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ORIGINAL ARTICLE

Novel *Spiroplasma* Spp. Cultured From Brains and Lymph Nodes From Ruminants Affected With Transmissible Spongiform Encephalopathy

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Abstract

Spiroplasma spp., tiny filterable wall-less bacteria, are consistently associated with the transmissible spongiform encephalopathies (TSE). Spiral forms have been transiently isolated from TSE-affected brain tissues in SP4 growth media designed for isolation of *Spiroplasma* spp., but the isolate could not be propagated in SP4 media. A bacterium must grow in vitro in cell-free cultures to allow full characterization of a suspect pathogen. Here, a novel *Spiroplasma* sp. was isolated from scrapie- and chronic wasting disease (CWD)-affected brains and lymph nodes. Filtrates of tissue homogenates inoculated into Brucella media incubated for 14 days at 35 °C resulted in high titers of spiroplasma as shown by dark-field microscopy. A drop assay of infected media on Bacto Schaedler agar showed spiroplasma isolates forming unique subsurface colonies after 21 days incubation. Spiroplasma coils, coccoid forms and clumps of entwined spiroplasma filaments were seen on the agar by scanning electron microscopy. Since Brucella media has a sodium bisulfite additive that lowers oxygen tension, TSE spiroplasma growth requires media with low oxygen tension. Brucella media allows for isolation and propagation of spiroplasma from TSE-affected tissues, which will lead to complete characterization of this TSE pathogen and determine its role as a candidate causative agent of TSE.

Key Words: Bacto Shaedler agar, Brucella special media, Chronic wasting disease, Creutzfeldt–Jakob disease, Prion, Scrapie, Spiroplasma, Transmissible spongiform encephalopathy (TSE).

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INTRODUCTION

The discovery by transmission electron microscopy (TEM) of spiroplasma in brain biopsy and autopsy tissues from Creutzfeldt–Jakob disease (CJD) patients (1, 2), and not in control brains suggested involvement of these tiny filterable wall-less bacteria in the pathogenesis of the transmissible spongiform encephalopathies (TSE). Polymerase chain reaction studies have revealed spiroplasma-related ribosomal rDNA in TSE-affected brain tissues including CJD patients, scrapie-affected sheep, and chronic wasting disease (CWD)-affected deer (3). Furthermore, there is close morphological resemblance of the internal fibril network of *Spiroplasma* spp. to scrapie-associated fibrils (SAF), which are consistent ultrastructural markers of TSE infection (4). These internal fibril proteins are unique to *Spiroplasma* spp. (5) and are responsible for motility of the bacterium (6). The SAF link of spiroplasma to TSE was confirmed by immune reaction of hyper-immune rabbit anti-SAF sera (courtesy of Pat Merz, Institute for Basic Research in Developmental Disabilities, NY) against spiroplasma fibril proteins in protease-treated *Spiroplasma mirum* broth cultures (7). Suckling rats inoculated intracranially with the GT-48 strain of *S. mirum* developed neurodegeneration (8) manifested by spongiform encephalopathy, the neuropathological hallmark of CJD and other TSEs (9). The GT-48 spiroplasma were readily recovered from the rat brain tissues showing a dose response (10). The suckling mouse cataract agent strain of *S. mirum* inoculated intracranially into neonatal deer induced a clinical syndrome at $3^1/_2$ months post inoculation identical to naturally occurring CWD with spongiform encephalopathy seen in the deer brain tissues at necropsy (11). Suckling mouse cataract agents were identified in the diseased deer obex by immunohistochemistry and TEM.

Spiroplasma sp. has been isolated in SP4 media from TSE-affected tissues including scrapie-affected sheep, CWD-affected deer and CJD-affected cases (12, 13) supporting our premise that spiroplasma are involved in the pathogenesis of TSE. Long spirals phenotypically consistent with spiroplasma were seen in the SP4 preparations by dark-field microscopy but appeared inactive (13). These observations of TSE-related spiroplasma in SP4 media were transient since the TSE-related *Spiroplasma* sp. could not be propagated in the SP4 media. Although SP4 media was designed specifically for

isolation and propagation of fastidious *Spiroplasma* spp. (14), it was apparent that the SP4 media was inadequate for culture of TSE-associated spiroplasma unlike other *Spiroplasma* spp.

In order to link spiroplasma to the TSEs, it is essential these TSE-related *Spiroplasma* sp. isolates be propagated in vitro before there can be progress in further linking spiroplasma to the TSEs (15). Here, we show TSE *Spiroplasma* sp. has been reproducibly isolated from scrapie- and CWD-affected ruminant brains and lymph nodes (LN) and propagated through several passages in Brucella special media (BBL™ Brucella Broth 8806541 2015) and as typical spiroplasma subsurface colonies (16) on Bacto Schaedler agar plates. Brucella special broth was designed for isolation and propagation of anaerobic bacteria including *Brucella* spp. and *Campylobacter* spp. (17, 18) and differs from SP4 media by presence of sodium bisulfite additive (an oxygen scavenger) in Brucella special media (18). Therefore, TSE spiroplasma isolates are only cultivable under low oxygen tension; an observation that is key for isolation and propagation of the TSE-related spiroplasma.

MATERIALS AND METHODS

Isolation Method

The method detailed in this report for isolation of spiroplasma from TSE tissues is based upon the use of Brucella special media to provide the proper growth conditions necessary for propagation of the TSE spiroplasma isolates. Samples of 4 frozen scrapie-affected sheep brains (purchased from the Caine Research Center, University of Idaho) and frozen retropharyngeal LN) from 5 deer affected with CWD (courtesy of Dr. Philip Bochsler, University of Wisconsin) were prepared as tissue homogenates. All TSE-affected tissues were positive for prion amyloid by immunohistochemistry. Briefly, small tissue samples (25 mg) selected from different regions of the brain affected with scrapie or from LN affected with CWD are homogenized using glass tissue grinders in a small volume of Brucella special media (3 ml). The homogenates were centrifuged for 10 minutes at 4,000*g*, the pellets discarded, and the supernatants filtered through either 0.2 or 0.45 μm cellulose syringe filters. The filtrates were placed in stoppered 50 ml tubes and an equal volume of Brucella media was added. The samples were incubated in a humidified incubator in closed tubes at 35 °C. At 6 days post inoculation, the media became turbid and the sample was examined by dark-field microscopy. These examinations were repeated at 14 and 21 days incubation. At that juncture, 10-μl drops of the TSE-inoculated Brucella media were placed on Bacto Schaedler agar plates containing antibiotics and antifungals. Samples of 6 normal sheep brains, 1 normal deer brain, and 1 normal deer LN were prepared as above as controls.

Dark-Field Microscopy

Spiroplasma growth was documented in the Brucella media by observation of motile spiral and filamentous forms under dark-field microscopy. Cultured samples in Brucella media were prepared for dark-field examination by placing a 5-μl drop of sample on a clean glass microscope slide, covered with a #1.5 cover slip, and sealed on all 4 sides with clear finger nail polish. Slides and cover slips were examined with a Zeiss Axio Imager A1 microscope equipped with a dark-field top lens condenser 1.2–1.4 and an EC Plan-NeoFluar 100 Å∼ oil immersion objective with the 1.3 iris closed to its lowest setting (0.7).

Negative Stain Electron Microscopy

A drop of log phase culture (as determined by dark-field examination of a 19-day-old culture) was placed on a formvar-coated copper grid and stained with PTAH. The grid was examined when dry using a JEOL transmission electron microscope (JEM1011).

Identification of the Spiroplasma Isolate on Bacto Schaedler Agar Plates

Seeding of the spiroplasma TSE isolate grown in Brucella media on Bacto Schaedler agar plates produced typical spiroplasma subsurface colonies after 21 days incubation at 35 °C (16). In order to confirm that the colonies seen on agar contained typical spiroplasma, the colonies on the Bacto Schaedler agar plates were scraped and mixed with 200 μl of 1× PBS then a drop was placed on a glass disc (13 mm in diameter). After allowing sedimentation of the fragments of the colonies on the glass, the preparation was fixed in 1.25% glutaraldehyde in cacodylate buffer for 1 hour then placed in the cacodylate buffer until the glass disc was prepared for scanning electron microscopy (SEM).

Biofilm Formation

Evidence of TSE spiroplasma to form biofilm was studied by incubating nickel wire in a culture of the TSE spiroplasma isolate in the Brucella media for examination by SEM. The nickel wires exposed to spiroplasma cultures or media alone were rinsed 3× with 10 mM PBS pH 7.5. The wires were fixed by adding cold 1.25% glutaraldehyde in cacodylate buffer for 1 hour and then maintained in the same buffer until processing. Each sample was mounted on a metal stub with adhesive and sputter coated by platinum. Specimens were examined by SEM using an FEI Quanta 200 high-resolution environmental microscope. Wires were also washed with sterile PBS and laid on Bacto Shaedler agar plates containing antibiotics and antifungals and incubated for 21 days to determine if the spiroplasma in the biofilm were viable.

Isolation of Scrapie Spiroplasma sp. On Embryonic Mosquito Cell Cultures

A filtrate of scrapie-affected brain or eye tissues were layered on embryonic mosquito cell cultures (Aedes albopictus clone c6/36 [ATCC CRL-1660]) and incubated at 28 °C, the goal being to determine efficiency of isolation of the scrapie spiroplasma by SEM in a tissue culture system. A portion of the embryonic mosquito cells were grown on sterile 13-mm glass discs, infected with the scrapie spiroplasma isolate, then prepared for SEM as above.

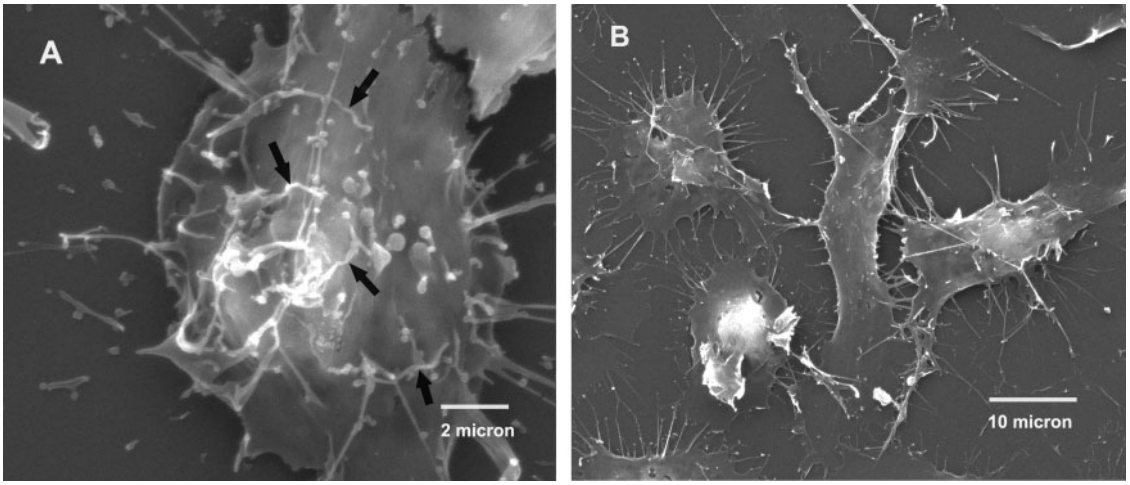

FIGURE 1. Isolation of scrapie associated spiroplasma on mosquito embryo cell culture. **(A)** Filaments of Spiroplasma sp. (black arrows) shown by SEM on surface of embryo cell. Note disruption of cellular attachments to flask. (**B)** Mosquito embryo cell culture control.

Review of Case Material From 2005

Since *Spiroplasma* sp. from TSE-affected tissues was consistently isolated and propagated in Brucella media, we determined whether spiroplasma could be reisolated from frozen archived brain tissues from sheep and goats that had been inoculated intracranially with a spiroplasma isolate from sheep brain-affected with scrapie from USDA, Washington State or with a spiroplasma isolate derived from CWD-affected deer brain from Colorado State University; studies carried out in 2005 (11). The frozen archived brains from this experimental study stored at −20 °C were processed as above and the filtered brain homogenates were inoculated into Brucella media and incubated as above.

RESULTS

The evidences showing the association of spiroplasma infection with the TSEs (9, 13) has been buoyed by repeated isolations of *Spiroplasma* sp. in cell-free SP4 media from brain and eyes of scrapie-affected sheep from University of Idaho and from brains and LN affected with CWD (deer) from Colorado State University and brains-affected with CJD (humans) from LSU Health Science Center, New Orleans, LA (12, 13, 19). SP4 media (14) and a modification referred to as M1D are specialized medias of high osmolality used for routine isolation and characterization of *Spiroplasma* spp. However, *Spiroplasma* sp. isolated from TSE-affected tissues survived only briefly in these specialized spiroplasma medias as documented by dark-field microscopy (11, 12, 19) or negative stain TEM (11). The long spirals of the scrapie spiroplasma isolate seen on dark-field microscopy (12, 13, 19) were inactive suggesting the SP4 media was deficient in some critical ingredient needed to support the growth of the TSE spiroplasma isolates.

The scrapie spiroplasma isolate replicated and showed persistent infection in bovine corneal endothelial cells including 4 scrapie-affected brain samples from University of Idaho (samples- #296, #6000, #301, and #5061) and in mouse neuroblastoma cell culture (12, 13) from scrapie-affected sheep brains from USDA, Washington State. We undertook a study to cultivate the *Spiroplasma* sp. from scrapie-affected sheep brain samples from University of Idaho (#296 and #6000) in insect embryo cells with plans to separate the scrapie spiroplasma isolates from the cultures based upon ultracentrifugation methods separating TSE infectivity from prion (20). The scrapie spiroplasma isolate grew in the embryo mosquito cell cultures as shown in Figure 1. This objective was abandoned when we discovered the TSE spiroplasma are easily isolated and propagated in Brucella special media and as colonies on Bacto Scheidler agar plates.

New Strategy

In a search for a media containing proper ingredients for growing the TSE spiroplasma isolates, we tested whether this novel TSE related *Spiroplasma* sp. would propagate in Brucella special media designed for cultivating fastidious anaerobic bacteria including *Brucella* spp. and *Campylobacter* spp. (17, 18). Inoculation of Brucella special media with a filtrate from a scrapie-affected brain homogenate and incubating at 35 °C led to slight turbidity after 7 days incubation with continued turbidity to 11 days postinoculation. Examination of the inoculated Brucella media samples by dark-field microscopy revealed a complement of tiny motile bacteria consistent with spiroplasma as shown in Figure 2, while none were seen in other samples containing SP4 media inoculated with the TSE spiroplasma brain homogenate. Identical dark-field microscopic findings were seen in cultures of CWD-affected LN in Brucella special media. Supplementary Data Movies S1 and S2 show motile spiroplasma isolates from CWD-affected LN: Supplementary Data Movie S1 shows CWD spiroplasma isolate in Brucella media showing motile spiroplasma in dark-field microscopic study. Multiple motile spiroplasma in 5-ml sample from Brucella media inoculated with CWD-affected brains and LN are seen after 19 days incubation at 35 °C by dark-field microscopy (original magnification: 1200×). Supplementary Data Movie S2 shows CWD spiroplasma

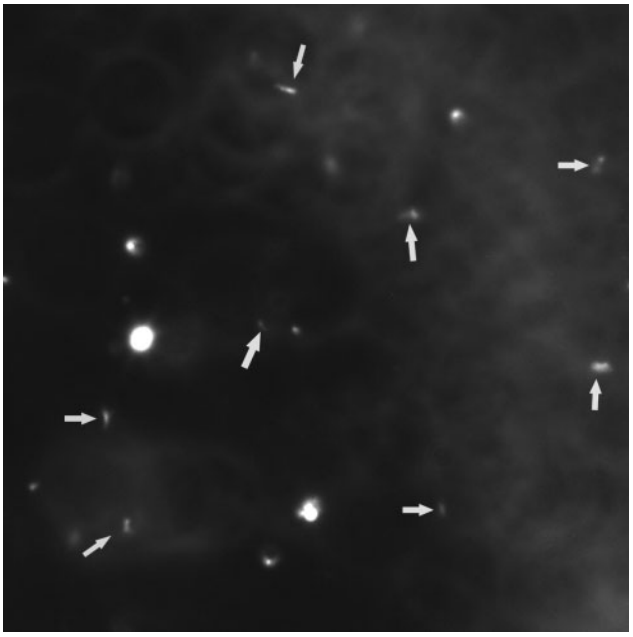

FIGURE 2. *Spiroplasma* sp. is isolated in Brucella media inoculated with filtrates of scrapie brain homogenates. Numerous motile spiroplasma coils (arrows) are seen in the Brucella media after 11 days incubation at 35 °C by dark-field microscopy. Similar findings were seen in Brucella media cultures of CWD brain and LN homogenates. No spiroplasma were identified in Brucella media inoculated with normal ruminant tissue homogenates. Original magnification: 1200×.

isolate in Brucella media showing complex spiroplasma forms with several spiral buds from a central core gyrating in dark-field microscopic study. The complex forms from a central core are characteristically seen in log phase cultures of *Spiroplasma* spp. as shown in reference # 23. These buds disappear when the culture is subjected to environmental stress (original magnification: 1200×). Spiroplasma filaments measuring 60 nm in diameter and 1.5 µm in length (Fig. 3) were seen by negative stain electron microscopy in Brucella media inoculated with CWD spiroplasma isolate. The spiroplasma morphology corresponded to TEM of the scrapie spiroplasma isolate seen in tissue sections and in tissue culture (12, 13). The dark-field observations were consistently reproduced in isolations from several scrapie-affected brains (University of Idaho) (Table 2). *Spiroplasma* sp. were isolated and propagated through several passages in Brucella media from brain samples of deer affected with CWD and from prion-positive LN from deer affected with CWD (courtesy Dr. Terry Spraker, Colorado State University) and from LN from 5 individual deer affected with CWD (courtesy of Dr. Philip Bochsler, Veterinary Diagnostic Laboratory, University of Wisconsin).

Seeding of Bacto Schaedler Agar Plates

After successful propagation of the TSE spiroplasma isolate in Brucella special media to near log phase growth as documented by dark-field microscopy, Bacto Schaedler agar plates were seeded with mini drop samples (10 ml) of these

FIGURE 3. Spiroplasma filaments are seen in Brucella media inoculated with filtrate of scrapie brain homogenate at 19 days incubation. The *Spiroplasma* sp. isolated from scrapie-affected brain measure 60 nanometers in diameter and 1.5 microns in length as seen by negative stain electron microscopy. Two bacteria appear coiled on one another.

TSE-related spiroplasma-infected Brucella media. Typical spiroplasma subsurface colonies (16) were seen on the agar surface after 21 days incubation at 35 °C (Fig. 4). Both scrapie and CWD spiroplasma isolates grew on Bacto Schaedler agar plates supplemented with antibiotics and antifungals (Table 1). The phenotypic identification of spiroplasma colonies on the Bacto Schaedler agar plates was done after 21 days incubation wherein colonies were scraped off the agar surface and allowed to settle on a glass disc and examined by SEM. Typical spiral spiroplasma coils and coccoid forms measuring 100 nm in diameter were seen by SEM (21) on the glass surface and clumps of entwined spiroplasma filaments were identified representing a core of a colony (16) (Fig. 5). These data confirmed the phenotypic nature of the scrapie isolate as a *Spiroplasma* sp. in the colonies (16). The spiroplasma colonies related to the TSE isolates are clearly identifiable by light microscopy since they are uniquely subsurface burying into the agar with indistinct deep margins since the spiroplasma are motile (16, 22).

Evaluation of Normal Controls

In order to determine the specificity of this in vitro isolation and propagation of TSE-associated *Spiroplasma* sp. in

Brucella media and on Bacto Schaedler agar plates, ruminant brains and LN from normal sheep (5 individual cases) and a normal deer were examined. Filtered brain homogenates were prepared from 5 normal sheep brains and from brain and LN from a normal deer, and filtered preparations were inoculated into Brucella special media and on Bacto Schaedler agar plates. The Brucella media preparations showed no turbidity after being incubated in closed tubes at 35 °C for 11 days and no motile spiroplasma were seen in those preparations by dark-field microscopy. The Bacto Schaedler agar plates seeded with preparations of normal sheep brain and LN samples incubated at 35 °C for 21 days revealed no spiroplasma colonies indicating absence of spiroplasma proliferation in the control ruminant preparations.

TABLE 1. Antibiotics and Antifungals in Bacto Schaedler Agar Plates

Polymyxin B (as SO4)	2,500 I.U.
Bacitracin	12,500 I.U.
Cycloheximide	50 mg
Nalidixic acid	2.5 mg
Nystatin	50,000 I.U.
Vancomycin (as HCl)	10 mg

The TSE spiroplasma isolates are completely resistant to these antibiotics and antifungals.

In fact, all passages of the TSE-spiroplasma isolates are fed with Brucella media containing these antibiotic and antifungal additives.

Formation of Biofilm by Scrapie Spiroplasma Isolate

Biofilm formation on nickel wire is a significant biologic property of laboratory *S. mirum* strains (21) so the capability of a scrapie spiroplasma isolate to produce biofilm was tested. Nickel wire was incubated for 14 days in a Brucella media culture of scrapie spiroplasma isolate and examined by SEM revealing abundant biofilm formation on the wire (Fig. 6). The viability of the spiroplasma attached to the wire

TABLE 2. Primary Isolation and Passage of *Spiroplasma* sp. From TSE Brains and Lymph Nodes

Tissue Samples	Positive Isolations	Passages	Negative Cultures
[Scrapie-affected brain, eye and blood samples from Idaho]			
296 scrapie sheep brain	10	8 at least to p3	0
1012 scrapie sheep brain	1	n/a	0
7045 scrapie sheep brain	6	15 at least to p6	
1 isolate from agar	0		
5045 scrapie sheep brain	1	2 at least to p2	0
[CWD-affected Deer samples of pharyngeal lymph nodes from Wisconsin]			
324551 CWD Deer LN	2	5 at least to p2	
3 isolations from agar	0		
325325 CWD Deer LN	1	3 at least to p1	
2 isolations from agar	0		
325308 CWD Deer LN	1	2 isolations from agar	0
323789 CWD Deer LN	1	2 isolations from agar	0
324535 CWD Deer LN	1	2 isolations from agar	0
[TSE isolates passaged in sheep and goats in 2005 study (scrapie-affected sheep from Washington State, and CWD-affected deer brains from Colorado)]			
Y-11 scrapie sheep	2	4 at least to p4	0
M6Blk CWD Goat	2	n/a	0
M6Brn CWD Goat	1	n/a	0
178-F CWD Goat	1	n/a	0
178-M CWD Goat	1	n/a	0
189-R CWD Goat	1	4 at least to p4	0
Y-13 CWD sheep	1	2 at least to p2	0
175-F Control Goat	0	n/a	2
[Normal sheep and lymph node controls]			
Normal sheep brains-5 individual cases	0	n/a	6
Normal sheep LN	0	n/a	1
Normal Deer 73 LN	0	n/a	2
Normal Deer 73 Brain	0	n/a	1

Repeated isolations of TSE-associated spiroplasma have been accomplished and the table shows a summary of the combined work including passage history and controls. **Positive Isolation:** several spiroplasma were identified by dark-field microscopy after primary isolation from tissue sample and incubation for 1–3 weeks. Brain or lymph node from 1 case processed at same time was counted as 1 isolation.

Passages: Spiroplasma culture passaged by adding fresh media in 2 to 1 ratio. Passaging tubes from same isolation was counted as just one passage. Passage via agar inoculation was also listed as a passage. N/a indicates that passage was not attempted. **Negative Cultures:** Number of times spiroplasma were not identified in the culture by dark-field microscopy after 3 weeks incubation.

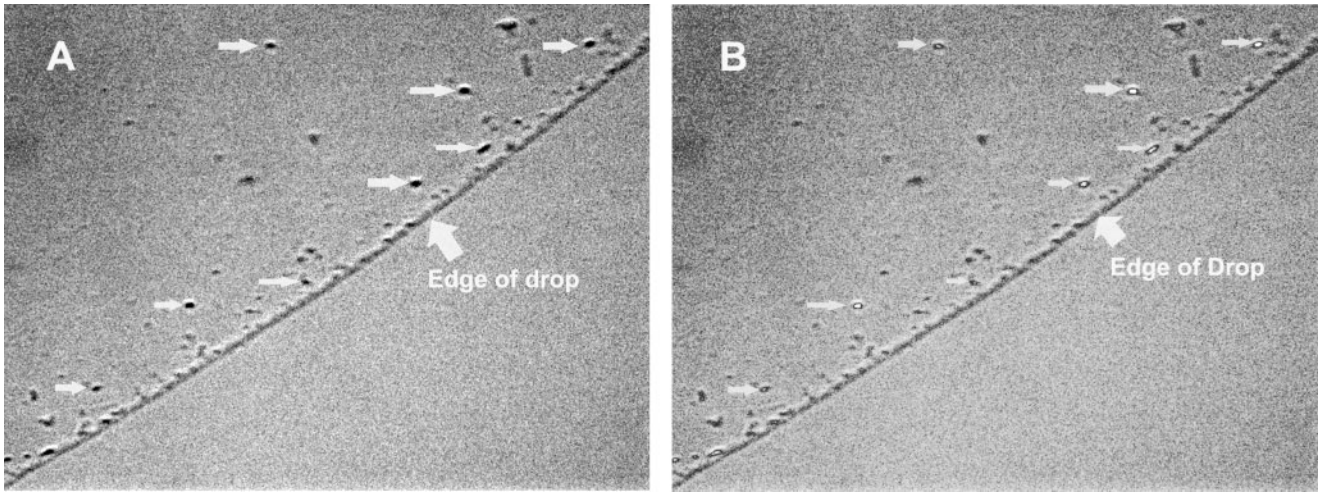

FIGURE 4. Typical subsurface spiroplasma colonies are seen on Bacto Schaedler Agar plates inoculated with samples from 11 day old Brucella media culture infected with CWD-affected LN filtrate. The plates were examined after incubation at 35 °C for 21 days. **(A)** Drop assay on the Bacto Schaedler plates revealed subsurface spiroplasma colonies localized along the leading edge of the drop (arrows). In this plane, the colonies appear as black spots. **(B)** Same field photographed in another plane showed enhancement of edges of the colonies (arrows) buried in the agar surface. The morphology of these spiroplasma subsurface colonies are unique and diagnostic of spiroplasma infection. Original magnification: 200×.

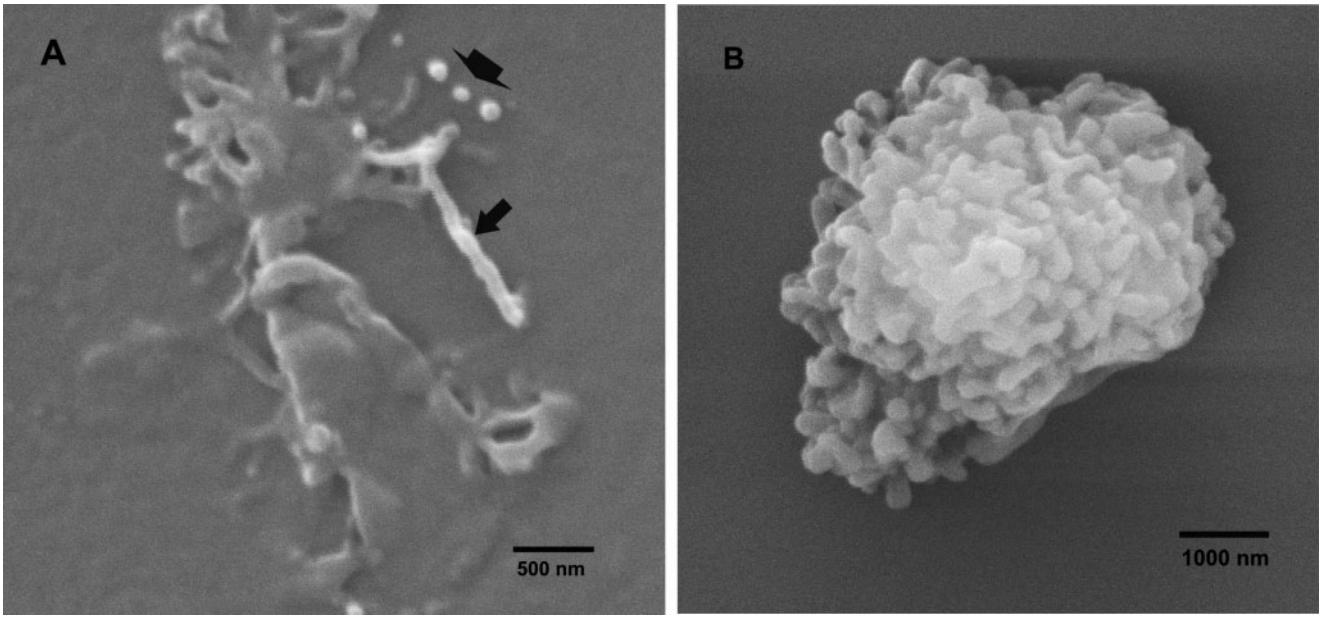

FIGURE 5. Spiroplasma phenotypically identified in disrupted subsurface colonies on Bacto Schaedler Agar plates inoculated with sample of 11 day old Brucella media culture infected with scrapie-affected brain homogenates. **(A)** A typical spiroplasma spiral (thin arrow) has been disrupted from a colony as shown by SEM. Spiropasma coccoid forms are seen as well (fat arrows). **(B)** An entwined clump of spiroplasma filaments is seen by SEM representing the center of a colony dislodged by the scraping.

was demonstrated by placing the wires on the surface of a Bacto Schaedler agar plate and after 21 days incubation typical spiroplasma colonies were seen migrating off the wire into the surrounding agar (Fig. 7).

Koch's Postulates?

In a prior 2005 study, scrapie and CWD spiroplasma isolates in SP4 media obtained in our Tulane laboratory prior to Katrina were inoculated intracranially into sheep and goats at

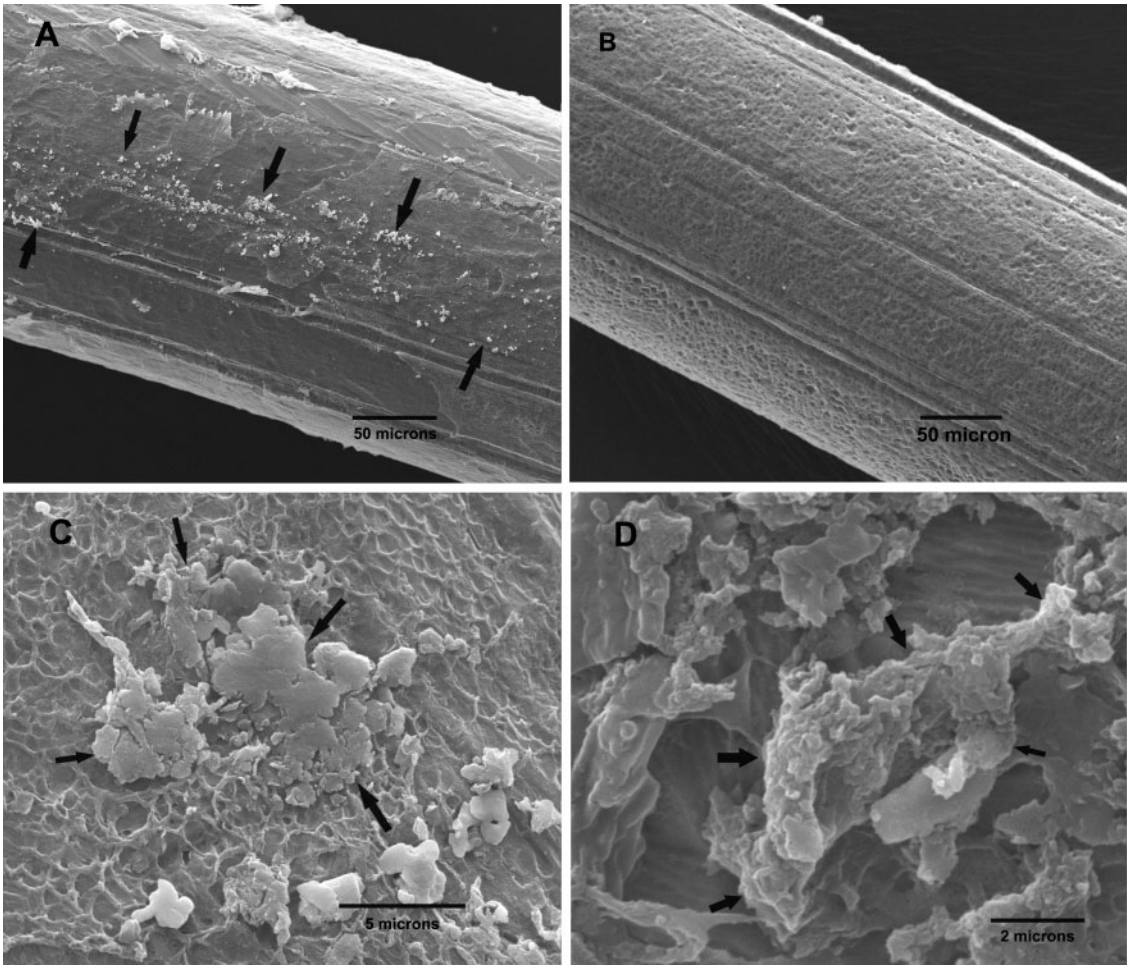

FIGURE 6. Scrapie *Spiroplasma* sp. formed biofilm on nickel wire incorporated in Brucella media culture inoculated with filtrate of scrapie brain homogenate after 14-day incubation at 35 °C. **(A)** Nickel wire incubated in Brucella media inoculated with scrapie spiroplasma isolate shows extensive biofilm on the wire surface (a large deposit is outlined by arrows); **(B)** Nickel wire exposed to brucella special media without scrapie spiroplasma inoculatioon showed no biofilm formation representing no growth; similarly, no biofilm was observed on wires exposed to normal sheep brain. **(C)** Biofilm formation on nickel wire exposure to Brucella media culture of scrapie spiroplasma isolate shows granular biofilm matrix typical of spiroplasma-related biofilm (arrows). **(D)** Typical spiroplasma filaments and clumps of entwined spiroplasma filaments are seen embedded in biofilm on the wire surface (the largest clump is outlined with arrows closely resembles the clump of entwined spiroplasma filaments in a spiroplasma colony shown in Figure 5B).

the LSU Agricultural facility in Baton Rouge LA (11). However, the animals were killed at 12 months post inoculation before any clinical signs appeared. At necropsy, the neuropathology of these animals revealed classic vacuolar lesions in the obices identical to naturally occurring lesions in scrapie or CWD affected ruminants (11). However, attempts to reisolate the spiroplasma in SP4 media failed, questioning the reliability of the reported neuropathology findings. In the current study, spiroplasma were isolated from archived frozen brains from those experimental animals by inoculation of filtered brain homogenates into Brucella media. After incubation in Brucella media for 11 days, motile spiroplasma were seen by dark-field microscopy (Fig. 8). Seeding of these cultures on Bacto Schaedler agar plates and incubating for 14 days at 35 °C revealed numerous typical subsurface spiroplasma colonies (16) (data not shown). The reisolation of experimental samples from that 2005 study (11) worked for inoculation of both scrapie and CWD spiroplasma isolates in sheep and goats, supporting our premise that *Spiroplasma* sp. has a causal role in the TSEs (23).

DISCUSSION

Spiroplasma sp. from scrapie- and CWD-affected ruminant brains and LN grow in Brucella special media showing the requirement of media with low oxygen tension for isolation and propagation of these novel TSE-related bacteria. The TSE isolates grown in the Brucella media showed distinctive spiroplasma phenotypic features and form typical spiroplasma subsurface colonies on Bacto Schaedler agar plates (16). Numerous separate replicates of isolation and propagation of the scrapie and CWD prion-positive tissue samples in the Brucella

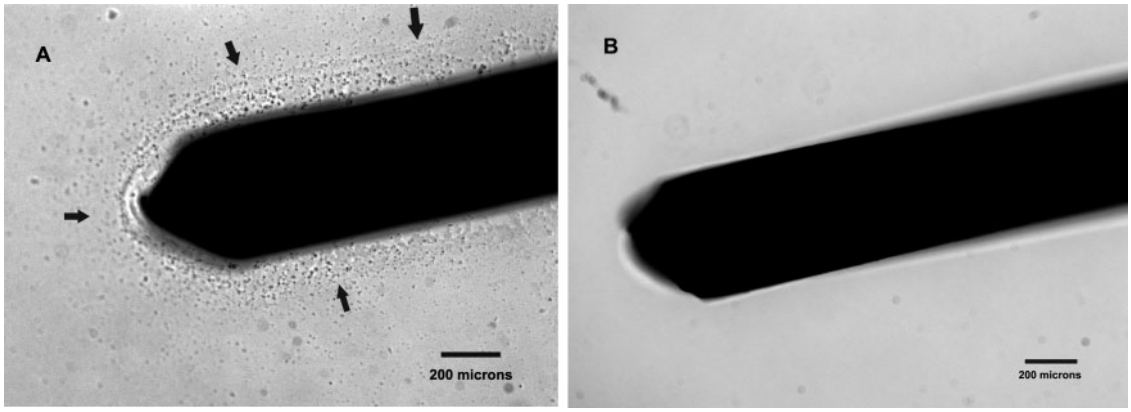

FIGURE 7. Spiroplasma colonies grow into Bacto Schaedler media surrounding nickel wire containing biofilm indicate viability of spiroplasma within the biofilm. **(A)** Nickel wire exposed to scrapie spiroplasma isolate in Brucella media after 11 days incubation is placed on Bacto Schaedler agar plate and incubated for 21 days. Note outgrowth of typical spiroplasma colonies (arrows) from the biofilm on wire into adjacent agar showing the viability of the spiroplasma. **(B)** shows no growth from wire exposed to Brucella media alone. Examinations done by inverted light microscopy.

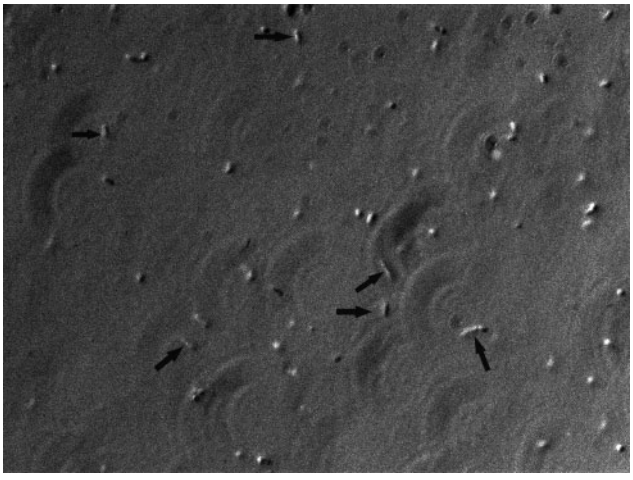

FIGURE 8. Scrapie-associated *Spiroplasma* sp. was reisolated from brain of a sheep inoculated in 2005 experiment with spiroplasma isolate derived from scrapie-affected sheep (courtesy-USDA, Washington State). The isolate consists of motile spiroplasma with numerous coiled forms (arrows) as shown by dark-field microscopy. Original magnification: 1200×.

special showed the consistency of this growth requirement (Table 2). Furthermore, application of this method to archived brain tissues from a prior 2005 study showed reisolation of *Spiroplasma* sp. from both scrapie and CWD-associated spiroplasma inoculated ruminants previously unrecoverable in SP4 media (11). The isolations of unique *Spiroplasma* spp. from several TSE-affected brains and LN in Brucella special media, on Bacto Schaedler agar and on mammalian (12, 13) and mosquito embryo cultures, strongly support the association of spiroplasma infection with the TSEs. The association of spiroplasma infection with the TSEs is not related to immunosuppression secondary to the TSE disease process since scrapie infection does not affect T-cell responses in vitro (24). Additionally, spiroplasma are not considered to be opportunistic organisms in immunosuppressed conditions. Therefore, the consistent recovery of *Spiroplasma* spp. from TSE tissues indicates that this bacterium is likely important in the pathogenesis of TSE diseases.

The revelation why the TSE spiroplasma isolate propagates in Brucella media and not in SP4 media was apparent when these media formulations are compared (Table 3). The sodium bisulfite additive in Brucella special media lowers oxygen tension, therein indicating that *Brucella* spp. (18, 19), *Campylobacter* spp. (19), and now this novel TSE-related *Spiroplasma* sp. require low oxygen tension for growth. The selective nature of oxygen levels for bacterial growth can be dramatic in that *Heliobacter* will not grow in Brucella media supplemented with sodium bisulfite, while they flourish in Brucella media lacking the additive (25). The complex nature of propagation of these microaerophilic microbes is represented by a brief 18-hour survival of *Campylobacter jejeuni* under ambient oxygen conditions but where viability of the bacteria precipitously drops after that time (18). The limited survival of *Campylobacter* spp. in presence of oxygen simulates the transient recovery of the scrapie spiroplasma isolate in SP4 broth. *Campylobacter* sp. survives under aerobic conditions in mixed bacterial infection (18) simulating *Spiroplasma citri* growth enhancement in mixed bacterial infection in insects (26). The absolute requirement of TSE spiroplasma isolates for propagation in growth media with low oxygen tension and the lack of immune cross reactivity with *S. mirum* indicates that these novel TSE-related *Spiroplasma* sp. are unlike any known cultivable spiroplasma (22).

Genome sequencing now possible with cultivation of the TSE spiroplasma isolates in cell free Brucella media will determine whether this spiroplasma is distinct from the *S. mirum* clade (22) and provide the basis for developing a future serological test for TSE. The availability of cell free spiroplasma isolates from TSE-affected tissues grown in large quantities in Brucella media will allow determination whether TSE *Spiroplasma* sp. possess biologic properties similar to the transmissible agent of TSE (9). Planned studies include testing

TABLE 3. Comparison of Formulae of SP4 Media Designed for Culture of *Spiroplasma* spp. and Brucella Special Media Designed for Culture of Fastidious *Brucella* sp. and *Campylobacter* sp

SP4 media formula	Brucella special media formula
Tryptone 10.0 g	Tryptone 10.0 g (Pancreatic digest of casein
Yeast Extract 8.75 g	Yeast Extract 2.0 g
Peptone 5.3 g	Peptone A 10.0g (Peptic digest of animal tissue
PPLO broth base * 3.5 g (beef heart infusion[250 g/L] peptic digest of animal tissue [10.0 g], NaCl [5.0g])	
Glucose 5.0 g	Dextrose 1.0 g
Yeastolate * 2.0 g (autolyzed yeast = peptides, amino acids, carbs, vitamins)	
CMRL 1066 * 0.49 g (Defined media with amino acids)	
Thalium acetate * 0.25 g (suppress gram+ and gram− bacteria)	
	NaCl 5.0 g
	Sodium bisulphite ** 0.1 g
Serum and antibiotics	Serum and antibiotics

The nutrients are similar in both media formulations but the most significant difference is the presence of sodium bisulfite additive in the Brucella broth that reduces the oxygen tension of the media. The low oxygen tension of the media appears to support growth of scrapie and CWD spiroplasma isolates.

resistance of these spiroplasma TSE isolates to environmental challenges such as X-ray radiation and chemicals. Prior studies have already shown that the *S. mirum* laboratory strains are remarkably resistant to glutaraldehyde (21). Experiments using rodent or ruminant animal models are necessary to determine whether experimental inoculation of these TSE spiroplasma isolates into animal models will induce classical clinical and pathological findings of naturally occurring TSE. It is also essential to determine whether TSE spiroplasma isolates will induce prion amyloid tissue deposits in those animal models. It is noteworthy that *S. mirum* induces formation of α-synuclein in tissue culture (27). The formation of an amyloid deposit associated with Parkninson disease in humans by spiroplasma is similar to amyloid formation by other bacteria in vitro (28, 29), which would provide a link to other human neurodegenerative brain diseases and perhaps explain occurrence of combined CJD/Alzheimer disease cases (30).

The discovery that TSE spiroplasma isolates produce typical spiroplasma colonies on Bacto Schaedler agar plates indicate that even at this early stage we have a workable live diagnostic test for TSE. The uniqueness of the colony formation by the TSE spiroplasma isolates on Bacto Schaedler agar plates provides a straightforward way to potentially determine presence of this pathogen in a test sample such as tissues, urine, blood, or feces (16). We have used this method already to show that the CWD spiroplasma isolate is resistant low concentrations of formalin followed by heat designed for killing the bacterium while preserving antigenicity for purpose of preparation of a vaccine. Following this treatment numerous typical spiroplasma colonies were seen on the Bacto Schaedler agar plates inoculated with the formalin-treated CWD-affected LN. The unique preference of the TSE isolates to propagate under low oxygen tension likely enhances biofilm formation by the TSE spiroplasma isolate therein inducing chronicity (31). This property of growth of the TSE spiroplasma isolates in low oxygen would facilitate passage of spiroplasma from the gut through oxygen deficient Peyer's patches and would support potential growth within macrophages and dendritic cells (31) considered essential for transmission of the transmissible agent in naturally occurring TSE infection (9). The ease of isolation of spiroplasma associated with the TSEs in the Brucella special media suggests that prion researchers can now readily retrieve spiroplasma from their experimental TSE specimens, which should result in a needed shift in TSE research direction.

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