2011

Characterization of Spiroplasma mirum and its role in transmissible spongiform encephalopathies

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CHARACTERIZATION OF *SPIROPLASMA MIRUM* AND ITS ROLE IN TRANSMISSIBLE
SPONGIFORM ENCEPHALOPATHIES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of Pathobiological Sciences

by
Hilari French
B.S., Louisiana Tech University, 2006
D.V.M, Louisiana State University, 2009
August 2011

ACKNOWLEDGEMENTS

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ABSTRACT

There are many contradictory reports in the literature involving the culture of *Spiroplasma mirum*, its resistance to disinfectants and antibiotics, and its potential role in Transmissible Spongiform Encephalopathies (TSEs). These contradictions led to an interest in a possible link between spiroplasma and TSEs and the development of a research plan to elucidate this connection.   It was hypothesized that *Spiroplasma* was associated with a neurodegenerative disease such as TSE. In this work we further characterized *S. mirum* laboratory strains and continued to evaluate the possible correlations they have with TSE infections and the prion.

To optimize recovery of *S. mirum* from experimental infections, we established reproducible culture conditions in M1D media, embryonated eggs, and SP4 plates. Overall, results indicated that SP4 plates are the most accurate quantitative method for *S. mirum*. However, plate counts should be accompanied by qualitative assessments of cultures in liquid M1D as well.

A susceptibility profile for physical and environmental disinfectants and antibiotics for spiroplasma was also determined. The three species of *Spiroplasma* tested were susceptible to minimal dilutions of common laboratory disinfectants. They were also susceptible to many of the antibiotics in use for other mollicutes. Although *Spiroplasma* and prions, the presumed infectious agent of TSEs, may both persist in neurologic tissues for extended periods of time, the two do not share the same properties of resistance.

Experimental neonatal goat infections with a *S. mirum* laboratory strain SMCA using three different inoculation routes did not result in pathology, clinical signs, or an immune response over a two year time period. In addition, the organism was not detected via PCR or culture. The same strain did cause minimal clinical signs in one animal when inoculated into five month old white-tailed deer and was recovered from the cortex of the clinically-affected deer after multiple passages in culture. Although the goat experiments did not result in TSE-like disease, an improved methodology for a spiroplasmosis animal model was developed, and further research should be conducted using similar methods in neonatal white-tailed deer.

CHAPTER 1: INTRODUCTION/ REVIEW OF LITERATURE

Introduction

Mollicutes are the smallest known self-replicating organisms. Classified by their genome size, sterol requirement, morphology, urea utilization, and other biochemical properties, they are divided into five genera: *Acholeplasma*, *Anaeroplasma*, *Mycoplasma*, *Spiroplasma* and *Ureaplasma* (Murray and Schleifer, 1994). This group of organisms is believed to have resulted from degenerative evolution of Gram-positive bacteria (Davis et al., 1972; Woese et al., 1980). The members of the genus *Spiroplasma* were first recognized as helical, wall less bacteria in 1972 (Davis et al., 1972). Taxonomically, spiroplasmas are in the Domain Bacteria, Phylum *Firmicutes*, Class *Mollicutes*, Order *Entomoplasmatales*, Family *Sprioplasmataceae*, and Genus *Spiroplasma*. They received their name from a description of their motility in 1973 (Davis et al., 1972). The first named spiroplasma was *Spiroplasma citri*, the causative agent of citrus stubborn disease, which was originally considered a virus (Saglio et al., 1973). The Suckling Mouse Cataract Agent (SMCA), isolated from rabbit ticks, was originally considered a virus because it was filtered through such a small pore size (Clark, 1964). Spiroplasmas have also been misidentified as spirochetes because of their similar appearance in dark field microscopy. The sex ratio organism, SRO, which causes a disease fatal to only males of certain *Drosophila* species, was originally considered a spirochete in 1961 but was later identified as a spiroplasma (Williamson et al., 1983).

Spiroplasma were classified as Mollicutes (renamed from mycoplasma) because they form "fried egg colonies" on solid media, pass through 0.22µm pore filters, fail to revert to walled bacteria, are completely resistant to penicillin, and lack cell wall or murien cell wall precursors (Bove, 1984a; Saglio et al., 1973). When first defined, spiroplasmas were classified by G+C content, their ability to utilize glucose and arginine, their host/habitat, serological tests, metabolism inhibition, and spiroplasma deformation tests (Whitcomb, 1981). As more isolates were characterized, serological cross reactions were helpful in establishing groupings; however, the results needed to be duplicated in the deformation and metabolism inhibition tests in order to fully establish a connection within a group (Whitcomb et al., 1983). Williams, et al. suggested that as a last criterion, DNA-DNA homology should be used to determine an isolate's grouping into a single species (Davis et al., 1979; Williamson and Tully, 1982; Williamson et al., 1998). In 2007 the requirements were made more stringent to prevent inaccurate classifications. Currently the major requirements for placement to order and family include: tests for sterol requirements, lack of cell wall as shown by transmission electron microscopy, ability to form colonies on solid media, quantification of the ability to pass a graded series of ultra filters, demonstration of the ability of the organism to grow at various temperatures with determination of an approximate optimal temperature, determination of the base composition of organismal DNA, and demonstration of antigenic relationships by using the spiroplasma deformation and metabolism inhibition tests (Whitcomb, 2007). To date, *Spiroplasmas* have been classified into 34 groups and 14 subgroups (Padovan et al., 2000 ; Pettersson et al., 1996; Williamson et al., 1998). Thirty-six representative members of these groups or subgroups have been described as species.

Spiroplasmas have a variety of hosts including plants and arthropods. There are three pathogenic plant spiroplasmas: *S. citri, S. kunkelii* and *S. phoeniceum,* which are all found within group I. These plant pathogens are most commonly found within plant phloem and flower nectar. *Spiroplasma melliferum,* a honeybee pathogen not pathogenic to plants, is also a member of group I (subgroup 1-2) and is closely related to the three pathogenic plant spiroplasmas. The other four spiroplasmas in this group are either plant surface- or arthropod-associated organisms. Plant surface spiroplasmas are thought to be deposited by insects, which are the major hosts of members of groups II to XXXIV (Williamson et al., 1998). Spiroplasmas residing in arthropods are transmitted to plant surfaces by defecation and/or regurgitation of fluids on plant surfaces or may be introduced into the phloem by plant-sucking insects (Gasparich, 2002).

All members of the genus *Spiroplasma* are obligately associated with insects, either as commensals, pathogens or mutualists (Gasparich, 2002). Most insect spiroplasmas are found within the following orders: Hymenoptera (bees and wasps), Diptera (flies, horseflies, fruit flies, mosquitoes), Coleoptera (beetles), Lepidoptera (butterflies), Homoptera (leafhoppers), Hemiptera (green leaf bugs), or Odonta (dragonflies) (Tully and Whitcomb, 1991; Williamson et al., 1997). Spiroplasmas are most commonly found in the gut, less frequently in the hemolymph, and occasionally in salivary glands and other organs (Bove, 1997). In some insects, spiroplasmas that are ingested may be highly invasive and pathogenic or lethal to the host. In addition to *S. melliferum,* lethal infections in honeybees were also observed following exposure to *S. apis* (Tully and Whitcomb, 1991). *S. poulsonii* is associated with sex ratio abnormalities in the progeny of *Drosophila* spp. (Williamson et al., 1999), and *S. floricola* is associated with a lethargy disease of the scarabaeid beetle *Melolontha melolontha* (Seemuller et al., 2002). Spiroplasmas were also isolated from blood sucking insects such as mosquitoes and tabanid flies (Tully and Whitcomb, 1991; Williamson et al., 1998).

Spiroplasmas have also been isolated from ticks and rabbit ticks have yielded two spiroplasmas: *S. mirum* (SMCA, GT48, and TP-2) and *Spiroplasma* sp. strain 277F (Tully et al., 1982b). SMCA causes cataracts and persistent neurological infections in a variety of small mammals when inoculated intracerebrally (Clark, 1964). GT48 causes encephalitis when inoculated intracerebrally and cataracts in experimental intraperitoneal inoculated rat infections (Bastian et al., 1984; Tully et al., 1984). *Spiroplasma sp*. strain 277F caused mortality within three days after intracerebral inoculation in suckling rats. Similar experiments with diluted cultures did not produce any clinical or pathological changes. Strain 277F was not recovered from any experimental animals (Stalheim et al., 1978). Eight serologically related isolates were also isolated from an ixodes tick. A representative strain Y32 was characterized and designated *S. ixodetis* (Tully et al., 1995).

Recently a spiroplasma has been implicated as the causative agent of tremor disease (TD) that affects the central and surrounding nerve systems of the Chinese mitten crabs, *Eriocheir sinensis* (Wang et al., 2004). This disease has had disastrous effects on aquaculture in China (Wei, 1999). Based on morphological and pathological studies (Wang and Gu, 2002; Wang et al., 2002; Zhang et al., 2000), the TD agent (TDA) was previously thought to be a rickettsia-like organism. Analysis using universal primers for bacterial 16S rRNA gene sequences, however, showed that the agent was not a rickettsia but a spiroplasma, showing 98% 16S rRNA gene sequence similarity to *S. mirum* (Wang et al., 2003; Wang et al., 2004). Spiroplasma has also been isolated

from the American crayfish (*Procambarus clarkii*) and freshwater-cultured Pacific white shrimp (*Litopenaeus vannamei*) in provinces of China (Wang et al., 2005). It was also associated with mortalities in Pacific white shrimp of South America (Nunan et al., 2005; Nunan et al., 2004). The report of a spiroplasma in a fresh water species has changed the understanding of this organisms host range (Regassa and Gasparich, 2006).

Spiroplasma is a difficult organism to cultivate and isolate, adding to the classification difficulties. Initially media was made for cultivation of spiroplasma with ingredients similar to plant phloem (Saglio et al., 1973). As more spiroplasmas were identified from different hosts, media began to reflect insect hemolymph (Tully et al., 1977). Most medias now contain 10-20% fetal bovine serum, PPLO broth base and/or peptone and tryptone supplements, tissue culture medium such as CMRL-1066 or Schneider's Drosophila media, carbohydrates, fresh yeast extract, and yeastolate (Whitcomb, 1981).

The first formulation for *Spiroplasma* growth was similar to mycoplasma media with the addition of soribitol, fructose, sucrose and DNA (Whitcomb, 1981). Researchers were able to characterize *S. citri* using mycoplasma media but ran into difficulties characterizing a similar spiroplasma, the corn stunt spiroplasma (Saglio et al., 1971; Saglio et al., 1973). This led to the formation of more complex media. M1 medium was designed to be a very rich media and with slight variations has supported all spiroplasmas to date. The SP-4 medium used CMRL-1066 tissue culture supplement and was hypothesized to be best for those Spiroplasma with possible vertebrate affinities (Whitcomb, 1981). This formulation was used to cultivate SMCA (Tully et al., 1976). Table 1.1 lists the ingredients found in the most common spiroplasma media. Mycoplasma medium was originally used for characterizing spiroplasma isolates. As research developed, M1A use resulted in better growth for insect/flower isolates. The addition of yeast extract was made for M1D media, which allowed for better growth for some spiroplasma isolates and SP4 was developed for the tick isolates.

The literature has ample evidence that spiroplasma may be isolated from a variety of different hosts. However, the isolation methods are tedious depending on the inoculum (Whitcomb, 1981). Isolating the corn stunt spiroplasma (CSS) required blind passage of sap-inoculated cultures in contrast to easy primary isolation from infected maize (Jones et al., 1977). The formulation of media also proved to be a critical factor in primary isolation. Jones demonstrated that components of M1A medium are factors that allow for primary isolation of CSS from insect hemolymph in contrast to attempts with SP-4 (Jones et al., 1977). Similarly, the isolation of SMCA was more readily observed in SP-4 rather than M1A medium (Tully et al., 1981). Research showed that different formulations are best for isolation and maintenance (Chen and Davis, 1979; Chen and Granados, 1970). SRO is easily maintained in M1A, but attempts at primary isolation in the same media were futile (Williamson and Poulson, 1979).

The research presented throughout this work will focus on *S. citri*, *S. mirum*, and *S. melliferum*. *Spiroplasma citri*, a flower pathogen, was the first spiroplasma identified and fully characterized. *Spiroplasma mirum*, isolated from ticks, was shown to be pathogenic to vertebrates. *Spiroplasma melliferum*, an insect pathogen, is associated with pathology leading to death in honey bees and has also been shown to multiply in vertebrates (LeGoff and Humphery-Smith,

1991). Table 1.2 summarizes the host and pathology seen with these three species (Williamson et al., 1998).

Table 1.1 Spiroplasma media (yields 300 mLs)

Ingredients	Standard Mycoplasma Medium	M1A	M1D	SP4
Autoclave at 15psi for 30min				
Mycoplasma broth base	4.5 g	2.1 g		1 g
Bacto-Tryptone		1 g		3 g
Bacto-Peptone		0.8 g		1.6 g
Glucose		0.1 g		
Fructose		0.1 g		
Sucrose		1 g		
Sorbitol		7 g		
Distilled water	200 mL	70 mL		197 mL
For solid media	**Add 1.6 g Difco Noble Agar to autoclavable portion**	**Add 2.4 g to 6.8 g Difco Noble agar to autoclavable portion**		
Sterile filter				
Schneiders Drosophilia medium		160 mL		
CMRL-1066 (10x w/glutamine, w/out NaHCO3)				15 mL
Fetal Bovine Serum		50 mL		50 mL
Horse Serum	60 mL			
Fresh yeast extract (25%)	30 mL		10 mL	30 mL
Phenol Red 0.5%	(0.1 %) 6mL	1.2 mL		1.2 mL
Potassium Penicillin (100,000 U/ml)	1.5 mL	2.5 mL		3 mL
Yeastolate 2%				30 mL
Glucose (50% solution)	3 mL			3 mL
L-arginine-HCl 42%	1.5 mL			

Table 1.2 Summary of differences seen in separate species of *Spiroplasmas*.

Identification Date	Organism	Group	Isolation method	Media	Pathology
1973	*S. citri*	I-1	Mycoplasma broth	M1D	Citrus stubborn disease
1982	*S. mirum*	V	Embryonated eggs	SP4	Cataracts in suckling mice, spongiform encephalopathy
1985	*S. melliferum*	I-2	M1D broth	M1D	Death in honey bees

Spiroplasma citri

Host(s): *Spiroplasma citri*, known for its economic impact on citrus plants as the causative agent of citrus stubborn disease, can infect a wide variety of woody and herbaceous hosts (Whitcomb, 1980). It is restricted to the phloem sieve tubes and is transmitted by phloem sap-feeding insects (Bove et al., 2003). *Spiroplasma citri* is naturally transmitted by leafhoppers*: Circulifer tenellus*, *Scaphytopius nitrides and S. acutus delongi* in California (Oldfield, 1988); *Neoaliturus haemactoceps* (Bove et al., 1987) and *C. tenellus* (Klein et al., 1988) in the Mediterranean area. Other insects may acquire the spiroplasma but do not transmit it (Bove and Saillard, 1979; Rana et al., 1975).   *Spiroplasma citri* is dependent on insect vectors for transmission and therefore the distribution follows the patterns of the leafhoppers' movements (Golino and Oldfield, 1990).

Isolation: *Spiroplasma citri* was isolated in 1971 from a Washington naval sweet orange tree afflicted with stubborn disease (Bove and Saillard, 1979). Stubborn disease was described in 1944, and the causative agent was assumed to be a virus until this observation (Bove, 1984b).

Media: *Spiroplasma citri* was originally isolated in a moderately simple mycoplasma medium (see Table 1.1) (Whitcomb, 1983). It is now most commonly cultivated in M1D medium. It has a temperature range of 20^oC - 37^oC with 32^oC being optimal and a 4.1 hour doubling time (Konai et al., 1996).

Disease/Pathology: In the leafhopper, *S. citri* adheres to receptors within the gut and are taken into the cytoplasm by endocytosis.   After migrating through the cell, it is released by exocytosis into the hemolymph, where it circulates and replicates. *Spiroplasma citri* is then transported from the hemolymph to the saliva by passing through the basal lamina and adhering to receptors on the plasmalemma outer surface, after which the spiroplasma are endocytosed, passed through the cell, and exocytosed into the salivary ducts. From the ducts, they are introduced into the phloem of plants with the saliva of feeding leafhoppers. The latency period in the insect is at least two weeks (Liu et al., 1983).

Once in the plants, *S. citri* remains restricted to the phloem tissue where it spreads in the plant. It causes severe symptoms, such as stunting, yellowing of leaves, and yield losses, in a variety of economically important crops: grapefruits, lemons, mandarins, and oranges (Calavan, 1980). The spiroplasma develops best in citrus under hot conditions (28^oC - 32^oC) and may not give rise to symptoms at lower temperatures (Bove, 1988).   These organisms are not transmitted via plant seeds and are not transovarially transmitted to next-generation leafhoppers. Therefore, they cannot survive outside their hosts and are dependent upon transmission for survival and dispersal (Gasparich, 2002).

Other information: *Spiroplasma citri* utilizes three sugars: glucose and fructose, presumably from the sieve tubes of the plant, and trehalose from the insect hemolymph (Bove et al., 2003). At least two mechanisms are utilized for sugar uptake by *S. citri*: the phosphoenolpyruvate:phosphotransferase system (PTS) and the SBP-ABC transporter system (Bai and Hogenhout, 2002; Gaurivaud et al., 2000a). This could be characteristic of multiple-host bacteria that need to rapidly adapt from a host or host-compartment that uses one sugar to a host or host-compartment that uses a different sugar (Bove et al., 2003). The identification of

these systems within the spiroplasma genome may serve to determine the different hosts that other isolates might inhabit.

Spiroplasma melliferum

Host(s): *Spiroplasma melliferum,* the first spiroplasma to be isolated from an insect, was originally isolated from honey bees in Maryland in 1976 (Whitcomb, 1980). The organism has also been isolated from bumble bees, andrenids, anthophors, robber flies, and one butterfly species (Clark et al., 1985).

Isolation: In 1976, Clark isolated *S. melliferum* from diseased honey bees, *Apis mellifera,* in Maryland (Clark, 1978) in M1D.

Media: *Spiroplasma melliferum* growth is supported well in M1D medium. It has a temperature range of 20 - 41°C with 37°C being its optimal temperature with a 1.5 hour doubling time (Konai et al., 1996).

Disease/Pathology: *Spiroplasma melliferum* is able to cross the gut barrier of the honey bee, replicates at high numbers within the hemolymph and eventually kills its host (Bove, 1997). *Spiroplasma melliferum*-infected bees frequently exhibit neurological signs (Clark, 1978). It can be found on flower surfaces that are frequented by infected honey bees (Whitcomb, 1981) but only causes fatality in the bee.

Spiroplasma melliferum also persists in suckling mice post-intracerebral inoculation. Spiroplasma was found within the brain at high titers at 70 days post-inoculation and had limited recovery 285 days post inoculation (LeGoff and Humphery-Smith, 1991).

Spiroplasma mirum

Host(s): *Spiroplasma mirum* was isolated from *Haemaphysalis leporis-palustris*, a rabbit tick (Clark, 1964). Experimental inoculation of honey bees, mice, rats, and chicks all showed evidence of a persistent infection (Bastian et al., 1984; Clark, 1964; Clark, 1969; Clark, 1974; Kirchhoff et al., 1981; Tully et al., 1984)

Isolation: The Suckling Mouse Cataract Agent (SMCA) was originally isolated from a small pool of rabbit ticks removed from a rabbit found dead on the road in Georgia in 1962. Researchers were searching for rickettsiae in wild animals when they came across this organism. SMCA was lethal to embryonated eggs but was nonculturable on a variety of bacterial or mycoplasmal media. Due to its resistance to penicillin and streptomycin and its filterability through a 220nm pore, it was assumed to be a virus (Clark, 1964). In 1973 it was described to have mycoplasma-like characteristics when seen via electron microscopy (Zeigel and Clark, 1974).

Strain GT48 was also isolated from a pool of rabbit ticks collected in Georgia in 1962, hence its name Georgia Tick 48 (Kotani et al., 1986). It was determined that GT48 was antigenically indistinguishable from SMCA. This organism was also lethal to embryonated eggs, exhibited a

similar host range in experimentally infected animals, and had similar morphology and physicochemical characteristics as SMCA (Tully et al., 1984; Zeigel and Clark, 1974).

Media: SMCA and GT48 are usually cultured in SP4 medium; however, most laboratory strains adapt well to M1D also (Regassa and Gasparich, 2006). Growth occurs from 25°C - 41°C with the optimal temperature being 37°C. *S. mirum* does represent the more fastidious spiroplasmas and, even at its optimal temperature, has a doubling time of 8 hours (Konai et al., 1996).

Due to its fastidious nature, attempts were made to cultivate *S. mirum* in a variety of mammalian, avian and tick cell cultures but were unsuccessful due to cytopathic effects and slow growth from the organism (Bastardo et al., 1974; Clark, 1964; Clark, 1974; Fabiyi et al., 1971; Yunker et al., 1987). Chick embryo chorioallantoic membrane and yolk sac fragment cultures failed to support growth (Clark, 1964). Successful cultures were made in a number of mosquito cell lines, and the growth was equivalent to that seen in SP4 media with minimal cytopathic effects (Yunker et al., 1987). *Spiroplasma mirum* was also able to grow in Drosophila Dm-1 cells, creating a chronic infection when maintained at 25ºC but showed cytopathic effects at 30ºC (Steiner et al., 1982). SMCA was also found to permanently transform NIH 3T3 mouse embryo fibroblast cells. Transformed 3T3 cells exhibited loss of contact inhibition, aggregating into one foci on the monolayer (Bove, 1997; Kotani et al., 1986). SMCA also transformed monkey kidney CV-1 cells (Kotani et al., 1986). Due to the formation of cataracts in suckling mice, Megraud et al. experimented with ocular tissues and were able to establish spiroplasma growth on rabbit lens cultures that were comparable to the growth seen in SP4 media (Megraud et al., 1983).

Disease/Pathology: *Spiroplasma mirum* is the only species found to be pathogenic to vertebrates (Bove, 1997). As the name describes, SMCA, when inoculated intracerebrally, caused suckling mice to develop cataracts (Clark, 1964). The organism was able to persist in suckling mice, specifically the brain tissues, for up to two years (Clark and Karson, 1968). Due to this observation, it was tentatively included in the slow viruses or chronic infectious neuropathic agents (Hotchin, 1967). When inoculated into suckling rats, 70% developed cataracts and 36% developed mild to severe hydrocephalus (Elizan et al., 1972). The organism causes death between four and seven days post inoculation in seven day old embryonated eggs (Clark, 1964). In one day old chicks inoculated intracerebrally or subcutaneously, the organism caused death within five days (Clark, 1974). When inoculated intracerebrally into day old hamsters, CNS signs and severe weight loss were observed (Kirchhoff et al., 1981). Death and severe microphthalmia were seen in rabbits inoculated intracerebrally less than twenty-four hours after birth. No clinical signs were seen when *S. mirum* was inoculated in adult mice, roosters, hamsters, rabbits, guinea pigs, or rhesus monkeys (Clark, 1974).

Strain GT48 induces fatal encephalitis when inoculated intracerebrally into newborn mice and rats (Clark, 1964). Suckling mice did not develop cataracts after inoculation with GT48 at even low doses (Clark and Karson, 1968). Suckling rats developed cataracts as well as severe weight loss after intraperitoneal or subcutaneous inoculation with GT48 (Bastian et al., 1987b). Strain GT48 also causes death in seven day old embryonated eggs, although the deaths are more sporadic and over a greater range of days (Clark, 1964). Table 1.3 summarizes clinical findings found post *S. mirum* inoculation.

Transmission: *Spiroplasma mirum* has only been isolated naturally from the rabbit tick. The true pathogenicity has been questioned since the organism has only experimentally induced disease either by inoculation into the yolk sac of seven day old chick embryos; intracerebral injection into newborn rats, mice or hamsters; or by inoculation into the scarified cornea of adult rabbits (Bove, 1997). The fastidious nature of the organism makes primary isolation challenging and leaves researchers questioning other possible hosts.

Other information: Its persistence for long periods of time within the brains of experimentally inoculated animals led researchers to presume that SMCA should be categorized as "slow virus." This family included the infectious agent for scrapie in sheep and the agent of Kuru in man (Schwartz and Elizan, 1972).   Tully suggested that this organism may be related to the causative agent of scrapie which had not yet been identified (Tully et al., 1976). In 1979 Bastian reported spiral membranous inclusions seen by electron microscopy in brain biopsy tissues from a patient with Creutzfeldt-Jakob disease (CJD)(Bastian, 1979). Following this initial finding of spiroplasma in CJD tissues, Reyes and Hoenig (Reyes and Hoenig, 1981) published two case reports of patients that were suspected of CJD to have similar inclusions. Electron microscopy revealed numerous membrane-bound vacuoles within the cell bodies of neurons and astrocytes. Elongated, spiral, membranous inclusions were occasionally present within cortical cell processes, as well as in synaptic terminals. Reyes also reported successful transmission of CJD to spider monkeys from portions of brain tissues from one of these reported patients. In 1980 Gray reported a case of CJD to have spiral membranous inclusions, indicative of spiroplasma, with five to eight twists seen via electron microscopy (Gray et al., 1980). In 1981 Bastian reported two more cases of CJD with similar spiral-like inclusions seen through electron microscopy (Bastian et al., 1981).

After these case findings, more research was committed to spiroplasma in association with CJD cases. Since morphological evidence was all that suggested an association between spiroplasma and CJD, other techniques, namely cultivation of the spiroplasma and serological tests, were used to identify spiroplasmas from confirmed cases of CJD. These techniques failed to detect the organism. No spiroplasma or other mollicutes were cultivated in SP4 medium from brain tissue in eighteen cases, and no antibodies to several recognized *Spiroplasma* spp., including *S. mirum,* were detected in sera from fifteen patients by immunofluorescence and metabolism inhibition tests.(Leach et al., 1983)

In 1988 Humphery-Smith and Chastel proposed that the spiral-like inclusions seen in Gray and Reyes' cases were actually crystalline artifacts (Humphery-Smith and Chastel, 1988). Connolly reported a number of unidentifiable structures in a CJD case via electron microscopy including

Table 1.3 Summary of *Spiroplasma mirum* experimentally infected animals

Species	Age	Route	Death	Disease	Persistence of organism	Ref
Chicks	<24 h	IC	4 days pi	Yellow livers and swollen spleens	NT	(Clark, 1974)
	<24 h	SQ	5-6 days pi		NT	(Clark, 1974)
Mice	<96 h	IC	Rarely	Cataracts [1] Stunting Death	827 days	(Clark and Karson, 1968)
	>5 days	IC	No	None	133 days	(Clark and Karson, 1968)
	<96 h	IP	No	None	9 days [2]	(Clark and Karson, 1968)
	<96 h	IV	No	None	NT	(Clark and Karson, 1968)
	Adult	IC	No	None	497 days	(Clark and Karson, 1968)
		IV, IN, IP, cornea			NT	
Rats	<96 h	IC	Yes	Cataracts [3]	60 days	(Clark, 1974)
	<24 h	SQ/IP[4]	No	Cataracts Weight loss Alopecia	50 days	(Bastian et al., 1984) (Bastian et al., 1987b)
Deer Mouse	Adult	SQ	No	None	NT	(Clark and Karson, 1968)
Roosters	Adult	IM	No	None	NT	(Clark, 1974)
Hamsters	<24 h	IC	Variable	CNS signs Dramatic loss of weight Runting	NT	(Kirchhoff et al., 1981)
	Adult	IV	No	None	NT	(Clark, 1974)
Rabbits	<24 h	IC	12 days	Death Severe microphthalmia	12 days	(Kirchhoff et al., 1981)
	Adult	IV, SQ	No	None	NT	(Kirchhoff et al., 1981)
		Scarified cornea	No	Ocular lesion	AB negative	(Clark, 1974)
Guinea Pigs	Adult	IP, IM, SQ, cornea	No	None	NT	(Clark, 1974)
Box Turtle	Adult	IP	No	None	NT	(Clark and Karson, 1968)
Opossum	Adult	IP	No	None	NT	(Clark and Karson, 1968)
Rhesus Monkey	Adult	IV, SQ, cornea	No	None	NT	(Clark, 1974)

IC: Intracereberal, SQ: Subcutaneous, IP: Intraperitoneal, IV: Intravenous, IN: Intranasal, IM: Intramuscular, NT: Not tested

1 Cataracts were the most common clinical manifestation of SMCA inoculation

2 Organism was not recovered from brain or eye tissues but was found in liver-spleen suspension

3 Cataracts in suckling rats were more severe with a higher inoculation levels

4 Rats were inoculated with GT48

one which was tightly coiled, intranuclear, and which measured 570nm in length by 45 nm in width, but specified that no spiroplasma-like shapes were seen (Connolly et al., 1988).

Further research was conducted to show similarities between the transmissible agent of CJD and spiroplasma. Bastian reported that the tissue response to experimental spiroplasma infection in the suckling rat brain resembles the spongiform degenerative brain diseases of both man and other mammals. The degenerative changes are localized to the gray matter, as seen in CJD cases (Bastian and Jennings, 1984). The optimal growth temperature for *S. mirum* and other spiroplasma isolates was 37°C, suggesting that spiroplasmas could be found in a mammalian host (Bastian and Jennings, 1984; Konai et al., 1996). The organism's proven ability to produce an acute or persistent brain infection in small mammals was also used as evidence. Subsequent studies have shown some resistance of spiroplasma organisms to chemical factors with some resemblance to the resistance exhibited by the transmissible agent of CJD (Bastian and Fermin, 2005). Bastian concluded that the size and membranous nature of spiroplasma organisms would best fit with the data regarding the elusive CJD agent (Bastian et al., 1984).

In 1987 a scrapie-associated fibril was identified as a consistent finding and possible infectious unit of the transmissible spongiform encephalopathies. Cross reactions with this fibril to *S. mirum* fibril proteins supported evidence of an association between spiroplasma and TSEs (Bastian et al., 1987a). In 2001 Bastian and Foster (Bastian and Foster, 2001) reported the presence of spiroplasma ribosomal DNA in CJD and scrapie-infected brains. In 2004 eight of ten scrapie infected brains, five of seven CWD infected brains, and two of two CJD brains were found to be positive for *S. mirum* via southern blotting of PCR products (Bastian et al., 2004). PCR products obtained from the scrapie, CWD, and CJD cases had 99% homology with published *S. mirum* 16S rDNA sequences (Bastian et al., 2004). In this study, it was reported that the mode of DNA extraction was critical. The most reliable method involved initial solubilization with guanidine thiocyanate followed by phenol/chloroform extraction (Bastian et al., 2004). Southern blotting was also acknowledged as the more sensitive method of detection, allowing detection of spiroplasma DNA where PCR product was not readily apparent (Bastian et al., 2004). This study is in contrast to the 2006 study of experimentally-infected scrapie hamsters that found no evidence of bacterial 16S rRNA (Alexeeva et al., 2006). It should be noted that Bastian's tissues were all from naturally infected animals, and those in Alexeeva's study were experimentally infected.

In 2005 Bastian et al. (Bastian et al., 2005) isolated a spiroplasma from homogenates of CWD and scrapie infected brains. The brains were homogenized in SP4 media and then inoculated into eight day old embryonated eggs. Allantoic fluids from the inoculated eggs were then passaged into SP4 media and incubated for sixteen days at 37ºC. Evidence of spiroplasma growth was found via phase microscopy, electron microscopy, and PCR. DNA sequence analysis showed 98% homology with *S. mirum* but revealed nucleotide substitutions unlike any known spiroplasma strain. The isolates from this experiment were later inoculated into neonatal goats and sheep (Bastian et al., 2007). No clinical signs were seen, but all brains had evidence of spongiform changes. At eleven months post inoculation, one goat and one sheep had severe cerebella and hippocampal spongiform degeneration with neuronal vacuolization in the brain stem. All other

animals were reported to have spongiform changes to a lesser degree. It was noted that the intraneuronal vacuoles in the cerebellar cortex and brain stem seen in the *Spiroplasma* spp. inoculated ruminants were identical to lesions typical of naturally occurring TSE (Bastian et al., 2007).

In 2007 Bastian et al. induced spongiform encephalopathy in neonatal deer inoculated intracerebrally with SMCA. Three of four deer developed clinical signs of neurological deterioration between 1.5 months and 5.5 months post inoculation. One deer brain showed signs of hydrocephalus, while all others were grossly normal. Histological sections of all deer showed spongiform encephalopathy in the cerebral cortex, cerebellar cortex, and the brain stem. Severity of neuropathology was noted as incubation time increased. In the same publication, sheep were inoculated intracerebrally with varying dilutions of SMCA. No clinical signs were noted, but spongiform encephalopathy was seen histologically. Severity of lesions was decreased as the dosage of the inoculum decreased.   Neonatal goats were also inoculated intracerebrally with SMCA. After five months no clinical signs were seen and only minimal vacuolization was noted histologically (Bastian et al., 2007).

In another study, *S. mirum* failed to induce spongiform encephalopathy when experimentally inoculated into 5.5 month old raccoons. Raccoons are used as an experimental model for transmissible mink encephalopathy (TME). In this study, one group of raccoons was inoculated intracerebrally with low doses of SMCA and another group was inoculated intracerebrally with high doses of GT48. The strains of *S. mirum* used in this study were adapted to growth at 31ºC with minimal passages in artificial media. None of the raccoons had any clinical signs and no significant histological changes could be found >900 days later (Hamir et al., 2011).

Most recently, a spiroplasma isolate has been recovered from ocular fluid taken aseptically from scrapie-infected sheep. Minced corneal tissues were inoculated into M1D, and spiroplasma organisms were evident via dark field microscopy. These organisms did not propagate and the culture was lost after several passages (Bastian et al., 2011). This has recently been repeated and efforts are ongoing to passage the spiroplasma to a substantial culture (data not published).

Transmissible Spongiform Encephalopathies

Transmissible Spongiform Encephalopathies (TSEs) encompass a condition that affects humans and a variety of animals with a fatal neurodegenerative disease and associated neurological clinical signs. The disease presents with morphological and pathophysiological features that parallel other progressive encephalopathies such as Alzheimer's and Parkinson's diseases (Aguzzi and Haase, 2003). Research currently supports evidence that the agent responsible for TSE is a prion, a small proteinaceous infectious particle (Prusiner, 1982). The prion is a cell surface glycoprotein (Stahl et al., 1987) with an unknown function. The pathogenesis for the prion is still widely unknown; however it is accepted that the normal prion PrP^C undergoes some sort of posttranslational modification that converts it to a pathogenic prion PrP^{SC}. PrP^{SC} is an insoluble and partially protease-resistant isoform that propagates itself by imposing its abnormal conformation onto other PrP^C molecules (Aguzzi and Calella, 2009). The

human prion diseases include Kuru, CJD, variant CJD, Gerstmann-Straussler-Scheinker (GSS) disease, and fatal familial insomnia (FFI). The most common animal TSEs are scrapie, which affects sheep and goats; bovine spongiform encephalopathy (BSE); and chronic wasting disease (CWD), which affects deer and elk.

Scrapie, the TSE disease of sheep and goats, was characterized as early as 1738 (Parry, 1983). The etiology of the disease can be traced back to a vaccination of sheep for louping-ill virus with formalin-treated extracts of ovine lymphoid tissues that were unknowingly contaminated with scrapie prions (Gordon, 1946). In 1998 atypical scrapie was described in a herd of Norway sheep. Following this disease characterization, a more active surveillance program was instituted in European countries and far more cases of atypical scrapie were reported (A Buschmann, 2004; SJ Everest, 2006). Scrapie and atypical scrapie can be easily transmitted through flocks, but the mechanism for disease spread is still not understood (Aguzzi and Calella, 2009).

In 1986 an epidemic of BSE or "mad cow" disease occurred in Great Britain (Westaway et al., 1987). The source of this disease spread was traced back to the meat and bone meal nutritional supplements used in cattle feed (Wilesmith et al., 1992). The BSE epidemic reached its peak in 1992 with over 35,000 cattle affected. Cases of BSE are now rarely reported. Brain extracts of BSE cattle have transmitted disease to mice, cattle, sheep, pigs, and non human primate marmoset after intracerebral inoculation (Baker et al., 1993; Bruce, 1993; Dawson et al., 1990a; Dawson et al., 1990b; Fraser et al., 1988).

Chronic wasting disease (CWD), the TSE affecting deer and elk, was initially reported in a captive research herd of deer in Colorado (Williams and Young, 1980). Cases of CWD have since been detected in free-ranging mule deer (*Odoncoileus hemionus*), white-tailed deer (*O. virginanus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) in areas close to Colorado (Nebraska, New Mexico, and Utah) but also in distant states (Wisconsin, Illinois, Canada) (Aguzzi and Calella, 2009). Research indicates that CWD is easily transmitted among cervids through grazing in areas contaminated with prion-infected secretions, excretions, tissues or decomposed carcasses (Miller and Williams, 2003; Miller et al., 2004).

The human prion diseases have been classified as infectious, inherited, or sporadic, depending on the clinical, genetic, and neuropathological findings. The clinical signs include progressive dementia, myoclonus, visual or cerebellar impairment, pyramidal/extrapyramidal signs, and akinetic mutism. With the exception of one case, all reported CJD cases have been in people over 40 (Packer et al., 1980). The first documented case of iatrogenic prion transmission occurred in 1974 from a corneal transplantation from a CJD patient (Duffy et al., 1974). Iatrogenic transmission has also been reported after neurosurgery with contaminated instruments (Bernoulli et al., 1977; Davanipour et al., 1984; Masters and Richardson, 1978), human growth hormone (Brown et al., 1985; Buchanan et al., 1991; Fradkin et al., 1991), and human pituitary gonadotropin (Healy and Evans, 1993). Familial TSEs were characterized after discovery of an autosomal dominant PRNP gene alteration, the gene encoding cellular PrP. The three familial TSEs can be described clinically as fCJD: rapid progressive dementia with myoclonus and pseudoperiodic discharges of electroencephalogram; GSS: slow progression of ataxia followed by later onset dementia; FFI: refractory insomnia,

hallucinations, dysautonomia, and motor signs. Sporadic CJD (sCJD) accounts for 85% of all human prion diseases (Aguzzi and Calella, 2009). With sCJD there is no association with mutant PRNP allele or any epidemiological evidence for TSE agent exposure. New variant CJD (vCJD) was first described in 1996 and has been linked to BSE (Will and Zeidler, 1996). New variant CJD is clinically characterized by psychiatric abnormalities, sensory symptoms, and ataxia (Aguzzi and Calella, 2009). It is distinguished by the abnormal age of onset (19y -39y) as opposed to sCJD (55y-70y) and its shorter duration of illness.

Shortcomings to the Prion Theory

Opponents of the prion theory have argued that a protein alone cannot account for the pathology caused in TSE diseases. Studies have shown that neuropathology and clinical signs can be transmitted without the presence of the pathologic prion (Manuelidis and Fritch, 1996; Sklaviadis et al., 1989). Manuelidis (Manuelidis and Fritch 1996) experimentally infected thirty mice with BSE-infected cattle brain homogenates. All of the mice exhibited clinical signs, but 55% of the mice had no detectable abnormal prion PrP^{res} accumulation. Brain homogenates from PrP^{res} negative mice were inoculated into a new set of mice which resulted in a mixture of PrP^{res} negative and positive mice. After the third passage of PrP^{res} negative brain homogenates, all mice were PrP^{res} positive. In another prion study (Race et al., 2001), there was a one year period post-scrapie infection where there was no evidence of replication of PrP^{res} titers in mouse brains. Following this period of inactivity a period of active replication of infectivity as well as adaptation of new strains of agent capable of causing disease in mice was seen. In most mice, neither the early phase nor the later replicative phase could be detected by immunoblot assay for protease-resistant prion protein. The transmission of TSE without detectable PrP^{res} accumulation supports the theory that in addition to the abnormal prion an infectious agent is likely.

With the development of a new cyclic amplification method for prions, the protein-misfolding cyclic amplification (PMCA), more information has been learned about prions in general. The PMCA is a method in which a subthreshold amount of any abnormal strain of prion PrP^{TSE} can, by repeated incubation and sonication in the presence of a normal tissue homogenate, be increased to detectable levels (Castilla et al., 2005; Saa et al., 2006a; Saa et al., 2006b). The sensitivity of the PMCA has contributed information about the possible presence of misfolded protein species in normal brains. Experimental studies on brain tissue from normal rodents (hamster, mice, and bank voles) subjected to many rounds of PMCA cycling have yielded detectable amounts of PrP^{TSE} and infectivity. It has been suggested that misfolded PrP molecules are regularly produced in normal brain tissue and require only one or more as yet unidentified triggers to unleash the cascade of molecular events that terminate in symptomatic disease (Brown, 2008).

Manuelidis et al. suggests a virus is associated with the infectious agent of TSE (Manuelidis, 1997). In one experiment, an attenuated strain of CJD was inoculated into mice and provided interference in PrP^{res} prion development and subsequent development of clinical signs. Manuelidis also suggested that some populations could harbor an attenuated CJD agent that makes them less susceptible to low-challenge doses of more virulent CJD agents, citing the new variant CJD outbreak from BSE infected meat that

only affected a few, although millions were exposed (Manuelidis, 1997). Researchers have also reported that the inactivation, behavior and size of the scrapie agent are very similar when compared to viral controls (Rowher, 1984). Studies of the scrapie agent's sedimentation behavior (Prusiner, 1978; Prusiner et al., 1980), exclusion size in permeation chromatography (Diringer and Kimberlin, 1983; Kimberlin et al., 1971), and buoyant density also suggest a virus (Diringer and Kimberlin, 1983; Kimberlin et al., 1971). These studies are in contrast to previous research that showed the transmissible agent to be more resistant to heat and fixatives than most viruses (Alper et al., 1966; Burger and Gorham, 1977).

There is evidence that a normal prion isoform on the cell surface may be used as a receptor for a bacterium (Watarai et al., 2003). This study suggests that the abnormally folded prion is a product of the infection rather than the cause. The study was based on the concept that intracellular bacteria use lipid rafts preferentially to enter the cell (Manes et al., 2003). Entering the cell in this method, the bacteria are able to avoid degradation pathways and lysosomes and both the adaptive and innate immune response (Manes et al., 2003).

Further characterization of the pathogenic prion led to the discovery that this protein is resistant to the potent protease potential of proteinase K (McKinley et al., 1983). Resistance to proteinase K is one of the distinguishing characteristics of the prion. In 1987 Bastian et al. (Bastian et al., 1987a) showed that *S. mirum* also contained proteinase K resistant proteins of 28, 30, 66, and 76 kDa. It was also suggested there is an immunological cross-reactivity and morphological similarities to the scrapie prion. In 1991 evidence Butler (Butler et al., 1991) reported that many of the mollicutes contained proteins that were also proteinase K resistant.

Other spiroplasmas are also implicated in neurological disease. Leafhoppers infected with *S. citri* (Western-X disease) develop notable lesions in the optic lobes and salivary glands (Nasu et al., 1970; Whitcomb et al., 1967). Simultaneous involvement of the optic lobes and salivary glands in Western-X is similar to the occurrence of either Sjorgren's syndrome or mucosal inflammation with ocular manifestations in multiple sclerosis (MS) (Coyle and Bulbank, 1989; Sandberg-Wollheim et al., 1992). Brown suggested that infection of both plant phloem and insect axons indicates a predilection of the organism for components of electrochemical conduction (Brown, 2003). There are also clinical correlations with bee and wasp stings and the subsequent development of MS (Dionne et al., 2000). These reports attribute the disease to bee venom; but bees and wasps are known carriers of spiroplasmas, and Brown suggests that the inoculation of humans with *S. citri* by bee or wasp stings could be the mechanism causing MS (Brown, 2003). Humphery-Smith et al. (Humphery-Smith et al., 1992) suggested that *S. melliferum* is a potential model for studying central nervous system degenerative disorders because of its ability to multiply and survive for up to nine months in intracerebrally inoculated mice. It was also found that this spiroplasma causes pathology in neuronal mitochondria in vitro and in vivo. In 1992 a spiroplasma isolated from a tabanid also had the capability of multiplication and survival in the suckling mouse brain (Humphery-Smith et al., 1992).

There is also clinical evidence that scrapie can be transmitted via hay mites in sheep. In one experiment, mites from a known infected farm were collected, pooled and

homogenized, and inoculated into mice. Prion disease developed 626 days later. In subsequent passages the incubation time was reduced, and the author concluded that mites could be a vector for the disease (Carp et al., 2000). Although mites are not one of the major hosts for spiroplasma, Jaenike (Jaenike et al., 2007) showed that mites could support different S*piroplasma* spp. and therefore could act as a vector for transmission of *Spiroplasma* spp. from host to host.

Discrepancies Seen in *Spiroplasma mirum*/TSE Publications

There have been many contradictory reports in the literature involving the culturing of *S. mirum*, its resistance to disinfectants and antibiotics, and its role in TSE. This section will be used to highlight those areas of interest. The contradictions will be the focus of the research to follow.

In 1979 Bastian was the first to publish a spiral-like inclusion in CJD brains seen via electron microscopy (Bastian, 1979). Gray and Reyes' also reported similar findings and published pictures (Figure 1.1 A) of the spiral inclusion (Gray et al., 1980; Reyes and Hoenig, 1981). In 1988, Humphery-Smith reported that the inclusions seen in the Gray and Reyes' publication had very similar morphology to crystalline artifacts (Figure 1.1 B and C)(Humphery-Smith and Chastel, 1988). In 1984 Bastian reported visualization of the spiroplasma within vacuolated cells of GT48-inoculated brains. The morphology of these organisms was drastically different from those identified in his CJD patients. These findings were of filaments or crescent shapes, both which were also seen in a pelleted sample of GT48 for comparison (Bastian et al., 1984).

Leach (Leach et al., 1983) followed up on the association of CJD and spiroplasma, based solely on morphologic appearance via electron microscopy, with an attempt to isolate spiroplasma from eighteen CJD patients. The brain tissues were homogenized in SP4 and diluted. The dilutions were plated on SP4 plates, and the remaining cultures were allowed to incubate for two weeks. Sera were also tested for spiroplasma by indirect immunofluorescence and metabolism inhibition tests.
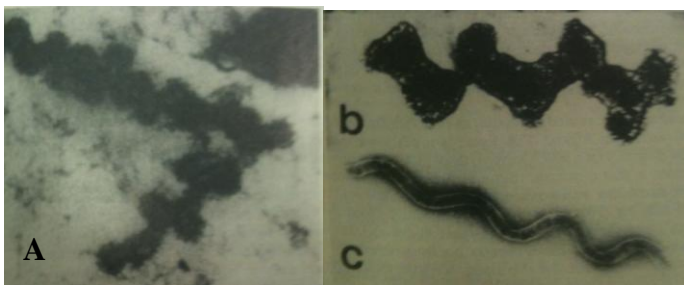
Figure 1.1 Spiroplasma-like inclusions
A) Spiroplasma-like inclusion seen in CJD brains by Gray B) Crystalline artifacts labele and C) an example of Spiroplasma labeled all seen via electron microscopy.

The experimental design was possibly flawed in Leach's experiments, in that incubation was for two weeks only; and a culture was diluted, that if positive most likely had very few organisms originally. The cultures were not reported to be passaged, and no other

culture tissues (embryonated eggs) were used. Furthermore, no antibodies are found circulating in experimentally infected animals (Clark, 1964), so the lack of inhibition from the serum tested is not indicative of a non-infection. This is not to argue that spiroplasma could be found in these CJD patients but to show the drawbacks in experiments that question the *S. mirum*/TSE correlation.

In a 1984 publication, Bastian (Bastian et al., 1984) experimentally inoculated suckling rats intracerebrally with GT48. He reported that at day 15, organisms were at a titer of 10^7 to 10^9; but those animals necropsied at day 60 had considerably lower titers. The brains examined histologically at day 14 revealed microcystic encephalitis. The neuropil was studded with vacuoles and showed a prominent inflammatory infiltrate of polymorphonuclear leukocytes and mononuclear cells. Histological examination of brain tissues obtained from rats at 25 days revealed minimal pathologic alteration. Bastian also reported that organisms were readily identified via electron microscopy for rats sacrificed at day 14 but not for rats sacrificed at day 25. In the 2005 publication, Bastian cited the previous 1984 study as stating that infection with GT48 spiroplasma produces perineuronal vacuoles, lack of inflammation and scattered microglia (Bastian and Fermin, 2005). The 1984 publication only found pathology with prominent inflammatory infiltrates at day 14 and reported a lack of pathology by day 25 (Bastian et al., 1984). Bastian also referenced this experiment again in the same publication stating in the rat there is extensive spongiform degeneration without inflammatory response when in 1984 he reported prominent inflammatory infiltrates of white blood cells and pathology was resolved by day 25 (Bastian and Fermin, 2005).

In 1984 another publication from Tully, Bastian and Rose reported on experimental spiroplasma infections in rats (Tully et al., 1984). In this study, three different rat species were inoculated intracerebrally with low or high doses of GT48. Histological sections were not reported, only the persistence of the organism over 60 days for the three different species. Responses were not uniform and the authors reported that some rats receiving moderate to high levels of GT48 did not harbor viable organisms even in early periods after challenge. They went on to suggest caution in assuming that *S. mirum* strains always induce a consistent brain infection in the rat model (Tully et al., 1984).

In the 2005 publication listed above, Bastian also reports that *Spiroplasma* can resist exposure to temperatures near boiling, as well as glutaraldehyde and other fixatives (Bastian and Fermin, 2005). In 1964, Clark reported that SMCA was ether- and sodium desoxycholate- sensitive and was efficiently inactivated by 0.5% phenol, 0.2% formalin, and 0.05% B-propiolactone (Clark, 1964). Clark also found that SMCA was completely inactivated following 30 minutes of heating at $56^{o}C$ (Clark, 1964).

Although Bastian et al. was able to produce clinical and neuropathological signs with SMCA-inoculated neonatal deer similar to those of CWD in his 2007 publication (Bastian et al., 2007), attempts to reproduce this in other species has not been successful (Hamir et al., 2011). Furthermore, evidence of recovery of the organism by culture, antibodies, or PCR was not reported and allows further questions regarding spiroplasma and its role in neurodegenerative diseases.

The discrepancies listed above led to the interest in the association of spiroplasma and TSEs and the development of the research proposed in this dissertation. Based on the results reported by Bastian et al. in 2007, it is hypothesized that spiroplasma are associated with neurodegenerative diseases similar to TSE. In this work we further characterized *S. mirum* and continued to evaluate the possible correlations found in TSE infections. In order to establish the best overall picture of *S. mirum* infections, we looked at culture conditions, physical and environmental disinfectant resistance, pathology and disease in experimental animals, and the immunological reactions found in experimental animals. The following outlines the work in this dissertation.

In chapter two, we determined the optimal growth conditions for the laboratory strains of *S. mirum*, SMCA and GT48. In M1D, both SMCA and GT48 grew better at 30ºC and a typical growth curve was established. In embryonated egg cultures, SMCA caused death in a reliable time frame and GT48 had sporadic death rates. Both results coincided with previous research. Growth on solid media was established with SP4 plates, and colony forming units were used to compare growth rates in liquid and solid media for both strains of *S. mirum*.

Chapter three focuses on the resistance of spiroplasma to a variety of physical and chemical disinfectants as well as antibiotics. Susceptibility was assessed by dilution and time for common laboratory disinfectants and an antibiotic profile was established for SMCA, GT48, *S. citri*, and *S. melliferum*. Information reported in this chapter was used to compare the TSE infectious agent and spiroplasma.

Chapter four reports the clinical and pathological findings of our animal experiments. In order to compare natural TSE infections to spiroplasma-induced disease, we established a more long term animal model. It has been proven that the mouse model does not always accurately portray a natural infection and so the ruminant model was chosen for disease establishment. Neonatal goats were inoculated intracerebrally with SMCA and monitored for clinical signs for two years. At the end of the experiment, various tissues were collected and examined for pathology and persistence of the organism. Deer were also inoculated at five months and monitored for clinical signs. Seven months post-inoculation various tissues were collected and examined for pathology and persistence of the organisms. Information from this experiment was compared to that of Bastian et al. (Bastian et al., 2007) and their neonatal deer experiment. In order to test possible routes of transmission SMCA was delivered by intravenous and intradermal routes in neonatal goats to evaluate possible vector routes to establish an infection. Goats were monitored for four months for clinical signs and persistence of the organism via various tissues. After four months, various tissues were collected and examined for pathology.

CHAPTER 2:
A COMPARISON OF CULTURE MEDIA FOR *SPIROPLASMA* SPP

Introduction

The successful recovery of mollicutes from clinical specimens is dependent upon a number of factors, including host-dependent components (inhibitory enzymes, host antibodies, etc) and a variety of cultural or technical factors that may either enhance or limit isolation (Tully et al., 1983). It is established that no single culture medium or cultivation procedure is adequate for the isolation of all or even most of the known mollicutes (Tully et al., 1983). This is especially true for the unique and varied genus of *Spiroplasma*. Spiroplasmas were classified as Mollicutes (then Mycoplasmas) in 1972 because they form "fried egg" colonies on solid media, pass through 0.22 μm filters, fail to revert to walled bacteria, are completely resistant to penicillin, and lack cell wall or murien cell wall precursors (Bove and Saillard, 1979; Saglio et al., 1971).

Spiroplasmas are a unique group of organisms. Originally thought to be viruses and then mis-identified as spirochetes, spiroplasmas have been difficult to characterize. They can be found in a variety of hosts including plants, insects, and ticks. They were first recognized as helical, wall-less bacteria in 1972 and received their name from a description of their motility in 1973 (Davis et al., 1972). The first named spiroplasma was *S. citri,* the causative agent of citrus stubborn disease, which was originally considered a virus (Saglio et al., 1971). *Spiroplasma mirum*, strain Suckling Mouse Cataract Agent (SMCA), isolated from rabbit ticks, was first considered a virus because it was filtered through such a small pore size (Clark, 1964). Spiroplasmas have also been misidentified as spirochetes because of their similar appearance in dark field microscopy. The sex ratio organism (SRO), a disease fatal to only males of certain *Drosophila* species, was originally identified as a spirochete in 1961 but was later re-identified as a spiroplasma (Williamson et al., 1983).

Spiroplasmas vary greatly in their habitats and therefore require a wide variety of culture mediums for isolation and growth. Media designed for isolates from vertebrates stress sodium concentrations and reduced potassium and supply nucleic acid precursors (Whitcomb, 1983). Media designed for insect and flower isolates have much higher concentrations of all amino acids. All spiroplasma media must contain a sterol or lipid ingredient in order to support growth (Whitcomb, 1980). In general spiroplasma media usually contain combinations of 10-20% serum, beef heart infusion, peptone and tryptone, carbohydrates, and amino acids (Bove and Saillard, 1979; Chen and Davis, 1979; Whitcomb, 1980; Whitcomb, 1983). The first sustained cultivation of a spiroplasma was *S. citri*, a flower pathogen, on a moderately simple mycoplasma medium (see Table 2.1) (Whitcomb, 1983). The development of M1A resulted in enhanced growth for insect/flower isolates. The addition of yeast extract for M1D media allowed for better growth for some spiroplasma isolates. In order to cultivate SMCA, a fastidious organism, a much more complex medium SP4 (see Table 2.1), with added nutrition, especially amino acids, was required (Whitcomb, 1980). Isolates identified from arthropods were thought to be non-cultivatable for years (Tully et al., 1982a). Clark stated that one-half of the spiroplasmas observed in hemolymph of insects were non-

cultivatable with the media known in 1982 (Clark, 1982). Similar media are being used in spiroplasma research today, making further isolation attempts challenging to researchers.

Table 2.1 Spiroplasma media (yields 300 mLs)

Ingredients	Standard Mycoplasma Medium	M1A	M1D	SP4
Autoclave at 15 psi for 30 min				
Mycoplasma broth base	4.5 g	2.1 g		1 g
Bacto-Tryptone		1 g		3 g
Bacto-Peptone		0.8 g		1.6 g
Glucose		0.1 g		
Fructose		0.1 g		
Sucrose		1 g		
Sorbitol		7 g		
Distilled water	200 mL	70 mL		197 mL
For solid media	**Add 1.6 g Difco Noble Agar to autoclavable portion**	**Add 2.4 g to 6.8 g Difco Noble agar to autoclavable portion**		
Sterile filter				
Schneiders Drosophilia medium		160 mL		
CMRL-1066 (10x w/glutamine, w/out NaHCO3)				15 mL
Fetal Bovine Serum		50 mL		50 mL
Horse Serum	60 mL			
Fresh yeast extract (25%)	30 mL		10 mL	10.5 mL
Phenol Red 0.5%	(0.1 %) 6mL	1.2 mL		1.2 mL
Potassium Penicillin (100,000 U/ml)	1.5 mL	2.5 mL		3 mL
Yeastolate 2%				30 mL
Glucose (50% solution)	3 mL			3 mL
L-arginine-HCl 42%	1.5 mL			

A defined media was also developed for *S. mirum*. After the growth experiments in SP4, researchers began experimenting with a variety of different elements within the recipe in order to determine the best recipe for *S. mirum* growth enhancement. H-1, a medium containing sphingomyelin, was created (Hackett et al., 1987). This medium contains lower concentrations of inorganic salts and higher concentrations of amino acids, nucleic acid precursors, and cofactors. The addition of sphingomyelin and modifications made in H-1 allowed SMCA's doubling time to decrease from 25 hours to 7 hours with higher growth yields (Hackett et al., 1987).

Culture media has also been modified for different phases of the organisms' growth. Media used for isolation of the organism usually contain a rich mixture of nutrients. However, some researchers suggest that for some isolates, the large amount of nutrients could be toxic for isolation (Whitcomb, 1983). Primary isolation also depends on the organism, hosts, and the number of organisms available for isolation (Friedlaender et al., 1976). This was proven with repeated attempts to isolate spiroplasma from primary tick material in different media (Tully et al., 1981; Yunker et al., 1987). In the case of *S. mirum* isolation, it is speculated that an inoculum of low numbers may benefit from the interaction with host cells. However, after four weeks of various tick cell line cultures, all tissue cultures were not viable due to cytopathic effects on host cells and an artificial media (SP4) was needed to sustain growth (Yunker et al., 1987). Once isolated, it is common for the spiroplasmas to adapt to a simplified media for continued passage (Whitcomb, 1983).

Spiroplasmas vary greatly in their ability to produce colonies on solid media. Although it was one of the distinguishing characteristics in determining *Spiroplasma* is a mollicute (mycoplasma), typical fried-egg or umbonate colony morphology is only observed under poor growth conditions (Townsend et al., 1980; Whitcomb, 1983). Instead, some spiroplasma produce small satellite colonies in the vicinity of primary colonies; others produce granular-like colonies while still others form extremely diffuse colonies (Whitcomb, 1980). The formation of colonies on solid media is dependent on a number of factors, the greatest being the agar content. An effective strategy for obtaining countable colonies with highly motile spiroplasmas is the incorporation of higher concentrations of agar (Whitcomb, 1983). Interestingly, spiroplasmas have been found to move faster with more viscous/more gelatinous media (Gilad et al., 2003; Wolgemuth and Charon, 2005). The incubation conditions of the agar plates can also affect the growth of spiroplasma colonies. Many isolates require a gaseous environment of 95% N_2 and 5% CO_2 for as long as 14-20 days (Bove and Saillard, 1979; Chen and Davis, 1979). Variations in composition of solid media can also affect the ability of spiroplasmas to form colonies (Whitcomb, 1980).

Qualitative and quantitative assessments of growth are required for spiroplasma cultures. Qualitative monitoring of growth is important especially during continuous passage. If media, temperature, pH, or osmolarity are not optimized, abnormal growth and morphology may result (Chen and Davis, 1979; Liao and Chen, 1978). Phenol red has been added as an indicator of growth based on acidic changes in the media because cultures entering stationary phase reach a minimal pH (Whitcomb, 1980). SMCA, one of the more fastidious organisms, is slow to acidify media and may reach stationary phase of growth before acidifying the media. For this reason growth of cultures should be confirmed by dark field or phase microscopy (Whitcomb, 1980).

Direct quantification of spiroplasmas is accomplished by counting helical organisms under dark field microscopy (Liao and Chen, 1977; Williamson, 1969). This can be time consuming and requires the use of a dark field microscope. Colony-forming units

(CFUs) can be obtained by plating liquid culture and counting colonies 7-10 days after incubation (Bove and Saillard, 1979; Whitcomb, 1980). Accurate counts of CFUs may be difficult to obtain with spiroplasmas. Viable organisms from older cultures are less likely to form colonies than those in log phase (Whitcomb, 1980). As stated above, spiroplasmas vary in their ability to produce consistent colonies on solid media for various reasons. Some researchers have found that using titration methods resulted in accurate counts of spiroplasma cultures. In a dilution series, the highest dilution that shows spiroplasma growth as exhibited via a color change is considered to contain one color changing unit (CCU) (Whitcomb, 1980). This is a statistically crude estimate of viable spiroplasma cells but has worked well for estimating organism numbers for the more fastidious organisms (Whitcomb, 1980).

Indirect quantification methods have been developed for some spiroplasmas. Absorbance at 660 nm may be used to measure growth of *S. citri* (Whitcomb, 1980); however the measurements of absorbance or turbidity in most spiroplasma cultures may be affected by nonspecific changes in the growth media, some which are not necessarily growth related (Whitcomb, 1980). Incorporation of radioactive precursors into nucleic acids has also been used to indirectly quantify *S. citri* (Bove and Saillard, 1979). This method measures the radioactivity of trichloracteic acid insoluble precipitates during logarithmic growth. ATP, total adenylate pools, and amounts of incorporated radioactive thymidine and phenylalanine also increase during the logarithmic growth phase (Saglio et al., 1971; Saglio and Whitcomb, 1979). Thymidine uptake as a measurement of growth was evaluated for *S. mirum*, one of the more fastidious spiroplasmas. The growth yields were not as high as the compared CFU counts of the same inoculum, but the same growth curve pattern was seen in both media (Bastian et al., 1988).

Spiroplasma growth depends on temperature, medium composition, history of inoculum, and history of passage in other mediums (Chen and Davis, 1979; Davis, 1978; Liao and Chen, 1978). Growth rates appear to correspond closely with the organism's natural environment (Junca et al., 1980). Those spiroplasma found on flower surfaces have rapid growth rates with doubling times of about 1.8 hr (Whitcomb, 1980). *Spiroplasma citri*, which can be found in insects and within plants, grows less rapidly with a doubling time of 5-6 hr (Saglio and Whitcomb, 1979). Those spiroplasma that have specialized habitats grow slowly with doubling times of 20 hr or more (Liao and Chen, 1977).

The focus of this chapter is to establish optimal culture methods for laboratory strains of *S. mirum*. A stock solution of SMCA and GT48 are used in all experiments. Stocks of *S. melliferum (*ATCC strain BC-3 #33219) and *S. citri* (ATCC strain R8A2 #27556) were also used in broth experiments for growth comparisons. In this laboratory, SMCA and GT48 were better adapted to M1D broth media than SP4 for continued passages for experimental purposes. To optimize the best culture method for laboratory strains, spiroplasma growth studies in M1D broth, SP4 solid media, and embryonated eggs were compared. Direct quantification was assessed via dark field microscopy in broth cultures and embryonated egg cultures. Colony forming units were used for quantification from SP4 plates.

Materials and Methods

Bacteria: Two strains of *S. mirum* were used, SMCA and GT48. Strain SMCA was obtained from Dr. Frank Bastian, Tulane Medical Center, New Orleans, LA. GT48, ATCC strain #29334, was obtained from ATCC Bioresource Center. *Spiroplasma melliferrum (*ATCC strain BC-3 #33219 Vc161, created on 17MAR90, pass # 3) and *S. citri* (ATCC strain R8A2 #27556 130 Allen, created on 15Mar95, pass # 4) were also obtained from the ATCC Bioresource Center.

M1D Media: The ingredients for M1D are seen in Table 2.1.   Media was filter sterilized through a 0.2 µm filter and stored at -4°C. Media was heated to room temperature and containers were sterilized with 70% ethanol before use to reduce possible bacterial contamination. Once inoculated with bacteria, media was monitored for growth via dark field microscopy and/or color change.

Embryonated Eggs: Pathogen-free eggs were obtained from the Poultry Science Unit at Louisiana State University Agricultural Center. Prior to use, eggs were stored at 18°C and then incubated for 7 days in a humidified 37°C chamber. After 7 days, eggs were candled for viability (Figure 2.1). Viable eggs were cleaned with 70% ethanol or 10% betadine, and a sharp metal pick was used to make a small hole at the crown of the egg above the air sac (Figure 2.2).
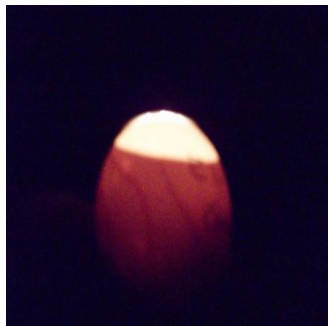
Figure 2.1 Egg Candling
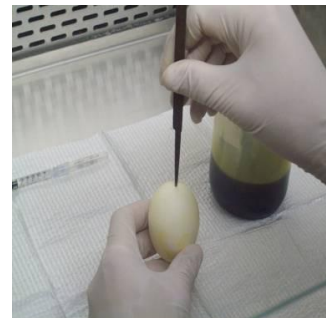
Figure 2.2 Egg inoculation

A 10 µl sample was injected via a 22 gauge 1 inch needle into the allantoic cavity by inserting the needle 3/4th of the way into the egg. Samples were injected into the yolk sac by inserting a 22 gauge 1 inch needle at a 90° angle 3/4th of the way into the egg. The injection opening was closed using candle wax or finger nail polish. Figure 2.3 details the placement of each injection route. The blue line represents the path of chorioallantoic inoculation; the red line, the path of yolk sac inoculation.
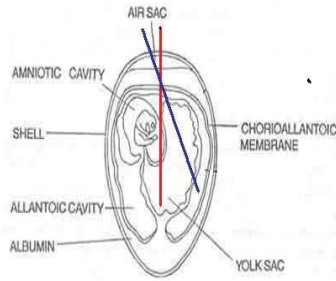
Figure 2.3 Egg inoculation routes   **Figure 2.4** Allantoic fluid collection
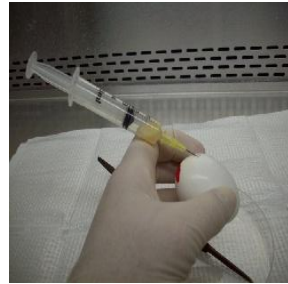
After injection, eggs were placed in a humidifying 37°C incubator (rocking or non-rocking) and candled daily for viability. On day 18 (11 days post inoculation) or when determined to be non-viable, eggs were cooled to -4°C. Eggs were cleaned again with 70% ethanol. Approximately 1 mL of allantoic fluid was aspirated from each egg using a 22 gauge needle placed at a 45° angle through the sealed hole used for injection (Figure 2.4). Collected allantoic fluid was observed via dark field microscopy. Fluid was either frozen at -80°C or passaged into new embryonated eggs or M1D.

SP4 Plates: A modified SP4 recipe was used for the plates in these experiments. Ingredients for plates can be found in Table 2.2. The base solution was autoclaved for 30 min at 121°C, 15 psi and then cooled to 56°C. Supplements were combined and filter sterilized through a 0.2 µm filter and then heated to 56°C. Once combined, media was poured immediately into plates which were left under a hood until dry. Recipe makes approximately 18 100 x 15 mm plates. Plates were incubated overnight at 37° to check for bacterial contaminates.

Table 2.2 ATCC SP4 recipe

Base Solution	
Mycoplasma Broth Base	5.5 g
Tryptone	5 g
Distilled H_2O	262.5 mL
Noble Agar	4 g
Supplements	
Fetal Bovine Serum	85 mL
Glucose (5%)	50 mL
Phenol Red (0.1%)	10 mL
Yeastolate (2%)	45 mL
CMRL 1066 (10X)	25 mL
Fresh Yeast Extract (25%)	17.5 mL

Dark Field Microscopy: A 5 µL culture sample was placed on a clean glass microscope slide, mounted with a #1.5 cover slip and sealed on all 4 sides with clear finger nail polish. Slides were examined with a Ziess Axio Imager A1 microscope (Carl Zeiss, Inc. North America, Thornwood, NY, USA) equipped with a dark-field top lens condenser 1.2-1.4 and an EC Paln-NeoFluar x100 oil immersion objective (Carl Zeiss, Inc. North America, Thornwood, NY, USA) with 1.3 iris closed to its lowest setting (0.7).

Dark Field Enumeration: The average of at least 3 separate counts taken from various areas of the 5 µl sample was multiplied by the area of the viewing space. This yielded the approximate number of organisms per 5 µl samples. Cultures were assumed to be uniform, and calculations were made to establish amount of spiral organisms per mL. The grid size used to enumerate samples was determined and a formula (484 x 100 x the average of three counts) was used to calculate the amount of organisms per 5 µL. This number was used to calculate the total number of organisms per mL and reported in Tables 2.4 – 2.11. Organisms per mL were log 10 trasformed for graphing purposes and reported in Figures 2.6 – 2.8.

PCR Methods: PCR was carried out using oligonucleotide primers that specifically identified a 1460 bp portion of the *S. mirum*-related adhesin gene. The forward and reverse primers were F3 (5'-TCTAGTCTTAATCATTTTACTTATTTATTAGAA -3') and R4 (5'-TTATTAAGTCATTCACTCTCTTTCTTT -3') respectively.
The reaction mixture (50 µL total) contained 2 µL of each the forward and reverse primers (10 µM each), 1 unit (0.2 µL) of *Taq* DNA Polymerase (New England BioLabs, Ipswich, MA), 2 µL of 10 mM dNTP (AmpliTaq Gold, Applied Biosystems, Foster City, CA), and 5 µL of 10x Standard *Taq* Buffer (New England BioLabs), which contains 15 mM of $MgCl_2$. For M1D cultures, 2 µL of culture was added to the reaction mixture without prior DNA extraction. For SP4 plates, agar plugs were taken from 0.8% plates and mixed with PBS. 2 µL of this solution was added to the reaction mixture. Plates with higher concentrations allowed cotton swabbing of the surface. The cotton swab was vigorously mixed in PBS and 2 µL of this solution was added to the reaction mixture. A variety of allantoic fluid dilutions were used for PCR from embryonated eggs. Each reaction was brought to a final volume of 50 µL with sterile, nuclease-free H_2O. PCR's were performed using a MyCycler Thermal Cycler (Bio-rad). The thermal cycler program used was 94ºC for 5 minutes, then 30 cycles at 94ºC for 30 seconds, 57ºC for 20 seconds, and 72ºC for 120 seconds followed by a final extension of 72ºC for 10 minutes. All PCR products were electrophoresed on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer, and the DNA was visualized by SYBR Safe (Invitrogen, Eugene, OR) DNA gel staining under UV fluorescence.

Statistical Analysis: To determine the best growth conditions we used an analysis of variance of a factorial arrangement of treatments was conducted. Treatment groups included time, temperature, organisms, and daily growth counts. When overall differences were found post-hoc comparisons were conducted with Tukey's HSD test for main effects and pairwise t-test for interaction effects. All comparisons were considered significant when $p \leq 0.05$.

Experiment 1. Growth in M1D

- 1a. Growth at 30°C vs. 37°C: To assess optimal growth temperature, all three species of *Spiroplasma* were incubated at 30°C and 37°C temperatures. Cultures were initiated by adding 100 µL of thawed bacteria stock cultures to 900 µL of M1D in a 3 mL polystyrene tube. Tubes were placed in a 30°C or 37°C incubator and checked daily for color change. A 5 µL sample of each culture was assessed via dark field microscopy daily for 7 days and then once weekly for a total of 21 days.

- 1b. Growth patterns of diluted cultures: Tenfold dilutions out to 10^{-8} were made from a fresh 7 day old culture for both SMCA and GT48 strains and were incubated as above. Growth was monitored daily via dark field microscopy, color change, and PCR for 21 days.

- 1c. Medusa formation: While monitoring cultures daily for growth, it was observed that SMCA and GT48 aggregated to form medusa-like structures which could affect accurate counts. In order to test this, a fresh culture was made by 1:100 dilution of a 21 day culture and monitored. When five or more medusas were noted within the daily sample taken for dark field microscopy, the culture was vortexed for a varied amount of time: 1 minute, 5 minutes, and 10 minutes. Culture was checked after vortexing for the presence of medusa-like aggregates by dark field microscopy. The culture was monitored for growth daily for 7 days and compared to a culture not vortexed.

Experiment 2. Growth in Embryonated Eggs

- 2a. Injection site and incubation: To optimize the culture method in embryonated eggs, both injection site and type of incubator was assessed. 36 pathogen-free eggs were incubated at 37°C in a rocking humidifying incubator for 7 days. A 10 µL sample of 7 day old SMCA cultures was injected into 24 eggs via either the yolk (12) or allantoic cavity (12) of the eggs. A 10 µLsample of M1D was injected into 12 eggs via either the yolk (6) or allantoic cavity (6) of the control eggs. Six eggs from each injection route and controls were placed in a 37°C non-rocking humidifying incubator. The remaining eggs were returned to the rocking incubator. Eggs were candled daily to check viability. On day 18 (11 days post inoculation) or at time of non viability, eggs were cooled to -4°C. Allantoic fluid was collected and observed via dark field microscopy. Egg deaths and dark field results were recorded per day. Egg deaths that occurred 1 day post inoculation were attributed to technical problems rather than bacterial deaths.

- 2b. Comparison of egg deaths for GT48 and SMCA: Tenfold dilutions out to 10^{-8} were made from fresh 7 day old cultures for both SMCA and GT48 strains. Ninety eggs were divided into groups of 6 and incubated at 37°C in a rocking humidifying incubator for 7 days.   A 10 µL sample of each dilution (7 dilutions of each strain: SMCA and GT48) was injected into 7 day old embryonated eggs. A 10 µL sample of M1D was injected into 6, 7 day old embryonated eggs to serve as controls. All eggs were placed in a 37°C non-rocking humidifying incubator. Eggs were candled daily

to check viability. On day 18 (11 days post inoculation) or at time of death eggs were cooled to -4°C. Allantoic fluid was collected and observed for presence of organisms via dark field microscopy and PCR. Experiment was repeated for accuracy of results. Egg deaths and dark field results were recorded per day. Egg deaths that occurred 1 day post-inoculation were attributed to technical problems rather than bacterial deaths.

- 2c. SMCA effects on embryonated eggs: The development of the chick causes a great deal of changes within the embryonated egg. In order to assess the effects SMCA has on the development of embryonated eggs, 20 eggs were monitored daily for pH changes. Twelve eggs were injected with 10 µL of viable SMCA culture. To serve as controls, 3 eggs were injected with 10 µL of M1D and 3 eggs were not inoculated. At time of inoculation a small aspirate of allantoic fluid was taken and pH was assessed with a 6-10 pH strip. All eggs were placed in a 37°C non-rocking humidifying incubator. Eggs were candled daily to check viability. A sample of allantoic fluid was taken daily from all viable eggs for 7 days and tested on pH strip. All samples were also assessed for presence of bacteria via dark field microscopy.

Experiment 3. Growth on SP4 Plates

- 3a. Bacteria growth characteristics: Four 1 mL aliquots of 7 day old cultures (SMCA, GT48, *S. citri*, and *S. melliferum*) were spun down at 16000xg for 1 hour. The pellet was re-suspended in varying amounts of PBS, and each was inoculated onto modified SP4 plates containing 4 grams of noble agar (Table 2.2). Plates were incubated at 30°C and 37°C for 14 days, and colonies were observed and counted when possible.

- 3b. Agar concentration experiments: Plates were made following the ATCC SP4 recipe with 4 grams (0.8%), 8 grams (1.6%), and 12 grams (2.4%) of Noble agar. Tenfold dilutions of SMCA and GT48 were made in M1D, and 50 µL of each dilution was placed on the agar plates. Plates were allowed to incubate for 14 days at 30°C and 37°C, and colonies were observed and counted.

Results

Experiment 1. Growth in M1D

- 1a. Growth at 30°C vs. 37°C : Incubation at 30°C proved to be significantly better (p≤0.05) for all four species of spiroplasma based on daily dark field enumeration. *Spiroplasma citri* had limited growth at 37°C. *Spiroplasma melliferum* cultures were sustained at both temperatures, with 30°C yielding higher numbers. SMCA and GT48 had very similar growth patterns at 30°C and 37°C, with 30°C sustaining cultures for longer time period. Figure 2.6 and Table 2.3 highlights the dark field counts obtained at various time points.
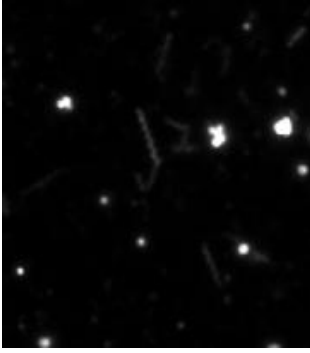
Figure 2.5 Spiroplasma organisms as seen via dark field microscopy.
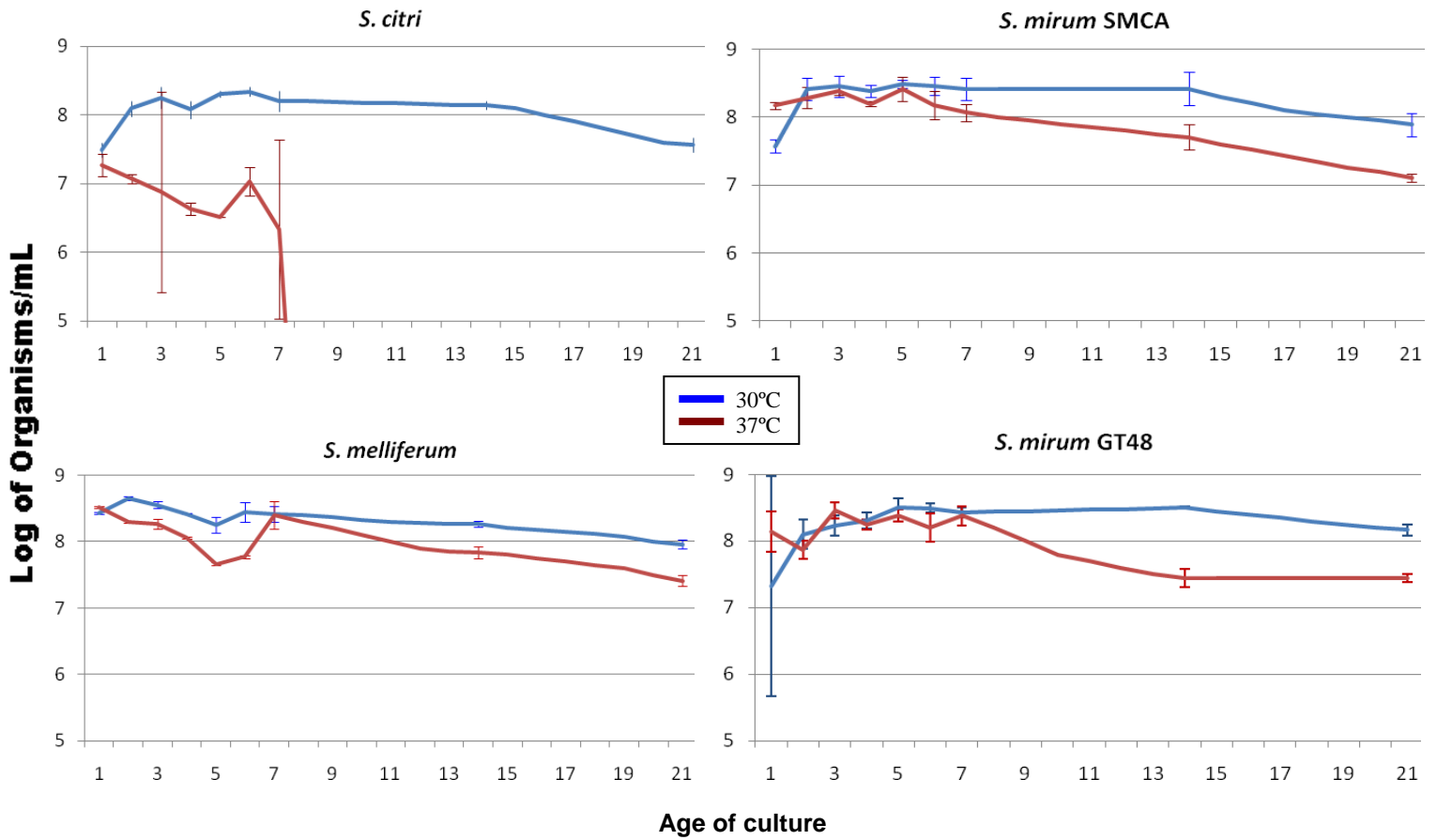
Figure 2.6 Comparisons of *Spiroplasma* growth yields over 3 weeks at 30°C and 37°C. Growth yields were determined by dark field observations and compared by pairwise t-test of least squares means for significance where p≤0.05. *Spiroplasma citri*, *S. melliferum*, GT48, and SMCA had significantly better growth at 30°C.

Table 2.3 Summary of *Spiroplasma* growth. Dark field counts at various time points throughout the experiment for all three *Spiroplasma* species at each temperature tested. *: No organisms found via dark field microscopy.

Organism	Temp	Organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	3.7E+07	2.8E+08	3.1E+08	2.6E+08	2.6E+08	7.7E+07
SMCA	37°C	1.5E+08	2.4E+08	2.6E+08	1.2E+08	5.1E+07	1.3E+07
GT48	30°C	2.1E+07	1.7E+08	3.2E+08	2.7E+08	3.2E+08	1.5E+08
GT48	37°C	1.4E+08	2.9E+08	2.4E+08	2.4E+08	2.8E+07	2.8E+07
S. melliferum	30°C	2.7E+08	3.6E+08	1.8E+08	2.6E+08	1.8E+08	9.0E+07
S. melliferum	37°C	3.3E+08	1.8E+08	4.6E+07	2.5E+08	6.8E+07	1.2E+08
S. citri	30°C	3.1E+07	1.8E+08	2.1E+08	1.6E+08	1.4E+08	3.7E+07
S. citri	37°C	1.8E+07	7.5E+06	3.2E+06	2.2E+06	*	*

- 1b. Growth patterns of diluted cultures: Growth for all dilutions of GT48 and SMCA at 30°C and 37°C can be seen in the following figures (Figure 2.8 and 2.9) and tables (Tables 2.4 – 2.11). For GT48 and SMCA cultures between 1-7 days, growth at 37°C was significantly better (time points highlighted with * when p≤0.05) or the same as growth at 30°C. However, after day 7, growth at 30°C was significantly better (time points highlighted with * when p≤0.05).

  Cultures were also monitored via PCR. The PCR results from 3 days post-inoculation are seen in Figure 2.7. As seen via dark field, more organisms were seen in diluted cultures earlier in the 37°C cultures. Figure 2.10 reveals PCR results for 8 days post-inoculation. SMCA has reached higher numbers at 30°C as seen with banding in the lower dilutions compared to the bands for 37°C. Positive bands are seen at 1460 bp, correlating with the *S. mirum*-related adhesin gene.
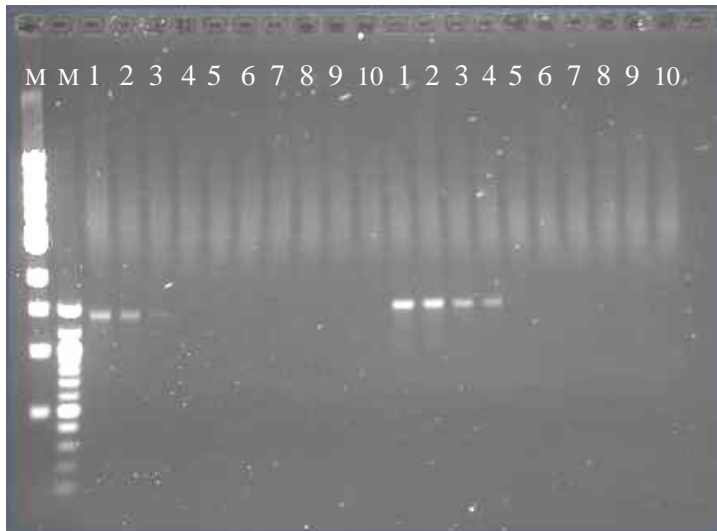
Figure 2.7 PCR results for tenfold dilutions of SMCA at 30°C and 37°C three days post-inoculation. The first two lanes are markers. The next ten lines represent tenfold dilutions of SMCA cultures at 30°C, followed by tenfold dilutions of SMCA at 37°C. Positive bands are seen at 1460 bp, correlating with the *S. mirum*-related adhesin gene.
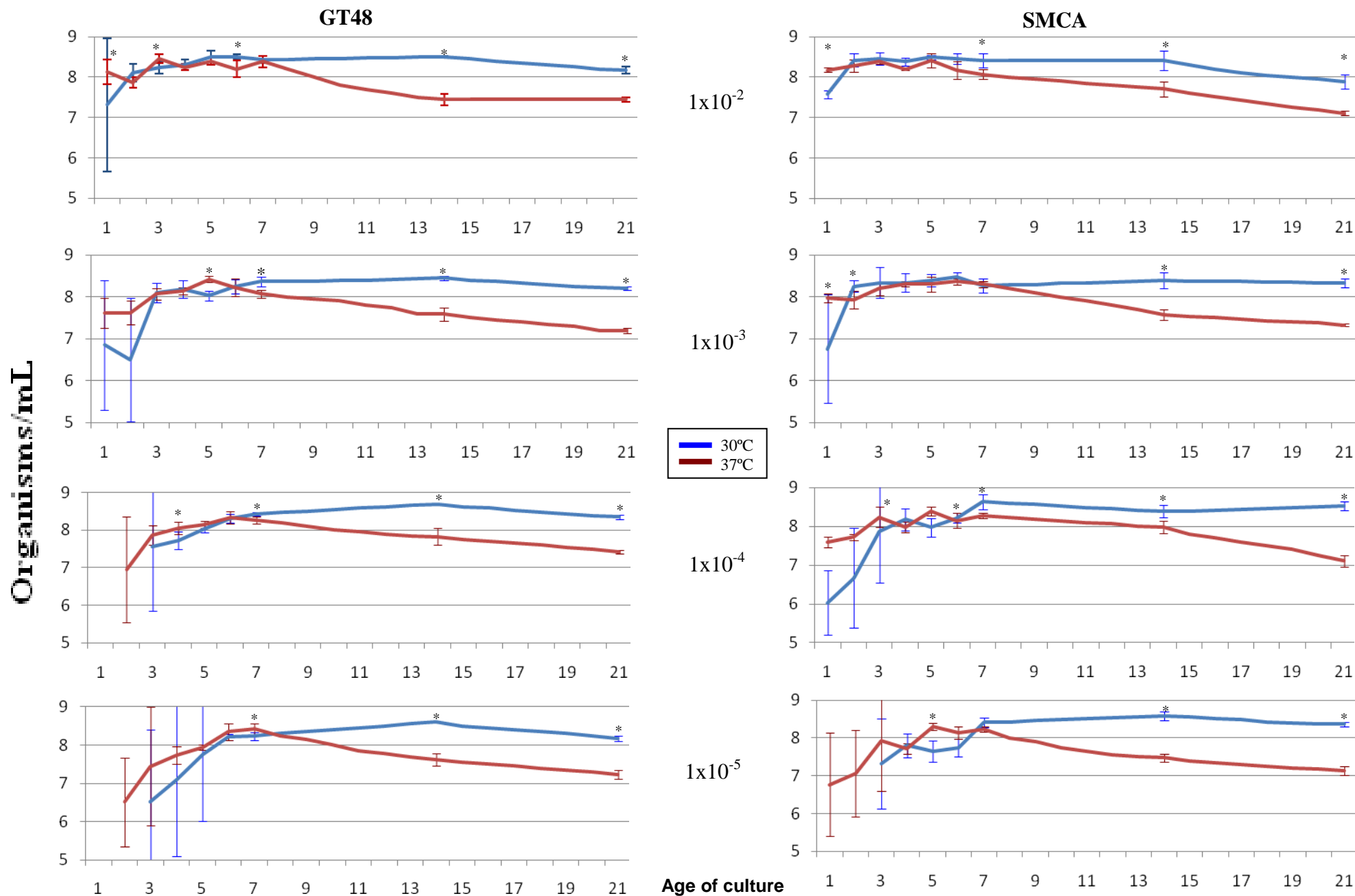
Figure 2.8 Growth of diluted (1×10^{-2} - 1×10^{-5}) GT48 and SMCA cultures over 3 weeks at 30°C and 37°C. Growth yields were determined by dark field observations and log transformed data was compared by pairwise t-test of least squares means for significance where p≤0.05 (time points designated with * when p≤0.05).

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Table 2.4 Dark field counts for diluted (1×10^{-2}) SMCA and GT48 cultures

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	2.1E+07	1.7E+08	3.2E+08	2.7E+08	3.2E+08	1.5E+08
SMCA	37°C	1.4E+08	2.9E+08	2.4E+08	2.4E+08	2.8E+07	2.8E+07
GT48	30°C	3.7E+07	2.8E+08	3.1E+08	2.6E+08	2.6E+08	7.7E+07
GT48	37°C	1.5E+08	2.4E+08	2.6E+08	1.2E+08	5.1E+07	1.3E+07

Table 2.5 Dark field counts for diluted (1×10^{-3}) SMCA and GT48 cultures

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	5.7E+06	2.2E+08	2.5E+08	1.9E+08	2.5E+08	2.2E+08
SMCA	37°C	9.4E+07	1.6E+08	2.0E+08	2.0E+08	3.7E+07	2.1E+07
GT48	30°C	7.1E+06	1.2E+08	1.1E+08	2.3E+08	2.8E+08	1.6E+08
GT48	37°C	4.1E+07	1.2E+08	2.6E+08	1.2E+08	3.9E+07	1.5E+07

Table 2.6 Dark field counts for diluted (1×10^{-4}) SMCA and GT48 cultures
*: No organisms seen via dark field microscopy

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	1.1E+06	7.3E+07	9.4E+07	4.3E+08	2.5E+08	3.3E+08
SMCA	37°C	3.9E+07	1.7E+08	2.5E+08	1.9E+08	9.6E+07	1.3E+07
GT48	30°C	*	3.6E+07	1.1E+08	2.7E+08	5.0E+08	2.3E+08
GT48	37°C	*	7.4E+07	1.4E+08	1.9E+08	6.7E+07	2.6E+07

Table 2.7 Dark field counts for diluted (1×10^{-5}) SMCA and GT48 cultures
*: No organisms seen via dark field microscopy

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	*	2.1E+07	4.4E+07	2.6E+08	3.8E+08	2.3E+08
SMCA	37°C	5.8E+06	8.5E+07	2.1E+08	1.7E+08	3.0E+07	1.4E+07
GT48	30°C	*	3.2E+06	5.8E+07	1.7E+08	4.0E+08	1.5E+08
GT48	37°C	*	2.8E+07	8.8E+07	2.6E+08	4.1E+07	1.7E+07
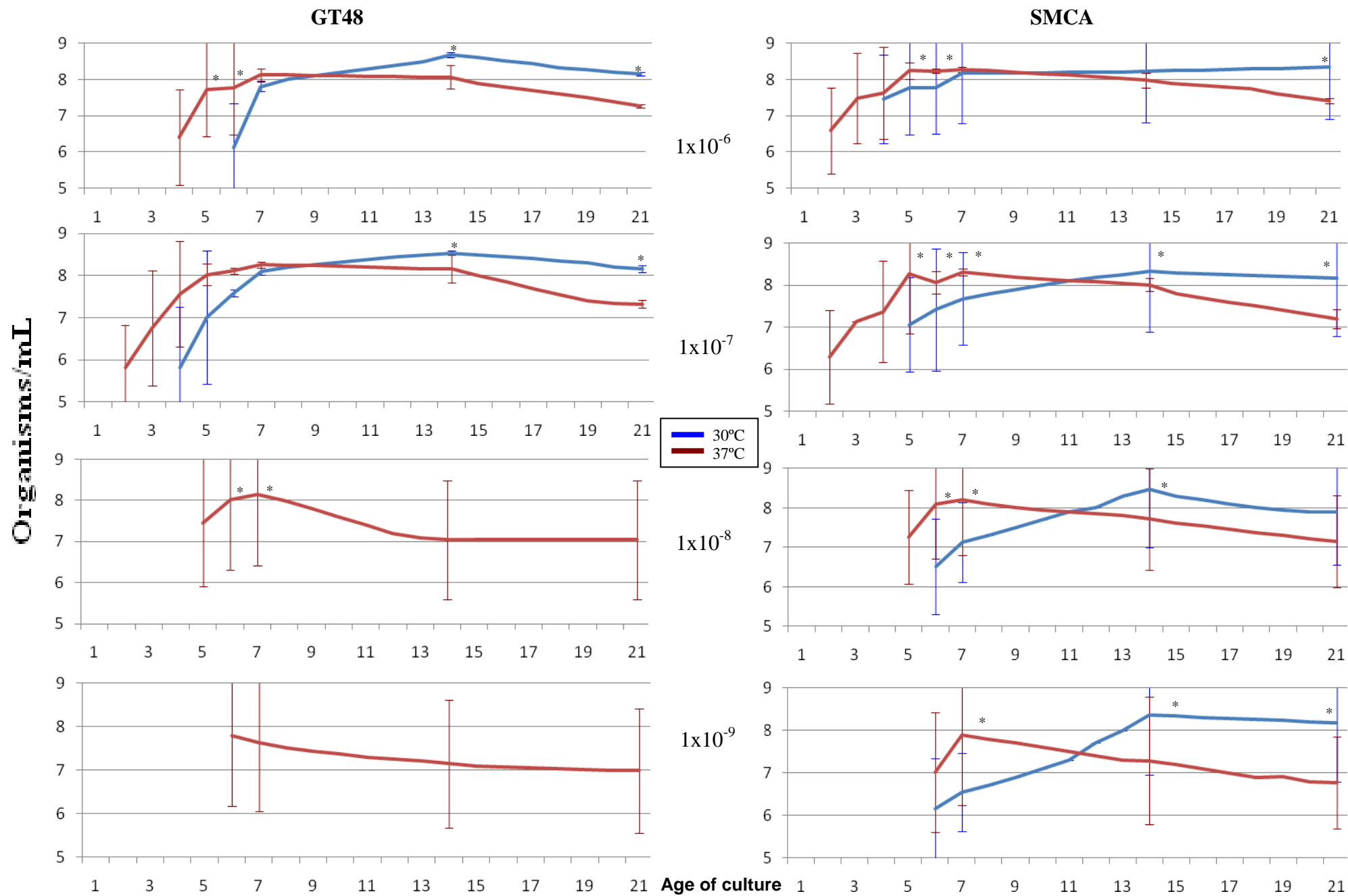
Figure 2.9 Growth of diluted (1×10^{-6} - 1×10^{-9}) GT48 and SMCA cultures over 3 weeks at 30°C and 37°C. Growth yields were determined by dark field observations and log transformed data was compared by pairwise t-test of least squares means for significance where $p \leq 0.05$ (time points designated with * when $p \leq 0.05$).

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Table 2.8 Dark field counts for diluted (1×10^{-6}) SMCA and GT48 cultures
*: No organisms seen via dark field microscopy

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	*	*	6.0E+07	1.5E+08	1.7E+08	2.2E+08
SMCA	37°C	*	3.0E+07	1.8E+08	1.9E+08	9.5E+07	2.6E+07
GT48	30°C	*	*	1.0E+07	1.3E+08	3.5E+08	1.5E+08
GT48	37°C	*	5.8E+06	1.1E+08	1.8E+08	1.4E+08	2.1E+07

Table 2.9 Dark field counts for diluted (1×10^{-7}) SMCA and GT48 cultures
*: No organisms seen via dark field microscopy

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	*	*	1.1E+07	4.8E+07	2.1E+08	1.5E+08
SMCA	37°C	*	*	1.9E+08	2.1E+08	1.0E+08	1.6E+07
GT48	30°C	*	*	*	6.3E+07	4.7E+08	1.5E+08
GT48	37°C	*	*	5.4E+07	1.4E+08	1.2E+08	1.9E+07

Table 2.10 Dark field counts for diluted (1×10^{-8}) SMCA and GT48 cultures
*: No organisms seen via dark field microscopy

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	*	*	*	1.4E+07	2.9E+08	7.7E+07
SMCA	37°C	*	*	1.8E+07	1.6E+08	5.2E+07	1.4E+07
GT48	30°C	*	*	*	9.0E+06	*	*
GT48	37°C	*	*	2.8E+07	1.4E+08	1.1E+07	1.1E+07

Table 2.11 Dark field counts for diluted (1×10^{-9}) SMCA and GT48 cultures
*: No organisms seen via dark field microscopy

Organism	Temp	Dark field counts: organisms/mL					
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21
SMCA	30°C	*	*	*	3.6E+06	2.3E+08	1.5E+08
SMCA	37°C	*	*	*	7.9E+07	1.9E+07	5.8E+06
GT48	30°C	*	*	*	*	*	*
GT48	37°C	*	*	*	4.4E+07	1.4E+07	9.7E+06
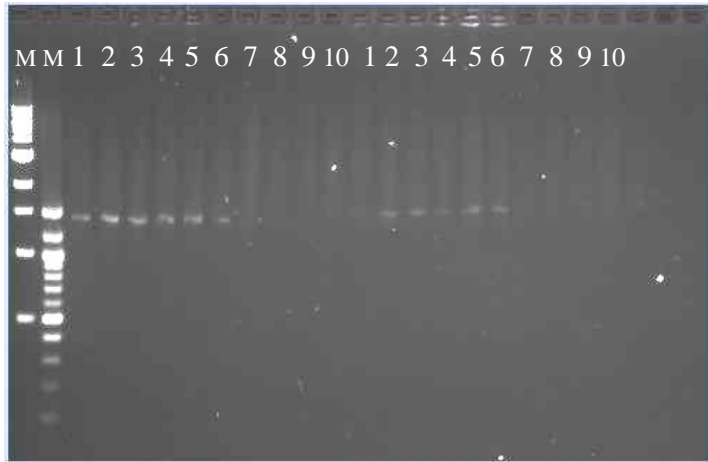
Figure 2.10 PCR results for tenfold dilutions of SMCA at 30°C and 37°C eight days post-inoculation. The first two lanes are markers. The next ten lines represent tenfold dilutions of SMCA cultures at 30°C, followed by tenfold dilutions of SMCA at 37°C. Positive bands are seen at 1460 bp, correlating with the *S. mirum*-related adhesin gene.

- 1c. Medusa formation: After 1 minute of vortexing, large medusas were still present when examined with dark field microscopy. After 5 minutes, only small to medium medusas (Figure 2.11) were still present. After 10 minutes, no medusas were present. The vortexed culture was monitored for growth daily for 7 days and compared to a non-vortexed control culture. The growth curve for vortexed and non-vortexed cultures had no significant differences.
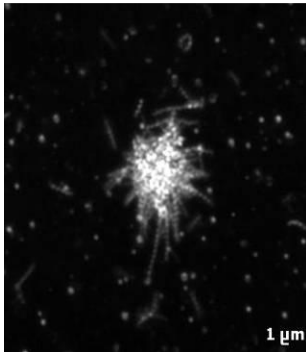
Figure 2.11 An example of a medusa or aggregate of spiroplasma organisms

Experiment 2. Growth in Embryonated Eggs

- 2a. Injection site and incubation: 100% (6/6) of yolk-injected embryonated eggs and 33% (2/6) of allantoic-inoculated eggs that were placed in a non-rocking incubator died within 4-8 days post inoculation. Only 33% (2/6) yolk-injected eggs and 16% (1/6) of allantoic-inoculated eggs died after continuing to incubate in a rocking incubator for 11 days post inoculation. There were no deaths in control eggs with either treatment. Figure 2.12 depicts the results from this experiment.
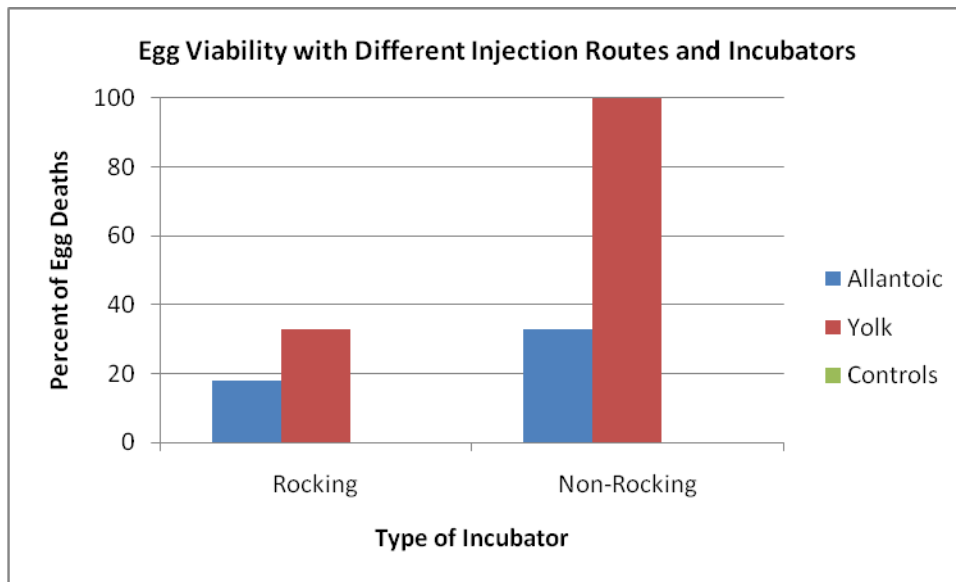
Figure 2.12 Comparison of egg viability when injected via allantoic or yolk sac inoculations and incubated in a rocking or non-rocking incubator.

- 2b. Comparison of egg deaths for GT48 and SMCA: SMCA had a lethality rate of over 70% for all 7 dilutions. GT48 inoculation resulted in fewer egg deaths as the culture was diluted. Visual evidence of spiroplasma in the allantoic fluid via dark field microscopy from non viable eggs was higher for GT48 deaths than for SMCA deaths. Figures 2.13 and 2.14 depict the percentages for deaths and those with positive dark field microscopy.
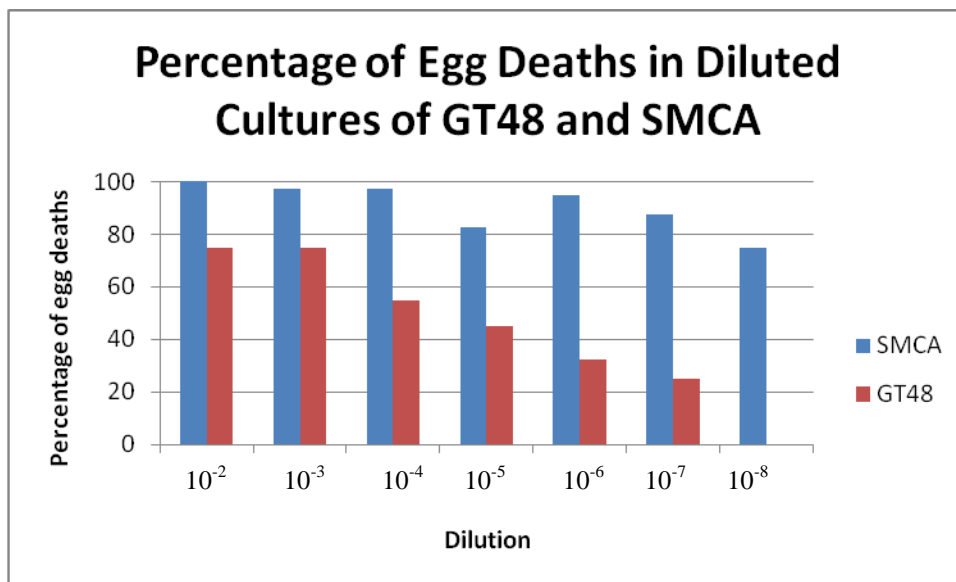
Figure 2.13 Comparison of pathogenicity of SMCA and GT48 in embryonated eggs. Results are shown from tenfold dilutions of SMCA and GT48 cultures inoculated into embryonated eggs via the yolk sac inoculation route and incubated in a non-rocking incubator.
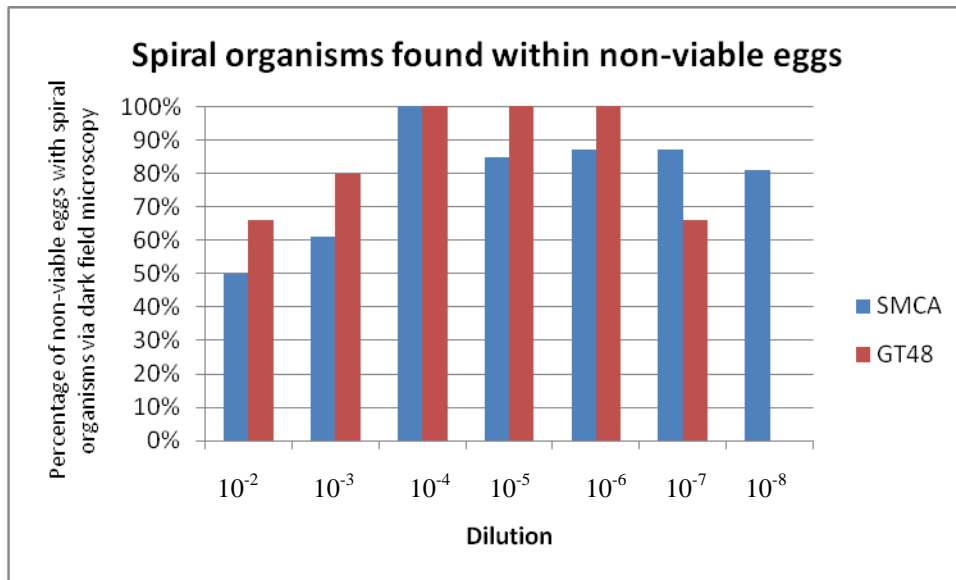
Figure 2.14 Comparison of observed *Spiroplasma* organisms from non-viable embryonated eggs.

- 2c. SMCA effects on embryonated eggs: Figure 2.15 depicts the difference in daily readings of pH in SMCA infected eggs and control eggs. Results from M1D and non inoculated eggs are included for the control pH. The average of all eggs within each group was calculated for these results. If egg death occurred, a note was made and calculations were adjusted based on viable eggs at day of reading. Embryonated eggs inoculated with SMCA resulted in a lower pH than the control eggs. The difference in pH was not found to be significant.
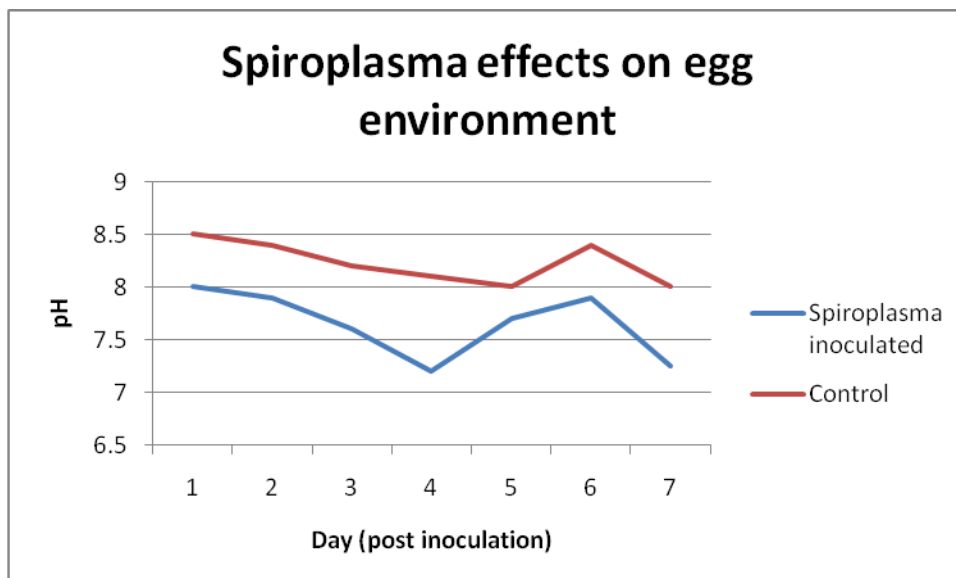
Figure 2.15 Comparison of pH readings in control embryonated eggs and *Spiroplasma* inoculated embryonated eggs.

<u>Experiment 3. Growth on SP4 Plates</u>

- 3a. Bacteria growth characteristics: SP4 plates made with 4 grams (0.8%) of Noble agar revealed diffuse colonies for both SMCA and GT48. *S. melliferum* and *S. citri* SP4 plates had evidence of multiple small pinpoint colonies throughout the plate. SMCA and GT48 inoculated plates revealed circular bodies with multiple satellite bodies when examined under light microscopy which could be found at multiple levels within the agar. Results revealed that dilution would be necessary to allow for individual colony growth, and better growth may be observed on a more solid media. An example of GT48 and *S. melliferum* growth can be seen in Figure 2.16. Figure 2.16 A shows 4 inoculation sites of 20 µL GT48 culture (not diluted). Figure 2.16 B shows a view of colonies at 10x under light microscopy. Figure 2.16 C shows the growth of 50 µL of non-diluted *S. melliferum* culture placed in the center of the plate and allowed to incubate for 10 days at 30°C.
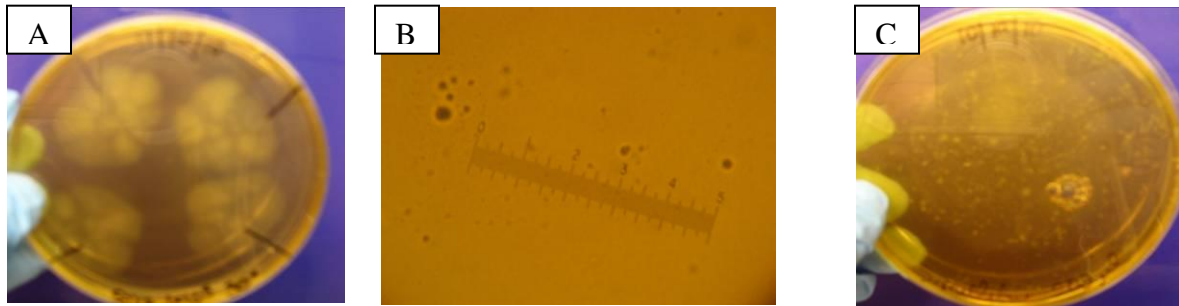
Figure 2.16 GT48 and *S. melliferum* growth on 0.8% Noble agar SP4 plates. Plates were incubated at 30°C for 10 days. A) 4 inoculation sites of 20 µL GT48 culture (not diluted). B) view of colonies at 10x under light microscopy. C) Growth of 50 µL of non diluted *S. melliferum* culture placed in the center of the plate and allowed to incubate for 10 days at 30°C.

- 3b. Agar concentration experiments: Results for 0.8% agar plates are seen in Figure 2.16. SP4 plates made with 1.6% of noble agar revealed small somewhat irregular colonies for both SMCA and GT48 (Figure 2.17). All colonies appeared to be on or near the surface of the agar. Counts were made and recorded in Table 2.12. Based on pairwise t-test, colony forming units were significantly ($p \leq 0.05$) higher at 37°C than 30°C for both GT48 and SMCA on 1.6% agar SP4 plates. SP4 plates made with 2.4% agar revealed "fried egg" colonies for SMCA and GT48. Colony formation was irregular and sparse for even the less diluted cultures. An example of SMCA culture diluted to 1×10^{-3} can be seen in Figure 2.18.

  Positive PCR bands were found from both an agar plug (0.8% plates) and a cotton swab from the surface of a SMCA inoculated 1.6% plate (Data not shown).
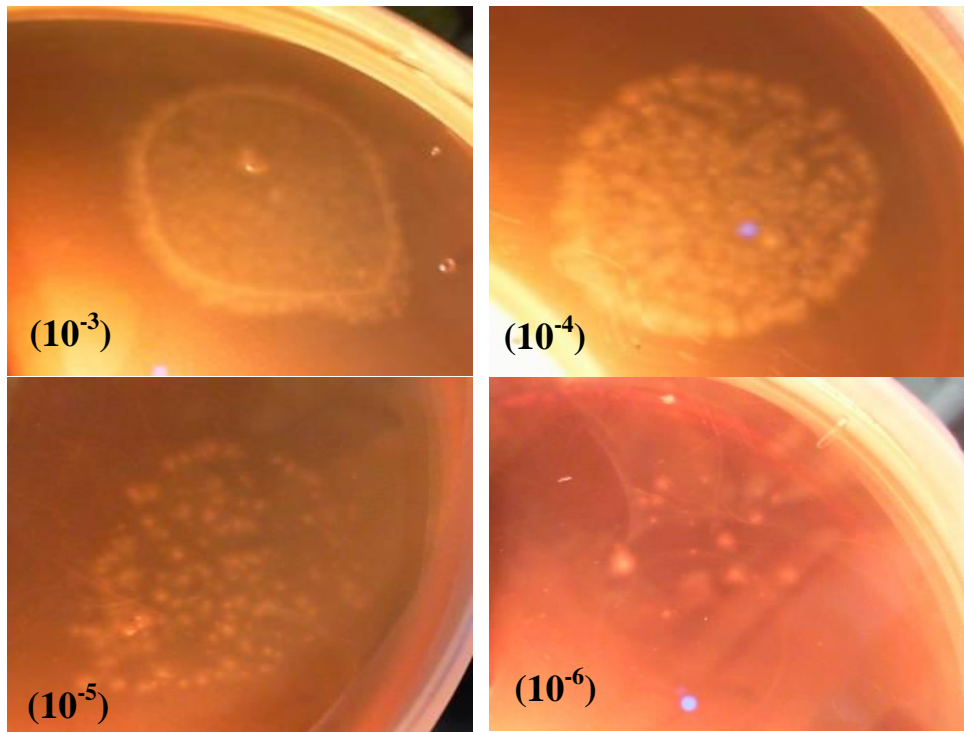
Figure 2.17 Growth of diluted SMCA on SP4 plates made with 1.6% Noble agar.

Table 2.12 Summary of CFUs from diluted 1 day old cultures on modified SP4 plates with 1.6% Noble agar.

Dilution	SCMA 30°C	SMCA 37°C	GT48 30°C	GT48 37°C
10^{-1}	TNTC	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC	TNTC
10^{-3}	TNTC	TNTC	TNTC	TNTC
10^{-4}	TNTC	TNTC	TNTC	TNTC
10^{-5}	1.3E+08	1.6E+08	1.6E+08	2.2E+08

Figure 2.18 Growth of diluted SMCA 10^{-3} on SP4 plates made with 2.4% Noble agar.

Discussion

As the literature indicates, spiroplasmas adapt to different temperatures and media by changing their typical growth characteristics (Bove and Saillard, 1979; Konai et al., 1996; Tully et al., 1983; Whitcomb, 1983). This lab predominantly uses M1D media and therefore needed to assess the optimal growth conditions for laboratory strains of SMCA and GT48 in order to properly study their growth behaviors. M1D media has been used for primary isolation and maintenance of cultures for both *S. citri* and *S. melliferum* (Whitcomb, 1983), and these two spiroplasmas were used for comparison in growth yields.

The temperature range for spiroplasma growth is 5°- 41°C (Konai et al., 1996). These experiments compare 30°C and 37°C. In a previous study, *S. citri* had optimal doubling time at 32°C; *S. melliferum* and *S. mirum* both had optimal doubling times at 37°C (Konai et al., 1996). Doubling time was based on color change alone. The literature has evidence that color change can be a poor indication of growth for SMCA, since it is slow to acidify media and may reach stationary phase of growth before acidifying the media (Whitcomb, 1980). For this reason, we chose to use dark field enumeration to monitor the spiroplasma growth. Based on dark field results, the calculations of organisms per mL are limited to an approximate range of 1×10^{5} to 1×10^{8}. Without being able to detect a true beginning point in the log phase of the growth curve, accurate doubling time cannot be calculated. This limitation in detection of organisms may also limit the accuracy for the peak of growth to determine when stationary phase begins.

All four strains exhibited significantly ($p\leq0.05$) better growth at 30°C based on dark field enumeration. Detection of the logarimthmic growth phase for *S. citri* and *S. melliferum* was limited before they appeared to move into a stationary phase. GT48 cultures had sporadic growth at 37°C, and cultures quickly diminished after reaching the high point in our calculation range. SMCA appeared to grow well at both temperatures, with higher yields at 30°C. This finding is probably an indication of the laboratory strains' adaptation to grow at both temperatures in M1D.

Although an accurate growth curve could not be determined due to the limitations of dark field microscopy, the death phase can be predicted to start sometime after 7 days for cultures at 37°C and 14 days for cultures at 30°C, regardless of dilution. For GT48 cultures between 1-7 days, growth at 37°C was significantly better or the same as growth at 30°C. However, after day 7, growth at 30°C was significantly better. The same can be said regarding SMCA cultures. This finding indicates that *S. mirum* does not have a long stationary phase at 37°C but cultures at 30°C could sustain high levels of organisms for approximatly 1 week before moving into the death phase.

The formation of medusa structures has been seen in other spiroplasmas as well. Whitcomb found that in later stages of *S. citri* cultures, which probably corresponded to the stationary phase, aggregates of bodies appeared in which longer forms of spiroplasma were entangled. He hypothesized that these forms arose by simple aggregation of multiple organisms or from repeated branching of a single organism (Whitcomb, 1980). After disrupting the aggregation by vortexing for 10 minutes, the organisms were not killed from this manipulation, since dark field enumerations post-vortexing were similar to those numbers calculated for a non manipulated

culture. This method appears to be appropriate for disruption of aggregates in order to get accurate dark field counts.

SMCA was originally isolated and characterized using embryonated egg cultures (Clark, 1964). In order to compare the laboratory strains of *S. mirum* to the literature, the embryonated egg research techniques from previous reports were repeated. The first step was to optimize the methods by comparing inoculation site and incubation methods. Yolk sac inoculations followed by non-rocking incubation of embryonated eggs proved to be the best method for embryonated egg experiments based on egg viability results. This followed the findings reported by Clark where the most consistent results were found from yolk sac inoculations compared to allantoic, amniotic, and chorioallantoic membrane inoculations (Clark, 1964).

The literature states that pathogenicity of spiroplasma can be lost due to repeated passages in artificial media (Tully et al., 1982a; Whitcomb, 1980). It was necessary to determine if these laboratory strains had become attenuated after repeated passages in M1D. Overall, SMCA-inoculated eggs had a higher death rate than those inoculated with GT48. These findings also correlate well with results found in literature. Clark (Clark, 1964) found that eggs inoculated with SMCA died within four to seven days post yolk sac inoculation. Eggs inoculated with GT48 exhibited a death curve which was delayed and less uniform then that produced by SMCA. These findings support that the laboratory strains have not been attenuated and still produce reliable results in embryonated eggs.

The visualization of GT48 and SMCA from embryonated eggs was complicated by the gelatinous state of the allantoic fluid causing difficulties in dark field microscopy. PCR results were also inhibited by factors within the egg fluids and could not be used as a reliable method for presence of the organism. The negative results for observed organisms in non-viable eggs are not a reliable indicator for positive presence of the organism. A better method for testing for presence of the organism within non-viable eggs would be passaging the egg fluids into M1D or SP4.

Knowing that spiroplasma acidifies artificial media, it was speculated that the same would be true in embryonated eggs. Spiroplasma did appear to lower the pH within the embryonated egg, however, it was not a significant difference ($p \leq 0.05$).

Obtaining colony plate counts has been historically difficult for spiroplasmas. Not only do the organisms vary in their ability to form colonies, but they are also highly affected by agar content and environmental conditions (Whitcomb, 1980). In experiments conducted by Tully, SMCA and GT48 produced colonies on 3.5% noble agar SP4 plates (original recipe). Typical colonies were somewhat diffuse and irregular, and much of their growth occurred within the agar after 14 to 17 days of incubation at 30°C in an atmosphere of 95% N_2 and 5% CO_2. Similar results were obtained from plates made with 0.8% noble agar. Following the ATCC SP4 recipe with the addition of Noble agar (1.6%) produced consistent countable colonies on or near the surface of the agar. Plates incubated at 37°C did produce a significantly ($p \leq 0.05$) higher number of CFUs. It should be noted that plates should be used immediately for best results.

Whitcomb stated that an effective strategy for obtaining countable colonies was the incorporation of higher concentrations of agar (Whitcomb, 1983). The addition of excess Noble agar produced "fried egg" like colonies, which were described as an indication for poor growth conditions (Whitcomb, 1983). These plates produced a much lower colony count that did not correlate well with our dark field results.

Conclusion

Growth in M1D media at 30°C proved to be significantly better ($p \leq 0.05$), yielding higher dark field counts than cultures at 37°C. Dark field enumeration appears to be limited to a range of $1x10^5$ to $1x10^8$ organisms/mL and therefore an accurate growth curve could not be calculated using this method. PCR was used to check for growth daily and results were comparable to dark field counts. Color changes were evident in all dilutions after 7 days, though the more diluted cultures were less obvious. Embryonated egg results were comparable to that in the literature; however, the artificial media is better for experimental purposes. The organism is difficult to detect after egg fatality by both dark field and PCR. PCR results may be skewed due to inhibitory factors in the egg fluids. Embryonated egg experiments support that the laboratory strains were not attenuated by multiple passages and still produce similar virulence in embryonated egg cultures as seen in literature. A method for obtaining colony forming units was also determined for SMCA and GT48. Following the ATCC SP4 recipe with the addition of Noble agar (1.6%), *S. mirum* produced consistent countable colonies on or near the surface of the agar. Those plates incubated at 37°C yielded higher growth counts and significantly better growth ($p \leq 0.05$) than those at 30°C.

Although the media used in these experiments supported spiroplasma growth, other media should still be explored. Research has been promising for some tissue cultures but none have been successful. The establishment of a culture system may better elucidate the organism's potential hosts and pathogenic nature.

CHAPTER 3:
EFFECTS OF DISINFECTANTS AND ANTIBIOTICS ON *SPIROPLASMA* SPP.

Introduction

Mollicutes are a class of bacteria distinguished by the absence of a cell wall. The word "Mollicutes" is derived from the Latin mollis (meaning "soft" or "pliable") and cutis (meaning "skin"). They are very small, typically only 0.2–0.3 μm in size and have a very small genome. *Mollicutes* are the smallest self-replicating organisms known. They are classified into five genera: *Acholeplasma*, *Anaeroplasma*, *Mycoplasma*, *Spiroplasma* and *Ureaplasma* (Murray and Schleifer, 1994). All members of the genus *Spiroplasma* are obligately associated with insects, either as commensals, pathogens or mutualists (Gasparich, 2002). Spiroplasmas are also plant pathogens implicated in many economical crop diseases. *Spiroplasma mirum,* isolated from a rabbit tick, is the only spiroplasma that has been found to be experimentally pathogenic to vertebrates (Bove, 1997). Suckling mouse cataract agent (SMCA) represents one of the strains of *S*. *mirum*. As the name describes, SMCA inoculated intracerebrally caused suckling mice to develop cataracts (Clark, 1964). The organism was able to persist in suckling mice, specifically in the brain tissues for up to two years (Clark and Karson, 1968). It was able to cross the blood brain barrier, assumingly without provoking an immune response, and caused a persistent neurologic infection (Bastian, 2005; Bastian and Jennings, 1984; Clark and Karzon, 1968; Tully et al., 1984). Due to this observation, it was tentatively included with the slow viruses or chronic infectious neuropathic agents (Hotchin, 1967). It was implicated as a possible causative agent for transmissible spongiform encephalopathies (TSEs) (Bastian, 2005).

TSEs encompass a condition that affects humans and a variety of animals with a fatal neurodegenerative disease and associated neurological clinical signs. The disease presents with morphological and pathophysiological features that parallel other progressive encephalopathies such as Alzheimer's and Parkinson's disease (Aguzzi and Haase, 2003). Research currently supports evidence that the agent responsible for TSE is a prion, a small proteinaceous infectious particle (Prusiner, 1982). The prion is a cell surface glycoprotein (Stahl et al., 1987) with an unknown function. The pathogenesis of the prion is still widely unknown; however it is accepted that the normal prion PrP^c undergoes some sort of posttranslational modification that converts it to a pathogenic prion PrP^{sc}. PrP^{sc} is an insoluble and partially protease-resistant isoform that propagates itself by imposing its abnormal conformation onto other PrP^c molecules (Aguzzi and Calella, 2009). The human prion diseases include Kuru, Creutzfeldt-Jakob disease (CJD), variant CJD, Gerstmann-Straussler-Scheinker (GSS) disease, and fatal familial insomnia (FFI). The most common animal TSEs are scrapie, which affects sheep and goats; bovine spongiform encephalopathy (BSE); and chronic wasting disease (CWD), which affects deer and elk.

The difficulty of inactivating TSE agents first became apparent when sheep developed scrapie post-vaccination against louping-ill. The vaccine was contaminated with the scrapie agent that had survived exposure to 0.35% formalin (Taylor et al., 1999). Iatrogenic CJD has been described in humans in three circumstances: in patients for whom medical equipment was used during intracranial placement of contaminated electroencephalography electrodes or neurosurgical procedures; in patients who received hormone therapy with cadaveric human growth hormone or gonadotropin; and in patients who received an implant of contaminated

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grafts. All known instances of iatrogenic CJD have resulted from exposure to infectious brain, pituitary, or eye tissue (Rutala and Weber, 2010). It is generally accepted that BSE was caused by the transmission of the scrapie agent to bovines via foodstuff. It is now known that the heating process used in many of the rendering procedures used traditionally to manufacture meat and bone meal do not completely inactivate BSE or scrapie agents (Taylor et al., 1999).

A multitude of research has been dedicated to determining inactivation procedures for prion-infected tissues. Table 3.1 summarizes the research on effective and ineffective decontamination procedures (Rutala and Weber, 2010).

Research has also been directed towards determining pre-mortem therapy for TSE infections. Forloni found that several compounds have been found to antagonize prion propagation in cellular and/or animal models of disease. These include polyanions, polyene antibiotics, congo red, iododoxorubicin, tetrapyrroles, branched polyamines, tetracyclines, and modified PrP peptides. Unfortunately most of these compounds do not cross the blood-brain barrier and are therefore useless in TSE therapy (Forloni et al., 2002).

Resistance to a variety of chemical and physical disinfectants has been cited as a common factor between *Spiroplasma* and TSE infectious agents (Bastian, 2005). Bastian reported that *Spiroplasma* can resist exposure to temperatures near boiling, glutaraldehyde and other fixatives (Bastian, 2005). This chapter will focus on *Spiroplasma's* resistance to a variety of physical and chemical disinfectants as well as antibiotics. Susceptibility will be assessed by dilution and time of exposure for common laboratory disinfectants, and an antibiotic profile for SMCA, GT48, *S. citri*, and *S. melliferum* will be determined. The information gathered from these experiments will aid in further evaluating *Spiroplasma's* possible role in TSEs.

Spiroplasma's ability to cross the blood brain barrier, assumingly without provoking an immune response, will also be further tested in this chapter. Complement plays a role in clearing invading foreign antigens as part of the body's innate immune system or is activated by the adaptive immune system via antibodies. In this chapter, we will test complement's innate activity against spiroplasmas using normal goat complement. Antibody-mediated complement fixation will also be tested using goats hyperimmunized with killed SMCA antigens. This will help determine the immune systems role in spiroplasma clearance.

Materials and Methods

Bacteria: Two strains of *S. mirum* were used, SMCA and GT48. Strain SMCA was obtained from Dr. Frank Bastian, Tulane Medical Center, New Orleans, LA. GT48, ATCC strain #29334, was obtained from the ATCC Bioresource Center. *Spiroplasma melliferrum (*ATCC strain BC-3 #33219 Vc161, created on 17MAR90, pass # 3) and *S. citri* (ATCC strain R8A2 #27556 130 Allen, created on 15Mar95, pass # 4) were also obtained from the ATCC Bioresource Center.

Stock cultures of all strains of spiroplasma (approximately $1x10^8$ organisms per mL) were used for the following experiments. Cultures were pelleted by centrifugation for 1 hour at 16000xg

Table 3.1 Efficacy of decontamination procedures for inactivating prions (Rutala and Weber, 2010)

Ineffective (< 3 log reduction)	Effective (> 3 log reduction)
	Autoclave at 121°C–132°C for 1 hour (gravity displacement sterilizer) or 121°C for 30 minutes (prevacuum sterilizer)
	Autoclave at 134°C for 18 minutes (prevacuum sterilizer)
	Autoclave at 134°C for 18 minutes immersed in water
Autoclave at standard exposure conditions (121°C for 15 minutes)	Sodium dodecyl sulfate, 2%, plus acetic acid, 1%, plus autoclave at 121°C for 15–30 minutes
	Sodium hydroxide (NaOH), 0.09 N or 0.9 N, for 2 hours plus autoclave at 121°C for 1 hour (gravity displacement sterilizer)
Acetone	
Alcohol, 50%–100%	
Ammonia, 1.0 M	
Boiling	
Chlorine dioxide, 50 ppm	Chlorine, 11,000 ppm
Dry heat	
Ethylene oxide	
	Guanidine thiocyanate, 13 M
Formaldehyde, 3.7%	
Glutaraldehyde, 5%	
Hydrochloric acid, 1.0 N	
	Copper, 0.5 mmol/L, and hydrogen peroxide, 100 mmol/L
Hydrogen peroxide, 0.2%, 3%, 6%, 30%, 60%	Hydrogen peroxide, 59%
	Vaporized hydrogen peroxide, 1.5–2 mg/L
Hydrogen peroxide gas plasma, Sterrad 100S (ASP)	Hydrogen peroxide gas plasma (Sterrad NX)
Iodine, 2%	
Microwave	
Ortho-phthalaldehyde, 0.55%	
Peracetic acid, 0.2%–19%	Peracetic acid, 0.2%
Phenol/phenolics (concentration variable)	Phenolic disinfectant (specific formulation), 10.9%
Potassium permanganate, 0.1%–0.8	
Sodium dodecyl sulfate, 1%–5%	Sodium dodecyl sulfate, 2%, and acetic acid, 1%
	Sodium hydroxide, x1 N
	Sodium metaperiodate, 0.01 M
Sodium deoxycholate 5%	
	Radiofrequency gas plasma
Tego (dodecyl-di[aminoethyl]-glycine), 5%	
Triton X-100, 1%	
Urea, 4–8 M	
UV light Ionizing radiation	

and resuspended in sterile PBS. Growth of *Spiroplasma* in M1D media was determined by color change indicated by phenol red acidification and confirmed by dark field microscopy.

M1D Media: The ingredients for M1D are seen in Table 3.2. Media was filter sterilized through a 0.2 μm filter and stored at -4°C. Media was heated to room temperature and containers

were sterilized with 70% ethanol before use to reduce possible bacterial contamination. Once inoculated with bacteria, media was monitored for growth via dark field microscopy and/or color change.

Table 3.2 M1D media (yields 300 mLs)

Ingredients	
Mycoplasma broth base	2.1 g
Bacto-Tryptone	1 g
Bacto-Peptone	0.8 g
Glucose	0.1 g
Fructose	0.1 g
Sucrose	1 g
Sorbitol	7 g
Distilled water	70 mL
Schneiders Drosophilia medium	160 mL
Fetal Bovine Serum	50 mL
Fresh yeast extract (25%)	10 mL
Phenol Red 0.5%	1.2 mL
Potassium Penicillin (100,000 U/ml)	2.5 mL

Dark Field Microscopy: A 5 µL culture sample was placed on a clean glass microscope slide, mounted with a #1.5 cover slip and sealed on all four sides with clear finger nail polish. Slides were examined with a Ziess Axio Imager A1 microscope (Carl Zeiss, Inc. North America, Thornwood, NY, USA) equipped with a dark-field top lens condenser 1.2-1.4 and an EC Paln-NeoFluar x 100 oil immersion objective (Carl Zeiss, Inc. North America, Thornwood, NY, USA) with 1.3 iris closed to its lowest setting (0.7).

Disinfectant experiments: Broth dilution and especially microbroth dilution methods for testing disinfectant or antibiotic susceptibility have been modified for mollicutes. The method employs spiroplasma media with decreasing disinfectant concentrations, inoculated with a standardized number of microorganisms in a 96-well microtiter plate (Bebear and Bebear, 2002). The mean inhibitory concentration (MIC) is defined as the lowest concentration of disinfectant that prevents a color change at the time when the color in the control without disinfectant has changed. Turbidity or inappropriate color change in broth control indicates bacterial contamination.   The following experiments will follow these procedures.

100 µL of a specific species of spiroplasma (approximately 1×10^8 organisms per mL) resuspended in PBS was placed in all wells of a 96 well plate. 100 µL of disinfectant was then added to the first vertical column of the microtiter plate. Original concentrations of disinfectants are indicated in Table 3.2. 100 µL was then transferred through the adjacent row of wells to create 1:2 dilutions. At set time points (1, 5, 10, 15, 30, and 60 minutes), 20 µL of each dilution was transferred into 280 µL fresh M1D media in a separate 96 well plate. A row in the plate was

left with media alone, and the last row was seeded with normal Spiroplasma culture as a positive control. All transferred cultures were incubated at 30°C for 7 days. Growth was confirmed via dark field microscopy. A list of disinfectants used can be found in Table 3.3. The same procedure was followed for all liquid disinfectants. Experiments were duplicated to establish reproducibility.

For heat lability experiments, 1 mL of *Spiroplasma* culture resuspended in PBS was placed in a 1-mL centrifuge tube and submerged in a water bath at varying temperatures (45°C, 50°C, 56°C). For ultraviolet experiments, 1 mL of *Spiroplasma* culture resuspended in PBS was left in an open 1 mL polystyrene centrifuge tube and placed under a UV light. At set time points(1, 5, 10, 15, 30, and 60 minutes), 20 µL of culture was removed and placed into 280 µL fresh media in a separate 96 well plate and incubated as above. A row in the plate was left with media alone, and the last row was seeded with normal Spiroplasma culture as a positive control. For autoclaving experiments, 1ml of *Spiroplasma* culture was placed in a glass test tube and autoclaved for 15 minutes at 121ºC. 100 µl of culture was removed prior to autoclaving and placed in 900 µl of fresh M1D to serve as a control.

Table 3.3 Summary of disinfectants tested for spiroplasma susceptibility

Disinfectant		Range of dilution
Alcohols	Ethanol	70-0.02%
	Methanol	100-0.02%
	Isopropanol	100-0.02%
Aldehydes	Formalin	20-0.002%
	Gluteraldehyde	100-0.02%
Oxidizing Agents	Sodium Hypochlorite	5.25-0.001%
	Povidone-iodine	100-0.02%
	Hydrogen Peroxide	3-0.001%
Solvent	Acetone	100-0.02%
Surfactants	Chlorhexidine	2-0.001%
	Sodium Hydroxide (1 Molar)	100-0.02%
	SDS	10-0.002%
	Roccal-D Plus	100-0.02%
Buffers	Tris (pH 7)	100-0.02%
	Tween-20	100-0.02%
Ethylenediaminetetraacetic acid (EDTA) (1 Molar)		100-0.02%
Irradiation	Ultraviolet Light	0-60 minutes of exposure
Temperature Changes	Autoclave	121°C for 15 minutes
	Heat	45°C, 50°C, 56°C

Antibiotic experiments: A fresh 1:100 dilution stock culture was made of each species. 200 µL of culture was placed in a TREK Diagnostic Systems Sensititre Avian plate, Cleveland, Ohio. Three wells within the diagnostic plate were not seeded with any antimicrobials to serve as controls. A list of antimicrobial agents and the ranges tested can be found in Table 3.4. Each plate was incubated at 30°C for 7 days. Growth was confirmed by dark field microscopy. Experiments were duplicated to establish reproducibility.

Table 3.4 Summary of antibiotics used to test spiroplasma susceptibility

Antibiotic		Range (µg/mL)	Bacteriocidal or Bacteriostatic	Mechanism of action
Fluorquinolones	Enrofloxacin	2-0.12	Bacteriocidal	Inhibits DNA gyrase
Aminocoumarin	Novobiocin	4-0.5	Bacteriocidal	Inhibits DNA gyrase
Penicillins	Penicillin	8-0.5	Bacteriocidal	Inhibit cell wall synthesis
	Amoxicillin	16-0.25		
Cephalorsporins	Cetfiofur	4-0.25	Bacteriocidal	Inhibits cell wall synthesis
Aminoglycosides	Streptomycin	1024-8	Bacteriocidal	Bind 30S ribosome and inhibit protein synthesis
	Neomycin	32-2		
	Gentamicin	8-0.5		
Aminocyclitols	Spectinomycin	64-8	Bacteriostatic	Bind 30S ribosome and inhibit protein synthesis
Tetracyclines	Tetracycline	8-0.25	Bacteriostatic	Bind 30S ribosome and inhibit protein synthesis
	Oxytetracycline	8-0.25		
Macrolides	Erythromycin	4-0.12	Bacteriostatic	Bind 50S ribosome and inhibit protein synthesis
	Tylosin tartrate	20-2.5		
Florfenicol		8-1	Bacteriostatic	Bind 50S ribosome and inhibit protein synthesis
Lincosamides	Clindamycin	4-0.5	Bacteriostatic	Bind 50S ribosome and inhibit protein synthesis
Sulfonamides	Sulfadimethoxine	256-32	Bacteriostatic	Inhibits bacterial synthesis of folic acid
	Sulphathiazole	256-32		
	Trimethoprim/ sulfamethoxazole	2/38-0.5/9.5		

Complement experiments: Complement susceptibility tests were conducted on all *Spiroplasma* to evaluate its role in the immune system response to spiroplasma. Blood was collected from the jugular vein of a normal goat in a red top vacutainer tube (no additives). The tubes were immediately put on ice to preserve complement. Tubes were spun at what speed after icing overnight, and the complement-preserved serum was removed and stored at -20°C. Blood was also collected following the same procedures from goats hyperimmunized with killed SMCA antigen. Previously, two goats were immunized with boiled SMCA antigen. 1 mL of the solution was mixed with 1 mL of incomplete freund's adjuvant. The 2 mL mixture was injected intramuscularly into two different goats. Goats were boosted with 0.5 mL SMCA and 0.5 mL incomplete freund's adjuvant 4 and 6 weeks later. To serve as controls, 1 mL of complement-preserved serum from normal goats and hyperimmunized goats was heated in a water bath at 56°C for 1 hour. 500 µL of *Spiroplasma* culture was mixed with 500 µL of complement-preserved normal serum, 500 µL of complement-preserved hyperimmunized serum, or the 500 µLof the heat inactivated controls. At set time points, 20 uL of culture was removed and placed into 280 µL fresh media in a separate 96-well plate and incubated as above.

Results

Disinfectant experiments: Following a two-fold dilution series, the concentration listed in the results below is the smallest dilution of disinfectant which inhibited spiroplasma growth.

- Alcohols: *Spiroplasma* spp. were susceptible to low levels of alcohol treatment. After 1 minute of exposure to 35% ethanol, all spiroplasma growth was inhibited (Table 3.5). Methanol and isopropanol were also effective on contact and inhibited growth at 50% concentration (Table 3.6 and 3.7).

Table 3.5 Concentration of ethanol (70-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	**35%**	**35%**	**35%**	**35%**
5 minutes	35%	35%	35%	35%
10 minutes	35%	35%	35%	35%
15 minutes	35%	35%	35%	35%
30 minutes	35%	35%	35%	35%
60 minutes	35%	35%	35%	35%

Table 3.6 Concentration of methanol (100-0.02%) that inhibited spiroplasma growth

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	**50%**	**50%**	100%	50%
5 minutes	50%	50%	**50%**	50%
10 minutes	50%	50%	50%	50%
15 minutes	50%	50%	50%	50%
30 minutes	50%	50%	50%	50%
60 minutes	50%	50%	50%	**25%**

Table 3.7 Concentration of isopropanol (100-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	50%	**50%**	50%	50%
5 minutes	50%	50%	**25%**	50%
10 minutes	50%	50%	25%	50%
15 minutes	50%	50%	25%	50%
30 minutes	25%	50%	25%	25%
60 minutes	**12.5%**	50%	25%	**12.5%**

- Aldehydes: *Spiroplasmas* were susceptible to 3-0.04% formalin (Table 3.8). GT48 was able to grow in 50% gluteraldehyde, but all other *Spiroplasma* tested were inactivated by 0.8% gluteraldehyde or less (Table 3.9).

Table 3.8 Concentration of formalin (20-0.002%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	0.6%	2.5%	0.3%	0.04%
5 minutes	0.6%	2.5%	0.3%	0.04%
10 minutes	0.6%	2.5%	0.3%	0.04%
15 minutes	0.6%	2.5%	0.3%	0.04%
30 minutes	0.6%	2.5%	0.3%	0.04%
60 minutes	0.6%	2.5%	0.3%	0.04%

Table 3.9 Concentration of gluteraldehyde (100-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48*	*S. melliferum*	*S. citri*
1 minute	1.6%	50%	6.2%	1.6%
5 minutes	1.6%	50%	1.6%	1.6%
10 minutes	0.8%	50%	1.6%	1.6%
15 minutes	0.8%	50%	1.6%	0.8%
30 minutes	0.4%	50%	0.8%	0.8%
60 minutes	0.2%	50%	0.8%	0.4%

* GT48 survived in all dilutions of Gluteraldehyde.

- Oxidizing Agents: The oxidizing agents had a major impact on *Spiroplasma* growth. Iodine did not allow growth at even the lowest dilution (0.02%) for any spiroplasma species (Table 3.11). Sodium hypochlorite inhibited growth of all *Spiroplasma* at very small dilutions (Table 3.10) as did hydrogen peroxide (Table 3.12)

Table 3.10 Concentration of sodium hypochlorite (5.25-0.001%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri**
1 minute	0.04%	0.04%	0.006%	0.003%
5 minutes	0.04%	0.04%	0.006%	0.003%
10 minutes	0.04%	0.04%	0.006%	0.003%
15 minutes	0.02%	0.04%	0.006%	0.003%
30 minutes	0.02%	0.04%	0.003%	0.003%
60 minutes	0.02%	0.04%	0.003%	0.003%

* *S. citri* had no growth. The lowest dilution tested is listed.
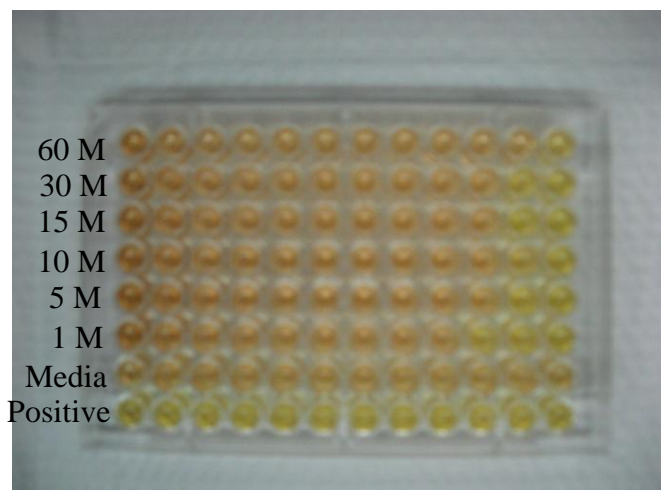
Figure 3.1 Example of 96-well plate used to study the effects of sodium hypochlorite on spiroplasma growth

Table 3.11 Concentration of povidone-iodine (100-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri**
1 minute	1.6%	0.05%	1.6%	**0.025%**
5 minutes	0.4%	0.05%	0.4%	0.025%
10 minutes	0.4%	0.05%	0.4%	0.025%
15 minutes	0.1%	0.05%	0.1%	0.025%
30 minutes	0.1%	**0.025%****	0.1%	0.025%
60 minutes	**0.025%****	**0.025%****	**0.025%****	0.025%

* *S. citri* had no growth. The lowest dilution tested is listed.
**No growth in the lowest dilution tested.

Table 3.12 Concentration of hydrogen peroxide (3-0.001%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	0.09%	**0.09%**	**0.18%**	0.09%
5 minutes	0.09%	0.09%	0.18%	0.09%
10 minutes	0.09%	0.09%	0.18%	0.09%
15 minutes	0.09%	0.09%	0.18%	**0.05%**
30 minutes	0.09%	0.09%	0.18%	0.05%
60 minutes	**0.05%**	0.09%	0.18%	0.05%

- Solvent: Acetone (50%) was effective against *Spiroplasma* growth (Table 3.13).

Table 3.13 Concentration of acetone (100-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	**50%**	**25%**	**50%**	**50%**
5 minutes	50%	25%	50%	50%
10 minutes	50%	25%	50%	50%
15 minutes	50%	25%	50%	50%
30 minutes	50%	25%	50%	50%
60 minutes	50%	25%	50%	50%

- Surfactants: All surfactants tested were able to inhibit spiroplasma growth at low dilutions. Chlorhexidine (Table 3.14) and SDS (Table 3.16) were able to inhibit most species tested at all dilutions. Sodium hydroxide, an effective agent for prions, inhibited growth at 3.2% for all *Spiroplasma* (Table 3.15). Roccal-D Plus, used around herd animals, was also very effective, inhibiting growth at dilutions of 0.4% and less (Table 3.17).

Table 3.14 Concentration of chlorhexidine (2-0.001%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	0.004%	0.002%	0.008%	0.002%
5 minutes	0.002%	0.002%.	**0.004%**	0.002%
10 minutes	0.002%	0.002%.	0.004%	0.002%
15 minutes	0.002%	0.002%	0.004%	**0.001%****
30 minutes	0.002%	**0.001%****	0.004%	**0.001%****
60 minutes	**0.001%****	**0.001%****	0.004%	**0.001%****

.**No growth in the lowest dilution tested

Table 3.15 Concentration of sodium hydroxide (1 mol) (100-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	6.2%	**3.13%**	6.2%	6.2%
5 minutes	3.13%	3.13%	6.2%	3.13%
10 minutes	3.13%	3.13%	**3.13%**	3.13%
15 minutes	3.13%	3.13%	3.13%	1.6%
30 minutes	3.13%	3.13%	3.13%	**0.8%**
60 minutes	**1.6%**	3.13%	3.13%	0.8%

Table 3.16 Concentration of SDS (10-0.002%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	0.01%	**0.02%**	0.02%	0.005%
5 minutes	0.005%	0.02%	0.02%	0.005%
10 minutes	0.005%	0.02%	0.02%	0.005%
15 minutes	**0.002%****	0.02%	**0.01%**	**0.002%****
30 minutes	**0.002%****	0.02%	0.01%	**0.002%****
60 minutes	**0.002%****	0.02%	0.01%	**0.002%****

**No growth in the lowest dilution tested.

Table 3.17 Concentration of Roccal-D Plus (100-0.02%) that inhibited spiroplasma growth.

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	**0.4%**	**0.1%**	0.8%	0.4%
5 minutes	0.4%	0.1%	0.8%	0.4%
10 minutes	0.4%	0.1%	0.4%	0.4%
15 minutes	0.4%	0.1%	0.4%	0.4%
30 minutes	0.4%	0.1%	**0.2%**	0.4%
60 minutes	0.4%	0.1%	0.2%	**0.2%**

- Buffers: Tris (pH 7) and Tween-20 allowed growth for all *Spiroplasmas* at all dilutions and all time points (data not shown).

- EDTA: EDTA (1 mol) did not inhibit growth of any *Spiroplasma* strain tested at any dilution or time point (data not shown).

- Irradiation: With the exception of GT48, *Spiroplasmas* are also susceptible to UV irradiation after 1 hour (Table 3.18).

Table 3.18 Spiroplasma growth in the presence of ultraviolet irradiation at various times

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	Growth	Growth	Growth	Growth
5 minutes	Growth	Growth	Growth	Growth
10 minutes	Growth	Growth	Growth	Growth
15 minutes	Growth	Growth	Growth	Growth
30 minutes	Growth	Growth	Growth	Growth
60 minutes	**No Growth**	Growth	**No Growth**	**No Growth**

- Temperature: All *Spiroplasma* species were inactivated following autoclaving at 121ºC, 15 psi (Table 3.19). SMCA was able to survive 1 hour at 40°C and 50°C but only 10 minutes at 56°C (Tables 3.20-3.22). *Spiroplasma melliferum* and *S. citri* survived only 30 minutes at both 50°C and 56°C (Tables 3.20-3.22). GT48 was able to survive 1 hour at all temperatures tested (Tables 3.20-3.22).

Table 3.19 Spiroplasma growth following autoclaving (121°, 15psi)

Time	SMCA	GT48	*S. melliferum*	*S. citri*
15 minutes	No Growth	No Growth	No Growth	No Growth

Table 3.20 Spiroplasma growth in the presence of heat (45° C) at various times

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	Growth	Growth	Growth	Growth
5 minutes	Growth	Growth	Growth	Growth
10 minutes	Growth	Growth	Growth	Growth
15 minutes	Growth	Growth	Growth	Growth
30 minutes	Growth	Growth	Growth	Growth
60 minutes	Growth	Growth	Growth	Growth

Table 3.21 Spiroplasma growth in the presence of heat (50° C) at various times

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	Growth	Growth	Growth	Growth
5 minutes	Growth	Growth	Growth	Growth
10 minutes	Growth	Growth	Growth	Growth
15 minutes	Growth	Growth	Growth	Growth
30 minutes	Growth	Growth	Growth	Growth
60 minutes	Growth	Growth	No Growth	No Growth

Table 3.22 Spiroplasma growth in the presence of heat (56° C) at various times

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	Growth	Growth	Growth	Growth
5 minutes	Growth	Growth	Growth	Growth
10 minutes	Growth	Growth	Growth	Growth
15 minutes	No Growth	Growth	No Growth	Growth
30 minutes	No Growth	Growth	No Growth	No Growth
60 minutes	No Growth	Growth	No Growth	No Growth
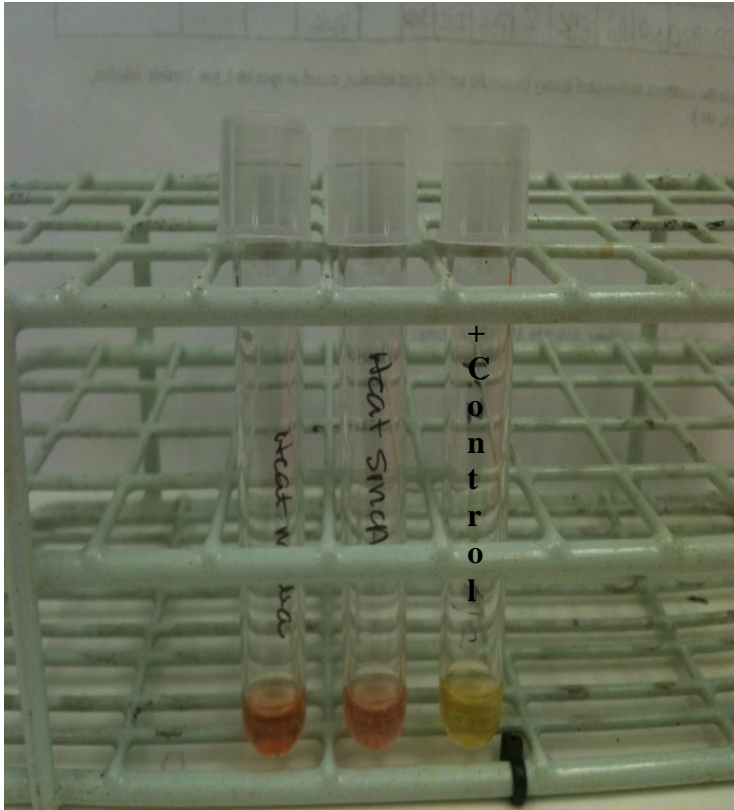
Figure 3.2 Examples of cultures growing at 30°C after heat experiments. The center tube contains SMCA after 1 hour at 56°C. A positive control containing normally incubated bacteria in media (yellow fluid) is found to the right and was used to help correlate color change. A negative control of media alone (orange fluid) was heated along with SMCA culture to be sure that no color change would occur due to the heating process.

Overall *Spiroplasma* was susceptible to most of the common laboratory disinfectant methods. Furthermore we found that the majority were effective at very low concentrations and at immediate contact (<1minute). GT48 was the most resilient of the *Spiroplasma* tested, showing growth at higher concentrations and higher temperatures than the other *Spiroplasma*. Table 3.23 summarizes the smallest percentage of disinfectant positive for *Spiroplasma* growth and the contact time required to reach this end point. Table 3.24 summarizes the percentage of disinfectant positive for *Spiroplasma* growth after 60 minutes.

Table 3.23 Minimal inhibitory concentration and time required of disinfectants for spiroplasma.
G: Growth, NG: No growth

Disinfectant		Range of dilution	SMCA		GT48		*S. melliferum*		*S. citri*	
Alcohols	Ethanol	70-0.02%	35%	1m	35%	1m	35%	1m	35%	1m
	Methanol	100-0.02%	50%	1m	50%	1m	50%	5m	25%	60m
	Isopropanol	100-0.02%	12.5%	60m	50%	1m	25%	5m	12.5%	60m
Aldehydes	Formalin	20-0.002%	0.6%	1m	2.5%	1m	0.4%	1m	0.04%	1m
	Gluteraldehyde	100-0.02%	0.2%	60m	G	60	0.8%	30m	0.4%	60m
Oxidizing Agents	Sodium Hypochlorite	5.25-0.001%	0.02%	15m	0.04%	1m	0.002%	30m	NG	1m
	Povidone-iodine	100-0.02%	NG	60m	NG	30m	NG	60m	NG	1m
	Hydrogen Peroxide	3-0.001%	0.1%	5m	0.1%	1m	0.18%	1m	0.04%	15m
Solvent	Acetone	100-0.02%	50%	1m	25%	1m	50%	1m	50%	1m
Surfactants	Chlorhexidine	2-0.001%	NG	60m	NG	30m	0.004%	5m	NG	15m
	Sodium Hydroxide (1 Molar)	100-0.02%	1.6%	60m	3.2%	1m	3.2%	10m	0.8%	30m
	SDS	10-0.002%	NG	15m	0.02%	1m	0.01%	15m	NG	15m
	Roccal-D Plus	100-0.02%	0.4%	1m	0.1%	1m	0.2%	30m	0.2%	60m
Buffers	Tris (pH 7)	100-0.02%	G	60m	G	60m	G	60m	G	60m
	Tween-20	100-0.02%	G	60m	G	60m	G	60m	G	60m
EDTA (1 Molar)		100-0.02%	G	60m	G	60m	G	60m	G	60m
Irradiation	Ultraviolet Light	0-60 minutes of exposure	G	30m	G	60m	G	30m	G	30m
Temperature Changes	Autoclave	121°C for 15 minutes	NG	-	NG	-	NG	-	NG	-
	Heat	45°C	G	60m	G	60m	G	60m	G	60m
		50°C	G	60m	G	60m	G	30m	G	30m
		56°C	G	10m	G	60m	G	10m	G	15m

Table 3.24 Minimal inhibitory concentration of disinfectant for spiroplasma after 60 minutes
G: Growth, NG: No growth

Disinfectant		Range of dilution	SMCA	GT48	*S. melliferum*	*S. citri*
Alcohols	Ethanol	70-0.02%	35%	35%	35%	35%
	Methanol	100-0.02%	50%	50%	50%	25%
	Isopropanol	100-0.02%	12.5%	50%	25%	12.5%
Aldehydes	Formalin	20-0.002%	0.6%	2.5%	0.3%	0.04%
	Gluteraldehyde	100-0.02%	0.2%	G	0.8%	0.8%
Oxidizing Agents	Sodium Hypochlorite	5.25-0.001%	0.02%	0.04%	0.002%	NG
	Povidone-iodine	100-0.02%	NG	NG	NG	NG
	Hydrogen Peroxide	3-0.001%	0.04%	0.1%	0.18%	0.04%
Solvent	Acetone	100-0.02%	50%	25%	50%	50%
Surfactants	Chlorhexidine	2-0.001%	NG	NG	0.004%	NG
	Sodium Hydroxide (1 Molar)	100-0.02%	1.6%	3%	3%	0.8%
	SDS	10-0.002%	NG	0.02%	0.01%	NG
	Roccal-D Plus	100-0.02%	0.4%	0.1%	0.2%	0.2%
Buffers	Tris (pH 7)	100-0.02%	G	G	G	G
	Tween-20	100-0.02%	G	G	G	G
EDTA (1 Molar)		100-0.02%	G	G	G	G
Irradiation	Ultraviolet Light	0-60 minutes of exposure	NG	G	NG	NG
Temperature Changes	Autoclave	121°C for 15 minutes	NG	NG	NG	NG
	Heat	45°C	G	G	G	G
		50°C	G	G	NG	NG
		56°C	NG	G	NG	NG

Antibiotic experiments: Overall we found *Spiroplasma* to be susceptible to macrolides, florfenicol, and tetracylcines. They were most affected by bacteriostatic antibiotics that targeted the 50S ribosome. *Spiroplasmas* were most susceptible to the macrolide tylosine tartrate that inhibited growth at all concentrations. Table 3.25 summarizes *Spiroplasma* susceptibility to all antibiotics tested.

Table 3.25 Spiroplasma susceptibility to antibiotics.

Antibiotic		Range (ug/ml)	S/C	Mechanism of action	*Spiroplasma* susceptibility			
					S	G	*S.m*	*S.c*
Fluorquinolones	Enrofloxacin	2-0.12	C	Inhibits DNA gyrase	R	R	1	1
Aminocoumarin	Novobiocin	4-0.5	C	Inhibits DNA gyrase	R	R	R	R
Penicillins	Penicillin	8-0.5	C	Inhibit cell wall synthesis	R	R	R	R
	Amoxicillin	16-0.25			R	R	R	R
Cephalosporins	Cetfiofur	4-0.25	C	Inhibits cell wall synthesis	R	R	R	R
Aminoglycosides	Streptomycin	1024-8	C	Bind 30S and inhibit protein synthesis	512	R	512	256
	Neomycin	32-2			R	R	R	R
	Gentamicin	8-0.5			R	R	R	R
Aminocyclitols	Spectinomycin	64-8	S	Bind 30S and inhibit protein synthesis	R	R	R	R
Tetracyclines	Tetracycline	8-0.25	S	Bind 30S and inhibit protein synthesis	2	4	4	4
	Oxytetracycline	8-0.25			2	4	4	2
Macrolides	Erythromycin	4-0.12	S	Bind 50S and inhibit protein synthesis	0.25	0.5	2	0.5
	Tylosin tartrate	20-2.5			2.5	2.5	2.5	2.5
Florfenicol		8-1	S	Bind 50S and inhibit protein synthesis	2	4	R	R
Lincosamides	Clindamycin	4-0.5	S	Bind 50S and inhibit protein synthesis	0.5	0.5	4	1
Sulfonamides	Sulfadimethoxine	256-32	S	Inhibits bacterial synthesis of folic acid	R	R	R	R
	Sulphathiazole	256-32			R	R	R	R
	Trimethoprim/ sulfamethoxazole	2/38-0.5/9.5			R	R	R	R

Antibiotics are listed in their categories and the different antibiotics that were tested. The range of antibiotics is listed as well as their mechanisms of action. Bactericidal and bacteriostatic are indicated with C (bacteriocidal) and S (bacteriostatic). The lowest concentration of antibiotic that inhibited growth is listed for each *Spiroplasma*. Those antibiotics that allowed sprioplasma growth at all levels of antibiotic tested are indicated with an R for resistant. Three different species of Spiroplasma were tested: *S. mirum*'s (S) SMCA; *S. mirum*'s (G) GT48; (*S.m*) *S. melliferum*; and (*S.c*) *S.citri*.

Complement experiments: *Spiroplasma* spp. were not susceptible to normal goat complement (Table 3.26). All three species were susceptible to goat complement following opsonization with hyperimmune serum (Table 3.27). *Spiroplasma* spp. grew in all heat inactivated complement controls (data not shown).

Table 3.26 Spiroplasma growth in the presence of normal goat complement

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	Growth	Growth	Growth	Growth
5 minutes	Growth	Growth	Growth	Growth
10 minutes	Growth	Growth	Growth	Growth
15 minutes	Growth	Growth	Growth	Growth
30 minutes	Growth	Growth	Growth	Growth
60 minutes	Growth	Growth	Growth	Growth

Table 3.27 Spiroplasma growth in the presence of hyperimmunized goat complement

Time	SMCA	GT48	*S. melliferum*	*S. citri*
1 minute	Growth	Growth	Growth	Growth
5 minutes	Growth	Growth	Growth	Growth
10 minutes	Growth	Growth	Growth	Growth
15 minutes	Growth	Growth	Growth	Growth
30 minutes	**No Growth**	**No Growth**	**No Growth**	**No Growth**
60 minutes	**No Growth**	**No Growth**	**No Growth**	**No Growth**

Discussion

The lack of cell wall in spiroplasmas explains most of the susceptibility seen for the disinfectants tested. However, GT48 was resistant to higher dilutions of many of the disinfectants as well as higher temperatures and requires more testing to determine its virulence factors. In general, the spiroplasmas were susceptible to most disinfectants that are ineffective for prions.

Alcohols are a common disinfectant used in everyday laboratory use. They have limited residual activity due to evaporation, which results in brief contact times unless the surface is submerged, and have a limited activity in the presence of organic material. Alcohols are most effective when combined with purified water to facilitate diffusion through the cell membrane; 100% alcohol typically denatures only external membrane proteins. Ethyl alcohol (C_2H_5OH), isopropyl alcohol (($CH_3)_2CHOH$), and methanol (CH_3OH) cause membrane damage, rapid denaturation of proteins, and subsequent interference on metabolism resulting in cell lysis. Alcohols are generally effective against vegetative bacteria, viruses, and fungi (McDonnell and Russell, 1999). They are reported to be an ineffective disinfectant for prions at concentrations of 50-100% (Rutala and Weber, 2010). *Spiroplasma*, however, were susceptible to low levels of alcohol treatment. After 1 minute of exposure to 35% ethanol, all spiroplasma growth was inhibited. Methanol and isopropanol were also effective on contact and inhibited growth at 50% concentration. This can be attributed to the membrane damage and rapid denaturation of proteins that alcohols cause on contact. This study correlates well with Stanek's findings in

1981. He reported that *S. citri*, *S. melliferum*, and SMCA were susceptible to ranges of ethanol from 40-20% over a 40 minute time period (Stanek et al., 1981).

Aldehydes, such as formaldehyde (CH_2O) and glutaraldehyde ($CH_2(CH_2CHO)_2$), have wide microbiocidal activity and are sporocidal and fungicidal. Formaldehyde mixed in an aqueous solution is commonly referred to as formalin. Both of these aldehydes kill cells quickly by crosslinking proteins and may be used as a disinfectant or fixative for infected tissues. Formaldehyde (3.7%) and gluteraldehyde (5%) have been shown to be ineffective against prion fixation. *Spiroplasmas* were susceptible to 3-0.02% formalin which agrees with Clark and Stanek's publications reporting inactivation of *Spiroplasma* after exposure to 2-0.03% formalin (Clark, 1964; Stanek et al., 1981). GT48 was able to grow in 50% gluteraldehyde (see Table 3.28), and Bastian also reported *Spiroplasma* could resist gluteraldehyde (Bastian, 2005). All other *Spiroplasma* were inactivated by 0.8% gluteraldehyde or less. This also correlates with Stanek's findings for gluteraldehyde (inactivation range: 0.5-0.06%) (Stanek et al., 1981). Formaldehyde appears to be the more suitable fixative for spiroplasma-infected tissues due to its ability to cross link proteins and kill the organisms. Gluteraldehyde was less effective, especially for GT48, and more research is needed to determine the strain's survival factors.

Oxidizing agents act by oxidizing the cell membrane of microorganisms, which results in a loss of structure and leads to cell lysis and death. A large number of disinfectants function in this way. Sodium hypochlorite (NaClO), povidone-iodine, and hydrogen peroxide (H_2O_2) are examples of oxidizing agents that were studied in this paper. Household bleach, sodium hypochlorite normally sold as a 5.25% concentration, is used at 10% solution in laboratory conditions to keep harmful corrosive effects to a minimum. It is effective against 99% of bacteria, viruses and some types of mold (Estrela et al., 2002). Povidone-iodine contains 9-12% available iodine (I_2) and is effective against bacteria, viruses, fungus, and protozoan cysts (Reimer et al., 1998). Hydrogen peroxide, diluted to 3%, is a common disinfectant in laboratories and households. These agents had a major impact on *Spiroplasma* survival. Iodine did not allow growth at even the lowest dilution (0.02%) for any spiroplasma species. Sodium hypochlorite inhibited growth of all *Spiroplasma* at very small dilutions (0.04%) as did hydrogen peroxide (0.1%). The oxidizing agents' efficacy against *Spiroplasmas* is most likely attributed to the lack of a cell wall and easy access for oxidation of the cell membrane. Prions can be inactivated by hydrogen peroxide but only at higher percentages (59%). They are not affected by iodine (2%) (Rutala and Weber, 2010).

A variety of surfactants are used in the laboratory for disinfecting glassware and work surfaces. Chlorhexidine (N',N''''-hexane-1,6-diylbis[N-(4-chlorophenyl) (imidodicarbonimidic diamide)) is commonly used as a topical antiseptic. It works by damaging the outer cell layers and attacks the bacterial cytoplasmic or inner membrane ((McDonnell and Russell, 1999). Sodium hydroxide (NaOH), also known as lye, is a caustic cleaning agent. Its mechanism of action is poorly understood, but it is effective against most bacterial agents. Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}SO_4Na$) is an anionic surfactant used in many cleaning agents. The mode of action is attributed to the positive charge, which forms an electrostatic bond with negatively charged sites on microbial cell walls. Those electrostatic bonds create stress in the cell wall, leading to cell lysis and death. (Simoes et al., 2006). Roccal (Didecyl dimethyl ammonium chloride 9.2%, Alkyl (C12, 61%; C14, 23%; C16, 11%; C18, 2.5%; C8 & C10, 2.5%), dimethyl benzyl

ammonium chloride 9.2%, Alkyl (C12, 40%; C14, 50%; C16, 10%) dimethyl benzyl ammonium chloride 4.6%, bis-n-tributyltin oxide 1.0%, Inert Ingredients: 76.0%) is a commercial cationic surfactant. It is a bactericide, fungicide, and virucide for veterinary, laboratory animal, kennel and animal breeder facilities. It is also a residual bacteriostat and inhibits bacterial growth on moist surfaces and contains rust corrosion inhibitors (Drugs.com). All surfactants tested were able to inhibit spiroplasma growth at low dilutions. Chlorhexidine and SDS were able to inhibit most species tested at all dilutions. Sodium hydroxide, an effective agent for prions, inhibited growth at 3.2% for all spiroplasma. Roccal, used around veterinary operations and herd animals, was also very effective inhibiting growth at dilutions of 0.4% and less. Surfactants' efficacy for *Spiroplasma*, like the oxidizing agents, is also attributed to their attack on the outer cell layers, leading to cell lysis and death.

Acetone is an organic compound with the formula $(CH_3)_2CO$. In the laboratory, acetone is used as a polar aprotic solvent in a variety of organic reactions, such as SN_2 reactions. It is typically the solvent of choice for cleaning purposes of laboratory glassware because of its low cost and volatility (McDonnell and Russell, 1999). Acetone (50%) was effective against *Spiroplasma* survival, due to its quick denaturing properties. It is not effective at any percentage for prion inactivation.

Tris $((HOCH_2)_3CNH_2)$ and Tween-20 are common compounds found in many laboratory buffers. It has reported that Tris inhibits a number of enzymes; and therefore, it should be used with care when studying proteins. (Desmarais et al., 2002). EDTA is a chelating agent that is mainly used to sequester metal ions in aqueous solution. Animal experiments frequently use blood collecting tubes that contain EDTA as an anticoagulant. As a precaution, dilutions of EDTA were also used in the disinfectant studies to be sure that there was no interference in spiroplasma recovery. *Spiroplasma* spp. were able to grow in the presence of all concentrations of Tris, Tween, and EDTA, supporting the presumption that all other experiments with these compounds will not be affected by their use. EDTA has also been suggested as a disrupting agent for biofilms. *S. citri* has the potential to create a biofilm because it uses the phosphoenolpyruvate:phosphotransferase system (PTS) (Bove et al., 2003). Although we did not test the efficacy of EDTA's ability to disrupt *Spiroplasma* biofilms, our results indicate that it will not cause *Spiroplasma* death.

When subjected to changing temperatures, various bacteria have different susceptibilities. *Spiroplasma* have been reported to grow at temperature ranges from 5-43°C (Konai et al., 1996). Konai also found that SMCA had no color change at 43°C, but slow growth may have occurred without acidifying the media. SMCA was able to survive 1 hour at 40°C and 50°C but only 10 minutes at 56°C. *S. melliferum* and *S. citri* survived only 30 minutes at both 50°C and 56°C. GT48 was able to survive 1 hour at all temperatures tested. The lack of cell wall is a reasonable explanation for the vulnerability to higher temperatures. GT48's ability to survive at the higher temperatures is another reason for further testing of its resistant properties. Prions are able to sustain a wide range of dry heat temperatures (Rutala and Weber, 2010).

An autoclave is used for sterilization purposes in many laboratories. It works by subjecting items placed inside to high pressure steam at 121 °C for 15–20 minutes depending on the size of the load and the contents. This inactivates all bacteria, viruses, spores, and fungi. Its efficacy

against prions has not been proven. All *Spiroplasma* spp. were inactivated following autoclaving, another example of their vulnerability to high temperatures.

Ultraviolet lamps are used to sterilize workspaces and tools used in biology laboratories and medical facilities. UV light at germicidal wavelengths causes adjacent thymine molecules on DNA to dimerize; if enough of these defects accumulate on a microorganism's DNA, its replication is inhibited, thereby rendering it harmless. With the exception of GT48, *Spiroplasmas* are also susceptible to UV irradiation after 1 hour. UV irradiation is ineffective for GT48 as well as prions. GT48's resistance to UV irradiation gives further evidence for protective mechanisms not seen in the other spiroplasmas tested.

Spiroplasma's lack of cell wall was a contributing factor to its susceptibility to many of the disinfectants tested, but it is a survival factor against many of the antibiotics used in today's therapies. *Mollicutes'* lack a cell wall that makes them innately resistant to all antimicrobials which target the cell wall. These antimicrobials include polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin (Bebear and Bebear, 2002). With this explanation, the resistance found for the penicillins and cephalosporins tested was not surprising. Although results in this study agreed with the research for resistance to sulfonamides and trimethoprim, in 1984 Bastian found trimethoprim/sulfamethoxazole (TMP/SMX) inhibited clinical and pathological signs in SMCA-inoculated suckling rats. Suckling rats received 200 mg/kg SMX and 40 mg/kg TMP in 5% sterile dextrose by IV infusion from day 5-15 post SMCA-inoculation. Rats were followed to day 25 of the infection. The use of TMP/SMX treatment reduced the mortality rate significantly. Spiroplasma was recovered from TMP/SMX treated rats, but the titers were significantly lower (Bastian et al., 1989). Bastian did report that this data disagreed with their preliminary in vitro findings. He went on to suggest that mortality was not an appropriate end point and that only viability titers in the brain should be considered.

Spiroplasma was also resistant to the aminoglycosides neomycin and gentamicin, and the aminocyclitols spectinomycin. Aminoglycosides are bactericidal antibiotics that are used in the treatment of infections caused by Gram-negative aerobes. They bind to the 30S ribosome and inhibit the rate of protein synthesis and the fidelity of messenger ribonucleic acid (mRNA) translation, resulting in the synthesis of abnormal proteins. Aminoglycoside uptake by bacteria involves an energy-dependent step that is oxygen-linked. Therefore uptake is inhibited by an anaerobic or acidic environment. Streptomycin represents one of the oldest members of this group. Neomycin, another aminoglycoside, is used to treat enteric infections and topically to treat skin, ear, and eye infections. Gentamicin and kanamycin are extended-spectrum aminoglycosides that are used for the treatment of septicemia and infections of the skin, respiratory tract, ear, eye and urinary tract. Aminocyclitols are chemically related to the aminoglycocides. They also bind to the 30S ribosome and inhibit protein synthesis. Unlike aminoglycosides, they are bacteriostatic. They are effective against Gram-negative and *Mycoplasma* bacteria. Spectinomycin is an aminocyclitol used for treatment of enteric and respiratory disease (Papich, 2007). These antibiotics have been used for treatment of other genera of mollicutes, the *Mycoplasma*. Resistance was also found when different concentrations of the broad-spectrum antibiotics kanamycin and novobiocin failed to inhibit the multiplication of SMCA in chick embryos and rabbit lens organ cultures (Bastardo et al., 1974; Schwartz and Elizan, 1972).

Spiroplasmas, with the exception of GT48, were susceptible to streptomycin, one of the oldest members of the aminoglycosides. This antibiotic works on the 30S ribosome as do the tetracyclines. Tetracyclines are bacteriostatic inhibitors of a broad spectrum of bacteria including, rickettsia, spirochetes, chlamydiae, mycoplasmae, and some protozoa. They inhibit bacterial protein synthesis by binding to the 30S ribosome and preventing attachment of the aminoacyl transfer ribonucleic acid (tRNA) to the mRNA-ribosome complex. They block the addition of amino acids to the growing peptide chain. Tetracyline and oxytetracycline are used in the treatment of local and systemic bacterial, chlamydial, rickettsial, and protozoal infections (Papich, 2007). All three species of *Spiroplasma* were also susceptible to tetracyclines in this study. However, *in vivo*, tetracycline did not protect suckling rats against SMCA-induced cataracts when inoculated (50 µg/animal, repeated 4 days pi) at time of SMCA inoculation or 4 hours prior (Clark, 1974). Rats born to mothers given tetracycline in drinking water (approximately 500 µg/ml) prior to parturition and during the nursing period were not protected against SMCA-induced cataracts (Clark, 1974).   Tetracyclines have been studied as possible therapy for TSE infections. Tetracyclines interact with aggregates obtained by synthetic PrP peptides or pathological PrP (PrP^sc) extracted from TSE brains, and they destabilize the structure of amyloid fibrils, reducing their resistance to digestion by proteinase K. Tetracyclines also interact with peptide oligomeric structures and inhibit the protein misfolding associated with PrP^sc formation (Forloni et al., 2002).

There were mixed reactions for *Spiroplasma* susceptibility to enrofloxacin and florfenicol. Enrofloxacin is a fluoroquinolone that inhibits bacterial DNA gyrase. *In vivo*, it is distributed throughout the CNS, bone, and prostate and has broad spectrum bactericidal effects on most bacteria. Florfenicol is a thiamphenicol derivative bacteriostatic agent that also binds to the bacterial 50S ribosome unit to inhibit peptide bond formation and protein synthesis. It has broad spectrum against most anaerobic bacteria and *Mycoplasmas* and is used for respiratory, CNS, and ocular infections (Papich, 2007). *S. melliferum* and *S. citri* were susceptible to enrofloxacin. SMCA and GT48 were susceptible to florfenicol.   Both of these antibiotics can reach high intracellular concentrations, which may be an advantage for spiroplasma infections.

Novobiocin is an aminocoumarin antibiotic that is a very potent inhibitor of bacterial DNA gyrase.   It targets a different site on DNA gyrase than that of the flouroquinolones, targeting the GyrB subunit of the enzyme involved in energy transduction. Novobiocin as well as the other aminocoumarin antibiotics act as competitive inhibitors of the ATPase reaction catalysed by GyrB. It is bacteriocidal and is used predominantly for staphylococcal infections(Papich, 2007). Unlike florfenicol, all *Spiroplasmas* were resistant to novobiocin.

Spiroplasmas, like *Mycoplasmas* are susceptible to macrolides and lincosamides. Macrolides are bacteriostatic antibiotics that work primarily against Gram-positive bacteria and *Mycoplasma*. They inhibit bacterial protein synthesis by binding to the 50S ribosome, preventing translocation of amino acids to the growing peptide chain. Erythromycin, a macrolide, is used as an alternative to penicillin for the treatment of infections caused by Gram-positive bacteria. Tylosin, another macrolide, is used for the treatment of local and systemic infections caused by mycoplasma, Gram-positive bacteria, and some Gram-negative pathogens. Lincosamides represent another group of antibiotics that inhibit protein synthesis by binding to the 50S

ribosome. They are also bacteriostatic and are active against Gram-positive bacteria, *Toxoplasma*, and *Mycoplasma* species.   Clindamycin, a lincosamide antibiotic, is used for periodontal disease, osteomyelitis, dermatitis, and deep soft tissue infections caused by Gram-positive bacteria (Papich, 2007). Kotani (Kotani et al., 1986)Kotani, Phillips et al. 1986) found that a combination of tylosine (30 µg/ml) and gentamycin (50 µg/ml) inhibited the transformation of NIH-3T3 and CV-1 cells by SMCA. Furthermore, cells with history of spiroplasma infection were negative for the bacteria after the addition of these antibiotics and tylosine appeared to be the active inhibiting compound in that experiment.

Spiroplasma spp. were not adversely affected by normal goat complement showing that they do not activate the alternative pathway of complement. All three species were susceptible to SMCA-hyperimmunized goat serum in the presence of complement. This indicates that complement mediated lysis does occur via activation of the classical pathway. This provides evidence that an acquired immune response is required for killing of the organism by complement.

Conclusion

Although *Spiroplasma* are similar to prions in their persistence in neurologic tissues, *Spiroplasma* do not share the same resistant properties as prions. A comparison of efficacy for the disinfectants against *Spiroplasma* and prions can be seen in Table 3.28. The three species of *Spiroplasma* tested were susceptible to minimal dilutions of common laboratory disinfectants. They were also susceptible to many of the antibiotics in use for other mollicutes. We did find strain differences in *S. mirum* in that GT48 was more resistant to many of the disinfectants and antibiotics tested. More research is needed to determine if this resistance is associated with any virulence factors it may possess.

Table 3.28 Comparison of disinfectant efficacy against *Spiroplasma* and prions.
All disinfectants that were found to inhibit spiroplasma are indicated with effective. For a select few, all species of *Spiroplasma* were inhibited with the exception of GT48. These disinfectants are indicated with (GT48).

Disinfectant		Spiroplasma	Prions
Alcohols	Ethanol 70%	Effective	Ineffective
	Methanol	Effective	Ineffective
	Isopropanol	Effective	Ineffective
Aldehydes	Formalin 3%	Effective	Ineffective
	Gluteraldehyde 50%	Effective **(GT48)**	Ineffective
Oxidizing Agents	Sodium Hypochlorite	Effective	Not tested
	Povidone-iodine 2%	Effective	Ineffective
	Hydrogen Peroxide 3%	Effective	Ineffective
Solvent	Acetone	Effective	Ineffective
Surfactants	Chlorhexidine	Effective	Ineffective
	Sodium Hydroxide	**Effective**	**Effective**
	SDS 5%	Effective	Ineffective
	Roccal	Effective	Not tested
Irradiation	Ultraviolet Light	Effective **(GT48)**	Ineffective
Temperature Changes	Autoclave	Effective	Ineffective
	Heat 45°C	Ineffective	Ineffective
	Heat 50°C	Ineffective	Ineffective
	Heat 56°C	Effective **(GT48)**	Ineffective

CHAPTER 4:
CLINICAL SIGNS AND PATHOLOGY OF *SPIROPLASMA MIRUM* SUCKLING MOUSE CATARACT AGENT IN SMALL RUMINANTS

Introduction

Spiroplasma mirum is the only spiroplasma that has been found to be pathogenic to vertebrates (Bove, 1997). As the name describes, Suckling Mouse Cataract Agent (SMCA) when inoculated intracerebrally (IC) caused suckling mice to develop cataracts (Clark, 1964). The organism was able to persist in suckling mice, specifically the brain tissues, for up to two years (Clark and Karson, 1968). Due to this observation, it was tentatively included with the slow viruses or chronic infectious neuropathic agents (Hotchin, 1967). When inoculated into suckling rats, 70% developed cataracts and 36% developed mild to severe hydrocephalus (Elizan et al., 1972). The organism causes death between four to seven days post inoculation in seven day old embryonated eggs (Clark, 1964). In day-old chicks inoculated IC or subcutaneously (SQ), *S. mirum* caused death within five days (Clark, 1974). When inoculated IC into day old hamsters, CNS signs and severe weight loss were observed (Kirchhoff et al., 1981). Death and severe microophthalmia were seen in rabbits inoculated IC less than twenty-four hours after birth. No clinical signs were seen when *S. mirum* was inoculated into adult mice, roosters, hamsters, rabbits, guinea pigs, and rhesus monkeys (Clark, 1974).

The organism's persistence for long periods of time within the brains of animals led researchers to categorize SMCA as a "slow virus." This family included the infectious agent for scrapie in sheep and the agent of Kuru in man (Schwartz and Elizan, 1972). Tully suggested that this organism could be related to the causative agent of scrapie which had not yet been identified (Tully et al., 1976). In 1979 Bastian reported spiral membranous inclusions seen by electron microscopy in brain biopsy tissues from a patient with Creutzfeldt-Jakob disease (CJD) (Bastian, 1979). Following this initial report of spiroplasma-like inclusions in CJD tissues, Reyes published two case reports of patients that were suspected of CJD with similar findings. Electron microscopy revealed numerous membrane-bound vacuoles within the cell bodies of neurons and astrocytes. Elongated, spiral, membranous inclusions were occasionally present within cortical cell processes, as well as in synaptic terminals (Reyes and Hoenig, 1981). Reyes also reported that transmission of CJD to spider monkeys was successful from brain tissues of one of these reported patients by Dr. D. C. Gajdusek and C. J. Gibbs. In 1980 Gray reported a case of CJD to have spiral membranous inclusions, indicative of spiroplasma, with five to eight twists seen via electron microscopy (Gray et al., 1980). In 1981 Bastian reported two more cases of CJD with similar spiral-like inclusions seen through electron microscopy (Bastian et al., 1981).

Further research was conducted to show similarities between the transmissible agent of CJD and spiroplasma. Bastian reported that the tissue response to experimental spiroplasma infection in the suckling rat brain resembles the spongiform degenerative brain diseases of both man and other mammals. The degenerative changes are localized to the gray matter, as seen in CJD cases (Bastian et al., 1984). The optimal growth temperature for *S. mirum* and other spiroplasma isolates was 37ºC, suggesting that spiroplasma could survive in a mammalian host (Bastian, 2005; Konai et al., 1996). The organism's proven ability to produce an acute or

Table 4.1 Summary of *Spiroplasma mirum* experimentally infected animals

Species	Age	Route	Death	Disease	Persistence of organism	Ref
Chicks	<24 h	IC	4 days pi	Yellow livers and swollen spleens	NT	(Clark, 1974)
	<24 h	SQ	5-6 days pi		NT	(Clark, 1974)
Mice	<96 h	IC	Rarely	Cataracts [1] Stunting Death	827 days	(Clark and Karson, 1968)
	>5 days	IC	No	None	133 days	(Clark and Karson, 1968)
	<96 h	IP	No	None	9 days [2]	(Clark and Karson, 1968)
	<96 h	IV	No	None	NT	(Clark and Karson, 1968)
	Adult	IC	No	None	497 days	(Clark and Karson, 1968)
		IV, IN, IP, cornea			NT	
Rats	<96 h	IC	Yes	Cataracts [3]	60 days	(Clark, 1974)
	<24 h	SQ/IP [4]	No	Cataracts Weight loss Alopecia	50 days	(Bastian et al., 1984) (Bastian et al., 1987b)
Deer Mouse	Adult	SQ	No	None	NT	(Clark and Karson, 1968)
Roosters	Adult	IM	No	None	NT	(Clark, 1974)
Hamsters	<24 h	IC	Variable	CNS signs Dramatic loss of weight Runting	NT	(Kirchhoff et al., 1981)
	Adult	IV	No	None	NT	(Clark, 1974)
Rabbits	<24 h	IC	12 days	Death Severe microphthalmia	12 days	(Kirchhoff et al., 1981)
	Adult	IV, SQ	No	None	NT	(Kirchhoff et al., 1981)
		Scarified cornea	No	Ocular lesion	AB negative	(Clark, 1974)
Guinea Pigs	Adult	IP, IM, SQ, cornea	No	None	NT	(Clark, 1974)
Box Turtle	Adult	IP	No	None	NT	(Clark and Karson, 1968)
Opossum	Adult	IP	No	None	NT	(Clark and Karson, 1968)
Rhesus Monkey	Adult	IV, SQ, cornea	No	None	NT	(Clark, 1974)

IC: Intracereberal, SQ: Subcutaneous, IP: Intraperitoneal, IV: Intravenous, IN: Intranasal, IM: Intramuscular, NT: Not tested

1 Cataracts were the most common clinical manifestation of SMCA inoculation

2 Organism was not recovered from brain or eye tissues but was found in liver-spleen suspension

3 Cataracts in suckling rats were more severe with a higher inoculation levels

4 Rats were inoculated with *S. mirum* strain GT48

persistent brain infection in small mammals was also used as evidence. Subsequent studies have shown some resistance of spiroplasma organisms to chemical factors with some resemblance to the resistance patterns seen in the transmissible agent of CJD (Bastian, 2005). Bastian concluded that the size and membranous nature of spiroplasma would best fit with the data regarding the elusive CJD agent (Bastian et al., 1984).

In 1987 a scrapie-associated fibril was identified as a consistent finding and possible infectious unit of the transmissible spongiform encephalopathies (TSEs). Cross reactions with this fibril to *S. mirum* fibril proteins supported evidence of an association between spiroplasma and TSEs (Bastian et al., 1987a). In 2001 Bastian and Foster reported the presence of spiroplasma ribosomal DNA in CJD and scrapie-infected brains (Bastian and Foster, 2001). In 2004 eight of ten scrapie infected brains, five of seven CWD infected brains, and two of two CJD brains were found to be positive for *S. mirum* via southern blotting of PCR products (Bastian et al., 2004). PCR products obtained from the scrapie, CWD, and CJD cases had 99% homology with published *S. mirum* 16S rDNA sequences (Bastian et al., 2004). In this study, it was reported that the mode of DNA extraction was critical. The most reliable method involved initial solubilization with guanidine thiocyanate followed by phenol/chloroform extraction (Bastian et al., 2004). Southern blotting was also acknowledged as a more sensitive method of detection, revealing spiroplasma DNA where PCR product was not readily apparent (Bastian et al., 2004).

In 2005 Bastian (Bastian, 2005) cultivated a spiroplasma from homogenates of CWD and scrapie infected brains. The brains were homogenized in SP4 media and then inoculated into eight day old embryonated eggs. Allantoic fluids from the inoculated eggs were then passaged into SP4 media and incubated for sixteen days at 37ºC. Evidence of spiroplasma growth was found via phase microscopy, electron microscopy, and PCR. DNA sequence analysis showed 98% homology with *S. mirum* but revealed nucleotide substitutions unlike any known spiroplasma strain.   The isolates from this experiment were later inoculated into neonatal goats and sheep (Bastian et al., 2007). No clinical signs were seen but all brains had evidence of spongiform changes. At eleven months post inoculation, one goat and one sheep had severe cerebella and hippocampal spongiform degeneration with neuronal vacuolization in the brain stem. All other animals were reported to have spongiform changes at a lesser degree. It was noted that the intraneuronal vacuoles in the cerebella cortex and brain stem seen in the *Spiroplasma* spp. inoculated ruminants were identical to lesions typical of naturally occurring TSE (Bastian et al., 2007).

In 2007 Bastian et al. (Bastian et al., 2007) induced spongiform encephalopathy in neonatal deer inoculated intracerebrally with SMCA. Three of four deer developed clinical signs of neurological deterioration between 1.5 months and 5.5 months post inoculation. One deer showed signs of hydrocephalus, while all others were grossly normal. Histological sections of all deer showed spongiform encephalopathy in the cerebral cortex, cerebella cortex, and the brain stem. Severity of neuropathology was noted as incubation time increased. In the same publication, sheep were inoculated intracerebrally with varying dilutions of SMCA. No clinical signs were noted, but spongiform encephalopathy was seen histologically. Severity of lesions was decreased as the dosage of the inoculums decreased.   Neonatal goats were also inoculated

intracerebrally with SMCA. After five months no clinical signs were seen and only minimal vacuolization was noted histologically.

In order to compare natural TSE infections to *Spiroplasma*-induced disease, a long term animal model was established. It has been proven that the mouse model does not always accurately portray a natural infection and so the ruminant model was chosen for disease establishment. Goats are known for multiple births, ease of handling, and have also been used for scrapie research; for these reasons, a herd of research goats were used for experiments with *S. mirum*. Neonatal goats were inoculated intracerebrally with SMCA and monitored for clinical signs for two years. At the end of the experiment, various tissues were collected and examined for pathology and persistence of the organism.

Five month old deer were also inoculated and monitored for clinical signs. Seven months post-inoculation, various tissues were collected and examined for pathology and persistence of the organisms. Information from this experiment was used to compare to Bastian's 2007 neonatal deer experiment (Bastian et al., 2007).

In order to test possible routes of transmission, experiments were conducted with various inoculation routes of SMCA in neonatal goats. Intravenous (IV) and intradermal (ID) routes were used as possible vector routes to establish a mode of transmission. Goats were monitored for four months for clinical signs and persistence of the organism via various tissues. After four months, tissues were collected and examined for pathology.

Since dose-dependent variations have been reported, pathology and clinical signs seen post-SMCA inoculations of higher concentrations were compared. SMCA cultures were concentrated ten times the normal and inoculated into neonatal goats.   Goats were monitored for four months for clinical signs and persistence of the organism via various tissues. After four months, various tissues were collected and examined for pathology.

Materials and Methods

SMCA: SMCA strain of *S. mirum*, courtesy of Dr Gail Gasparich, Towson University, Baltimore, MD, USA, was used for the inoculums in all experiments and had undergone multiple passages in M1D broth. SMCA was grown to log phase (Bastian et al., 1984), aliquoted into 1 mL portions mixed 50/50 with sterilized 30% glycerol resulting in a 15% glycerol mixture and frozen at –80ºC. For all following experiments, a stock of SMCA (approximately $1x10^8$ organisms per mL) was made by multiple passages at a 1:100 dilution rate into M1D and frozen at -80ºC. Presence of spirals was checked via dark field microscopy for all cultures.

Cultures were diluted in M1D at a 1:10 or 1:100 dilution prior to inoculation. For ID inoculation routes, 2 mLs of SMCA was spun at 16000xg for 1 hour. The supernatant was discarded and the pellet was re-suspended in 20 µL of M1D.   For concentrated SMCA experiments, a stock of SMCA was grown to equal 100 mL and frozen in 20 mL aliquots. At time of inoculation, 20 mL were separated into 1 mL aliquots and spun at 16000xg for 1 hour. The supernatant was removed from each tube and the pellet was re-suspended in PBS. All 20 tubes were combined to equal 2 mLs. For heat-killed concentrated SMCA inoculums, the re-suspended 2 mL PBS

culture was heated at 56°C in a water bath for 1 hour. To check sterility, 100 µL of heat killed SMCA cultures were inoculated into 900 µL of fresh M1D and incubated for 14 days at 30°C to check for any viable organisms prior to inoculation into animals.

Animals: White tailed deer were chosen from the LSU AgCenter Idlewild Experiment Station. Goats were obtained from the LSU AgCenter BenHur Research herd. All studies were done according to protocols approved by the Louisiana State University Agricultural Center Institutional Animal Care and Use Committee. Experimental animals were housed on concrete slabs in an IBRDSC approved isolation research facility. Control deer were housed at the LSU AgCenter Idlewild Experiment Station.

Tissue Samples:
- Blood Samples: Jugular blood samples were collected and immediately stored in both EDTA coated tubes and serum collection tubes. 100 µL of EDTA mixed blood was then transferred to 900 µL of M1D and allowed to incubate at 30°C for at least 21 days. Non-additive samples were centrifuged at 400 RPMs for 10 minutes to separate serum. Serum was removed and stored at -20°C for immunoblotting.
- Brain Tissues: At time of necropsy, heads were separated immediately and the brain was removed using a stryker saw. The right half of the brain was immersed in 10% formalin and stored at room temperature. After two weeks, samples were taken from the cortex, hypothalamus, thalamus, hippocampus, cerebellum, midbrain, pons, obex, and spinal cord for histological processing. The cortex, hypothalamus, and cerebellum were sampled for culturing from the left half of the brain in both M1D and embryonated eggs. Samples were also taken for PCR. The remaining left half of the brain was frozen at -80°C.

Histology: Formalin-fixed sections were stained with haematoxylin and eosin. Using standard protocols, the following immunohistochemical stains were applied: Anti-SMCA staining as established by Bastian (Bastian et al., 2007) to detect presence of the organism; and glial fibrillary acidic protein (GFAP) to detect the presence of astrogliosis.

Dark Field Microscopy: 5 µL of test sample was placed on a clean glass microscope slide, mounted with a #1.5 cover slip and sealed on all 4 sides with clear finger nail polish. Slides were examined with a Ziess Axio Imager A1 microscope (Carl Zeiss, Inc. North America, Thornwood, NY, USA) equipped with a dark-field top lens condenser 1.2-1.4 and an EC Paln-NeoFluar x100 oil immersion objective (Carl Zeiss, Inc. North America, Thornwood, NY, USA) with 1.3 iris closed to its lowest setting (0.7).

Embryonated Egg cultures: Approximately 100 mg of tissue was taken from the cortex, hypothalamus, and cerebellum and homogenized in 1 mL of M1D with 10 mg of vancomycin. Cultures were spun at 1000 RPM for 5 minutes to remove tissue debris. The supernatant was removed, and 10 µL was inoculated into the yolk sac of 7 day old embryonated eggs via a 1½ inch 22 gauge needle. Like number of eggs were injected with M1D media alone to serve as controls. Eggs were candled daily to monitor viability. At death or time of harvest, allantoic fluid was collected by aspiration via 1 inch 20 gauge needle and 3 ml syringe. The fluid was observed via dark field microscopy for spiral organisms. 10 µL of allantoic fluid was passaged

back into 7 day old embryonated eggs as well as M1D. Second passage of eggs followed above protocol; M1D was monitored for a color change until 21 days.

M1D cultures: Approximately 100 mg of tissue was homogenized in 1 mL of M1D with 10 mg of vancomycin. Cultures were spun at 1000 RPM for 5 minutes to remove large chunks of tissue debris. The top half of culture (500 μL) was removed and combined with fresh M1D with vancomycin to 1 mL. M1D with vancomycin was added to the original homogenized tissue culture to 1 mL. Cultures as well as a media control were incubated at either 30ºC or 37ºC, dependent on the experiment, for at least 21 days. At 21 days, cultures were checked via dark field microscopy for presence of spiral organisms. All cultures were also checked by PCR for presence of SMCA organisms. Cultures were passaged at least once at a 1:100 dilution into fresh M1D with vancomycin. Second passages were checked for spiral organisms via dark field microscopy and PCR at 21 days as well.

PCR Methods: PCR was carried out using oligonucleotide primers that specifically aplified a 1460 bp portion of the *S. mirum*-related adhesin gene. The forward and reverse primers were F3 (5'-TCTAGTCTTAATCATTTTACTTATTTATTAGAA -3') and R4 (5'-TTATTAAGTCATTCACTCTCTTTCTTT -3') respectively.
The reaction mixture (50 μL total) contained 2 μL of each the forward and reverse primers (10 μM each), 1 unit (0.2 μL) of *Taq* DNA Polymerase (New England BioLabs, Ipswich, MA), 2 μL of 10 mM dNTP (AmpliTaq Gold, Applied Biosystems, Foster City, CA), and 5 μL of 10x Standard *Taq* Buffer (New England BioLabs), which contains 15 mM of $MgCl_2$. For M1D cultures, 2 μL of culture was added to the reaction mixture without prior DNA extraction. DNA extraction followed the QIAamp Blood and Tissue DNA Extraction protocol for all animal tissue PCR reactions. Each reaction was brought to a final volume of 50 μL with sterile, nuclease-free H_2O. PCR's were performed using a MyCycler Thermal Cycler (Bio-rad). The thermal cycler program used was 94ºC for 5 minutes, then 30 cycles at 94ºC for 30 seconds, 57ºC for 20 seconds, and 72ºC for 120 seconds followed by a final extension of 72ºC for 10 minutes. All PCR products were electrophoresed on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer, and the DNA was visualized by SYBR Safe (Invitrogen, Eugene, OR) DNA gel staining under UV fluorescence.

Western Blots: SMCA cell lysates were made as 3 different antigen preparations: boiled, guanidine thiocyanate, and sonicated. The boiled SMCA antigen preparation was made as follows. Six 1 mL aliquots of SMCA were spun at 16000xg for 1 hour. Supernatant was discarded and 1 mL of PBS was added to each pellet. Each 1 mL suspension was then boiled for 10 minutes. All vials were mixed together to create a stock solution. Laemmli buffer with 2-mercaptoethanol was added volume to volume to SMCA solution and boiled for ten minutes. The guanidine thiocyanate-SMCA preparation was as described by Bastian (Bastian et al., 2004). The preparation was made, and laemmli buffer with 2-mercaptoethanol was added to a like amount and boiled for 10 minutes. The sonicated SMCA preparation was made following the same procedures as the boiled preparation. However, after boiling for 10 minutes, each 1 mL aliquot was then sonicated for 10 minutes (Heat-Systems-Ultrasonic, Inc., cycle time 1 sec; duty cycle 50%). All vials were mixed together to create a stock solution and laemmli blue with 2-mercaptoethanol was added as stated previously. All solutions were stored at -20ºC.

In order to determine an antibody profile for SMCA, 2 goats were immunized with boiled SMCA antigen. 1 mL of the solution was mixed with 1 mL of incomplete freund's adjuvant. The 2 mL mixture was injected intramuscularly into 2 different goats. Goats were boosted with 0.5 mL SMCA and 0.5 mL incomplete freund's adjuvant 4 and 6 weeks later. The same procedures were followed for inoculation of 2 goats with the sonicated antigen preparation. Serum was collected at various time points post-immunization and stored at -20ºC for future analysis.

Western immunoblot analysis was performed using the three cell lysates of SMCA. Electrophoresis was performed with a BioRad Mini-Protean II unit (Hercules, California, USA) utilizing the Laemmli discontinuous SDS-PAGE method with 5 μL of cell lysate per 20 μL well. Nitrocellulose transfer was performed for one hour with 150V in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Following transfer the blots were blocked with a 5% nonfat milk buffer for one hour followed by washing with tris-buffered saline containing 0.5% Tween-20. Three adjoining membrane lanes containing the 3 cell lysates were then incubated for at least 8 hours at 37ºC with a 1:40 dilution of the test serum. Subsequently, the blots were washed and incubated with rabbit anti-bovine (deer studies) or anti-goat (goat and sheep studies) IgG horseradish peroxidase conjugate (Sigma Chemical Company, Saint Louis, Missouri, USA) at a dilution of 1:800 for 45 minutes at room temperature. After washing, the substrate 4-chloro-1-naphthol (Sigma) was added for at least 10 minutes for color development.

Experiment 1
One neonatal goat was inoculated IC with 2 mLs of SMCA and a tracer. Twenty-four hours post-inoculation, the goat was humanely euthanized and the skull was removed for gross examination of the brain. The tracer was located in order to establish the area of inoculation in vivo. Various tissue samples were cultured in M1D as described above. Samples were also tested for presence of spiroplasma via PCR.

Experiment 2
Four, 5 month old deer were tranquilized with Telazol (2.5 mg/kg) and xylazine (0.3 mg/kg) via a dart gun and inoculated through the open fontanel of the skull and into the left hemisphere with 2 mLs of SMCA via an 18 gauge needle. Four control deer of like age and genetics were inoculated with 2 mLs of MID media in the same manner and remained at Idlewild. Deer were observed daily and euthanized at time of clinical signs or 7 months post-inoculation. The control deer were euthanized at the same time as the SMCA-inoculated deer for control tissues. Brain and eye tissues were collected and processed as described above.

Experiment 3
Twenty-nine 1 month old goats were separated into the following groups: 12 SMCA-inoculated goats, 3 SMCA 1:10 dilution goats, 2 SMCA 1:100 dilution goats, 6 M1D inoculated goats, 6 control goats. All inoculations equaled 2 mLs and were administered IC through the fontanel of the skull. Goats were separated by sex and housed on concrete slab stalls. Jugular blood samples were obtained every 6 months for immunoblotting. All animals were observed daily and euthanized if exhibiting clinical signs or at 2 years. Brain and eye tissues were collected and processed as described above.

Experiment 4

Twelve newborn goats were injected within 48 hours after birth via different inoculation routes
and allowed to remain with mother.   Three newborns were injected with 2 mLs of SMCA via
the open fontanel of the skull; 5 were injected with 2 mLs of SMCA IV via the jugular vein; and
4 were injected with 0.2 mLs of concentrated SMCA with a 22 gauge needle ID on the lower
eyelid. A jugular blood sample was obtained daily for the first 7 days and then weekly. All were
housed on concrete slab stalls and observed daily for clinical signs. Goats were euthanized at
time of clinical signs or 2 months after inoculation.   Brain and eye tissues were collected and
processed as described above.

Experiment 5

Three newborn goats were injected 48 hours after birth with concentrated SMCA (10X) via the
open fontanel of the skull and allowed to remain with mothers. Two newborns were injected in
similar manner with heat-killed concentrated SMCA. All goats were housed on concrete slab
stalls and observed daily for clinical signs. Goats were euthanized at time of clinical signs or 4
months after inoculation. Brain and eye tissues were collected and processed as described above.

Table 4.2 Summary of Experimental Animals

Exp	Species	Inoculum	Length of Experiment
1	Caprine	SMCA	24 hours
2	Cervidae	SMCA	6 months
3	Caprine	SMCA (IC)	2 months
		SMCA (IV)	
		SMCA (ID)	
4	Caprine	10x Concentrated SMCA	4 months
		Heat-killed 10x Concentrated SMCA	
5	Caprine	SMCA	2 years

Results

Experiment 1

Spiroplasma was recovered at high numbers from the mid-cerebral cortex adjacent to the area of
inoculation. Organisms were recovered from the optic nerve, cerebral cortex, hypothalamus,
thalamus, and hippocampus. Swabs of the skull cavity were positive for *Spiroplasma,* providing
evidence that the inoculum pools within the brain cavity sufficiently soaking all areas of brain
tissue. No organisms were found in homogenized cultures of cerebellum, midbrain, pons, obex,
or spinal chord. PCR results were the same as culture results. Figure 4.1 depicts the areas of
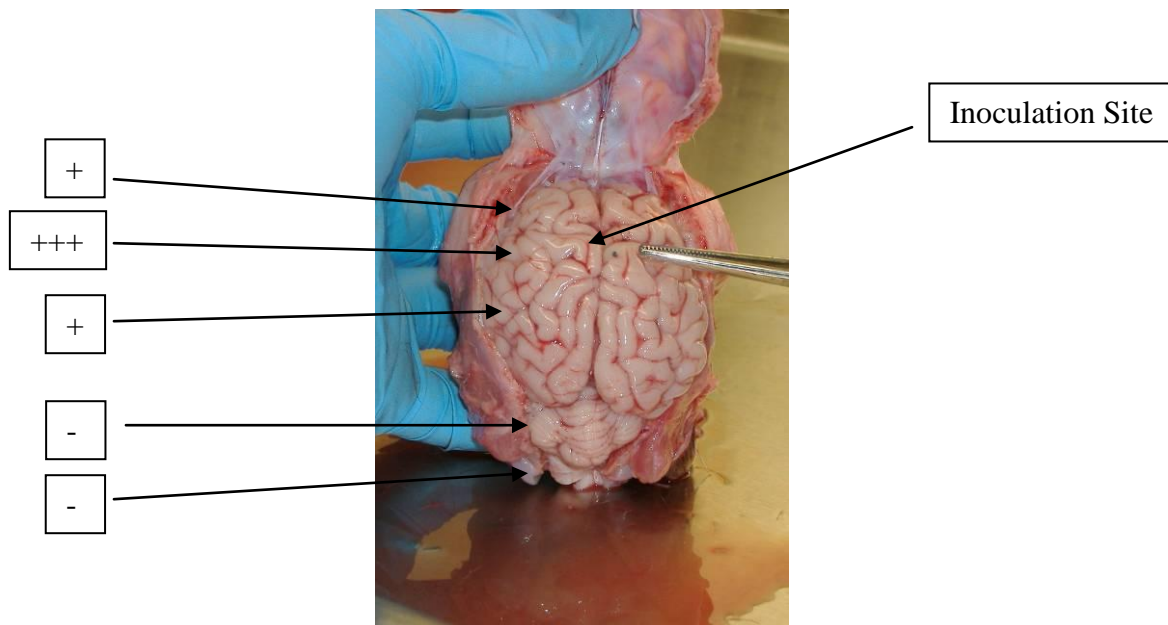spiroplasma recovery in this experiment.

Figure 4.1 *Spiroplasma* recovery 24 hours post-inoculation. Positive areas are indicated by +, negative areas are indicated by -.

Experiment 2-5

Clinical Signs: Prior to necropsy seven months post-inoculation, one deer showed minimal clinical signs (lethargy and slight weight loss). No goats exhibited any clinical signs regardless of experiment duration, inoculation route or dosage.

Histology: After extensive review, none of the experimentally infected animals appeared to have any pathological changes. No abnormalities were seen in H&E staining and no sections were positive for anti-SMCA antibody staining. One deer was positive for GFAP staining, however, it had been inoculated with M1D only (Figure 4.2 A). A comparison of SMCA-inoculated deer is seen in Figure 4.2 B.

Persistence of SMCA:
Blood: SMCA was recovered from the blood of neonatal goats IC and IV inoculated four days post inoculation. The bacteria could not be recovered after five days post inoculation. No organisms were recovered via the blood from any ID inoculated goats. PCR was conducted on all cultures to confirm presence of organisms.
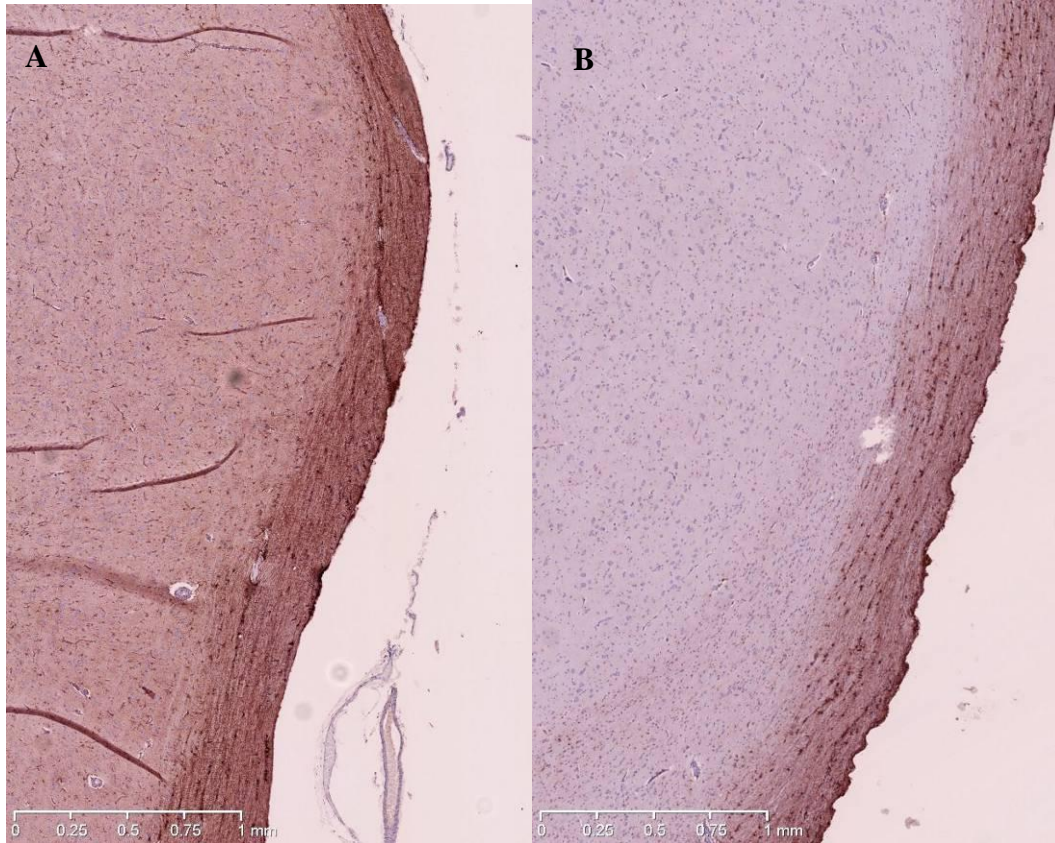
Figure 4.2 GFAP staining of experimental animals
A) GFAP staining from a M1D-inoculated deer six months post inoculation.
B) GFAP staining from a SMCA-inoculated deer six months post inoculation.
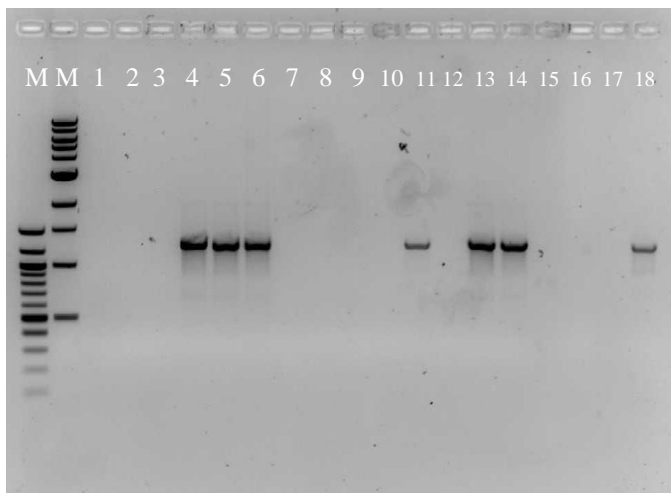
Figure 4.3 PCR results demonstrating cultures positive for SMCA. Lanes 1-3: IC goat cultures (14 days post-inoculation), Lanes 4-6: IV goat cultures (2 days post-inoculation), Lanes 7-9: ID goat cultures (2 days post-inoculation), Lanes 10-11: IC goat cultures (3 days post inoculation), Lane 12: water negative, Lanes 13-14: positive controls, Lanes 15-18: IV goats (4 days post inoculation). PCR primers are specific to the *S. mirum* adhesin gene.

Brain Tissues:
We were able to recover SMCA from one out of fifty-four animals. The cortex from an SMCA IC inoculated deer in experiment two was positive for spiroplasma organisms. The tissue was homogenized in M1D, and the supernatant inoculated into four embryonated eggs. Pooled allantoic samples were taken and inoculated into M1D media. After two passages in M1D, one helical organism was noticed via dark field microscopy (Figure 4.4). The culture was passaged into fresh M1D, but no *Spiroplasma* growth was found after any passages (M1D) or any embryonated eggs cultures that followed.

Figure 4.4 Spiroplasma seen via dark field microscopy from SMCA-inoculated deer.

PCR: No tissue samples from any of the fifty-four experimental animals tested were positive for spiroplasma via PCR. Figure 4.5 is an example PCR blot from SMCA-inoculated tissues. The positive banding seen is from the positive control. All other samples were negative.
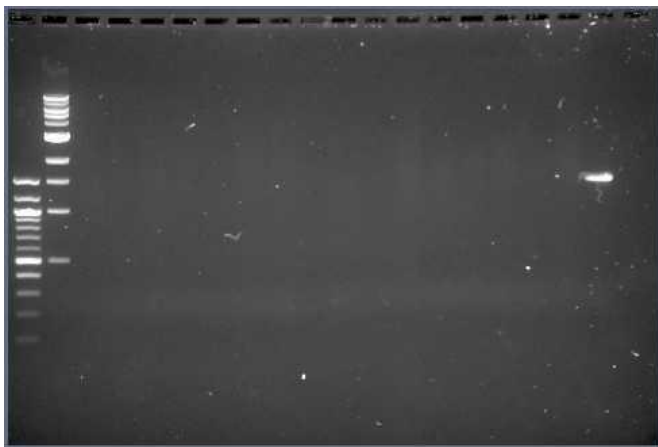

Figure 4.5 PCR for SMCA-inoculated tissues. PCR primers are specific to the *S. mirum* adhesin gene.

Antibody Production: Multiple serum samples were tested from each animal inoculated with SMCA. For short term experiments, serum samples from six weeks and eight weeks were tested. For long term experiments, serum samples were taken at six weeks and then once every six months. There was no spiroplasma-specific banding on any immunoblots for SMCA antibodies. There were minor non-specific bands found on SMCA-inoculated animals as well as M1D-inoculated animals. The antibody profiles from a goat hyperimmunized with boiled SMCA antigen can be seen in Figure 4.6 C below. Figure 4.6 A is an example of a western blot from a M1D-inoculated goat and B is from an SMCA-inoculated animal.
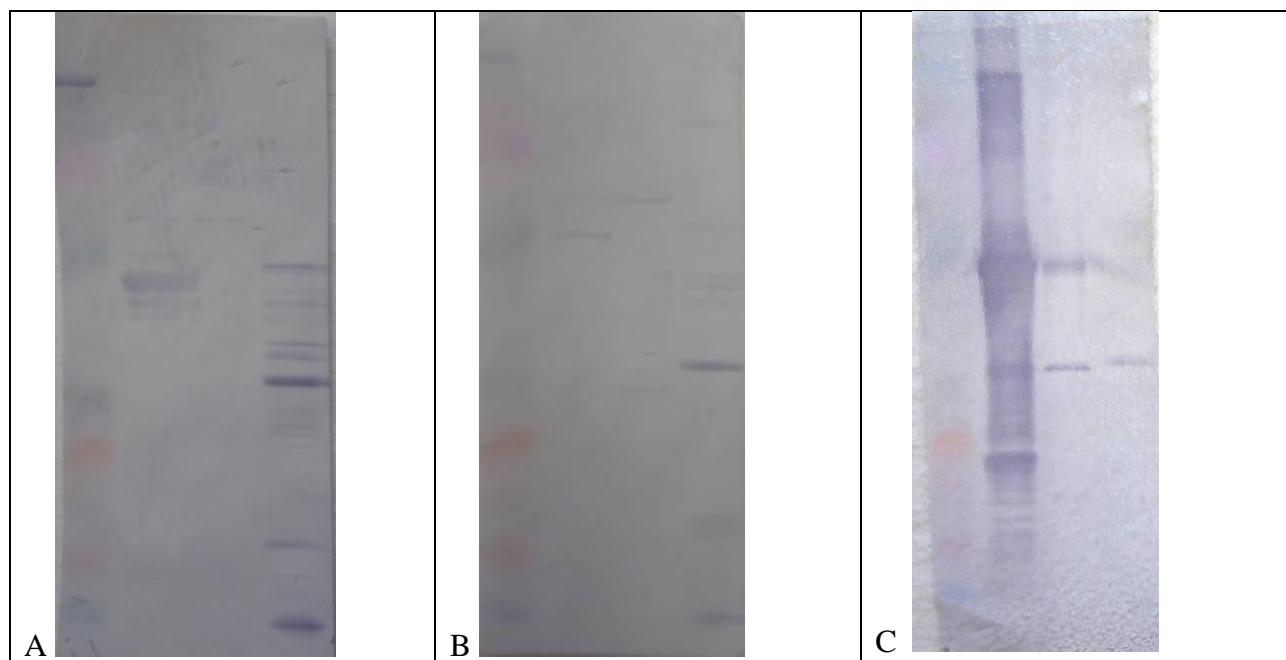

Figure 4.6 Immunoblots from experimental animals
A) Immunoblot from M1D-inoculated animal, B) Immunoblot from SMCA-inoculated animal.,
C) Immunoblot from goat hyperimmunized with boiled SMCA antigen.
The first line in all blots is the marker, the second line is the boiled antigen preparation, the third line is the guanidine thiocyanate-SMCA preparation. The last line is a from a Yesinia antigen preparation, used as a positive control.

Discussion

For the purpose of discussion, we will include all ruminant experiments done in this laboratory. This will include the experiments published by Bastian et al. in 2007 (Bastian et al., 2007). Table 4.3 summarizes all ruminant experiments. Figure 4.7 depicts an overall timeline for SMCA ruminant experiments.

Table 4.3 Summary of ruminant experiments

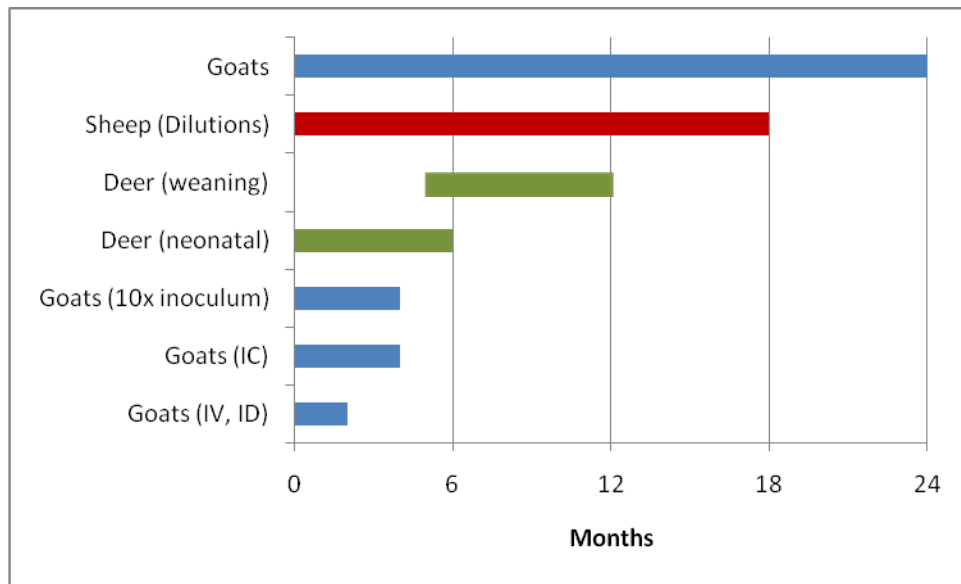| Exp	Species	Inoculum	Length of Experiment
Bastian 2007	Cervidae	SMCA	6 months
2	Cervidae	SMCA	6 months
Bastian 2007	Ovis	SMCA	1.5 years
		1:10 SMCA	
		1:100 SMCA	
	Caprine	SMCA	4 months
3	Caprine	SMCA (IC)	2 months
		SMCA (IV)	
		SMCA (ID)	
4	Caprine	10x Concentrated SMCA	4 months
		Heat-killed 10x Concentrated SMCA	
5	Caprine	SMCA	2 years

Figure 4.7 An overall timeline for SMCA ruminant experiments.

The experiments in this paper did not result in any evidence of pathology or persistence of SMCA. None of the fifty-four experimental animals were positive for spongiform encephalopathy, and only one was positive for the organism after six months of incubation. Tully suggested that pathogenicity is dependent on the *S. mirum* strain, the animal host, and the history of the inoculum (Tully et al., 1983). In the case of SMCA, after continued passage on artificial media, the LD_{50} for suckling rats increased; and the strain no longer induced cataracts, while the LD_{50} for chick embryos decreased (Bove, 1997). Loss of pathogenicity due to multiple passages could be an explanation for the contrasting results found in our experiments compared to those from 2007 (Bastian et al., 2007) however, virulence was seen in embryonated egg experiments previously reported in this body of work.

Clark found that SMCA pathology was dependent on dose, age, and host strain (Clark and Karson, 1968). This may account for the differences seen in deer experiments since our deer were five months old at time of inoculation. Clark also found that the larger the dose and the younger the host the greater pathology produced (Clark and Karson, 1968). This did not hold true in our neonatal goat experiments where we inoculated ten times the normal inoculum within seventy-two hours of birth. In 1984 Bastian reported microcystic encephalitis in rats one day after intracranial inoculation of GT48. The same experimental group of rats had minimal pathological alterations at day twenty-five (Bastian et al., 1984). Spiroplasma was still recovered in these tissues, but no pathology was associated in this experiment. These results could suggest that there is a transition time for pathology in different species. However, we tested goats at two months, four months, and two years and no pathology was detected at any time point.

For spiroplasma experiments and prion research, bacteria/prion strain has played a role in susceptibility. Mice strains also played a role in susceptibility. Our neonatal experiments were conducted using animals from the same genetic pool. One could argue that these strains have resistance to our organisms; however, Bastian's experiments resulted in pathology and the current study did not. Experiments using tissues from 2007 animals found no persistence of the organisms in brain tissues or production of antibodies at any time (data not published) which concurs with the present studies.

Spiroplasma was recovered from one experimentally infected animal. The results from experiment 1 (24 hour neonatal goat experiment) were used to determine the concentrations in certain areas for best options for organism recovery. Although spiroplasma was recovered from these areas in this experiment, the organism may migrate to different areas over time. In reported mice experiments, the maximal SMCA titer attained in the brain was at day fifteen post intracerebral inoculation. SMCA was recovered from blood of mice eighteen days post inoculation. No organisms were recovered after twenty-one days. Research found that the titer was always higher in the brain compared to blood (Clark, 1969). Titers peaked at day fifteen in brain tissues and subsequently decreased very slowly, with minimal titers of SMCA in mice sacrificed at after 800 days post inoculation (Clark and Karson, 1968). In experiments using other inoculation routes, no persistence of the organism was noted. One researcher has suggested that the failure to observe spiroplasma-like bodies in infected brains suggests that the site of localization of the infection has not yet been ascertained, or less likely but more intriguing, that the agent may be present in an unrecognizable form within the central nervous tissue (Clark, 1974).

SMCA was able to avoid the immune system resulting in a lack of antibody production in experimental animals. Immunoblots with three different SMCA antigen presentations were unable to detect any antibody from any time points in the experiments. In his 1984 rat experiments, Bastian (Bastian et al., 1984) also found a lack of antibody production and suggested that the presence of spiroplasma in the tissues without inflammatory response could be indicative of a unique adaptation of the organism to the host. This peculiar host adaptation may be related to the intracellular localization of the organisms. Clark did find evidence of antibody production in his mice experiments in 1968. Dams infected while pregnant did not transfer SMCA to their offspring (Clark and Karzon, 1968). The mothers were, however, able to transfer

protective antibodies to their offspring, who never developed cataracts post- intracerebral inoculation. Furthermore, following generations were able to pass protection to their offspring and prevent disease. The same experiments were repeated and found to hold true with GT48 (Clark and Karzon, 1968).

Spiroplasma mirum has only been isolated naturally from the rabbit tick. The true pathogenicity has been questioned since the organism has only experimentally induced disease either by inoculation into the yolk sac of seven day old chick embryos; intracerebral injection into newborn rats, mice or hamsters; or by inoculation into scarified cornea of adult rabbits (Bove, 1997). The fastidious nature of the organism makes primary isolation challenging and leaves researchers questioning other possible hosts. In these experiments different inoculation routes were tested in order to prove a hematogenous transmission route. No changes were seen within these experimental animals. In 1987 Bastian induced cataracts, weight reduction, and alopecia in rats with GT48 inoculated intraperitoneal or subcutaneously. The organism was recovered at low titers fifty days post inoculation. The brains of rats evaluated at day fifty showed minimal neuronal vacuolization in the hippocampus region. The peripheral route of inoculation failed to produce the moribund state and vacuolar encephalopathy shown when suckling rats were inoculated intracerebrally with spiroplasma (Bastian et al., 1987b). These findings do not support a hematogenous transmission route which would be needed for transmission from a tick to an animal.

Conclusion

The results reported in this chapter establish that the inoculation of this laboratory's *S. mirum* strain SMCA into neonatal goats did not mimic the clinical signs or pathology seen in transmissible spongiform encephalopathies. SMCA did not cause spongiform encephalopathy when inoculated intracerebrally, intravenous, or intradermally into neonatal goats. No pathology, clinical signs, or immune responses were noted over a two year time period; and the organism was not detected via PCR or culture from any experimentally inoculated animals. When inoculated into five month old white-tailed deer, SMCA did cause minimal clinical signs in one animal but did not cause spongiform encephalopathy after seven months. Spiroplasma was recovered from the cortex of the clinically-affected deer after multiple passages in culture.

Although the goat experiments in this chapter did not result in TSE-like disease, the methodology has been established for an animal model. Further research should be conducted using similar methods in neonatal white-tailed deer to better compare CWD and deer spiroplasmosis. Experiments using sheep may also allow for better comparisons to scrapie.

CONCLUSION

There have been many contradicting reports in the literature involving the culturing of *S. mirum*, its resistance to disinfectants and antibiotics, and its role in Transmissible Spongiform Encephalopathy (TSE). These discrepancies led to the interest in a possible connection between spiroplasma and TSEs and the development of the research in this document. Based on the results reported by Bastian et al. in 2007, it was hypothesized that *Spiroplasma* was associated with a neurodegenerative disease such as TSE. In this work we further characterized *S. mirum* and continued to evaluate the possible correlations found in TSE infections. In order to establish the best overall picture of *S. mirum* infections, we optimized culture conditions, determined a susceptibility profile for physical and environmental disinfectants and anitbiotics, and improved the methodology for creating an animal model for spiroplasmosis. From this information, a better comparison may be made to the presumed infectious agent of TSEs, the prion.

To optimize recovery of *S. mirum* from experimental infections, we established reproducible culture conditions in M1D media, embryonated eggs, and SP4 plates in chapter two. Growth in M1D media at 30°C proved to be significantly better (p$\leq$0.05), yielding higher dark field counts than cultures at 37°C. Dark field enumeration appears to be limited to a range of $1x10^5$ to $1x10^8$ organisms/mL and therefore an accurate growth curve could not be calculated using this method. PCR was used to check for growth daily and results were comparable to dark field counts. Color changes were evident in all dilutions after 7 days, though the more diluted cultures were less obvious. Embryonated egg results were comparable to that in the literature; however, the artificial media is better for experimental purposes. The organism is difficult to detect after egg fatality by both dark field and PCR. PCR results may be skewed due to inhibitory factors in the egg fluids. Embryonated egg experiments support that the laboratory strains were not attenuated by multiple passages and still produce similar virulence in embryonated egg cultures as seen in literature. A method for obtaining colony forming units was also determined for SMCA and GT48. Following the ATCC SP4 recipe with the addition of Noble agar (1.6%), *S. mirum* produced consistent countable colonies on or near the surface of the agar. Those plates incubated at 37°C yielded higher growth counts and significantly better growth (p$\leq$0.05) than those at 30°C.

Because of the organism's fastidious nature, tissue culture may be more appropriate for spiroplasma research. Attempts have been made to cultivate *S. mirum* in a variety of mammalian, avian and tick cell cultures, but these were unsuccessful due to cytopathic effects and slow growth of the organism (Bastardo et al., 1974; Clark, 1964; Fabiyi et al., 1971; Yunker et al., 1987). SMCA has also been found to permanently transform NIH 3T3 mouse embryo fibroblast cells (Bove, 1997; Kotani et al., 1986). SMCA also transformed monkey kidney CV-1 cells (Kotani et al., 1986). Due to the formation of cataracts in suckling mice, Megraud et al. experimented with the affinity the organism showed for ocular tissues (Megraud et al., 1983). Even though some research has been promising, successful tissue cultures have yet to be established. Although no tissue culture experiments were conducted in this body of work, the establishment of a tissue culture system may better elucidate the organism's potential hosts and pathogenic nature.

Chapter three establishes a susceptibility profile for disinfectants and antibiotics and compares these results to known prion susceptibility. Although *Spiroplasma* and prions may persist in murine neurologic tissues for a long period of time, the two do not share the same properties of resistance. The three species of *Spiroplasma* tested were susceptible to minimal dilutions of common laboratory disinfectants. They were also susceptible to many of the antibiotics in use for other mollicutes. We did find strain differences in *S. mirum* in that GT48 was more resistant than SMCA to many of the disinfectants and antibiotics tested. More research is needed to determine the factors it may possess in order to achieve this resistance.

The animal experiment results reported in chapter four establishes that the inoculation of this laboratory's *S. mirum* strain SMCA into neonatal goats did not mimic the clinical signs or pathology seen in TSEs. SMCA did not cause spongiform encephalopathy when inoculated intracerebrally, intravenous, or intradermally into neonatal goats. No pathology, clinical signs, or immune responses were noted over a two year time period; and the organism was not detected via PCR or culture from any experimentally inoculated animals. When inoculated into five month old white-tailed deer, SMCA did cause minimal clinical signs in one animal but did not cause spongiform encephalopathy after seven months. Spiroplasma was recovered from the cortex of the clinically-affected deer after multiple passages in culture. The tissue was homogenized in M1D and the supernatant inoculated into four embryonated eggs. Pooled allantoic samples were taken and inoculated into M1D media. After two passages in M1D, one helical organism was noticed via dark field microscopy. The culture was passaged into fresh M1D, but no growth was found after any passages (M1D or embryonated eggs).

Tully suggested that pathogenicity is dependent on the *S. mirum* strain, the animal host, and the history of the inoculum (Tully et al., 1983). Loss of pathogenicity due to multiple passages could be an explanation for the contrasting results found in our experiments compared to those from 2007 (Bastian et al., 2007); however, virulence was seen in embryonated egg experiments. Clark found that SMCA pathology was dependent on dose, age, and host strain (Clark and Karson, 1968). This may account for the differences seen in deer experiments since our deer were five months old at time of inoculation. Clark also found that the larger the dose and the younger the host the greater pathology produced (Clark and Karson, 1968). This did not hold true in our neonatal goat experiments where we inoculated ten times the normal inoculum within seventy-two hours of birth. Although the goat experiments in this chapter did not result in TSE-like disease, the methodology has been established for an animal model. Further research should be conducted using similar methods in neonatal white-tailed deer.

Spiroplasma mirum's growth at both 30°C and 37°C may suggest the possibility for two hosts. Research on *S. citri* has established its ability to survive in multiple hosts. At least two mechanisms are utilized for sugar uptake by *S. citri*: the phosphoenolpyruvate:phosphotransferase system (PTS) and the SBP-ABC transporter system (Bai and Hogenhout, 2002; Gaurivaud et al., 2000b). This could be characteristic of multiple-host bacteria that need to rapidly adapt from a host or host-compartment that uses one sugar to a host or host-compartment that uses a different sugar (Bove et al., 2003). As more spiroplasma genomes are completed, a better understanding of these organisms' ability to utilize different substrates in their environment will be obtained, which may determine the different hosts the organisms may inhabit. *Spiroplasma citri*'s use of the PTS system may also allow this organism

to create a biofilm (Bove et al., 2003). The creation of a biofilm may affect the virulence of this organism and may be the reason *Spiroplasma* is so difficult to recover from tissues. More research is needed to establish that *Spiroplasmas* create biofilms and what changes in resistance this could create.

Other strains of spiroplasma may also have the potential to cause neurodegenerative disease. *Spiroplasma melliferum* has also been shown to persist in suckling mice post intracerebral inoculation. Only one mouse exhibited CNS-like signs, and the organism was only isolated from one experimentally infected mouse after 284 days (not the clinical one). *Spiroplasma melliferum* was isolated at day seventy at a rate of 10^7 to 10^{10} CCU/ml in brain titers. These titers were lost after repeated passages in suckling mice. No neurological signs were observed, and histological changes were limited to discrete diffuse capillary congestion in brain sections. There was no antibody response from the suckling mice to *S. melliferum*, as is the case for *S. mirum* (Chastel et al., 1991). Leafhoppers infected with *S. citri* (Western-X disease) develop lesions, notably in the optic lobes and salivary glands (Nasu et al., 1970; Whitcomb et al., 1967). Simultaneous involvement of the optic lobes and salivary glands in Western-X has similarity to the occurrence of either Sjorgren's syndrome or mucosal inflammation with ocular manifestations in Multiple Sclerosis (MS) (Coyle and Bulbank, 1989; Sandberg-Wollheim et al., 1992). Brown suggested that the infection of both plant phloem and insect axons suggests a predilection of the organism for components of electrochemical conduction (Brown, 2003). Clinical correlations have been made with bee and wasp stings and the subsequent development of MS (Dionne et al., 2000). These reports attribute the disease to bee venom; but bees and wasps are known carriers of spiroplasmas, and Brown suggests that the inoculation of humans with *S. citri* by bee or wasp stings could be the mechanism causing MS (Brown, 2003). Using the methodology for an animal model set forth in chapter four, further research with these spiroplasma strains may provide insight into their potential association with neurodegenerative diseases.

Clark suggested that the SMCA system could provide a useful model for the study of intraocular infection (Clark, 1969). Bastian has isolated *Spiroplasma* from ocular fluid taken aseptically from scrapie-infected sheep. Minced corneal tissues were inoculated into M1D, and *Spiroplasma* organisms were evident via dark field microscopy. These organisms did not propagate and the culture was lost after several passages (Bastian et al., 2011). This has recently been repeated and efforts are ongoing to passage the spiroplasma to a substantial culture (data not published). There has been evidence in the literature that mycoplasma-like organisms were isolated from the aqueous humors of eleven of nineteen human eyes with unexplained uveitis (Zeigel and Clark, 1974) and *S. mirum* may be a causative agent of uveitis in ruminants. Its affinity for ocular tissue has been established, and more research is needed in unexplained uveitis cases in order to prove this association.

Based on the experiments performed in this body of work, further research should be conducted with *S. mirum*. The genomes of multiple strains of spiroplasma are presently being determined (Landry et al., 2010). Optimal growth characteristics have been established, and research can now move into genetic manipulations to evaluate possible virulence factors for spiroplasma. With the organism's affinity for neurologic and ocular tissues, spiroplasma could be genetically manipulated to be used as a potential therapeutic agent for infections affecting these tissues.

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VITA

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