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Short Paper

Goat as Potential Animal Model for Transmissible Spongiform Encephalopathy using *Spiroplasma mirum*

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Abstract

Objective

The objectives of the experiments presented in this study were: (i) create an animal model for *Spiroplasma* infections using small ruminants and compare neuropathological changes to those observed in natural Transmissible Spongiform Encephalopathy (TSE) infections, (ii) test possible inoculation routes in order to establish a mode of transmission; and (iii) titrate *Spiroplasma* in order to establish a dose dependent correlation with pathology and clinical signs seen post inoculation.

Animals

For each experiment age matched goats were used for control and treatment groups.

Procedures

Methods for inoculation, tissue retrieval, culture and PCR testing were evaluated to create an animal model using small ruminants.

Results

No clinical signs, bacterial persistence, or neuropathological changes were observed to validate inoculation route or dose dependent infections in goats.

Conclusions and Clinical Relevance

Unlike previous experimental results using deer, the goat experiments in this study did not result in TSE-like disease. Similar experiments should be conducted in neonatal white-tailed deer to better compare Chronic Wasting Disease to the deer spiroplasmosis model.

Keywords:
Suckling Mouse Cataract Agent (SMCA); Transmissible Spongiform Encephalopathy (TSE); *Spiroplasma*; Scrapie, Chronic Wasting Disease (CWD)

Abbreviations

TSE	:Transmissible Spongiform Encephalopathy
SMCA	:Suckling Mouse Cataract Agent
IC	:Intracerebral
CJD	:Creutzfeldt-Jakob Disease
CWD	:Chronic Wasting Disease
ID	:Intradermal
IV	:Intravenous
GFAP	:Glial Fibrillary Acidic Protein
M1D	:Specialized broth
TAE	:Tris-acetate-EDTA

Introduction

Spiroplasma mirum is the only species of *Spiroplasma* that has been found to be pathogenic to vertebrates [1]. As the name describes, a strain from this species Suckling Mouse Cataract Agent (SMCA), when inoculated intracerebrally (IC), caused suckling mice to develop cataracts [2]. The organism was able to persist in suckling mice, specifically the brain tissues, for up to two years [3]. In 1979 Bastian reported spiral membranous inclusions seen by electron microscopy in brain biopsy tissues from a patient with Creutzfeldt-Jakob disease (CJD) [4]. Following this initial report of *Spiroplasma*-like inclusions in CJD tissues, Reyes published two case reports from patients with similar findings that were suspected of CJD. 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 [5]. 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 [6]. In 1981 Bastian reported two more cases of CJD with similar spiral-like inclusions seen through electron microscopy [7].

In 2005 Bastian et al. cultivated a *Spiroplasma* from homogenates of Chronic Wasting Disease (CWD) and scrapie infected brains. These isolates were later inoculated into neonatal ruminants [8]. 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 the typical lesions of naturally occurring TSE [8].

The objectives of this study were: (i) create an animal model for *Spiroplasma* infections using small ruminants and compare any neuropathology noted to natural TSE infections, (ii) test possible inoculation routes in order to establish a mode of transmission; and (iii) varying doses of *Spiroplasma* in order to establish a dose dependent correlation with pathology and clinical signs seen post inoculation.

Materials and Methods

Experiment 1

Twenty-nine 1 month old goats were separated into the following groups: 12 undiluted SMCA-inoculated goats, 3 SMCA 1:10 dilution goats, 2 SMCA 1:100 dilution goats, 6 M1D inoculated goats, and 6 control goats. All inoculations equaled 2 mL 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 tissues were collected and processed as described below.

Experiment 2

Twelve newborn goats were injected within 48 hours after birth via different inoculation routes and allowed to remain with dams. Three newborns were injected with 2 mL of SMCA via the open fontanel of the skull; 5 were injected with 2 mL of SMCA IV via the jugular vein; and 4 were injected with 0.2 mL 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 animals 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 tissues were collected and processed as described below.

Experiment 3

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 tissues were collected and processed as described below.

SMCA: SMCA strain of S. mirum, courtesy of Dr Gail Gasparich,[a] was used for the inoculums in all experiments and had undergone multiple passages in M1D broth. SMCA was grown to log phase [9], aliquoted into 1 mL portions mixed 50/50 with sterile 30% glycerol giving a 15% glycerol mixture and frozen at –80ºC. For all following experiments, a stock of SMCA (approximately 1x108 organisms per mL) in M1D and frozen at -80ºC. Viability was checked via dark field microscopy for all cultures. SMCA stock cultures were diluted in M1D at a 1:10 or 1:100 for the dose dependent studies (Experiment 1). For intradermal (ID) inoculation routes (Experiment 2), the routine stock SMCA was concentrated into a 20ul volume in MID. For concentrated SMCA experiments (Experiment 3), a stock of SMCA was expanded and centrifuged to yield a 10X concentration in 2 ml of PBS. For heat-killed concentrated SMCA inoculums (Experiment 3), the re-suspended 2 mL PBS culture was heated at 56°C in a water bath for 1 hour and verified as being non-viable after

a 14 day incubation prior to use.

Animals: Previous experiments showed similar pathology in goats and sheep post-SMCA inoculation. A uniform population of goats was available to create a Spiroplasmosis model and was therefore used instead of sheep. Goats were obtained from the LSU AgCenter Ben Hur 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 separated by sex and housed on concrete slabs in an IBRDSC approved isolation research facility. Euthanasia was performed following pentobarbital administration and exsanguination, and all efforts were made to minimize suffering.

Tissue Samples

Blood Cultures (Experiment 1 and 2): Jugular blood samples were collected and immediately stored in both EDTA coated tubes and serum collection tubes. One hundred µL of EDTA mixed blood was then transferred to 900 µL of M1D and allowed to incubate at 30ºC 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 -20ºC for immunoblotting.

Brain Tissues (All experiments): 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 from the left half of the brain were sampled for culturing in both M1D and embryonated eggs. Samples were also taken for PCR. The remaining left half of the brain was frozen at -80ºC.

Histology (All experiments): Tissue sections were stained with haematoxylin and eosin. Using standard protocols, the following immunohistochemical stains were applied: Anti-SMCA staining as established by Bastian [8] to detect presence of the organism; and glial fibrillary acidic protein (GFAP) to detect the presence of astrogliosis.

Dark Field Microscopy (All experiments): Five 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 equipped with a dark-field top lens condenser 1.2-1.4 and an EC Plan-NeoFluar x100 oil immersion objective with 1.3 iris closed to its lowest setting (0.7).[b]

Embryonated Egg cultures (All experiments): Approximately 100 mg of the brain 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 large chunks of tissue. 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 (All experiments): Approximately 100 mg of the brain 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 large chunks of tissue. The top half of culture (500 µL) was removed and combined with fresh M1D with vancomycin to 1 mL. Cultures as well as a media control were incubated at either 30ºC 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 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,[c] 2 µL of 10 mM dNTP,[d] and 5 µL of 10x Standard Taq Bufferc which contains 15 mM of MgCl2. 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 H2O. PCR's were performed using a MyCycler Thermal Cycler. 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 DNA gel staining under UV fluorescence.[e]

Results

Clinical Signs:

No goats exhibited any clinical signs regardless of experiment duration, inoculation route or dosage.

Histology:

None of the experimentally infected animals appeared to have any pathological changes. No abnormalities were seen in H&E or GFAP staining and no sections were positive using anti-SMCA antibody staining.

Persistence of SMCA:

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 the presence of organisms.

Brain Tissues

No organisms were recovered from goat experiments.

Discussion

SMCA has been isolated 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 [1]. 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 investigate 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* [10]. These findings do not support a hematogenous transmission route which would be needed for transmission from a tick to an animal.

Unlike previous experimental results using deer, the goat experiments in this study did not result in TSE-like disease. Similar experiments should be conducted in neonatal white-tailed deer to better compare Chronic Wasting Disease to the deer spiroplasmosis model.

Footnotes

a. Dr Gail Gasparich, Towson University, Baltimore, MD, USA
b. Carl Zeiss, Inc. North America, Thornwood, NY, USA
c. New England BioLabs, Ipswich, MA
d. AmpliTaq Gold, Applied Biosystems, Foster City, CA
e. Invitrogen, Eugene, OR

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