



RESEARCH ARTICLE

**REVISED** Culture and identification of *Borrelia* spirochetes in human vaginal and seminal secretions [version 3; referees: 2 approved, 2 not approved]

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**Abstract**

**Background:** Recent reports indicate that more than 300,000 cases of Lyme disease are diagnosed yearly in the USA. Preliminary clinical, epidemiological and immunological studies suggest that infection with the Lyme disease spirochete *Borrelia burgdorferi* (Bb) could be transferred from person to person via intimate human contact without a tick vector. Failure to detect viable *Borrelia* spirochetes in vaginal and seminal secretions would argue against this hypothesis.

**Methods:** Patients with and without a history of Lyme disease were selected for the study after informed consent was obtained. Serological testing for Bb was performed on all subjects. Semen or vaginal secretions were inoculated into BSK-H medium and cultured for four weeks. Examination of genital cultures and culture concentrates for the presence of spirochetes was performed using light and darkfield microscopy, and spirochete concentrates were subjected to Dieterle silver staining, anti-Bb immunohistochemical staining, molecular hybridization and PCR analysis for further characterization. Immunohistochemical and molecular testing was performed in three independent laboratories in a blinded fashion. Positive and negative controls were included in all experiments.

**Results:** Control subjects who were asymptomatic and seronegative for Bb had no detectable spirochetes in genital secretions by PCR analysis. In contrast, spirochetes were observed in cultures of genital secretions from 11 of 13 subjects diagnosed with Lyme disease, and motile spirochetes were detected in genital culture concentrates from 12 of 13 Lyme disease patients using light and darkfield microscopy. Morphological features of spirochetes were confirmed by Dieterle silver staining and immunohistochemical staining of culture concentrates. Molecular hybridization and PCR testing confirmed that the spirochetes isolated from semen and vaginal secretions were strains of *Borrelia*, and all cultures were negative for treponemal spirochetes. PCR sequencing of cultured spirochetes from three couples having unprotected sex indicated that two couples had identical strains of Bb *sensu stricto* in their

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	Invited Referees			
	1	2	3	4
<b>REVISED</b>		✓	✗	✓
<b>version 3</b>		report	report	report
published 27 Apr 2015		↑	↑	
<b>REVISED</b>		✓	✗	
<b>version 2</b>		report	report	
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semen and vaginal secretions, while the third couple had identical strains of *B. hermsii* detected in their genital secretions.

**Conclusions:** The culture of viable *Borrelia* spirochetes in genital secretions suggests that Lyme disease could be transmitted by intimate contact from person to person. Further studies are needed to evaluate this hypothesis.

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**Competing interests:** The authors have no competing interests to declare. Preliminary results of the study were presented at the Western Regional Meeting of the American Federation for Medical Research, Carmel, CA, on January 25, 2014, and published in abstract form (*J Invest Med* 2014; 62: 280–1).

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**REVISED** Amendments from Version 2

We have addressed all of the latest referee comments. Please see the detailed response to those comments. In particular, we have added control data for the Dieterle silver staining and anti-Bb immunostaining. We have also provided details for the molecular hybridization testing and we have justified the variation in morphology in our samples with appropriate references from the medical literature. In addition, we have added a discussion of why our PCR results are not due to contamination. We wish to emphasize that our article demonstrates culture detection of live *Borrelia* spirochetes in genital secretions from Lyme disease patients using a combination of light and darkfield microscopy, silver staining, immunostaining, molecular hybridization and PCR techniques. Although any of these techniques could yield faulty results, the combination of all modalities performed in three independent laboratories provides substantial corroborative support for the findings of our study. We also wish to emphasize that our study does not claim to prove sexual transmission of Lyme disease. We are simply showing that *Borrelia* spirochetes can be cultured from genital secretions of Lyme disease patients. We hope that the referees will reconsider their negative reviews of the article after reading our detailed responses to their comments, in the true spirit of peer review.

See referee reports

## Introduction

Lyme disease is the most common human tick-borne disease in the world today (Stricker & Johnson, 2014). It is transmitted by *Ixodes* ticks and is caused by the spirochete *Borrelia burgdorferi* (Bb) (Burgdorfer *et al.*, 1982). Bb is phylogenetically related to the spirochetal agent of syphilis, *Treponema pallidum* (Gupta *et al.*, 2013). *T. pallidum* is transmitted sexually between partners through contact of mucosal membranes, gaining access to the bloodstream through microabrasions and then disseminating systemically (Ho & Lukehart, 2011; LaFond & Lukehart, 2006). The close phylogenetic relationship of Bb to *T. pallidum* suggests that this mode of transmission might be possible for Bb.

In addition to theoretical considerations, evidence for non-vector transmission of Bb is based on animal models. Proof of contact transmission of Bb – without involvement of an arthropod vector – was established by two studies in mice. Burgess *et al.* (1986) caged uninfected deer mice with experimentally-infected deer mice and demonstrated transmission of Bb by seroconversion of contact-exposed mice from negative to positive and by the isolation of Bb from the blood of one contact-exposed mouse 42 days after initial contact. A study by Wright & Nielsen (1990) demonstrated that white-footed mice were susceptible to oral infection and transmitted infection to each other through direct contact. Furthermore, sexual transmission of Bb has been proposed in a canine model. Bb was transmitted to uninfected female dogs in estrus via semen by natural breeding with male dogs infected experimentally with Bb (Gustafson, 1993). Successful transmission of infection from male dogs to female dogs was shown by seroconversion of female dogs from negative to positive as well as the detection of Bb DNA in the tissue of fetuses from resulting pregnancies. If contact transmission of Bb occurs in mice and sexual transfer occurs in dogs, it is not unreasonable to postulate similar routes of infection in humans.

We sought to determine if viable *Borrelia* spirochetes could be recovered from human vaginal and seminal secretions, an important first step to investigate whether sexual transmission of these spirochetes among humans is possible.

## Materials and methods

### 1. Research subject selection

Control subjects who were asymptomatic without a history of Lyme disease and patients with a history of Lyme disease were recruited for the study after written informed consent to collect and publish their data was obtained. Approval for sample collection was obtained from the Western Institutional Review Board, Olympia, WA (WIRB® #20141439). Further approval for sample testing was obtained from the Institutional Review Board of the University of New Haven, West Haven, CT. Serological testing of all participants after coding of their blood samples was performed by IGeneX Reference Laboratories, Palo Alto, CA in a blinded fashion.

Patients were considered positive for Lyme disease if they were serologically positive by CDC criteria and/or IGeneX criteria, as previously described (Engstrom *et al.*, 1995; Ma *et al.*, 1992), or if they had musculoskeletal, neurocognitive and/or cardiac symptoms clinically consistent with a Lyme disease diagnosis, as described elsewhere (Donta, 2014; Smith *et al.*, 2014). None of the patients were taking antibiotics at the time of testing.

### 2. *Borrelia* cultures

*Borrelia* spirochetes were cultured as previously described (Bankhead & Chaconas, 2007; Middelveen *et al.*, 2013b; Middelveen *et al.*, 2014a). The inoculum for blood culture was prepared as follows: 10 milliliters of whole blood was collected by sterile venipuncture from each patient. Samples sat at room temperature for 10 to 15 minutes allowing clotting to occur. Red blood cells (RBCs) were separated by low speed centrifugation. Barbour–Stoner–Kelly H (BSK-H) complete medium was used for cultures with the addition of 6% rabbit serum (Sigma Aldrich, #B8291) and the following antibiotics: phosphomycin (0.02 mg/ml), rifampicin (0.05 mg/ml), and amphotericin B (2.5 µg/ml) (Sigma Aldrich).

The culture medium described above was inoculated for blood culture with the spun serum containing white blood cells and some RBCs, and for genital culture with either ejaculated semen or vaginal secretions collected by intravaginal swabbing with a sterile cotton-tipped swab. Blood and genital cultures were incubated at 32°C in an Oxoid anaerobic jar (Thermo Scientific) containing an AnaeroGen sachet (Thermo Scientific) to provide an anaerobic environment. Cultures were incubated for four weeks and checked weekly by light and/or darkfield microscopy for visible motile spirochetes.

All cultures were processed for microscopic imaging and PCR by centrifuging the culture fluid at 15,000 g for 20 minutes to concentrate spirochetes. The supernatant was discarded and the pellet retained. The pellet samples were coded and processed in a blinded fashion for subsequent experiments.

### 3. Dieterle silver staining

Dieterle silver staining was performed using two fixation methods. In the standard method, formalin-fixed, paraffin-embedded pellets

were sectioned and stained with Dieterle silver stain as previously described (Aberer & Duray, 1991; Middelveen *et al.*, 2013a). In the newer method, culture fluid was spread and dried on a SuperFrost™ Plus microscope slide (Fisher Scientific) and fixed by incubating the slide in acetone for 10 minutes at -20°C, as previously described (Sapi *et al.*, 2013). Dieterle silver staining was performed on the acetone-fixed slide.

Positive and negative culture controls were prepared for comparison purposes with plasma from Bb-inoculated mice and uninfected mice followed by Dieterle silver staining using the standard method. Control cultures of mixed Gram-positive and mixed Gram-negative bacteria were also subjected to Dieterle staining. The control processing and staining was performed at McClain Laboratories LLC, Smithtown, NY.

#### 4. Anti-Bb immunostaining

**A. McClain Laboratories.** Blood and genital culture pellets from coded patient samples were processed in a blinded fashion for special staining at McClain Laboratories. Formalin-fixed, paraffin-embedded pellets were sectioned and stained with anti-Bb immunostain for spirochete detection, as previously described (Middelveen *et al.*, 2013a; Middelveen *et al.*, 2014a). In brief, immunostaining was performed using an unconjugated rabbit anti-Bb polyclonal antibody (Abcam ab20950), incubated with an alkaline phosphatase probe (Biocare Medical #UP536L), followed by a chromogen substrate (Biocare Medical #FR805CHC), and counterstained with hematoxylin. Positive and negative culture controls were prepared for comparison purposes with plasma from Bb-inoculated mice and uninfected mice followed by anti-Bb immunostaining. Culture pellets from fungal-infected human skin samples, mixed Gram-positive bacteria and mixed Gram-negative bacteria were also prepared for comparison purposes as negative anti-Bb immunostain controls to exclude cross-reactivity with commonly encountered microorganisms. Staining was titrated to determine optimal antibody dilutions to achieve positive staining of spirochetes while minimizing background staining (Middelveen *et al.*, 2013a; Middelveen *et al.*, 2014a).

**B. University of New Haven.** Coded samples were processed in a blinded fashion for Bb immunostaining as previously described (Sapi *et al.*, 2013). Culture fluid was spread and dried on a SuperFrost™ Plus microscope slide (Fisher Scientific) and fixed by incubating the slide in acetone for 10 minutes at -20°C. Dried, fixed culture fluid was submerged under 100 µl of polyclonal FITC-labeled rabbit anti-Bb antibody (Thermo Scientific #PA-1-73005) diluted 1:50 in 1× PBS buffer with 1% BSA (Sigma Aldrich #A9418). For negative controls, the antibody was omitted and replaced with normal rabbit serum. The slides were then incubated for 1 hour at 37°C in a humidified chamber, washed with 1× PBS for 5 minutes at room temperature, rinsed twice in double distilled water and dried in a laminar air-flow hood for 10 minutes. The slides were mounted with Vectashield mounting medium (Vector Labs) and viewed with fluorescent microscopy at 400× magnification with a Leica DM2500 microscope (Sapi *et al.*, 2013).

#### 5. Molecular hybridization using Bb DNA probe

The Bb molecular beacon DNA probe was generously provided by Dr. Alan MacDonald. Probe FlaB (sequence of 23 mer TGGGAGTT-TCTGGTAAGATTAAT) was derived from the Bb open reading frame

(ORF) BB0147 (approximately 1100 mer) of the flagellin B gene. A nucleotide Basic Local Alignment Search Tool (BLAST) search of the 23 mer sequence disclosed no matches in the human genome or in any other life form other than the Bb sequence of BB0147.

Bb detection with the molecular beacon was performed as previously described (Middelveen *et al.*, 2014a) on coded samples in a blinded fashion using the following protocol: paraffin sections were dewaxed by baking at 60°C, then immersed in serial 100% xylene baths followed by serial immersion through baths of 100% ethanol, 90% ethanol, 80% ethanol, and finally in distilled H<sub>2</sub>O, and then air-dried. Fixed sections were immersed in 20 µl of the working DNA beacon solution. The sectioned specimen was covered with a layer of plastic cut from a Ziploc® freezer bag and was heated at 90°C for 10 minutes to denature DNA and RNA. The heat was first reduced to 80°C for 10 minutes, then the slides were removed from heat and allowed to gradually cool to 24°C. The slides were washed in PBS, covered with 30% glycerol and a glass coverslip, then examined under an EPI Fluor microscope. Staining of test specimens was performed alongside staining of positive and negative controls. The positive control was prepared by embedding a known Bb strain in agarose, formalin-fixing the specimen then blocking in paraffin and staining sections as described above.

The specificity of the FlaB probe was validated in studies performed at the University of New Haven (Sapi *et al.*, unpublished observation 2014; see Supplemental Figure 1). The FlaB probe hybridized to *Bb sensu stricto*, yet failed to hybridize with *B. afzelii*, *B. garinii*, *B. hermsii*, *Treponema denticola* and *Escherichia coli*. Thus the probe appears to be specific for detection of *Bb sensu stricto*.

#### 6. PCR of cultures

Blood and genital culture pellets were first dissolved in 200 µl of Qiagen buffer, then forwarded to the University of New Haven, Department of Biology and Environmental Science, West Haven, CT, USA and Australian Biologics, Sydney, NSW, Australia for PCR detection of *Borrelia*. All control and patient samples were coded, and PCR testing was performed in a blinded fashion.

**A. Australian Biologics.** Detection of *Borrelia* by PCR was performed as previously described (Mayne *et al.*, 2012) using the Eco™ Real-Time PCR system with primers targeted to the genes encoding 16S rRNA (*Borrelia*), flA (*T. denticola*) and flIG1 (*T. pallidum*) and analyzed with the software version 3.0.16.0. DNA was extracted from the dissolved culture pellets using the QIAamp DNA Mini Kit (Qiagen) and 20 µl were used for each reaction. The thermal profile involved incubation for 2 minutes at 50°C, polymerase activation for 10 minutes at 95°C then PCR cycling for 40 cycles of 10 seconds at 95°C dropping to 60°C sustained for 45 seconds. All samples were run in duplicate with positive and negative controls. Positive controls were genomic DNA samples from *B. burgdorferi*, *B. garinii*, and *B. afzelii* (Amplirun DNA/RNA amplification controls, Vircell S.L, Granada, Spain). Negative controls were samples of non-template DNA in molecular-grade water. The magnitude of the PCR signal generated ( $\Delta R$ ) for each sample was interpreted as positive or negative compared to positive and negative controls.

In samples with sufficient DNA for sequencing, endpoint PCR amplification and Sanger sequencing of the *Borrelia* gene target

from cultures was followed by BLAST comparison with known *Borrelia* sequences, as previously described (Mayne *et al.*, 2012).

**B. University of New Haven.** DNA samples were extracted from blood, vaginal or seminal cultures by lysing cells overnight in 180 µl tissue lysis buffer (Qiagen) and 20 µl Proteinase K (Qiagen) at 56°C in a shaking water bath followed by phenol:chloroform extraction the next day. The DNA was resuspended in 50–100 µl 1×TE buffer.

A published TaqMan assay targeting a 139-bp fragment of the gene encoding the *Borrelia* 16S rRNA was used for the detection of *Borrelia* in DNA extracted from patient samples (O'Rourke *et al.*, 2013). All reactions were carried out at a final volume of 20 µl and consisted of 900 nM of each primer, 200 nM of probe, and 10 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 nanogram of DNA. Amplifications were carried out on a CFX96 Real-Time System (Bio-Rad), and cycling conditions consisted of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescent signals were recorded with CFX96 Real-Time software and Cq threshold was set automatically. The reactions were performed in triplicate with positive and negative controls.

Nested PCR primers for the genes encoding the *Borrelia* 16S rRNA, *fla* and *pyrG* loci were used as previously described (Clark *et al.*, 2013; Margos *et al.*, 2010; Sapi *et al.*, 2013). Reactions were carried out in a final volume of 50 µl using 10 µl template DNA. Final concentrations were 2× Buffer B (Promega), 2 mM MgCl<sub>2</sub>, 0.4 mM dNTP mix, 2 µM of each primer, and 2.5 U Taq polymerase (Invitrogen). “Outer” primers were used in the first reaction. “Inner” primers were used for the nested reaction, in which 1 µl of PCR product from the first reaction was used as template for the second. Cycling parameters were as follows: 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, annealing for 1 minute (temperature based on the primer set used), and extension at 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. PCR products were visualized on 1–2% agarose gels. Sanger sequencing was used for gene analysis, as previously described (Margos *et al.*, 2010).

## Results

### 1. Patient data

All patient data are shown in Table 1. The control group included four asymptomatic patients (two males and two females). All four were seronegative for Bb.

The patient group included six male subjects and seven female subjects, including four pairs of partners (Patients 6 and 7, 8 and 9, 10 and 11, and 12 and 13, respectively). Eleven of the 13 patients selected for the study were serologically positive for Lyme disease. Patient 1 was serologically equivocal and patient 8 was seronegative, although Bb plasmid DNA was detected in whole blood and serum from this patient.

### 2. Light and darkfield microscopy

Blood cultures from 11 patients were incubated for four weeks and checked weekly for spirochete growth using light and dark-field microscopy. Motile spirochetes and/or motile spherules were

observed in the culture fluid from all 11 patients after four weeks (Table 2). Genital cultures from the four controls were incubated for four weeks. None of the control cultures contained visible

**Table 1. Patient Data.** Patients 6 & 7 (\*), 8 & 9 (\*\*), 10 & 11 (†), and 12 & 13 (††) are sexual partners. Patients 8 and 11 were seronegative but clinically diagnosed with Lyme disease.

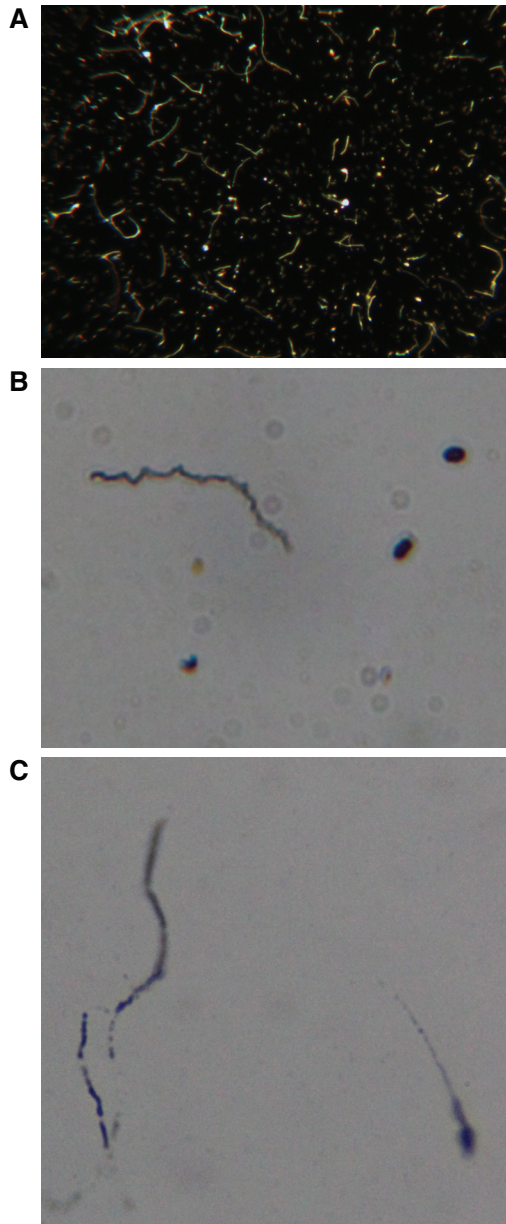
Control	Sex	Age	Serology
1 (M)	male	63	negative
2 (M)	male	53	negative
3 (F)	female	58	negative
4 (F)	female	43	negative
Patient	Sex	Age	Serology
1 (F)	female	56	equivocal
2 (M)	male	45	positive
3 (M)	male	35	positive
4 (F)	female	66	positive
5 (F)	female	27	positive
6 (M)*	male	63	positive
7 (F)*	female	53	positive
8 (M)**	male	42	negative
9 (F)**	female	40	positive
10 (M)†	male	56	positive
11 (F)†	female	54	negative
12 (M)††	male	65	positive
13 (F)††	female	54	positive

**Table 2. Microscopy results from fresh blood and genital culture fluid.** See Dataset, data file 1. ND, not done.

Patient number	Microscopy – fresh blood culture fluid	Microscopy – fresh genital culture fluid
1 (F)	motile spherules	vaginal – motile spirochetes
2 (M)	ND	seminal – ND
3 (M)	ND	seminal – ND
4 (F)	motile spherules	vaginal – motile spherules and spirochetes
5 (F)	motile spherules	vaginal – motile spirochetes, some yeast cells
6 (M)	motile spirochetes and spherules	seminal – motile spirochetes
7 (F)	motile spirochetes and spherules	vaginal – motile spherules and spirochetes
8 (M)	motile spherules	seminal – motile spirochetes
9 (F)	motile spherules	vaginal – motile spirochetes
10 (M)	motile spherules	seminal – motile spherules/spirochetes
11 (F)	motile spherules	vaginal – motile spherules/spirochetes
12 (M)	motile spherules	seminal – motile spherules/spirochetes
13 (F)	motile spherules	vaginal – motile spherules/spirochetes



spirochetes, and the cultures were sent for PCR testing. Genital cultures from the 11 patients were incubated for four weeks and checked weekly. Motile spirochetes were observed in the culture fluid from all 11 patients after four weeks (Figure 1A). See Dataset, data file 1.



**Figure 1.** **A:** Darkfield image of genital culture from Patient 1. Note numerous spirochetes. 400× magnification. See Dataset, data file 1. **B:** Diaterle silver stain of genital culture from Patient 12. Note darkly staining spirochete. Formalin fixed slide, 400× magnification. See Dataset, data file 2. **C:** Semen sample from Patient 10 showing *B. burgdorferi* spirochetes (left) and sperm cell (right). Diaterle silver stain of acetone fixed slide, 1000× magnification. See Dataset, data file 2.

Most genital cultures grew very well and contained abundant spirochetes, but some blood cultures contained few spirochetes. Therefore, to better document the presence of spirochetes in culture, the culture fluid was concentrated into pellets by centrifugation (Table 3). Spirochetes and/or spherules were detected by sectioning and special staining of paraffin blocked pellets in all the patient blood and genital cultures concentrated by centrifugation, except for blood and genital culture pellets from Patient 1 that were lost during paraffin blocking (Table 3). Control genital culture samples were sent directly for PCR testing and were not subjected to light and darkfield microscopy.

### 3. Immunohistochemistry

**A. Diaterle silver staining.** The culture samples of uninfected mouse plasma, mixed Gram-positive bacteria and mixed Gram-negative bacteria failed to stain with Diaterle silver stain using the standard staining method. In contrast, the culture sample of Bb-infected mouse plasma stained positive for spirochetes with Diaterle silver stain (Dataset, data file 2A).

Using standard Diaterle staining, spherules and/or spirochetal forms were visible in all patient genital cultures (Figure 1B). Spirochetes were detected in all patient genital culture pellets except for Patient 1, whose pellet was lost during processing (Table 3). Using

**Table 3. Microscopy results and Diaterle silver staining of genital culture concentrates.** See Dataset, data file 2.

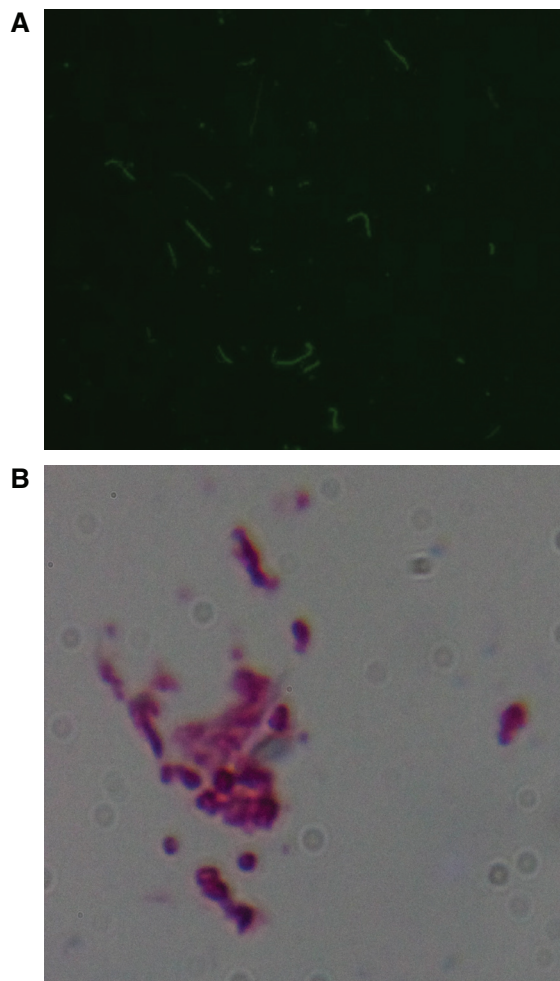
Patient number	Microscopy – genital culture pellet	Diaterle silver stain – genital culture pellet
1 (F)	pellet lost	pellet lost
2 (M)	seminal – spherules/spirochetes	seminal – spherules/spirochetes
3 (M)	seminal – spirochetes	seminal – spherules/spirochetes
4 (F)	vaginal – spirochetes	vaginal – spherules
5 (F)	vaginal – spirochetes, some yeast cells	vaginal – spherules
6 (M)	seminal – spirochetes	seminal – spherules/spirochetes
7 (F)	vaginal – spirochetes	vaginal – spherules/spirochetes
8 (M)	seminal – spirochetes	seminal – spherules/spirochetes
9 (F)	vaginal – spherules/spirochetes	vaginal – spherules
10 (M)	seminal – spirochetes	seminal – spherules/spirochetes
11 (F)	vaginal – spirochetes	vaginal – spherules/spirochetes
12 (M)	seminal – spirochetes	seminal – spherules/spirochetes
13 (F)	vaginal – spirochetes	vaginal – spherules/spirochetes

the newer fixation method, spirochetes and sperm cells were visible in semen samples and showed distinct morphology (Figure 1C). Sperm cells are known to stain with silver stains (Pathak *et al.*, 1979; Schmid *et al.*, 1983). Sperm cells were seen in all semen samples except for Patients 2 and 6, who had vasectomies (data not shown). Since control genital cultures had no visible spirochetes, the control samples were sent directly for PCR testing and were not subjected to Dieterle silver staining. See Dataset, data file 2.

### B. Anti-Bb immunostaining.

#### I. Culture fluid – University of New Haven

Genital culture fluid from Patient 1 was fixed on a SuperFrost™ Plus microscope slide and was stained with FITC-labelled polyclonal anti-Bb antibody. Staining was strongly positive, revealing well-defined spirochetes morphologically consistent with Bb (Figure 2A). The polyclonal antibody was not reactive to *T. denticola* (data not shown).



**Figure 2. A:** *B. burgdorferi* immunostaining of vaginal culture from Patient 1. Note intensely staining spiral and round forms in culture. 400× magnification. **B:** *B. burgdorferi* immunostaining of seminal culture from Patient 6. Note intensely staining spiral and round forms in culture. 400× magnification. See Dataset, data file 3.

#### II. Culture pellets – McClain laboratories

The culture sample of uninfected mouse plasma failed to stain with anti-Bb immunostain. In contrast, the culture sample of Bb-infected mouse plasma stained positive for spirochetes with anti-Bb immunostain (Dataset, data file 3A). Control fungalinfected human skin cultures, Gram-positive bacterial cultures and Gram-negative bacterial cultures all failed to stain for spirochetes with the anti-Bb immunostain (Dataset, data file 3A).

Anti-Bb immunostaining was positive for all genital cultures except for Patient 1, whose pellet was lost during processing (Table 4). Immunostaining revealed both spiral and globular Bb forms (Figure 2B). Since control genital cultures had no visible spirochetes, the control samples were sent directly for PCR testing and were not subjected to immunostaining. See Dataset, data file 3.

#### 4. Molecular hybridization

Hybridization with the Fla B probe was positive for genital culture pellets from Patients 2–9 (Table 4). The culture pellet from Patient 1 was lost during processing. The molecular probe showed intense staining in vaginal secretions and less intense staining in semen samples (Figure 3A and 3B). See Dataset, data file 4.

**Table 4. Results of *B. burgdorferi* immunostaining and FlaB molecular hybridization in genital culture concentrates.**

See Dataset, data files 3 and 4. ND, not done.

Patient number	Bb immunostaining – genital culture pellet	FlaB hybridization – genital culture pellet
1 (F)	pellet lost*	pellet lost
2 (M)	seminal – positive	seminal – positive
3 (M)	seminal – positive	seminal – positive
4 (F)	vaginal – positive	vaginal – positive
5 (F)	vaginal – positive	vaginal – positive
6 (M)	seminal – positive	seminal – positive
7 (F)	vaginal – positive	vaginal – positive
8 (M)	seminal – positive	seminal – positive
9 (F)	vaginal – positive	vaginal – positive
10 (M)	seminal – positive	ND
11 (F)	vaginal – positive	ND
12 (M)	seminal – positive	ND
13 (F)	vaginal – positive	ND

\*Positive Bb immunostaining of genital culture fluid. See Results section.

## 5. PCR testing

**A. Australian Biologics.** *Borrelia* 16S rRNA sequence was not detected by real-time PCR in any of the control genital culture pellets. In contrast, *Borrelia* 16S rRNA sequence was detected in genital culture pellets from 11 of 13 patients (Table 5A). Patient 2 had equivocal test results and Patient 3 had negative test results in seminal cultures. See Dataset, data file 5. Real-time PCR failed to detect treponemal gene sequences in any of the control or patient genital culture pellets. See Dataset, data file 5a. The 16S rRNA isolates from six patients were sequenced and subjected to BLAST analysis (see below).

**B. University of New Haven.** PCR testing using the TaqMan assay for *Borrelia* 16S rRNA sequence was positive in blood culture pellets from seven of nine patients tested (Table 5B). Patients 1 and 5 had negative results in blood culture pellets using the TaqMan assay, but both were positive by nested PCR for the *pyrG* gene. In addition, nested PCR targeting the *fla* gene was performed on blood culture pellets from Patients 2, 3 and 4, and nested PCR targeting the 16S rRNA gene was performed on the blood culture pellet from Patient 6. The samples were positive, and sequencing revealed

99–100% homology with *Bb sensu stricto* strain B-31 (Table 5B). See Dataset, data file 7.

PCR testing using the TaqMan assay for *Borrelia* 16S rRNA sequence was negative in all four control genital culture pellets, and nested PCR targeting the *pyrG* and *fla* genes was negative in all four control samples, confirming the results of the TaqMan assay (Table 5B). In contrast, eight of nine patients were positive for TaqMan 16S rRNA sequence in the genital culture pellets. Patient 6 was negative using the TaqMan assay for 16S rRNA sequence but positive using nested PCR targeting a different portion of the 16S rRNA gene (Table 5B). Nested PCR targeting the *fla* gene (Patient 3) and the 16S rRNA gene (Patients 3 and 7) was also performed on genital culture pellets and was positive in those patients, confirming the results of the TaqMan assay. Patient 12 had positive PCR targeting the *pyrG* gene with confirmatory sequencing (see below).

## 6. Sequencing of *Borrelia* detected in blood and genital cultures

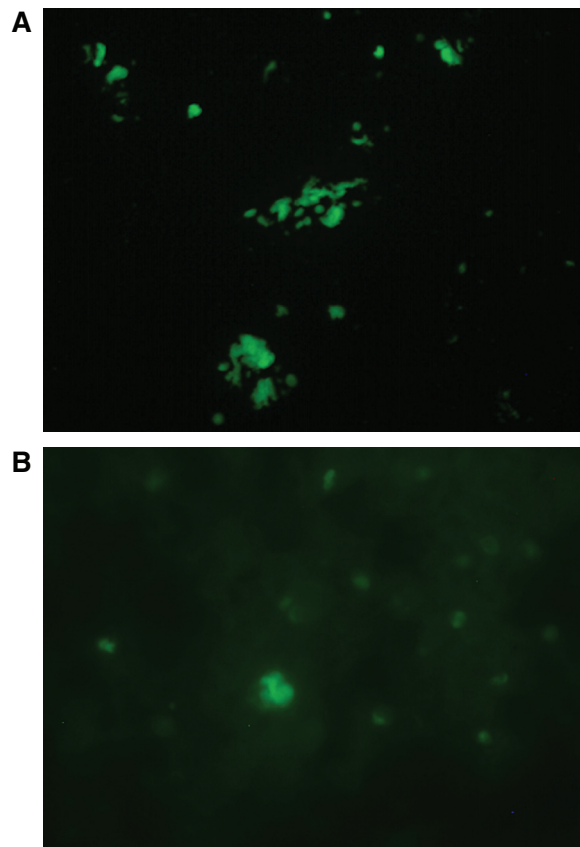
PCR isolates of the vaginal culture from Patient 1 (Australian Biologics) and the seminal culture from Patient 3 (University of New Haven) were subjected to Sanger sequencing and BLAST analysis and showed 97–99% homology with *Bb sensu stricto* strain B-31 (Table 5A and Table 5B). See Datasets, data files 6 and 7. PCR isolates of blood cultures from Patients 2, 3, 4 and 6 were subjected to Sanger sequencing and BLAST analysis at University of New Haven and showed 99–100% homology with *Bb sensu stricto* strain B-31 (Table 5B). See Dataset, data file 7.

PCR isolates of genital cultures from three couples having unprotected sex (Patients 6–7, 10–11 and 12–13) were subjected to Sanger sequencing and BLAST analysis. Patients 6, 7, 10, 11 and 13 had sequencing done at Australian Biologics, while Patient 12 had sequencing done at University of New Haven. Sequencing revealed that the first and third couples had *Borrelia* strains that matched *Bb sensu stricto* strain B-31 (Table 6). In contrast, the second couple had PCR sequences that matched *B. hermsii* strain YOR. Thus the *Borrelia* strain shared by this couple differed significantly from the strains identified in the other couples. See Dataset, data file 8.

## Discussion

In this study using standard and published culture, immunohistochemical, molecular hybridization and PCR techniques, we have shown that *Borrelia* strains are present in semen and vaginal secretions from patients with Lyme disease. Simultaneous testing for treponemal spirochetes was negative in genital secretions of all Lyme disease patients, confirming the specificity of *Borrelia* detection in these patients. Furthermore we have shown that couples having unprotected sex have virtually identical strains of *Borrelia* in their genital secretions, suggesting that *Borrelia* spirochetes might be transmitted from person to person without a tick vector.

As expected, PCR sequencing of cultured *Borrelia* from semen and vaginal secretions yielded primarily *Bb sensu stricto* strains, reflecting the North American origin of our study subjects. In addition, PCR sequencing of genital secretions from one couple yielded identical strains of *Bb sensu stricto* strains in two different laboratories. However, we were surprised to find one couple with



**Figure 3.** **A:** Molecular hybridization of *B. burgdorferi*-specific FlaB probe with seminal culture from Patient 6. Note intensely staining spiral and round forms in culture. 400× magnification. **B:** Molecular hybridization of *B. burgdorferi*-specific FlaB probe with vaginal culture from Patient 7. Note intensely staining spiral and round forms in culture. 400× magnification. See Dataset, data file 4.



**Table 5. A: Real time PCR testing of genital culture concentrates performed by Australian Biologics. ND, not done. See Dataset, data file 5. B: Real time and nested PCR testing of blood and genital culture concentrates performed by University of New Haven. See Dataset, data file 7. ND, not done.**

**Table 5A: Real-time PCR – Australian Biologics.**

Control number	Genital culture – Real-time <i>Borrelia</i> PCR	Genital culture – Real-time <i>T. pallidum</i> PCR	Genital culture – Real-time <i>T. denticola</i> PCR
1 (M) seminal	Negative	Negative	Negative
2 (M) seminal	Negative	Negative	Negative
3 (F) vaginal	Negative	Negative	Negative
4 (F) vaginal	Negative	Negative	Negative
Patient # sample	Genital culture – Real-time <i>Borrelia</i> PCR	Genital culture – Real-time <i>T. pallidum</i> PCR	Genital culture – Real-time <i>T. denticola</i> PCR
1 (F) vaginal	positive (sequenced 99% match B-31)	negative	negative
2 (M) seminal	equivocal	negative	negative
3 (M) seminal	negative	negative	negative
4 (F) vaginal	positive	negative	negative
5 (F) vaginal	positive	negative	negative
6 (M) seminal	positive (sequenced 100% match B-31)	negative	negative
7 (F) vaginal	positive (sequenced 98% match B-31)	negative	negative
8 (M) seminal	positive	negative	negative
9 (F) vaginal	positive	negative	negative
10 (M) seminal	positive (sequenced 100% match YOR)	ND	ND
11 (F) vaginal	positive (sequenced 100% match YOR)	ND	ND
12 (M) seminal	positive	ND	ND
13 (F) vaginal	positive (sequenced 100% match B-31)	ND	ND

**Table 5B: PCR – University of New Haven.**

Control number	Blood culture	Genital culture – 16S rRNA Taq Man PCR	Genital culture – Nested PCR
1 (M)	ND	Negative	pyrG negative, fla negative
2 (M)	ND	Negative	pyrG negative, fla negative
3 (F)	ND	Negative	pyrG negative, fla negative
4 (F)	ND	Negative	pyrG negative, fla negative
Patient number	Blood culture – primers with positive detection	Genital culture – primers with positive detection	
1 (F)	pyrG	16S rRNA Taq Man	
2 (M)	16S rRNA Taq Man, fla (sequenced, 100% match B-31)	16S rRNA Taq Man	
3 (M)	16S rRNA Taq Man, fla (sequenced, 100% match B-31)	16S rRNA Taq Man, fla, 16S rRNA (sequenced, 99% match B-31)	
4 (F)	16S rRNA Taq Man, fla (sequenced, 99% match B-31)	16S rRNA Taq Man	
5 (F)	pyrG	16S rRNA Taq Man	
6 (M)	16S rRNA Taq Man 16S rRNA (sequenced, 99% match B31)	16S rRNA	
7 (F)	16S rRNA Taq Man, pyrG	16S rRNA Taq Man, 16S rRNA, fla	
8 (M)	16S rRNA Taq Man	16S rRNA Taq Man	
9 (F)	16S rRNA Taq Man	16S rRNA Taq Man	
10 (M)	ND	ND	
11 (F)	ND	ND	
12 (M)	ND	pyrG (sequenced, 99% match B-31)	
13 (F)	ND	ND	

**Table 6. Comparison of seminal and vaginal *Borrelia* gene sequences using BLAST analysis.**

Sequencing for Patients 6, 7, 10, 11 and 13 was done at Australian Biologics. Sequencing for Patient 12 was done at University of New Haven. See [Dataset](#), data file 6.

Patient	Description	Maximum Score	Total Score	Query Cover	E Value	Reference Strain Match
6 (M)	Bb sensu stricto (B31)	230	230	84%	3e-57	100%
7 (F)	Bb sensu stricto (B31)	224	224	83%	2e-55	98%
10 (M)	B. hermsii (YOR)	32.2	1229	75%	1.5	100%
11 (F)	B. hermsii (YOR)	30.2	599	84%	2.1	100%
12 (M)	Bb sensu stricto (B31)	1218	1218	95%	1e-63	99%
13 (F)	Bb sensu stricto (B31)	97.6	4880	87%	1e-20	100%

**Dataset 1. Updated data of *Borrelia* spirochetes in human vaginal and seminal secretions**

<http://dx.doi.org/10.5256/f1000research.5778.d46058>

The dataset contains data files 1, 2, 2a, 3, 3a, 4, 5, 5a, 6 and 7. Detailed legends for each files can be found in the text file provided.

identical strains of *B. hermsii* in their genital secretions. The presence of a distinct *Borrelia* strain in semen and vaginal secretions from a sexually active couple that differs from strains found in other couples supports the premise of *Borrelia* transmission via shared genital secretions. The finding is analogous to sharing distinct human immunodeficiency virus (HIV) strains, which is well recognized in sexual partners with HIV/AIDS (Shaw & Hunter, 2012).

Animal models have provided compelling evidence for contact transmission of Bb without a tick vector in mice, ducks, cats and dogs (Burgess *et al.*, 1986; Burgess & Patrican, 1987; Burgess, 1989; Burgess, 1992; Wright & Neilsen, 1990). Bb has been shown to survive in stored semen from dogs, rams and bulls (Kumi-Diaka & Harris, 1995). Furthermore, seminal transmission of Bb has been noted in dogs, as described above (Gustafson, 1993). In contrast, contact transmission of Bb could not be demonstrated in Lewis rats and Syrian golden hamsters (Moody & Barthold, 1991; Woodrum & Oliver, 1999). Technical limitations in the study of these highly inbred rodents including limited contact between animals and failure to perform molecular testing may have contributed to the negative results.

While it is not possible to perform controlled sexual transmission studies of *Borrelia* in humans, several investigators have speculated that this mode of transmission is possible (Bach, 2001; Harvey & Salvato, 2003; Stricker *et al.*, 2004). The suggestion that Bb could be transmitted sexually was initially proposed by Bach in 2001. He observed that sexually active patients had a marked propensity for antibiotic failure and speculated that re-infection occurred by intimate person-to-person contact. Bb DNA was detected by PCR technology in human breast milk, umbilical cord blood, semen and vaginal secretions taken from patients presenting at his practice (Bach, 2001).

The study of a group of chronically ill Bb-seropositive and PCR-positive patients in Houston, Texas – a non-endemic area – provided epidemiological evidence that Lyme disease could spread in the absence of a suitable vector (Harvey & Salvato, 2003). In the absence of infected ticks, intimate person-to-person transfer was implicated as the probable means of transmission (Harvey & Salvato, 2003). A study by Stricker *et al.* provided clinical and immunological evidence for Bb transmission from partner to partner. In heterosexual seropositive couples with Lyme disease in which only one partner had a documented tick bite, the partner with the documented tick bite tended to have more severe clinical manifestations of the disease and a lower CD57 natural killer (NK) cell level (Stricker *et al.*, 2004). This difference in clinical severity and CD57 NK cell level was not noted in seropositive couples diagnosed with Lyme disease in which both partners had a documented

history of tick bite (Stricker *et al.*, 2004). Sexual transfer of *Borrelia* infection through mucosal contact therefore seems possible in humans. The fact that we have been able to culture motile, actively reproducing, viable spirochetes from human genital secretions supports this hypothesis.

Recent reports from the Centers for Disease Control and Prevention (CDC) indicate that more than 300,000 cases of Lyme disease are diagnosed yearly in the USA (CDC, 2013). Sexual transmission of *Borrelia* may partly explain the large number of annual cases that is almost two times higher than breast cancer and six times higher than HIV/AIDS (Stricker & Johnson, 2014). Recognition of possible sexual transmission of *Borrelia* in both humans and animals is fundamentally important because of the epidemiological implications. If sexual transmission of *Borrelia* occurs in both animals and humans, this mode of transmission is a possible means of introducing *Borrelia* infection into areas not considered endemic and of introducing the spirochete to new reservoirs. *Borrelia* would also join the list of other spirochetes that are either proven or postulated to be sexually transmitted, including the spirochetal agents of syphilis and leptospirosis (Harrison & Fitzgerald, 1988; Maatouk & Moutran, 2014). Of note, sexual transmission of other tickborne agents in animals and humans has also been proven or postulated (Facco *et al.*, 1992; Kruszezwska & Tylewska-Wierzbanowska, 1993; Metcalf, 2001; Miceli *et al.*, 2010; Milazzo *et al.*, 2001).

The number of spirochetes needed to infect an animal or human varies according to strain-specific biological and transmission factors. In mouse studies of experimental *Borrelia* infection, the 50% infectious dose was 18 spirochetes with tick salivary gland extract and 251 spirochetes with tick midgut extract (Cook, 2014). Transmission studies of syphilis using “human volunteers” found that the 50% infectious dose was approximately 57 organisms (LaFond & Lukehart, 2006). At present, the spirochetal load in genital secretions from Lyme disease patients is unknown, but it appears that genital infection could be induced by a relatively small number of organisms based on the studies outlined above. It is known that seminal plasma inhibits the immune response to Gram-negative pathogens (Brooks *et al.*, 1981), while the female genital tract induces immune factors that may be conducive to spirochete survival (Clark & Schust, 2013; Wira *et al.*, 2005). The role of the male and female genital tracts in tolerance and propagation of *Borrelia* infection merits further study.

Lyme disease diagnosis is based largely upon serological testing using CDC-sanctioned two-tier surveillance criteria supported by FDA-approved commercial test kits. While most patients in this study did have positive serological test results for Lyme borreliosis, some were considered serologically negative, and the majority of our study subjects did not meet the positive standard as defined by the CDC surveillance criteria (CDC, 2014a). We were able to detect *Borrelia* spirochetes in the blood and/or genital secretions of all patients who were clinically diagnosed with Lyme disease, demonstrating that the CDC surveillance protocol is inadequate diagnostically. Inadequate diagnostic methodology undoubtedly results in under-reporting of Lyme disease, and at least one group has speculated that this substandard methodology is considered

acceptable because *Borrelia* is not sexually transmitted (Lange & Sayyedi, 2002). In addition, if *Borrelia* spirochetes were transmitted sexually, then patients with false-negative results may unknowingly spread the infection to sexual partners.

The 2011 CDC case definition for Lyme disease states that a positive Bb culture confirms the diagnosis of the disease (CDC, 2014b). Although culture of *Borrelia* genital isolates may be a useful diagnostic laboratory methodology in the future, detecting and characterizing cultured *Borrelia* isolates is not straightforward, and both false-positive and false-negative results could occur. In our experience, human clinical isolates from genital secretions frequently propagate prolifically in culture, but on occasion they do not. In such instances, the culture must be concentrated and specific staining should be conducted to ascertain the presence of spirochetes. Once detected, spirochetes must be characterized genetically for specific identification. PCR is currently the most reliable means for correctly identifying cultured isolates, but even this methodology has drawbacks and limitations (Lange & Sayyedi, 2002; Nolte, 2012).

There are currently no standardized FDA-approved PCR protocols or kits available for Bb detection, so commercial PCR testing constitutes an array of “home brew” assays using different methodologies such as real-time PCR and nested PCR, with various primers targeting different genes, yielding wide differences in sensitivity and specificity (Nolte, 2012; Schmidt, 1997; Yang *et al.*, 2012). False negatives can result because primers may be strain-specific and may not detect all *Borrelia* genotypes, and fluids such as blood, semen and vaginal secretions may contain substances inhibitory to the PCR process (Lange & Sayyedi, 2002; Nolte, 2012; Yang *et al.*, 2012). The potential for false-positive PCR testing may also arise if there is DNA contamination in the laboratory, and appropriate positive and negative controls must be included in the assay (Lange & Sayyedi, 2002; Nolte, 2012). We experienced differences in primer specificity in our clinical isolates and also found that inhibition occurred, particularly in semen cultures.

Another complicating factor in *Borrelia* isolation is the morphological variation of the spirochete, which includes spherical, granular or cystic forms. Morphological variants of Bb, some of which are not culturable, are well documented in the medical literature (Barthold *et al.*, 2010; Hodzic *et al.*, 2014; Kurtti *et al.*, 1987; MacDonald, 2013; Meriläinen *et al.*, 2015; Mursic *et al.*, 1996). These variants may play a role in infection, enabling Bb and other pathogenic spirochetes to evade the immune system (Döpfer *et al.*, 2012; Menten-Dedoyart *et al.*, 2012; Mursic *et al.*, 1996). Limited Bb growth and non-spiral morphology are thought to be induced by unfavorable environmental conditions (Brorson *et al.*, 2009), and these features appear to be consistent with our observations. We found that *Borrelia* growth was more vigorous with more long slender morphological variants in cultures of genital secretions compared to cultures of blood, and we speculate that the human circulatory system is a more hostile environment for *Borrelia* than the human reproductive system.

The possibility of *Borrelia* contamination yielding false-positive PCR results in blood cultures from Lyme disease patients has been

suggested (Sapi *et al.*, 2013). This possibility is highly unlikely in our cultures of genital secretions for the following reasons: first, no reference strains of *Borrelia* that could cause contamination were present in the laboratory where cultures were performed. Second, the sequenced *Borrelia* strains were not 100% identical to the reference strains of *Borrelia*, implying that they were distinct from potentially contaminating reference strains. Third, testing was performed in three independent laboratories, and it would be highly unlikely to have contamination in all three locations. Fourth, negative controls were run with the molecular samples in the three independent laboratories, and the controls were consistently negative. Fifth, as noted above, one couple had a distinct strain of *Borrelia* in their genital secretions, so that selective contamination with two different reference strains would have had to occur in the PCR samples. Thus laboratory contamination yielding false-positive PCR results for *Borrelia* strains in the genital secretions is highly unlikely.

Several questions have been raised about the likelihood of *Borrelia* sexual transmission (Craig, 2014). First, according to the CDC surveillance system Lyme disease occurs most commonly in children and older adults. However, the CDC surveillance system only captures about 10% of Lyme disease patients, and the other 90% may have a different demographic distribution consistent with sexual transmission, as shown in a recent study from Australia (Mayne, 2015). A study from military treatment facilities in the USA “unexpectedly” found no association between the incidence of Lyme disease and the prevalence of infected ticks, and the rate of Lyme disease was 2.6 times higher in officers than enlisted men (Rossi *et al.*, 2015). Second, while sexually transmitted diseases like herpes simplex virus (HSV) and gonorrhea show an urban predominance, Lyme disease has a more rural distribution (Craig, 2014). However, Lyme disease is acquired in more ways than HSV and gonorrhea, and the rate of sexual transmission is unknown at present. Thus the epidemiology of Lyme disease may differ from other sexually transmitted diseases based on these undefined variables. Third, the transmission of HIV can be traced from one sex partner to another using HIV strain typing. Based on our study, a similar transmission pattern using *Borrelia* strain typing may be seen once larger studies are performed among couples having unprotected sex. In summary, sexual transmission of *Borrelia* is plausible in light of our limited knowledge about the risk of acquiring Lyme disease.

In conclusion, we have shown that *Borrelia* spirochetes are present in semen and vaginal secretions of patients with Lyme disease. Furthermore, virtually identical strains of *Borrelia* are present in couples having unprotected sex, suggesting that transmission via intimate contact without a tick vector may occur. The epidemiology and clinical risk of *Borrelia* sexual transmission remain to be determined.

#### Data availability

F1000Research: Dataset 1. Updated data of *Borrelia* spirochetes in human vaginal and seminal secretions., [10.5256/f1000research.5778.d46058](https://doi.org/10.5256/f1000research.5778.d46058) (Middelveen *et al.*, 2015).

#### Consent

Written informed consent to publish clinical details and study results was obtained from each participant.



### Author contributions

MJM recruited patients, performed the spirochete cultures and wrote the original manuscript. CB, KRF, AT and ES performed the IFA and PCR studies. JB, YW and AF performed the PCR studies. HAS and PJM provided patient samples and edited the manuscript. RBS recruited patients, coordinated all studies, revised the manuscript and edited it for publication. All authors approved the manuscript for publication.

### Competing interests

The authors have no competing interests to declare. Preliminary results of the study were presented at the Western Regional Meeting of the American Federation for Medical Research, Carmel, CA, on January 25, 2014, and published in abstract form (*J Invest Med* 2014; 62: 280–1).

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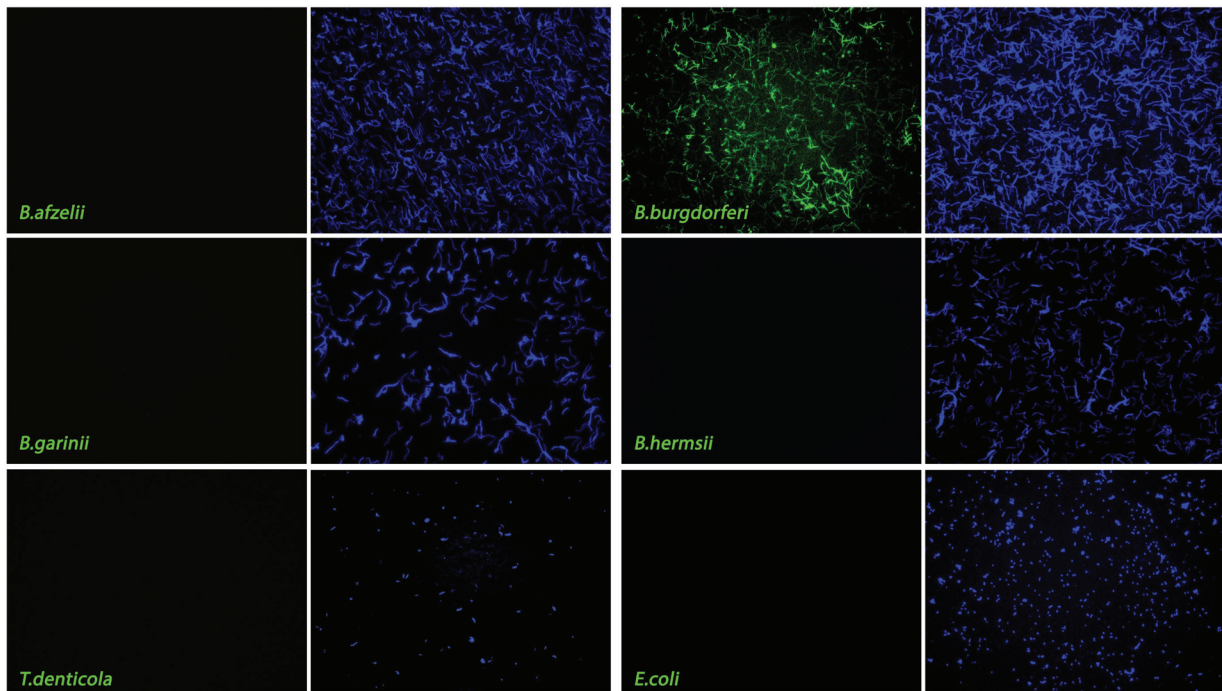
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## Supplementary material



**Supplemental Figure 1.** Molecular hybridization of FlaB probe with *Borrelia* strains, *T. denticola* and *E. coli*. FlaB hybridization is shown in green, while DAPI counterstain of bacterial targets is shown in blue. Note specific hybridization of FlaB probe with *B. burgdorferi sensu stricto* and lack of hybridization with other *Borrelia* strains, *T. denticola* or *E. coli*. 400x magnification. See [Dataset](#), data file 4.

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# Open Peer Review

Current Referee Status:    

Version 3

Referee Report 16 June 2017

doi:10.5256/f1000research.6856.r23250



**Nataliia Rudenko** , **Maryna Golovchenko**

Institute of Parasitology, Biology Centre Czech Academy of Sciences, České Budějovice, Czech Republic

The third version of the paper from Middelveen *et al.*, "Culture and identification of *Borrelia* spirochetes in human vaginal and seminal secretions" represents a well designed and nicely executed study strongly supported by multiple results that were obtained by three independent laboratories. The impact of the presented data is significant as it highlights the alternative way of the distribution of the causative agent of Lyme disease, *Borrelia burgdorferi sensu stricto* spirochetes, rather than the traditionally accepted vector-borne way. The well balanced study presents the cultivation of live and replicating spirochetes from genital secretions, confirming the possibility of person-to-person transmission of Lyme disease spirochetes. Supported by multiple techniques, the obtained results are clear, consistent and confirming. The authors do not bring the conclusion or speculate that spirochetes transmitted from person-to-person might trigger Lyme disease in humans, as this postulation needs to be deeply studied. However, confirmation of the fact that live *Borrelia* are present in semen and vaginal secretions of patients with Lyme disease and can be cultured in active and replicating form from them is the first and significant step in analysis of person-to-person transmission of the causative agent of Lyme disease. This may be one of the reasonable explanations of the increased numbers of confirmed cases of Lyme disease worldwide, in addition to the others that are not discovered yet.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes



**Competing Interests:** No competing interests were disclosed.

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Referee Report 11 May 2015

doi:10.5256/f1000research.6856.r8477



**Monica E. Embers**

Division of Bacteriology and Parasitology, Tulane National Primate Research Center, Tulane University Health Sciences, Covington, LA, USA

My concerns have not necessarily been allayed by the authors' rebuttal.

Please be aware that I have invested as much time as I can in the review process for this article, and will be unable to contribute further.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Referee Report 28 April 2015

doi:10.5256/f1000research.6856.r8478



**Robert Smith**

School of Life Sciences, University of Glasgow, Glasgow, UK

This current version of the paper is one which contributes significantly to the field. I re-affirm my original approval of what I consider is a well executed and well presented study and is worthy of indexation for rational scrutiny and further investigations by the wider medical and scientific communities.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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**Version 2**

Referee Report 09 April 2015

doi:10.5256/f1000research.6473.r8176



**Monica E. Embers**

Division of Bacteriology and Parasitology, Tulane National Primate Research Center, Tulane University Health Sciences, Covington, LA, USA

The design of these experiments to test the hypothesis that viable spirochetes exist in genital secretions is appropriate. However, the implementation of the experiments, presentation, and interpretation of data is questionable.

Culture of *B. burgdorferi* from secretions: These are not sterile sources, yet there is no mention of contamination with other microbes. The authors should show (supplemental data) staining of secretions and cultures from healthy controls. One would expect some level of background staining.

Images shown in Figures 1, 2 and 3 are not specifically detecting spirochetes and are questionable without appropriate controls. The darkfield and silver stain are of very little use, given that “pseudospirochetes” of flagellar bundles can form in body fluid cultures. No positive or negative controls are shown. The straight morphology and varying lengths of that depicted in Figure 1A (and in all of the dataset 1 darkfield samples provided) are inconsistent with *B. burgdorferi* morphology. Specific staining shown in Fig 2A is more consistent with spirochete morphology and a legitimate detection method (albeit a low quality image). However, the authors indicate in the Methods section that DAPI counterstain was used but no blue staining is apparent. Does this mean that there were no other sources of DNA in the culture (bacteria or cells)? Clearly, there is ample contamination with other bacteria (seen in darkfield images of dataset 1). Concerning immunostaining with the FlaB probe, the methods are not described and no positive or negative control samples are shown. With the exception of Patient 10, none of the silver stained samples resemble spirochetes.

This reviewer encourages readers to examine the images provided in supplementary datasets.

Concerning the molecular detection, the sequence for the 23mer FlaB DNA probe should be provided, as should evidence of validation with controls. If multiple genes were amplified from cultures, then those sequences should also be provided. Assuming there was a positive control, 98-100% homology cannot distinguish between a legitimate infection and PCR contamination (with introduction of errors from PCR). Sequences from patients 10 and 11 as *B. hermsii* is misleading, given that they are short, disconnected regions of homology. Given that 16s was used, the entire product should line up. BLAST input was not provided for Patient 12 and the region of homology for patient 13 was only 50 bp (where for patient 1, 6 and 7 it was ~120 bp).

In conclusion, this manuscript would be made much clearer if all of the control (positive and negative) data were also included. Validation of the assays employed is unapparent. The paper cited from the Australian Biologics assay indicated PCR detection of *Borrelia* in patients at a much higher frequency than IgG or IgM immunoblot detection of exposure. This is inconsistent with multiple reports. Furthermore, the PCR primers are listed as “proprietary” so an assessment of specificity cannot be made. Sending blinded samples to an impartial 3rd party for testing using validated assays is recommended.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 14 Apr 2015

**Raphael Stricker, USMA, USA**

- *The design of these experiments to test the hypothesis that viable spirochetes exist in genital secretions is appropriate. However, the implementation of the experiments, presentation, and interpretation of data is questionable.*

*Culture of B. burgdorferi from secretions: These are not sterile sources, yet there is no mention of contamination with other microbes. The authors should show (supplemental data) staining of secretions and cultures from healthy controls. One would expect some level of background staining.*

We acknowledge that these are not sterile sources. Three antibiotics were used to limit the growth of normal flora. Although *Borrelia* cultures may contain other flora, obtaining pure isolates was not the purpose of the study. The negative controls from healthy subjects did not show PCR evidence of spirochetal infection, as seen in Table 5 and Dataset 7. Real-time PCR failed to show spirochetal infection in genital cultures from negative controls (Table 5A), and both TaqMan PCR and nested PCR using a variety of targets failed to show *Borrelia* infection in genital cultures from negative controls (Table 5B). To address the referee's concern about control silver staining and immunostaining, however, we have performed additional control silver stains and immunostains using a culture from uninfected mouse plasma, which showed no staining for *Borrelia* with either technique. Cultures of fungal-infected human skin, mixed gram positive strep and staph (from dairy mastitis cultures) and mixed gram negative fecal coliforms (from canine feces cultured on McConkey Agar) also failed to show significant immunostaining for *Borrelia*. In contrast, plasma culture from a mouse infected with *Borrelia* spirochetes processed at McClain Laboratory showed positive silver staining and immunostaining. We have included this information in Datasets 2A and 3A and added a description in the Results section on Pages 8 and 9.

- *Images shown in Figures 1, 2 and 3 are not specifically detecting spirochetes and are questionable without appropriate controls. The darkfield and silver stain are of very little use, given that "pseudospirochetes" of flagellar bundles can form in body fluid cultures. No positive or negative controls are shown.*

Figure 1 shows darkfield imaging and silver staining indicating that cultures are living and viable. The Figure shows typical spirochetal morphology, and these viable spirochetes were confirmed to be *Borrelia* by PCR, as shown in Table 5 and Dataset 7. Figure 2 shows immunostaining that is specific for *Borrelia* and correlates with positive PCR detection in these samples. Figure 3 shows molecular hybridization with the FlaB probe that is highly specific for *Borrelia*. The positive and negative controls for this experiment are shown in Supplemental Figure 1. Although each figure by itself might not indicate the presence of *Borrelia*, the combination of darkfield imaging, silver staining, immunostaining, PCR testing using a range of targets and molecular hybridization provides strong evidence for viable *Borrelia* in these genital cultures, as stated repeatedly in the article.

- *The straight morphology and varying lengths of that depicted in Figure 1A (and in all of the dataset 1 darkfield samples provided) are inconsistent with B. burgdorferi morphology.*

We respectfully disagree with the referee, and we have included a discussion of

morphological variation and pleomorphism in *Borrelia* cultures in the Discussion section on Page 16 with multiple references. Furthermore, to our knowledge nobody has ever studied *Borrelia* in seminal or vaginal cultures, so nobody knows what morphological variants would be present. Since *Borrelia* morphological variation and pleomorphism is acknowledged in the medical literature, visual identification alone is not reliable and that is why we used silver staining, immunostaining and molecular detection to characterize the isolates as *Borrelia*.

- *Specific staining shown in Fig 2A is more consistent with spirochete morphology and a legitimate detection method (albeit a low quality image). However, the authors indicate in the Methods section that DAPI counterstain was used but no blue staining is apparent. Does this mean that there were no other sources of DNA in the culture (bacteria or cells)? Clearly, there is ample contamination with other bacteria (seen in darkfield images of dataset 1).*

We agree with the referee that immunostaining in Figure 2A is consistent with spirochete morphology. The DAPI counterstain was only used in the FlaB probe validation study, as shown in Supplemental Figure 1. We have modified the Methods description to reflect this fact.

- *Concerning immunostaining with the FlaB probe, the methods are not described and no positive or negative control samples are shown. With the exception of Patient 10, none of the silver stained samples resemble spirochetes.*

The FlaB probe is a DNA probe, not an immunostain. This DNA hybridization technique is described in detail in Methods section 5 (Page 4) with appropriate references, and positive and negative controls for the technique are shown in Supplemental Figure 1. Again, we have included references to studies showing morphological variation and pleomorphism in *Borrelia* cultures in the Discussion section on Page 16. As there are problems with specificity of visual identification using silver staining, we used this method to provide corroborative evidence only. It is the collective evidence -- including darkfield imaging, immunostaining and molecular detection of borrelial DNA that is important. We used a broad approach with various methods so that there was no reliance on any single methodology, as stated repeatedly in the article.

- *This reviewer encourages readers to examine the images provided in supplementary datasets.*

We encourage readers to do the same. The Dataset images support the findings described in the article.

- *Concerning the molecular detection, the sequence for the 23mer FlaB DNA probe should be provided, as should evidence of validation with controls.*

We have provided the FlaB probe sequence in Methods section 5 (Page 4), courtesy of Dr. Alan MacDonald. Again, this is a molecular technique intended to provide corroborative evidence -- along with all the other techniques. A detailed validation study was provided in Supplemental Figure 1 clearly showing that the molecular probe was specific for *Bb sensu stricto*.



- *If multiple genes were amplified from cultures, then those sequences should also be provided. Assuming there was a positive control, 98-100% homology cannot distinguish between a legitimate infection and PCR contamination (with introduction of errors from PCR).*

The gene sequences are described in Table 6, and BLAST analyses are shown in Dataset 6. The argument against PCR contamination is presented in the Discussion section on Page 17.

- *Sequences from patients 10 and 11 as *B. hermsii* is misleading, given that they are short, disconnected regions of homology. Given that 16s was used, the entire product should line up. BLAST input was not provided for Patient 12 and the region of homology for patient 13 was only 50 bp (where for patient 1, 6 and 7 it was ~120 bp).*

The *B. hermsii* sequences are short, but they were the closest match in those samples. The referee ignores the fact that this couple was the only one with a distinct *Borrelia* sequence that was detected in both sexual partners and not in any other individual or couple in our small study. The BLAST analysis for Patient 12 was performed using a different program, and labeling for the sequence input has been provided. For Patient 13, the region of homology was short, but it was virtually identical to the sequence in her sexual partner (Patient 12), as shown in Table 6.

- *In conclusion, this manuscript would be made much clearer if all of the control (positive and negative) data were also included. Validation of the assays employed is unapparent. The paper cited from the Australian Biologics assay indicated PCR detection of *Borrelia* in patients at a much higher frequency than IgG or IgM immunoblot detection of exposure. This is inconsistent with multiple reports. Furthermore, the PCR primers are listed as “proprietary” so an assessment of specificity cannot be made. Sending blinded samples to an impartial 3rd party for testing using validated assays is recommended.*

As outlined above, we have included control data for the PCR and molecular hybridization testing, which is the “acid test” for the presence of *Borrelia* in genital cultures. The assays described in our study are internally consistent since the darkfield imaging, silver staining and immunostaining have been validated by the PCR and molecular hybridization procedures. In contrast to the referee’s comment, there is ample evidence for “seronegative” Lyme disease, and we have included a discussion of this entity on Page 16 with appropriate references. We used two independent laboratories for PCR testing and molecular hybridization studies, and the results using a range of primers are consistent between the two laboratories. We intend to send blinded samples to independent laboratories for confirmation of our results. In light of our detailed responses to the referee comments, we believe that this preliminary study merits reconsideration for approval by the referee.

**Competing Interests:** None

doi:10.5256/f1000research.6473.r7696



**Robert Smith**

School of Life Sciences, University of Glasgow, Glasgow, UK

Middelveen *et al.* have produced a well written and informative account of their findings on the “Culture and identification of *Borrelia* spirochetes in human vaginal and seminal secretions” from subjects diagnosed as Lyme Disease patients by serological and clinical parameters compared to control individuals who were asymptomatic and serologically negative for the *Borrelia burgdorferi*. Of most interest is their report of the PCR sequencing of cultured spirochetes from three couples having unprotected sex which indicated that two couples had identical strains of *Bb sensu stricto* in their semen and vaginal secretions, while the third couple had identical strains of *B. hermsii*.

I believe the study is worthy of acceptance and approve it for the following reasons:

1. The rationale and hypothesis behind the investigation is clearly stated.
2. The work has been carefully planned, employing an array of pertinent experimental approaches: routine light microscopy, histological silver staining and immunohistological staining methodology, DNA probe analysis and PCR to demonstrate the presence of *Borrelia* spirochetes in cultures prepared from semen or vaginal secretions from Lyme disease subjects serologically tested for Bb, whilst samples from Bb negative control subjects did not yield spirochetes.
3. The study is well executed, with samples analysed following blind coding, and techniques performed in a number of independent laboratories.
4. The data sets, tables and figures are presented in a detailed and informative manner.
5. The authors demonstrate an authoritative awareness of previous work in the area, citing relevant animal studies which had investigated the possible sexual transmission of *Borrelia*, and give a plausible explanation for the discrepancies between the findings by Guftafson in dogs and those of Moody and Woodrun in inbred rodent models.
6. In this second version of the article, the authors have taken pains to address the main concerns of the reviewer Donta, and also answered the comments raised by Baker and others, to version one of the manuscript.
7. In their replies, Middelveen and Stricker have, in my opinion, more than allayed the criticisms with regard to technical details, and also those concerning aspects such as CDC surveillance criteria and of the unknown spirochetal load.
8. The conclusions drawn, that the culture of viable *Borrelia* spirochetes in human genital secretions suggests that Lyme disease could be transmitted by intimate contact from person to person, are justified and of significance. Their findings to date warrant a larger study and further attention by the scientific and medical community.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

## Version 1

Referee Report 05 January 2015

doi:10.5256/f1000research.6178.r7090

**Sam T. Donta**

Falmouth Hospital, Falmouth, MA, USA

There are a number of issues that mitigate against the authors' conclusion that Lyme disease can be transmitted sexually.

While there are conflicting reports from animal studies that there can be transmission by contact between animals and other studies that appear to better controlled that do not provide such evidence, there is no obvious epidemiological evidence in humans that this is a likely possibility. Apart from the sociologic implications of claiming that intimate or even casual transmission is possible, there needs to be more compelling evidence that this might be the case than is offered in this report.

Specific issues in this report:

1. No evidence that samples were blinded.
2. The numbers of patients were too small from which to draw meaningful conclusions.
3. The actual serologic data on patients should be presented in order to be more properly assessed.
4. PCR-DNA analyses should have been done on the original specimens; again in a blinded fashion, and weekly during the culture period. And, as PCR-DNA testing is much more sensitive than cultures, without this information, the validity of the presented information remains in question.
5. Concurrent samples of other body fluids, i.e. blood, saliva, should have been included.
6. Even if presuming the presence of the Lyme *borrelia* in vaginal secretions or semen, the numbers present would have not been sufficient to cause any transmission of infection, as, with any infectious process, there needs to be a critical inoculum to establish infection. This is the case with transmission by ticks.
7. *Borrelia* may be spirochetes as are *Treponemes* and *Leptospira*, but the transmission of the latter are obviously through body fluids, and not by tick or other vectors. It is not clear that infections transmitted by ticks are also transmitted by intimate or casual contact.
8. If it was true that 8/11 samples were positive, one would expect a much more obvious clinical picture of transmission by intimate or casual contact, which is not the case.
9. PCR-DNA analyses and long-term cultures can be subject to contamination, making the data here more difficult to interpret.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 12 Jan 2015

**Raphael Stricker, USMA, USA**

Co-written with Marianne J. Middelveen

- *There are a number of issues that mitigate against the authors' conclusion that Lyme disease can be transmitted sexually.*

We did not conclude that “*Lyme disease can be transmitted sexually*”. Based on preliminary editorial comments, we were careful to state that our microscopy, immunochemistry, molecular hybridization and PCR analysis showing live, culturable *Borrelia* in semen and vaginal secretions suggests that these spirochetes could be transmitted in that manner. Our study does not prove this form of transmission, and we do not make this claim anywhere in the text.

- *While there are conflicting reports from animal studies that there can be transmission by contact between animals and other studies that appear to better controlled that do not provide such evidence, there is no obvious epidemiological evidence in humans that this is a likely possibility. Apart from the sociologic implications of claiming that intimate or even casual transmission is possible, there needs to be more compelling evidence that this might be the case than is offered in this report.*

The statement that “better controlled” studies do not provide support for contact transmission of *Borrelia* in animals is contrary to the examples in mice and dogs described in the Introduction and Discussion sections of our article. We have pointed out that the two studies on highly inbred rodents that allegedly showed lack of such transmission did not use PCR techniques and therefore may have missed this transmission. The fact that the CDC now admits to more than 300,000 new cases of Lyme disease each year in the USA (and perhaps as many as one million new cases, as outlined in Stricker & Johnson, 2014) is suggestive that other forms of transmission occur, as noted in the Discussion section on page 10.

- *1. No evidence that samples were blinded.*

In response to the referee's comment, we have noted that the laboratory testing was performed on coded samples in a blinded fashion. This has been noted in the Abstract and reiterated throughout the Methods section.

- *2. The numbers of patients were too small from which to draw meaningful conclusions.*

The “*meaningful conclusions*” are that microscopy, immunochemistry, molecular hybridization and PCR analysis demonstrates live, culturable *Borrelia* in semen and vaginal secretions from Lyme disease patients. Although the numbers are relatively small, we feel that our detailed study supports this conclusion.

- *3. The actual serologic data on patients should be presented in order to be more properly assessed.*



We can include the actual serologic data as an original Dataset, but we don't see how that would alter the experimental findings in our study. The serologic data is presented in Table 1 and the Results section. In response to the referee's comment, we have added more detail about the serologic and diagnostic criteria in the Methods section on page 4, with supporting references.

- *4. PCR-DNA analyses should have been done on the original specimens; again in a blinded fashion, and weekly during the culture period. And, as PCR-DNA testing is much more sensitive than cultures, without this information, the validity of the presented information remains in question.*

PCR-DNA analysis was done on the "original specimens" in a blinded fashion in conjunction with the microscopy, immunochemistry and molecular hybridization analysis. Repeated testing at weekly or other intervals is beyond the scope of this pilot study, and this type of testing should certainly be explored in future Lyme disease studies.

- *5. Concurrent samples of other body fluids, i.e. blood, saliva, should have been included.*

As shown in Table 5, we did do PCR testing on concurrent blood samples in some patients. Saliva testing for *Borrelia* would have been of interest, but this form of *Borrelia* testing requires further investigation and is beyond the scope of our study.

- *6. Even if presuming the presence of the Lyme borrelia in vaginal secretions or semen, the numbers present would have not been sufficient to cause any transmission of infection, as, with any infectious process, there needs to be a critical inoculum to establish infection. This is the case with transmission by ticks.*

In response to the referee's comment, we have researched the number of spirochetes necessary for transmission of *B. burgdorferi* infection in mice and *T. pallidum* infection in humans. The results show that a very small number of spirochetes (as little as 18 organisms) are required for transmission of infection, and we have included this information in the Discussion on page 11. We have also noted that seminal plasma and the female genital tract may provide a relatively permissive environment for spirochetes compared to blood, skin and other immune sites, making transmission theoretically easier via the genital route.

- *Borrelia may be spirochetes as are Treponemes and Leptospira, but the transmission of the latter are obviously through body fluids, and not by tick or other vectors. It is not clear that infections transmitted by ticks are also transmitted by intimate or casual contact.*

In response to the referee's query, we have provided examples of other agents (*Babesia*, *Chlamydia*, *Coxiella*) that are proven or postulated to be transmitted by both tickbite and intimate contact. This information is included in the Discussion on page 11 with supporting references.

- *If it was true that 8/11 samples were positive, one would expect a much more obvious clinical picture of transmission by intimate or casual contact, which is not the case.*

As noted above, the substantial numbers of new Lyme disease cases each year suggests additional forms of transmission beyond a tickbite. At this time, the true epidemiology of Lyme disease is unknown because the CDC surveillance system only captures less than 10% of Lyme disease cases, as noted in the Discussion on page 11. Other epidemiological studies have suggested that some infected patients may be relatively asymptomatic (Harvey & Salvato, 2003), so transmission via intimate contact resulting in less obvious infection is plausible. The risk of this form of transmission and correlation with symptoms merits further study. Our report simply raises the possibility, and rejecting the report will shove this issue under the rug to the detriment of Lyme patients.

- 9. PCR-DNA analyses and long-term cultures can be subject to contamination, making the data here more difficult to interpret.

PCR-DNA analysis is subject to contamination. That is why we did blinded testing that always included negative controls in three different laboratories using microscopy, immunochemistry and molecular hybridization to confirm the PCR findings. Although PCR testing alone might be “difficult to interpret”, the combination of experimental techniques done in different laboratories makes interpretation much more reliable.

We hope that the referee will change his opinion after reading the revised manuscript and our responses to his comments.

**Competing Interests:** No competing interests were disclosed.

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## Discuss this Article

### Version 2

Reader Comment 16 Mar 2015

**Vett Lloyd**, Mount Allison University, Canada

It is probably not surprising that this work, reporting isolation of viable *Borrelia* in human vaginal and seminal fluid has incited such vigorous commentary; the epidemiological implication of sexual transmission is significant. However, the vigor of the commentary highlights the importance of evaluating the work based on its content rather than concern about the work's implications.

Phylogenetic analysis of spirochaetes places *Leptospiraceae* as the basal spirochaetal clade, suggesting that the ancestral mode of transmission for spirochaetes was through host urine (Radhey et al. 2013). While *Borrelia* has evolved mechanisms that allow efficient tick-vector transmission, there is no reason to expect that ancestral mode(s) of transmission would disappear. Incidences of contact or fluid-based transmission may simply be eclipsed by the more efficient arthropod-vector mode of transmission in most circumstances. Published evidence for non arthropod-vector contact transmission is extensive. These reports of non-arthropod vectored contact transmission indicate urine/fecal - oral infection routes.

There are multiple reports of viable *Borrelia burgdorferi* shed into the urine, in a variety of species. Bosler and Schultze (1986) report that bacteria can be cultured from the urine of the wild reservoir host, *Peromyscus leucopus*, although Wright and Neilsen (1990) were not able to find microscopic evidence of

spirochaetes in mouse urine. Similarly, live spirochaetes have been reported to have been cultured from urine from cows (Burgess 1988). Culture from canine urine has been found by some researchers (Grauer et al. 1988) but not others (Appel et al 1993).

The shedding of viable bacteria is not, in itself, sufficient for transmission. However, documentation of infection arising from oral exposure is strong. Ducks can acquire infection orally (Burgess 1989) and, in turn, the bacteria are shed in feces, providing the biological pre-requisites for non-arthropod vectored transmission. Cats also were able to be infected orally (Burgess 1983), detected by seroconversion, direct histology of tissue and bacterial culture from an orally-infected cat. There are multiple reports of seroconversion of uninfected dogs housed in arthropod-free clinical settings with infected dogs (Burgess 1986, Greene et al. 1988, Cerri et al. 1994). The mode of transmission was inferred to be via exposure to viable bacteria in their kennel-mate's urine. Appropriately, the most work on non-arthropod vectored *Borrelia* transmission has been with the wild reservoir species *Peromyscus maniculatus* and *Peromyscus leucopus*. Burgess and Patrican (1987) provide thorough documentation of oral acquisition of infection, assessed by seroconversion and xenotransmission to naive ticks in *Peromyscus maniculatus*.

Direct evidence for contact transmission is provided by carefully controlled experiments in which infected *P. leucopus* and *P. maniculatus* individuals were able to induce seroconversion in contact-exposed mice (Burgess et al. 1986). In both species, uninfected mice caged with infected mice became infected. Infection in the absence of an arthropod vector, i.e. contact transmission of infection, was detected by seroconversion and by culture of viable spirochaetes (identified as *B. burgdorferi* by immunofluorescence) from the blood of a contact-infected mouse. This work was extended by Wright and Nielsen (1990) who sought to determine whether infection was transmitted through sexual or body fluid contact. Wright and Nielsen (1990) confirmed that *P. leucopus* could be orally infected. Importantly, they showed that both contact with body fluids/waste and sexual contact were able to transmit infection to co-habiting previously uninfected mice. Infection was detected by seroconversion, histological analysis and IFA. Similarly, Lord et al. (1994) also conclude that contact transmission is taking place in *Peromyscus spp.* because infection was identified in wild mice in the winter when no ticks were active. These studies provide multiple examples of non-arthropod vectored infection in a variety of species. However, there is certainly evidence that contact-mediated infection may differ biologically from arthropod-vectored infection; Burgess et al. (1986), Wright and Nielsen (1990) and Appels et al (1993) all note differences in the serological profile of animals infected by ticks vs. other methods, with non-tick infected animals showing less seroreactivity. Clinical manifestations may also differ, which might reflect the role of other pathogens transmitted by the tick vectors versus pure cultures of *Borrelia burgdorferi*.

Given the number of published reports of contact transmission and the variety of animals in which this has been documented, this begs the question of why contact transmission was not found in the Moody and Barthold (1991) study using Lewis rats and in the Woodrum and Oliver (1999) study using Syrian hamsters. Although contact transmission was assessed with 30 days co-habitation, sexual transmission was assessed after the rats were in contact for one night. This was sufficient for mating but might not have been sufficient for transfer of *Borrelia* if sexual transmission is inefficient. In the work on hamsters, the authors caution against extrapolating the results from hamsters to the wild rodents that are the natural reservoir species for *Borrelia*. Presumably extrapolation of result to humans is similarly problematic.

If the conflicting results on contact and sexual transmission reflect species-specific differences in the immune response to *Borrelia*, and consequently the duration and location of infection, this raises the issue of whether the results of any animal model can be extrapolated to humans. Any animal model in which most individuals are asymptomatic or subclinical and/or spontaneously clear the infection are probably not going to be a good model for transmission studies in humans as both the duration of infection and

tissue/organ distribution of the bacteria will be different from that in humans. This makes the Middelveen et al. study that much more important. Notwithstanding the worrying question of the suitability of animal models for transmission studies, revisiting these animal models with an experimental design that would detect low frequency transmission would be very useful. And given the epidemiological importance of this work, independent verification of non-arthropod vectored transmission in humans is bound to follow.

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disease spirochete, *Borrelia burgdorferi*, in Syrian hamsters. *J Parasitol.* 1999; 85(3): 426–30.

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**Competing Interests:** No involvement in the work described here. Ongoing collaboration with some of the study's authors on an unrelated project.

Author Response 02 Feb 2015

**Raphael Stricker**, USMA, USA

Author Response to Phillip Baker 2/2/15

1. There was no culture contamination because there were no laboratory strains of *Borrelia* in the laboratory where the cultures were performed. We will state that emphatically in the next version of the article.
2. As shown in our article, there was no culture contamination because the BLAST analyses showed that the cultured organisms are not 100% matches with B-31 or other laboratory strains.
3. As shown in our article, we confirmed the physical presence of live spirochetes in cultures of genital secretions using darkfield microscopy and Dieterle silver staining that identifies spirochetes selectively. Then we did immunostaining and *in situ* molecular hybridization in two independent laboratories, again providing visual confirmation of *Borrelia* spirochetes in cultures of genital secretions. Then we did PCR DNA analysis in two independent laboratories that confirmed the presence of *Borrelia* spirochetes in cultures of genital secretions. Thus the collective evidence using different methods and performed in separate laboratories provides corroborative evidence for live culturable *Borrelia* spirochetes in semen and vaginal secretions from Lyme disease patients and makes it highly unlikely that there was contamination in our samples.
4. As stated in our article, we did controls for all experiments in three independent laboratories, and all controls were negative. This fact confirms that it is highly unlikely that there was contamination in our samples.
5. We do not yet know the numbers of spirochetes in semen and vaginal secretions of Lyme disease patients, but the fact that we can culture live spirochetes from a small swab suggests that there are more than “small numbers”. Furthermore, we have shown that only small numbers of spirochetes are needed to cause infection, as stated in our revised report. Again, we do not claim to have shown that sexual transmission of Lyme disease occurs in humans, but the possibility merits further investigation.

Raphael B. Stricker  
Marianne J. Middelveen

**Competing Interests:** None

Reader Comment 01 Feb 2015

**Phillip Baker**, American Lyme Disease Foundation, USA

This revised version still fails to provide evidence that just being able to detect small numbers of *Borrelia* in vaginal and seminal secretions indicates that such *Borrelia* cause disease by this route of entry. As I have



indicated in my previous comments, there is ample evidence that this is not likely to occur. My earlier reference to Koch's postulates is pertinent and quite appropriate in that context.

Furthermore, since these authors and their colleagues have a history of publishing results suggesting extensive laboratory contamination by *Borrelia* species<sup>1</sup>, these findings ought not to be accepted for indexation without independent confirmation by a third party using blinded specimens, even if they might be inconvenienced by such a reasonable request under such circumstances.

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**Competing Interests:** No competing interests were disclosed.

Author Response 25 Jan 2015

**Raphael Stricker, USMA, USA**

Co-written with Marianne J. Middelveen

We are sorry that Phillip Baker is upset by the inconvenient truth presented in our study showing that live, culturable *Borrelia* spirochetes are present in semen and vaginal secretions from patients with Lyme disease. As pointed out in a previous comment, his reference to Koch's postulates is inappropriate because according to Wikipedia these criteria for infection are largely obsolete in the age of molecular biology<sup>1</sup>. As for the epidemiology of Lyme disease, readers should judge for themselves whether the number of infected patients makes no difference in how we view the disease. The trivial illness that Baker and other "Lyme denialists" cling to has disappeared, and we need to accept that Lyme disease is a major epidemic no matter how inconvenient that truth may be<sup>2</sup>.

As for Baker's suggestion that *Borrelia* cannot survive the vaginal pH of 3.8-4.5, there are several major flaws in that argument: First, according to Wikipedia the vaginal pH in pre-menopausal women is in that range, but in post-menopausal women the pH increases to 6.5-7.0, which would be more permissive for spirochete survival<sup>3</sup>. Second, seminal plasma and vaginal mucus contain an alkaline medium that protects sperm and may also protect the spirochetal agents of syphilis and Lyme disease in the vagina<sup>3,4</sup>. Third, *Borrelia* is known to form cysts that can survive in extreme conditions, including low pH, and then revert to the spiral form in more favorable circumstances<sup>5,6</sup>. Fourth, as stated in our article the vaginal mucosa produces immune factors that would allow *Borrelia* to survive once it reaches that location. Thus *Borrelia* has ample means to survive in the "hostile" vaginal environment, and this important topic merits further study.

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**Competing Interests:** No competing interests were disclosed.

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## Version 1

Reader Comment 21 Jan 2015

**Phillip Baker**, American Lyme Disease Foundation, USA

Since the optimal pH for the growth of *Borrelia burgdorferi* is pH 7.6 and the pH of vaginal secretions is quite acidic, ranging from pH 3.8 - pH 4.5, perhaps Dr. Stricker would like to offer an explanation as to how long -- if at all-- *B. burgdorferi* is likely to survive in such a "hostile" environment.

**Competing Interests:** None

Reader Comment 19 Jan 2015

**Sin Hang Lee**, Milford Molecular Diagnostics , USA

Dr. Baker's comment linking Koch's postulates to the CDC Lyme disease case criteria which is based on the two-tier serology test results is not appropriate. The Koch's postulates<sup>1</sup> were published before the discovery of antibodies. In fact, attempts to rely on the Widal test (an antibody test) to diagnose typhoid fever were rejected more than 100 years ago<sup>2</sup>. A CDC document clearly states that serology tests are not reliable for the diagnosis of typhoid fever<sup>3</sup>. If the Koch's postulates are to be applied in the diagnosis of Lyme disease, alternative methods, e.g. DNA sequencing-based tests<sup>4</sup>, to detect causative agents must be used.

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**Competing Interests:** Competing Interests: Dr. Sin Hang Lee is the director of Milford Molecular Diagnostics, a CLIA-certified laboratory offering DNA sequencing-based tests for Lyme and related borrelioses

Reader Comment 08 Jan 2015

**Phillip Baker**, American Lyme Disease Foundation, USA

Despite Dr. Stricker's attempts to obfuscate the key issues, the fact remains -- as noted by Donta in [his review](#) - that the mere presence of *Borrelia* in seminal and vaginal secretions, if that finding indeed can be confirmed independently by other investigators, does not prove that they cause disease, especially in view of the fact that most of the patients examined were not seropositive by standard CDC test criteria. Perhaps Dr. Stricker ought to review Koch's postulates for guidance in that regard. In the absence of such proof, it is irresponsible and not in the best interest of the public health for Dr. Stricker to alarm the public by touting the results of preliminary and unconfirmed findings in the press.

Most recent data from the CDC still continue to show that, unlike the reported incidence of sexually transmitted diseases, reported cases of Lyme disease are concentrated in the same 13 States as in previous years. Obviously, the epidemiology is the same whether the reported number of cases is 30K or 300K per year.

**Competing Interests:** No competing interests were disclosed.

Author Response 03 Jan 2015

**Raphael Stricker**, USMA, USA

Co-written with Marianne J. Middelveen

We appreciate Phillip Baker's interest in our study showing live, culturable *Borrelia* spirochetes in semen and vaginal secretions from patients with Lyme disease. We used microscopy, immunochemistry, molecular hybridization and polymerase chain reaction (PCR) techniques to demonstrate the presence of these live spirochetes in cultures of genital secretions, and as stated in the study our results suggest that Lyme disease could be sexually transmitted. All of the concerns in Baker's commentary were previously aired on the Internet, and these concerns were addressed in our report. His objection has little merit for the reasons described below.

Baker represents a group of researchers who cling to the concept that Lyme disease is a trivial illness that is "hard to catch and easy to cure", and that chronic Lyme disease due to persistent infection with the Lyme spirochete, *Borrelia burgdorferi*, is rare or non-existent<sup>1,2</sup>. As discussed elsewhere, this "Lyme denialist" philosophy and disregard for opposing viewpoints has been a major factor in the epidemic spread of Lyme disease, which accounts for more than 300,000 new cases per year in the USA according to the latest Centers for Disease Control and Prevention (CDC) estimates<sup>2</sup>. As stated in our article, this alarming epidemic is twice as common as breast cancer and six times more common than HIV/AIDS, and its prevalence is hard to explain by tickbite transmission alone. Thus from an epidemiological perspective, sexual transmission of the live *Borrelia* spirochetes found in genital secretions is a plausible mechanism for

the spread of Lyme disease.

Baker's commentary on our article uses selective information that supports his point of view while ignoring contradictory evidence. He starts with two animal models that appear to refute contact or sexual transmission of Lyme disease, and he concludes with selective epidemiological evidence from the CDC that obliquely supports his view. Sandwiched in between is a flawed discussion of maternal-fetal transmission of Lyme disease that has no bearing on sexual transmission of the spirochetal infection. Although we have addressed the animal model and epidemiological issues in our article, we will address these issues in greater detail below. We will leave it to readers to find the flaws in Baker's denial of maternal-fetal transmission of Lyme disease, since this form of transmission has been documented in canine, bovine, murine and human reports from the peer-reviewed medical literature<sup>3-8</sup>.

Baker cites two animal studies as proof that Lyme disease cannot be sexually transmitted<sup>9,10</sup>. The study by Moody and Barthold was performed using Lewis rats, while the study by Woodrum and Oliver was performed using Syrian hamsters. In limiting his analysis to these two studies, Baker ignores the compelling observations in mice, birds, cats and dogs that support contact or sexual transmission of Lyme disease without a tick vector, as outlined in our report<sup>4,11-13</sup>. The Lewis rat and Syrian hamster studies had significant flaws. First, these rodents are highly inbred with all Lewis rats descended from a single breeding colony, and all Syrian hamsters descended from a single female breeder<sup>14,15</sup>. The effect of inbreeding on contact transmission of *Borrelia* is unknown. Second, these rodents are poor models for human Lyme disease. The Lewis rat develops a transient arthritis that fades without treatment following intraperitoneal injection of *Borrelia*, while the Syrian hamster may be colonized by *Borrelia* for long periods without showing any signs of disease<sup>16,17</sup>. Since the disease pattern in these animals differs significantly from the human pattern, it is not surprising that transmission of *Borrelia* would differ as well. In fact, the immunocompetent Syrian hamster has been abandoned as a model for human Lyme disease because of these differences. Third and perhaps most important, both studies used limited culture and immunological methods to determine sexual transmission of *Borrelia*, and more sensitive detection of spirochetes by molecular techniques might have yielded positive results, as shown in other rodent experiments using PCR<sup>4</sup>. Thus the selective animal models cited by Baker fail to refute the possibility of sexual transmission of Lyme disease in humans.

Baker attempts to use CDC surveillance statistics to further disprove sexual transmission of Lyme disease. He points to the fact that 95% of reported CDC surveillance cases occur in 12 states in the USA, and that these reported cases are seasonal and have a male and child predominance. As pointed out in our article, however, CDC surveillance cases account for less than 10% of total Lyme cases (about 30,000 cases out of more than 300,000 annual cases in the USA). Thus the epidemiology of the vast majority of Lyme cases is not covered by CDC surveillance reporting and may differ significantly from the epidemiology reported by the CDC. To support this view, as noted in our study, a recent report from Australia found that the prevalence of Lyme disease cases aligned with the sexually active population on that continent<sup>18</sup>. Baker also questions whether our Lyme disease testing was accurate since it differs from the serological parameters used for CDC surveillance criteria. However the CDC surveillance criteria rely on testing that misses more than half of Lyme disease cases<sup>19</sup>, and these criteria are not recommended for diagnosis of Lyme disease<sup>20</sup>. The reason why Baker thinks that insensitive and incomplete epidemiology statistics trump microscopy, immunochemistry, molecular hybridization and PCR testing that demonstrates live, culturable *Borrelia* spirochetes in genital secretions from our Lyme disease patients is unclear.

Baker bemoans the fact that because of the inconvenient evidence presented in our study, Lyme disease patients may suddenly be concerned about sexual transmission of their disease. Because Baker does not

represent or treat patients, he may be unaware that this question is being asked by patients in clinicians' offices every day as spouses and sexual partners become symptomatic. Should Lyme disease patients be concerned about sexual transmission? Absolutely. We currently treat Lyme disease with 60-year-old antibiotics that are marginally effective against a chronic infectious disease<sup>1,21</sup>. Baker and his research associates should focus on the risk of sexual transmission of *Borrelia* and strive to develop new treatments for this alarming epidemic infection along the lines of the global "Manhattan Project" that has successfully dealt with the HIV/AIDS epidemic<sup>2,19</sup>. Our suffering Lyme disease patients deserve nothing less.

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**Competing Interests:** None

Reader Comment 27 Dec 2014

**Phillip Baker**, American Lyme Disease Foundation, USA

The concept of sexual transmission of borreliosis, which has been resurrected recently by Middelveen *et al.*<sup>1</sup>, was refuted years ago by the well-designed and controlled studies of Moody and Barthold<sup>2</sup>, as well as Woodrum and Oliver<sup>3</sup>, internationally known experts on Lyme disease. These investigators used well-characterized animal models of borreliosis in which infection is much more disseminated and profound than it ever is in humans. It should be noted that, in the United States, Lyme borreliosis has historically been defined as a tick borne infection caused by *Borrelia burgdorferi sensu lato*<sup>4</sup>.

To determine if borreliosis can be transmitted by direct contact, Moody and Barthold<sup>2</sup> housed three-day-old - or three-week-old- Lewis rats, deliberately infected with *B. burgdorferi*, with normal, uninfected rats for 30 days. As expected, all deliberately infected rats continued to be actively infected, 30 days later; however, none of the uninfected rats acquired infection after 30 days of intimate direct contact with their infected house mates.

In other experiments, Moody and Barthold<sup>2</sup> were unable to demonstrate venereal transmission of borreliosis from seven infected females-or six infected males - to uninfected rats of the opposite sex.

In the work of Woodrum and Oliver<sup>3</sup>, six female Syrian hamsters infected with *B. burgdorferi* were mated with six uninfected males; conversely, three infected males were mated with six uninfected females. None of the uninfected hamsters became infected after mating with an infected partner of the opposite sex, indicating that borreliosis is not sexually transmitted. These investigators also failed to demonstrate contact transmission of *B. burgdorferi* between infected female -or male-hamsters and uninfected hamsters of the opposite sex. Also, it was not possible to transmit borreliosis to uninfected hamsters with urine or feces from infected hamsters.

Sadly, preliminary oral reports of the observations of Middelveen *et al.*<sup>1</sup> have already generated an inordinate amount of fear and anxiety within the lay community due to sensationalized reports of their unconfirmed findings by an uncritical - and often naïve - press. This has already caused much harm. To date, I have received numerous inquiries from distraught individuals, wondering if they now should even consider marrying their spouse-to-be for fear of contracting Lyme disease that some mistakenly believe to be incurable. Some fear the possibility of giving birth to an infected or congenitally deformed child, because their spouse or spouse-to-be had been diagnosed and treated for Lyme disease in the past.

To examine the issue of *in utero* transmission of infection, Moody and Barthold <sup>2</sup> inoculated pregnant female Lewis rats with viable *B. burgdorferi*, at four days of gestation. All of the inoculated pregnant females became seropositive as expected, and *B. burgdorferi* could be cultured from their spleens at 20 days of gestation; however, their placentas and fetuses were culture negative, indicating the lack of *in utero* transmission.

Moody and Barthold <sup>2</sup> used two different experimental protocols to determine if transplacental transmission of *B. burgdorferi* occurs. One protocol involved six non-pregnant infected females that were subsequently mated and became pregnant. Three of the females were allowed to carry to full term, whereas the remaining three were sacrificed just prior to parturition. All offspring and offspring-to-be were found to be culture negative for *B. burgdorferi*, as well as seronegative for antibody specific for *B. burgdorferi*, indicating that transplacental transmission of infection does not occur.

In the second protocol, six females were infected via tick bite after becoming pregnant, and were allowed to carry their fetuses to birth; all were negative for infection. The results of these studies likewise failed to provide evidence for the transplacental transmission of naturally acquired borreliosis.

Other investigators examined the possibility of congenital birth defects in humans with Lyme disease by doing a rather large comparative study involving 5,000 infants, half from an area in which Lyme disease was endemic and half as controls from an area without Lyme disease <sup>5</sup>. They found no significant differences in the overall incidence of congenital malformations between the two groups.

In another study, involving 1,500 subjects including controls, no increased risk of giving birth to a child with a congenital heart defect was noted in women who had either been bitten by a tick or had been treated for Lyme disease during or before pregnancy <sup>6</sup>.

Finally, an extensive analysis of the world literature revealed “that an adverse outcome due to maternal infection with *B. burgdorferi* at any point during pregnancy in humans is at most extremely rare” <sup>7</sup>.

In view of the above considerations, the work by Middelveen *et al.* <sup>1</sup> is not credible and must be view with much skepticism. There is no evidence to indicate that vaginal and seminal secretions provide a suitable environment for the growth of *Borrelia* <sup>8</sup> and that the mere presence of *Borrelia* in vaginal and seminal secretions – if such an observation is indeed valid and can be confirmed independently – results in disease. In the absence of such proof, no conclusions can be drawn as to the sexual transmission of Lyme disease by of *B. burgdorferi*.

#### Addendum added in proof:

It should be noted that the basic epidemiology of vector-borne Lyme disease is strikingly different from that for sexually transmitted diseases (STDs). For example, annual data provided by the CDC indicate that about 95% of all reported cases of Lyme disease occur in 12 States, and the incidence of Lyme disease is seasonal and peaks with increased tick activity. Also, in the case of STDs, there are more husband-wife paired cases rather than the preponderance of cases of males and children with Lyme disease. Most important, Middelveen *et al.* state that “... the majority of our study subjects **did not** meet the positive standard as defined by the CDC surveillance criteria.” One must therefore wonder if their study subjects ever had Lyme disease in the first place.

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