. Standard sequence of methods and materials, results, and discussion should be employed with tables and figures numbered in the order in which they are cited in the text. A disk in text format should accompany this.

Tables

Submit tables typed and double-spaced and provide a heading for all columns with a comprehensive title on separate sheets. A disk copy with a separate file for each table should be on the disk containing the text.

Illustrations

Photographs and figures should be submitted as glossy prints 5×7 in., with one copy of each print for each copy of the manuscript. Figure legends should be provided on a separate sheet with identification of the figure. The back of the glossy print should indicate the number of the figure.

References

References should be numbered in order of citation in the text, following the American Medical Association guidelines for references. The standard journal abbreviations from *Index Medicus* should be followed. Numbered references to personal communications, unpublished data, and manuscripts either "in preparation" or "submitted for publication" are unacceptable.

Drug Names

Generic names generally should be used. When proprietary brands are used in research, include the brand name in parentheses in the Methods section.

JERNIGAN Nutraceuticals

A New Option For A Multi-Modal Approach

Borrelogen™

By Jernigan Nutraceuticals

Research shows that Borrelogen (Bor-el-OH-jen) may be an effective complement for a multi-modal process in the care of patients with Borreliosis, Babesiosis, Ehrlichiosis and Leptospirosis. Borrelogen has been used by doctors and individuals around the world to aid in the recovery from spirochetal disorders with remarkable success. Individually or as a supplement to the protocol of treatment, many have seen that Borrelogen can most definitely be of benefit.

The following information is from a study conducted to determine if Borrelogen, an herbal formulation, is effective in providing positive Lyme Urine Antigen Tests. The LUAT (**L**yme **U**rine **A**ntigen **T**est) performed by IGeneX Reference Laboratories, utilizing a unique polyclonal antibody and a variation of the fluorescent ELISA has detected antigen in patients with early disease as well as those with persistent/recurrent/chronic disease.

During the period of this study, thirty-nine patients suffering from various chronic illnesses were selected through probable case history in combination with a positive clinical Bio-Resonance™ test. None of the participants had a previous diagnosis for any spirochetal illness. Each of the participants was given Borrelogen to be taken each day for one week prior to taking a three-day urine collection for the LUAT. The participants frozen urine was then submitted to IGeneX Reference Laboratories.

The laboratory reports only the highest score for each three-day series. In the participant group, the mean score was 154 ng/ml, median score of 125 ng/ml. with a standard deviation of 101 ng/ml. for the range of 33 ng/ml. to >400ng/ml. These participants had not taken an antibiotic of any form during the week before testing, nor had they any prior knowledge of possible infection with a spirochetal illness.

In Lyme Urine Antigen Testing it is necessary to kill some Borrelia burgdorferi bacteria so that the dead protein particles can be detected by combination of the antibody and florescent ELISA used by IGeneX. Normally, antibiotics are given to the patient for a week prior to the urine collection to increase the likelihood of a positive test. Reference laboratory standards have established that a positive LUAT is within the range of 32-45. Any score over 45 is considered highly positive. In the Borrelogen test group the majority of the results were above 100, and several >400 (which is the highest score possible).

Borrelogen was used instead of a prescription antibiotic with each of the participants. As you can see from the previously stated resulting test scores it is demonstrated that this herbal formula may be an effective alternative to antibiotics by effectively assisting the body in the elimination of the spirochetes, resulting in an increased probability of highly positive Lyme Urine Antigen Tests. Borrelogen has been verified by independent testing to not cause a false positive LUAT.

If you are interested in integrating Borrelogen into your clinical use, call a representative today for more information.

Please note:

These statements have not been evaluated by the FDA. This product is not intended to diagnose, treat, cure, or prevent any disease. Jernigan Nutraceuticals is not affiliated with the mentioned laboratory. Research was conducted in conjunction with the International Nutraceutical Research Group (INRGI).

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