A NEMATODE VECTOR AS A POSSIBLE MODE OF TRANSMISSION FOR *MYCOPLASMA PULMONIS*

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PREFACE

In the past attention has been given to the relationship of nematode larvae and pathogenic bacteria but mainly to synergestic effects that result in pathological changes in the host. The design of the present study was to investigate the potentiality of nematode larvae to serve as carriers of pathogenic bacteria.

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CHAPTER I

INTRODUCTION

Little is known about the natural means of survival during inter-epidemic periods of those *Mycoplasma* species that inhabit the lungs of mammals. Additionally, nothing is known of the different modes by which they gain access to the lungs of a new host. It is generally accepted that *Mycoplasma* species are transmitted from animal to animal by airborne means, but transmission by parasitic nematode larvae that migrate through the lungs of their host could be considered as a possible method of transmission (1).

Mycoplasma pulmonis is a bacterium frequently found in the lungs of rats. *M. pulmonis* and two nematodes (*Strongyloides ratti* and *Nippostrongylus brasiliensis*) that as adults inhabit the small intesttine of rats and have required larval migration through the lungs, were used as a model to study the biological and mechanical vector roles of *Mycoplasma* species transmission.

Evidence that nematodes are vectors of pathogenic *Mycoplasma* species has not been presented. Nematodes, as potential vectors for pathogens of animals, involve evolutionary adaptations among biological systems of parasitism. The ability of nematode larvae to transmit pathogenic bacteria is largely speculative and it is the purpose of this research to study an animal parasitic nematode as a potential vector of a *Mycoplasma* species.

CHAPTER II

LITERATURE SURVEY

Parasitic adult plant nematodes were found to contain bacteria as early as 1933 when Steiner showed that adult Rhabditis lambdiensis contained Bacterium tolaasi (2). Different investigators have since shown that adult Pristionchus lheritieri are capable of carrying Mycoplasma gallisepticum, Salmonella wichita, S. typhi, Chlamydomonas reinhardi, Ankistrodesmus species, Scendesmus species, Agrobacterium tumefaciens, Erwinia amylovora, E. carotovora, Pseudomonas phaseolicola, and Serratia marcescens (3, 4, 5, 6, 7, 8, 9). Adult rhabditids have been shown to carry Proteus mirabilis, Aerobacter aerogenes, A. clocoae, Pseudomonas species, Salmonella typhosa, S. paratyphi, S. typhimurium, Shigella sonnei, and S. dysenteriae II (9). Adult Diplogaster lheritieri is capable of carrying Fusarium oxysporium, Verticillium dahliae, Geotrichum species, Hormodendrum species, Penicillium species, and Stysanus species (10). Adult Mesodiplogaster lheritieri has been shown to carry Fusarium oxysporium f. lycopershici and F. dahliae (11). Adult Panagrolainus subelongatus have been shown to ingest and excrete M. gallisepticum (3).

A parasitic adult animal nematode, Stephanurus dentatus, has been shown to contain bacteria in different parts of its body (12). Escherchia species, Enterobacter species, and Streptococcus species were found in the cuticle, excretory glands, and in the ovaries.

Bacillus species and Streptococcus species were found in the intestine.

A plant parasitic nematode, Diplogaster lheritieri has been shown to have microorganisms in its larval forms (10). Fusarium oxysporum f. lycopersici, dahliae, and species of Geotrichum, Hormodendrum, Penicillium, and Stysanus were found in the second and third stage larvae.

Parasitic nematode larvae have been shown to be closely associated with pathogenic bacteria. Several species of Bacillus, B. coli (E. coli), B. viscosum, B. necrophorus and B. anthracis have been demonstrated in relation to third-stage larval migrations (8, 13, 14). The third-stage larvae of Ascaris species were shown to have no effect on the pathogenicity of a Mycobacterium species, whereas larvae of Brugia malayi have been shown to have a considerable enhancement on the pathogenicity of streptococci infections (15, 16). The third-stage larvae of Oesophagostomum columbianum when given per os are unable to transmit Corynebacterium pseudotuberculosis (7). Strongyloides papillosus is capable of carrying Erysipelothrix rhusiopathie through the skin of pigs and filariform larvae of Strongyloides stercoralis have been suspected of carrying Escherchia coli (17, 18).

CHAPTER III

MATERIALS AND METHODS

Cultivation of Nematodes and *Mycoplasma pulmonis*

Culturing of *Strongyloides ratti* in Charcoal Cultures

S. ratti cultures were prepared in culture dishes by mixing moist wood charcoal with egg laden feces. The cultures were maintained at 27 degrees Centigrade for five or more days (19).

Culturing of *Nippostrongylus brasiliensis* in Charcoal Cultures

N. brasiliensis cultures were prepared by mixing egg laden feces and intestinal contents from infected rats with moist wood charcoal in culture dishes. The cultures were maintained at room temperature and at 27 degrees Centigrade for five or more days. The cultures were sprayed daily with a fine mist of tap water (20).

Preparation of Non-inhibited Media (PPLO-NI) for Culturing *Mycoplasma pulmonis*

Broth medium was prepared in 100 ml quantities. Ninety ml of Difco PPLO broth was sterilized in an autoclave for 15 minutes at 15 pounds pressure, 121 degrees Centigrade. The medium was cooled at 56

degrees Centigrade and 10 ml of horse serum was added. The broth was then refrigerated at 18 degrees Centigrade.

The agar medium was prepared in 100 ml quantities. Ninety ml of Difco PPLO agar was dissolved by heating and one ml of a 5 percent solution of trypticase and 0.5 grams of soluble starch were added. The mixture was then sterilized in an autoclave for 15 minutes at 15 pounds of pressure, 121 degrees Centigrade. The medium was cooled to 56 degrees Centigrade and 10 ml of horse serum was added. The medium was poured into Petri dishes, allowed to solidify, and refrigerated at 18 degrees Centigrade (21).

Preparation of Inhibited Media (PPLO-I)
for Culturing Mycoplasma pulmonis

The broth medium was prepared in 100 ml quantities. Ninety ml of Difco PPLO broth, one ml of a one percent solution of cysteine HCl, and 0.25 ml of a 10 percent solution of thallium acetate were mixed. The mixture was sterilized in an autoclave for 15 minutes at 15 pounds pressure, 121 degrees Centigrade. The medium was cooled to 56 degrees Centigrade and 10 ml of horse serum and 100 units of solium penicillin were added. The broth was then refrigerated at 18 degrees Centigrade.

The agar medium was prepared in 100 ml quantities. Ninety ml of Difco PPLO agar was dissolved by heating and one ml of a 5 percent solution of trypticase, 0.5 grams of soluble starch, and 0.25 ml of a 10 percent solution of thallium acetate were added. The mixture was sterilized in an autoclave for 15 minutes at 15 pounds of pressure, 121 degrees Centigrade. The medium was cooled to 56 degrees Centigrade and 10 ml of horse serum and 100 units of penicillin were added. The

medium was then poured into Petri dishes, allowed to solidify, and refrigerated at 18 degrees Centigrade (21).

Culturing of Mycoplasma pulmonis

A pure culture of M. pulmonis (Ash strain) and a pure culture of a M. pulmonis isolated from the lung of a laboratory rat were maintained in PPLO-I broth medium and on PPLO-I agar plates. One ml of each M. pulmonis was transferred to 5 ml of PPLO-I broth every 4 to 6 days for maintainance of the cultures (22).

Collection of Mycoplasma pulmonis from Broth Cultures

Two-day-old M. pulmonis cells were collected by centrifugation in a Sorvall centrifuge at a speed of 23,500 x g in 50 ml tubes for 40 minutes using aseptic technique. The supernatant fluid was decanted and the pellet was used in experimental studies as a source of M. pulmonis.

Culturing of Strongyloides ratti and Nippostrongylus brasiliensis on PPLO-NI Culture Media

S. ratti and N. brasiliensis eggs were washed free of feces by floating them in a saturated sodium nitrate solution followed by several washings in saline. Between 400-600 washed eggs were placed on a PPLO-NI agar plate and maintained at room temperature for 5 or more days. The cultures were sprayed daily with a fine mist of tap water.

Collection of Nematode Larvae from Charcoal Cultures

Third-stage larvae were separated from the charcoal cultures using warm water and a Baermann apparatus. By using Kimwipes lining a wire sieve (Kimwipes obtained from Kimberly-Clark Corporation, Neenah, Wisconsin), clean yields of larvae free from particulate material were obtained for experimental use (23). These larvae were then used for *in vitro* or *in vivo* experiments.

Management of Rats

Maintainance of Rats for Nematode Passage

Four 21-day-old male Caesarean Originated-Barrier Sustained (COBS) rats were used to maintain the nematode infections. They were obtained at bi-weekly intervals and inoculated subcutaneously with 1000 third-stage larvae. The larvae were obtained from cultures of the preceding group of rats. The rats were kept in stainless steel cages and allowed to feed ad lib on rat chow and water. The environment of the rat room was maintained at 21 to 27 degrees Centigrade and at 52 to 87 percent humidity.

Handling of *Mycoplasma pulmonis* Free Rats in Isolators

M. pulmonis free 21-day-old rats were obtained from A. R. S. Spraque Dawley, Madison, Wisconsin, in special isolator units as needed. The rat shipping isolators were of a size that would fit into a holding unit that in turn led into a germ free environmental chamber.

Immediately upon receiving the rats, the isolator unit in which they were shipped was sterilized on the outside with a 50 percent aqueous Clorox solution and then placed into the holding unit of the germfree environmental chamber that had been fitted with rat cages and sterilized inside and out with a 50 percent Clorox and water solution. Immediately the holding unit was sealed and left for 30 minutes before the holding unit leading into the germfree environmental chamber was opened. The rats were then placed into cages or inoculated and placed into cages in the germfree environmental chamber.

The rats were maintained in the germfree environmental chambers at 21 to 27 degrees Centigrade and at 70 to 85 percent humidity for a predetermined period of time and then removed for further study. The rats were fed sterile water and rat chow while in the germfree environmental chamber.

Sterilization Processes

Clorox Treatment of Feces

Five to ten day old rat fecal-charcoal cultures free of nematode larvae were collected in 50 ml plastic centrifuge tubes. The feces were washed three times with distilled water by sedimentation and one-half ml aliquots of feces were treated with 9.5 ml of various Clorox-water solutions ranging from one to 20 percent Clorox. The treatment was for a 20 minute time period at room temperature. The Clorox treated feces was then washed 3 times with sterile saline using aseptic technique.

Clorox Treatment of Nematode Larvae

Rat fecal cultures were Baermannized for 20 minutes with warm water (30 to 35 degrees Centigrade) and the third-stage larvae were collected. The larvae were washed 3 times with distilled water by allowing the larvae to settle and then removing the supernatant fluid. One-half ml of settled larvae were treated with 50 ml of various strength Clorox-water solutions (1 to 20 percent) for a 20 minute time period at room temperature. Using aseptic technique, the Clorox-water treated larvae were washed 3 times with sterile saline. The larvae from the third washing were placed in a sterile Petri dish and left at 27 degrees Centigrade for 15 minutes, observed under a stereoscopic microscope, and survival percentages were calculated. The lowest percentage of Clorox necessary for bacteriological sterilization was determined using standard bacterial culture techniques.

Clorox Treatment of Nematode Eggs

Nematode eggs were floated in a saturated aqueous solution of sodium nitrate for 8 minutes at room temperature and drawn off with a Pasteur pipette. The sodium nitrate was removed from the eggs by repeated washings in saline with centrifugation at 120 x g in 50 ml tubes for 3 minutes. Between 400-600 eggs were treated with 10 ml of various strength Clorox solutions for 20 minutes at room temperature, shaking constantly at 80 shakes per minute. The Clorox treated eggs were washed 3 times with sterile saline using aseptic technique. The eggs of the third washing were placed in culture to check for viability. The eggs were also checked for bacterial sterility using bacteriological culture techniques. The method of bacterial sterilization of eggs was

based on the studies of Lawrence (24).

Bacteria-Nematode Exposure

Exposure of Mycoplasma pulmonis to Strongyloides ratti Larvae

A mixture of 5 ml of PPLO-NI broth and a pellet from 500 ml of a 2-day-old broth culture of M. pulmonis was transferred to a fresh S. ratti charcoal culture. The 3rd stage S. ratti larvae were collected and examined for ingested M. pulmonis by bacteriological and immunofluorescence techniques.

Exposure of Mycoplasma pulmonis to Nippostrongylus brasiliensis

A mixture of 5 ml of PPLO-NI broth and a pellet from 500 ml of a 2-day-old broth culture of M. pulmonis was transferred to a fresh N. brasiliensis charcoal culture. The newly hatched N. brasiliensis larvae were allowed to feed on the M. pulmonis for six to eight days at 32 degrees Centigrade and developed into third-stage larvae. The charcoal culture was then Baermannized and the third-stage N. brasiliensis larvae were collected and examined for ingested M. pulmonis by bacteriological and immunofluorescence techniques.

Preparation of Biological Materials for Tissue Sectioning

Nematode Larvae Preparation for Sectioning

Pellets of third-stage larvae were quick-frozen in an ethanol-

dry ice bath upon termination of M. pulmonis exposure. The larvae were thawed and refrozen onto the cryostat chuck with a glycerine solution containing 9 ml reagent grade glycerine and one ml 0.01 M phosphate buffered saline, pH 7.2. The larvae pellet was cut into 8 micron sections and every fifth section was placed onto a coverslip. The larvae-tissue coverslips were fixed in reagent grade acetone for 10 minutes at room temperature. Coverslips were labled, boxed, and frozen at -15 degrees Centigrade until examined.

Rat Lung Preparation for Sectioning

The lungs from experimental rats were removed immediately upon death. The lungs were placed in sterile cellophane and then into 15 ml sterile glass vials. The vials were sealed and quick frozen in dry ice and ethanol. The lung tissue was stored at -20 degrees Centigrade until tested for M. pulmonis.

Whole rat lungs were frozen onto the cryostat chuck with a glycerine solution containing 9 ml reagent grade glycerine and one ml 0.01 M phosphate buffered saline at a pH of 7.2. The lungs were cut into 8 micron sections and every fifth section was placed onto a clean coverslip. The tissue sections were placed into an incubator at 37 degrees Centigrade for 25 minutes. After incubation, the tissue coverslips were fixed in reagent grade acetone for 10 minutes at room temperature. These were then labeled, boxed, and placed in a freezer at -15 degrees Centigrade (1).

Preparation of Biological Materials for Bacteriological Culturing

Nematode Larvae Preparation for Bacteriological Culture Techniques

One-half ml aliquots of washed third-stage larvae were divided into two portions. One portion was frozen at -15 degrees Centigrade and the second portion was aseptically ground up with a mortar and pestle using sterile sand and 6 ml of sterile physiological saline (0.85 percent NaCl-water). The nematode larvae tissue suspensions were then diluted in two types of broth media, PPLO-I (as previously described) and Bacto-thiol broth made up according to manufacturers directions.

Rat Lung Preparation for Bacteriological Culture Techniques

The rats were swabbed with 70 percent alcohol after euthanasia and the lungs were aseptically removed. The three lobes of the right and left lungs were cut into and one-half of individual lobes were collected together. The lungs were divided into two portions this way. One portion was wrapped in cellophane and frozen at -15 degrees Centigrade. The other portion was aseptically ground up with a mortar and pestle using sterile sand and 10 ml of saline.

Analysis of Prepared Materials by Immunofluorescence Procedures

Conjugation of Gammaglobulin for Immunofluorescent Studies

Three ml samples of rabbit-anti M. pulmonis serum were placed into sterile 10 ml Erlenmeyer flasks along with magnets. The flasks were cooled in an ice bath. To each flask, 0.45 ml of pH 8.7 to 8.8 carbonatebicarbonate buffer was added and allowed to cool. Then, 4.4 mg of fluorescein isothiocyanate was added to each flask and allowed to stand on the fluid surface for 15 minutes in the ice bath. The Erlenmeyer flasks were then strapped to magnetic stirrers and allowed to run in a cold room for 18 to 24 hours.

Fifty grams of Cellex-D was added to 1000 ml of 0.5N NaOH and agitated on a magnetic stirrer for 10 minutes. The cellulose was moved through a Buchner filter with distilled water until the filtrate was pH 7.0 to 7.2. A 0.025 M phosphate buffer, pH 7.28 to 7.30, was run through the Buchner filter until the pH of the filtrate equalled 7.28 to 7.30. The Cellex-D was suspended in 0.025 M phosphate buffer and refrigerated. Fine grade glass wool was placed in 15 mm diameter columns followed by 10.5 cm of Cellex-D. The columns were washed with 0.025 M phosphate buffer, pH 7.28 to 7.30, until the pH of the buffer coming off of the column was 7.28 to 7.30.

The columns were then placed in a cold room and the buffer was run just to the top of the column of Cellex-D. The conjugated anti-serum from each flask was then pipetted onto each column. The anti-serum was run through the column until the last of the serum reached

the top of the column of Cellex-D. Cold 0.025 M phosphate buffer was added and the colored antiserum was collected in sterile tubes. The tubes were stoppered and frozen at -15° C. (26).

Immunofluorescent Staining of Mycoplasma pulmonis

Imprints of M. pulmonis or tissue sections on coverslips were made by removing cultures from plates and by frozen tissue sectioning. The imprints were placed in a solution of 0.1 percent Tween 80 and 0.1 M phosphate buffered saline for 10 minutes at room temperature.

Dilutions of rabbit anti-M. pulmonis serum (obtained from Merwin L. Frey, Oklahoma State University) and 0,01 M phosphate buffer were then prepared. The dilutions consisted of 1:2.5, 1:5, and 1:10. One-tenth ml of each dilution was then placed on a coverslip inprint. Control imprints consisting of M. meleagridis were stained with the 1:2.5 dilution of rabbit anti-M. pulmonis serum. The imprint coverslips were then placed on a wet gauze lined tray, covered, and inncubated at 37 degrees Centigrade for 25 minutes after incubation. The coverslips were placed in Coplin jars and rinsed in buffered saline at room temperature for 25 minutes. Then with the coverslips still in the Coplin jars, they were washed with distilled water by allowing them to stand for one minute followed by removing each imprint coverslip and shaking it to remove all excess water. One drop of buffered elvalsol (9 ml reagent grade glycerine and 1 ml 0.01 M phosphate buffered saline), pH 7.2, was placed on each imprint followed by placing it on a glass slide with the imprint down. The imprint preparations were then viewed with the fluorescent microscope (26).

Bacteriological Culture Analysis of Biological Materials

Analysis of Prepared Materials for Mycoplasma pulmonis

One-half of the ground up tissue suspension was diluted 1:3, 1:6, and 1:12 in PPLO-I broth. The dilutions were incubated aerobically at 37 degrees Centigrade. At intervals of 5, 7, 10, 14 and 21 days, subcultures were made to fresh PPLO-I broth media and PPLO-I agar plates. The plates were read after seven days incubation at 37 degrees Centigrade. All original tissue dilutions were subcultured 3 times at 7 day intervals (27).

Analysis of Prepared Materials for Bacteria Other than Mycoplasma pulmonis

One-half of the ground-up tissue suspension was diluted 1:3, 1:6, and 1:12 in thiol broth. The dilutions were incubated aerobically at 37 degrees Centigrade. At intervals of 1, 3, and 5 days, subcultures were made to fresh thiol broth media. All original tissue dilutions were subcultured 3 times and plated onto Bacto-heart infusion plates made according to manufacturers directions with 5 percent citrated bovine blood added. The plates were incubated aerobically and in candle jars. All plates were read on days 1, 3, and 7.

Inoculation of Rats with *Nippostrongylus brasiliensis* and *Mycoplasma pulmonis*

Inoculation of Rats with *Mycoplasma pulmonis*

A suspension of 10^6 colony forming units per ml of *M. pulmonis* broth culture in a fluid volume of 0.3 ml was inoculated subcutaneously along the back of each experimental rat (27).

Inoculation of Rats with *Nippostrongylus brasiliensis*

One thousand *N. brasiliensis* third-stage larvae in a volume of 0.3 ml of sterile saline was inoculated subcutaneously along the back of each experimental rat.

Inoculation of Rats with *Nippostrongylus brasiliensis* and *Mycoplasma pulmonis*

One thousand *N. brasiliensis* third-stage larvae and 10^6 colony forming units per ml of *M. pulmonis* broth contained in 0.3 ml was inoculated subcutaneously along the back of each experimental rat.

CHAPTER IV

RESULTS AND DISCUSSION

Several investigators have observed bacteria within nematode larvae but the possible role of this relationship in animal disease has received little attention.

Nematode larvae ingest bacteria in laboratory fecal cultures and the excreted bacteria are viable. The volume of inoculum in a bacteria-filled nematode larva is minor in comparison with the normal route of certain bacterial infections but small quantities of organisms could result in disease if carried by larvae in its tissue migration and released in susceptible tissue. The ingested bacteria become directed and mobilized by the nematode larvae which usually seeks specific organs of the mammalian host. Such movement, even if it were not extensive, would increase the probability that viable bacteria would gain access to the organs of the mammalian host.

Ingestion, passage and defecation of bacteria through the alimentary canal of nematode larvae gives the bacteria added protection against environmental factors that usually are lethal to bacteria. Additionally, ingestion and defecation of bacteria by nematode larvae favors wider dispersion of the ingested bacteria as well as a dissemination of bacteria which adhere to the external body surface of the larvae.

The primary scope of the present study was to develop procedures

which might detect bacterial transmission by nematode larvae. M. pulmonis was used as the bacterium and N. brasiliensis and S. ratti were used as the source of nematode larvae. The hypothesis tested was that nematode larvae can transmit M. pulmonis to rats if the larvae are exposed to M. pulmonis in the rhabditiform stage, prior to inoculation.

Preparation of Bacteria Free Feces, Nematode Eggs and Nematode Larvae

Clorox Treatment of Rat Fecal Material Without Nematode Larvae

The preparation of bacteria free feces without larvae was conducted using Clorox. Feces and feces frozen at -60 degrees Centigrade for 24 hours were treated with various strength Clorox-water solutions.

TABLE I

INACTIVATION OF THE BACTERIA OF FECAL MATERIAL WITHOUT NEMATODE LARVAE

	Percent Clorox In Solution					
	0.0	1.0	2.0	3.0	4.0	5.0
Bacterial Survival of Feces	+	-	-	-	-	-
Bacterial Survival of Frozen Feces	+	-	-	-	-	-

The results of the experiment are presented in Table I and show that a Clorox concentration of 1.0 percent frees feces from bacteria.

Determination of the Presence of Bacteria on Nematode Eggs After Treatment with Various Strength Clorox Solutions

Effects of various concentrations of Clorox-water solutions on the inactivation of the bacteria associated with the nematode eggs were determined.

The results of the experiments given in Table II indicate that a 2.0 percent Clorox solution will inactivate all bacteria on nematode eggs but with a high enough survivability of the eggs to use them in nematode growth studies.

TABLE II

BACTERIAL STERILIZATION OF NEMATODE EGGS IN VARIOUS STRENGTH CLOROX SOLUTIONS

	Percent Clorox in Solution				
	0.0	1.0	2.0	3.0	4.0
Bacterial Survival of Clorox Treated *S. ratti* Eggs	+	+	-	-	-
Bacterial Survival of Clorox Treated *N. brasiliensis* Eggs	+	+	-	-	-

Determination of the Presence of Bacteria in the 24 Hour Decantate of Viable Third-stage Nematode Larvae

Different researchers have used many solutions containing sodium hypochlorite for inactivation of bacteria on nematode larvae (24, 28, 29, 30). The method used for inactivation of bacteria on nematode larvae is a modification of the previous methods of other researchers who used other types of larvae.

Viable third-stage nematode larvae were washed three times with saline and treated with various strength Clorox-water solutions. The larvae were aseptically washed 5 times with sterile saline and placed in thiol broth for 24 hours at 23 degrees Centigrade. The decanted fluid from the larvae was incubated at 37 degrees Centigrade for seven days and streaked on blood agar plates. The blood agar plates were incubated aerobically and in CO_2 jars at 37 degrees Centigrade and were read on days 2, 4 and 7.

There seems to be a marked difference between the bacterial carrying capacities of S. ratti and N. brasiliensis as shown in Table III.

The data indicates that bacteria carried by S. ratti larvae can tolerate Clorox concentrations as high as 14 percent whereas N. brasiliensis larvae can protect bacteria in a Clorox concentration of only 2 percent.

TABLE III

INACTIVATION OF BACTERIA ASSOCIATED WITH VIABLE THIRD-STAGE NEMATODE LARVAE

Percent Clorox In Solution	Bacterial Survival of Clorox Treated *S. ratti* Larvae	Bacterial Survival of Clorox Treated *N. brasiliensis* Larvae
0.0	+	+
2.0	+	+
4.0	+	-
6.0	+	-
8.0	+	-
10.0	+	-
12.0	+	-
14.0	+	-
16.0	-	-

Determination of the Presence of Bacteria in the 24 Hour Decantation of Dead Third-Stage Nematode Larvae

Third-stage nematode larvae were killed by freezing at -60 degrees Centigrade for 24 hours. They were then washed three times with various strength Clorox-water solutions. The larvae were aseptically washed 5 times with sterile physiological saline and placed in thiol broth for 24 hours at 23 degrees Centigrade. The decanted fluid from the larvae was then incubated at 37 degrees Centigrade for 7

days and streaked on Bacto-heart infusion plates. The agar plates were read on days 1, 2, 4 and 7. The results of this experiment are given in Table IV.

The results from Table III and Table IV indicates that S. ratti larvae are capable of passing bacteria after treatment with a Clorox-water solution that is detrimental to cuticle bound bacteria.

TABLE IV

INACTIVATION OF BACTERIA ASSOCIATED WITH DEAD THIRD-STAGE NEMATODE LARVAE

	0.0	1.0	2.0	3.0	4.0
Bacterial Survival of Clorox Treated S. ratti Larvae	+	+	+	-	-
Bacterial Survival of Clorox Treated N. brasiliensis Larvae	+	+	-	-	-

Survivability of Mycoplasma pulmonis, Nematode Eggs and Nematode Larvae After Treatment with Various Strength Clorox Solutions

Survivability of Mycoplasma pulmonis in Various Strength Clorox Solutions

M. pulmonis PPLO-I broth containing 10^8 colony forming units per ml was divided into 1.0 ml aliquots and diluted with various concentrations of Clorox solutions at an exposure time of 20 minutes. It was found that the highest concentration of Clorox that was tolerated by M. pulmonis was 0.001 percent.

These data are shown in Table IV and demonstrate that a 0.01 percent Clorox solution will destroy external M. pulmonis on the cuticle of nematode larvae. A modification of this method was used by Jensen (4). He found 20 ppm free chlorine for 20 minutes to be bactericidal for M. gallisepticum on the cuticle of adult nematodes.

TABLE V

SURVIVABILITY OF MYCOPLASMA PULMONIS IN VARIOUS STRENGTH CLOROX-WATER SOLUTIONS

	Percent Clorox in Solution				
	0.0	0.001	0.01	0.1	1.0
Bacterial Survival	+	+	-	-	-

Survivability of Nematode Eggs in Various Strength Clorox-Water Solutions

The survivability of nematode eggs in various Clorox solutions is shown in Table VI. The results of this experiment demonstrate that a 2.0 percent Clorox solution will leave nematode eggs with a survivability level that is high enough for use as a model for nematode growth studies.

The egg survivability of S. ratti and N. brasiliensis percentages were based on 6 repetitions. The standard deviation for the 6 repetitions of each mean was calculated and a confidence interval for each mean was calculated to be at the 95 percent level (31).

TABLE VI

SURVIVABILITY OF NEMATODE EGGS IN VARIOUS STRENGTH CLOROX-WATER SOLUTIONS

Percent Clorox In Solution	Percent Egg Survivability of S. ratti	Percent Egg Survivability of N. brasiliensis
0.0	87.3 ± 6.3	91.3 ± 4.7
1.0	61.7 ± 7.1	70.1 ± 3.1
2.0	31.2 ± 7.2	41.3 ± 5.6
3.0	12.2 ± 5.1	18.6 ± 7.7
4.0	0.0 ± 0.0	0.0 ± 0.0
5.0	0.0 ± 0.0	0.0 ± 0.0

Survivability of Viable Third-stage Nematode Larvae in Various Strength Clorox Solutions

The results of viable third-stage nematode larvae exposed to various strength Clorox-water solutions are given in Table VII. A 2.0 percent solution of Clorox is the largest concentration of Clorox that viable S. ratti larvae can tolerate and survive in the 100 percent survival range in contrast to 4.0 percent Clorox for N. brasiliensis.

The S. ratti and N. brasiliensis survivability percentages were based on 6 repetitions. The standard deviation for the 6 repetitions of each mean was calculated and a confidence interval for each mean was calculated to be at the 95 percent level (31).

Disintegration of Freeze Killed Third-stage Nematode Larvae in Various Strength Clorox-water Solutions

Third-stage nematode larvae were killed by freezing at -60 degrees Centigrade for 24 hours. They were then treated with various strength Clorox-water solutions.

The results in Table VIII show that dead nematode larvae are disintegrated by less concentrated solutions of Clorox than are viable nematode larvae. Dead N. brasiliensis larvae will remain intact in a much stronger concentration of Clorox than S. ratti larvae.

TABLE VII

SURVIVAL OF VIABLE THIRD-STAGE NEMATODE LARVAE IN VARIOUS STRENGTH CLOROX-WATER SOLUTIONS

Percent Clorox In Solution	Percent of *S. ratti* Larvae That Survived	Percent of *N. brasiliensis* Larvae That Survived
0	98.7 ± 1.8	99.9 ± 2.3
1	96.3 ± 1.4	99.7 ± 1.6
2	96.0 ± 6.1	99.7 ± 2.1
3	89.0 ± 3.7	99.5 ± 1.7
4	89.7 ± 4.6	99.5 ± 6.3
5	80.3 ± 4.2	91.3 ± 2.2
6	67.3 ± 5.1	80.6 ± 5.3
7	61.0 ± 6.1	61.3 ± 4.1
8	40.7 ± 7.3	30.7 ± 2.9
9	35.7 ± 7.1	30.3 ± 4.3
10	28.3 ± 6.7	30.7 ± 4.7
11	15.7 ± 5.8	28.6 ± 5.4
12	5.1 ± 7.1	27.1 ± 7.6
13	3.3 ± 7.6	17.0 ± 7.7
14	0.0 ± 0.0	10.0 ± 6.3
15	0.0 ± 0.0	0.0 ± 0.0
16	0.0 ± 0.0	0.0 ± 0.0

TABLE VIII

DISINTEGRATION OF DEAD THIRD-STAGE NEMATODE LARVAE IN VARIOUS STRENGTH CLOROX-WATER SOLUTIONS

Percent Clorox In Solution	[1]Destruction of Dead *S. ratti* Larvae	[1]Destruction of Dead *N. brasiliensis* Larvae
0	-	-
1	-	-
2	-	-
3	-	-
4	-	-
5	+	-
6	+	-
7	+	-
8	+	-
.	.	.
.	.	.
.	.	.
20	+	-

[1]Destroyed larvae are larvae that are dissolved into pieces.

The Effect of the Age of Third-Stage Nematode Larvae on the Bacterial Flora of the Larvae Alimentary Tract

The Relationship of the Bacterial Content of Third-stage Nematode Larvae to Nematode Larvae Culture Age

The effect of nematode larvae from varying age cultures on the bacterial carrying capacity of third-stage larvae was investigated. Larvae recovered from charcoal cultures 5 days to 15 days old were used in the experiment. The third-stage larvae were collected from the cultures with a Baermann apparatus and washed three times in saline. The larvae were treated with 3.0 percent Clorox for 20 minutes and washed 5 times with sterile saline using aseptic technique. The larvae were placed into thiol broth and left for 24 hours at 23 degrees Centigrade. The decanted fluid from the larvae was streaked onto Bacto-heart infusion agar plates and incubated at 37 degrees Centigrade. The agar plates were read on days 1, 2, 4 and 7.

The results of the experiment are given in Table IX.

Biological Relationships of Nematode Larvae and *Mycoplasma pulmonis*

Determination of the Excretion of Viable *Mycoplasma pulmonis* by Third-stage Larvae

Five hundred ml precipitates of 2-day-old PPLO-NI broth cultures of *M. pulmonis* were placed in fecal cultures of nematode larvae at various times. The nematode larvae were harvested as soon as they

TABLE IX

THE EFFECT OF NEMATODE CULTURE AGE ON THE BACTERIAL CARRYING CAPACITY OF THIRD-STAGE NEMATODE LARVAE

Culture Age in Days	Bacterial Survival in *N. brasiliensis* Larvae	Bacterial Survival in *S. ratti* Larvae
5	+	+
6	+	+
7	+	+
8	+	-
9	+	-
10	+	-
11	-	-

became third-stage larvae and checked for the presence of *M. pulmonis*.

A paramount problem encountered with nematode larvae coexisting with *M. pulmonis* was a difference in growing temperature. *M. pulmonis* would not survive at a temperature below 35 degrees Centigrade and *N. brasiliensis* or *S. ratti* larvae would not develop to third-stage larvae above 32 degrees Centigrade. The optimum temperature for *N. brasiliensis* to develop was at 23 degrees Centigrade and *S. ratti* developed best at 27 degrees Centigrade. The optimum nematode growth temperatures were the temperatures at which *M. pulmonis* was placed into the nematode larvae cultures.

The results in Table X indicate that the larvae did not excrete viable *M. pulmonis* under *in vitro* conditions.

TABLE X

EXPOSURE OF FREE-LIVING NEMATODE LARVAE TO MYCOPLASMA PULMONIS

Length of Exposure (Days) of M. pulmonis	M. pulmonis Survival in S. ratti Larvae	M. pulmonis Survival in N. brasiliensis Larvae
0	-	-
1	-	-
2	-	-
3	-	-
4	-	-

The absence of viable excreted M. pulmonis by N. brasiliensis and S. ratti larvae could be due to several things. The change in culture temperature when the M. pulmonis was placed into the nematode larvae fecal cultures could have resulted in loss of viability of the bacteria or in death of the bacteria. There were a marked number of bacteria present in the fecal cultures besides M. pulmonis that could have resulted in the M. pulmonis being overgrown. In addition, the nematode larvae may not have fed on the M. pulmonis due to the presence of such large numbers of other bacteria.

McCoy (32) found that rhabditiform larvae of Ancylostoma caninum grew well in fecal cultures containing bacteria but very poorly in fecal cultures without bacteria. Apparently, substances aside from bacteria in the feces have some nutrititive value for the larvae or the

bacteria do something to the feces that converts the feces to acceptable nutrients. No larval development past the rhabditiform stage was observed in S. ratti or N. brasiliensis when bacteria free feces were mixed with bacteria free nematode eggs and M. pulmonis was added. Variations in the composition of the fecal material could possibly account for this difference.

Jensen (3) found that M. gallisepticum was ingested and excreted by adult nematodes that were fed only M. gallisepticum. The colonies of M. gallisepticum, however, were altered after the passage through the nematode gut, the colony growth was abnormal.

The numbers of M. pulmonis ingested by the nematode larvae may not have been large enough to allow safe passage of a few organisms through the intestinal tract without all organisms being digested. Another possibility for negative results maybe that the digestive enzymes or physical-chemical factors in the alimentary canal of S. ratti and N. brasiliensis larvae are selectively detrimental to M. pulmonis. Since there is no detailed information on digestive enzymes or other physical-chemical features in the alimentary canal of the larvae, further investigation is clearly indicated. Possibly Mycoplasma species highly tolerant of the digestive enzymes of S. ratti and N. brasiliensis could survive during ingestion in a lethal environment, while a Mycoplasma species with low tolerance would not survive.

The Potentiality of Nippostrongylus brasiliensis Serving as a Mechanical Vector for Mycoplasma pulmonis

Groups of 6 M. pulmonis free male rats, 21-days-old, were

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The lungs of the rats were macroscopically examined. The lungs of rats given saline or M. pulmonis did not show pathological changes. The lungs of the rats given N. brasiliensis third-stage larvae or N. brasiliensis third-stage larvae plus M. pulmonis showed small bright red hemorrhagic areas.

The emphasis in this research was upon potential biological rather than mechanical association of microbial organisms with nematode larvae. Previous experimentation has shown that pathogenic bacteria are most likely to be mechanically but least likely to be biologically transmitted by endoparasites (13, 14, 18). The evidence presented in this thesis indicates that biological transmission may not be the exception. The potential of nematodes to serve as biological vectors of pathogenic bacteria is still largely in the speculative stage, but the known instances of biological transmission of other disease agents encourages the exploration of nematodes as vectors or reservoirs of bacteria.

CHAPTER V

SUMMARY

The main scope of the present study was to develop procedures which could demonstrate if Nippostrongylus brasiliensis and Strongyloides ratti third-stage larvae were capable of carrying Mycoplasma pulmonis.

A technical problem which was not solved was to grow the nematode larvae and the M. pulmonis at the same temperature. The lowest temperature at which M. pulmonis would grow was lethal for the nematode larvae.

Inhibition of the normal bacterial flora in and on the third-stage nematode larvae was determined using a Clorox-water solution. The nematode larvae and eggs were freed of external bacteria and the larvae remained viable.

Eggs of N. brasiliensis and S. ratti whose bacterial flora was killed were successfully cultured in charcoal cultures but culturing with M. pulmonis as the sole source of larval nutrition was unsuccessful. Third-stage larvae of N. brasiliensis and S. ratti were shown to excrete ingested bacteria from charcoal cultures using feces as the nutritional source. The ingestion and excretion of M. pulmonis was not demonstrated using charcoal cultures containing feces and other bacteria as a nutritional source.

The ability of *N. brasiliensis* third-stage larvae to mechanically transmit *M. pulmonis* to the lungs of rats after subcutaneous inoculation of larvae and *M. pulmonis* was not demonstrated.

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