

Animal Model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*

UNIT 19.12

Nippostrongylus brasiliensis is an important parasite in biomedical research. It is a potent activator of both systemic and mucosal Th2 immune responses. To date, *N. brasiliensis* models have been extensively used to study host protective immunity and in vivo regulation of Th2 immune responses. The organism provides an acute model of gastrointestinal nematode parasite infection in which the parasitic larvae migrate through the tissues to the lung, are coughed up and swallowed, and reach the intestine within 3 to 4 days. Once in the intestine, the worms rapidly complete their development into adults, which mate and produce fertile eggs that are passed out in the feces. The host defenses eventually cause expulsion from the gut in a “self-cure” reaction, without killing the parasite. The entire infection life cycle is complete within 2 weeks.

N. brasiliensis (synonyms, *Heligmosomum muris* and *Nippostrongylus muris*) belongs to the phylum Nematoda and the superfamily Trichostrongyloidea, with the rat as its natural host, although it does infect a number of other rodent species. *N. brasiliensis* is sometimes referred to as the “rat hookworm.” Albeit not closely related, its development resembles that of hookworms.

The life cycle of *N. brasiliensis* is illustrated in Figure 19.12.1. The elliptical eggs are passed in the feces as a 16- to 20-cell-stage embryo and are 54 to 62 by 31 to 34 μm in size. There are three free-living larval stages, with the first-stage larvae hatching from the eggs after 24 hr at 26°C with a first molt 12 to 24 hr later, followed by a second molt within another 24 hr. The infective third-stage larvae (L3) are 620 to 750 μm in length, although L3 within a sheath (second-molt larvae) can be found that are 770 to 820 μm long. Infection can be by oral ingestion, but skin penetration is most efficient, with penetration within 5 min after placement on the skin. Parasitic larvae move deep into the epidermis, then migrate to the loose subcutaneous connective tissue where they find blood

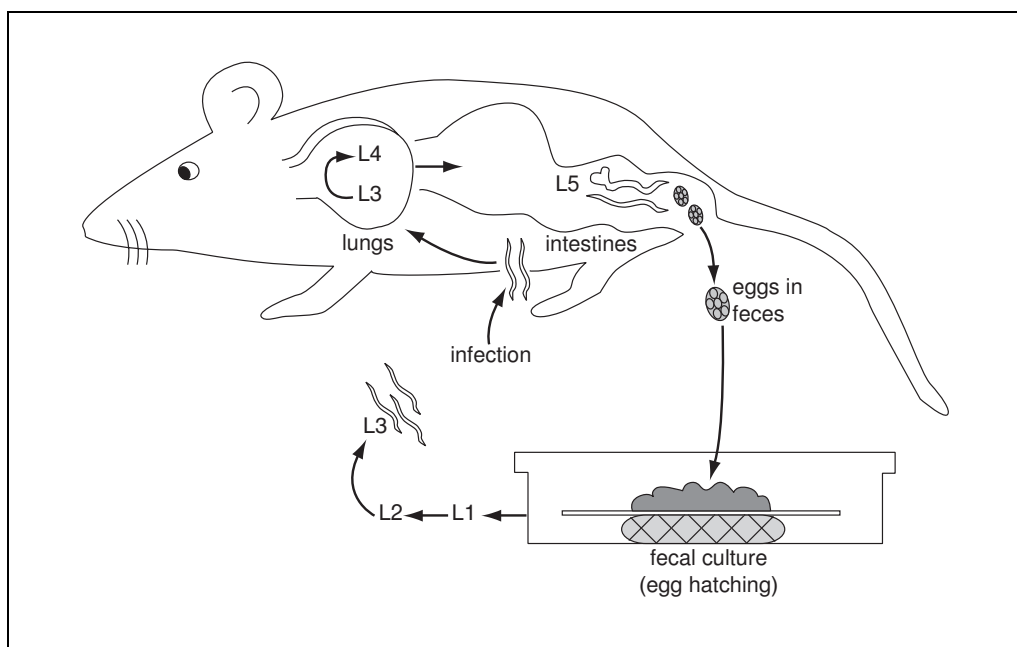


Figure 19.12.1 Life cycle of *Nippostrongylus brasiliensis* as carried out in the laboratory. Parasite stages are not drawn to scale. Approximate measurements for the various stages in length and width are: eggs, 60 $\times$ 35 μm , L3 infective larvae, 0.6 $\times$ 0.3 mm, adult male, 4 $\times$ 0.1 mm, adult female, 5 $\times$ 0.1 mm.

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vessels that carry them to the lungs as early as 11 hr after invasion. Experimental inoculations are typically given by subcutaneous injection of the infective L3. The third molt occurs in the lung between 19 and 32 hr, and the emerging L4 remain in the lung up to 50 hr post-infection before migrating via the trachea to the intestine. L4 larvae are morphologically similar in appearance to L3 infective larvae; however, the L4 larvae are longer, with darker pigmentation along the length of their bodies.

The final molt in the intestine begins at 90 to 108 hr post-inoculation and results in the L5, or mature adult. Adult worms are found loosely attached to the proximal half of the small intestine. The size of the male worm is from 3 to 4.5 mm in length, while the female ranges from 4 to 6 mm in length. The male has an asymmetrical fan-tailed bursa with long and slender spicules, while the caudal end of the female is hooked.

Eggs are produced at 6 days post-inoculation, reaching a maximum on days 8 and 9, followed by a gradual decline in egg production until the self-curing adult worm expulsion occurs between days 10 and 13 post-inoculation.

The first part of this unit describes the routine maintenance of *N. brasiliensis* in rats including inoculation of the rats with L3 infective larvae (see Basic Protocol 1), collection, counting, and culture of eggs and larvae (see Basic Protocol 2), and harvest (see Basic Protocol 3 and Alternate Protocol 1) and long-term storage (see Basic Protocol 4) of infective third-stage (L3) larvae.

Also described in this unit is the mouse model of *N. brasiliensis*, which is used for experimental purposes (see Basic Protocol 5 for inoculation of mice; see Basic Protocol 6 and Alternate Protocol 2 for enumeration of eggs). Collection points that are of immunological importance are also discussed (see Basic Protocols 7 to 9 and Alternate Protocol 3); photographs showing relevant morphological features seen at some collection points of interest are included in the unit. Techniques for preparing soluble antigenic preparations from the various developmental stages of the worm are presented in Support Protocol 1.

Basic Protocols 10 to 13 and Support Protocol 2 contain information on the propagation of and mouse model for infection with *Heligmosomoides polygyrus*. Many of the procedures used for *H. polygyrus* are similar to that used for *N. brasiliensis* and only the unique aspects of the life cycle or experimental manipulations are described. Finally, Support Protocol 3 discusses the evaluation of a range of immunological phenomena associated with *N. brasiliensis* and *H. polygyrus* infection.

Safety

Skin penetration by *N. brasiliensis* L3 larvae, but not *H. polygyrus*, can occur in humans, although any infection is limited. The other free-living forms of the parasites are harmless, as are the mature L4 and L5 larvae. Personnel should wear laboratory coats and gloves when working with this parasite. Decontaminate work surfaces after use and after spills with standard agents including 70% ethanol, 1% hypochlorite, or soap solutions.

Animal Facility Containment

Because the infective stage of both *N. brasiliensis* and *H. polygyrus* require free-living development that takes 5 to 6 days under optimal conditions of temperature and humidity, the parasites are not transmitted to other mice in a facility under normal cleaning and management procedures. Cross-transmission is not seen even when infected mice are mixed with uninfected mice in the same cage. The eggs and larvae are sensitive to hot water and detergent cleaning of rodent cages. Twice-weekly cleaning of cages will preclude transmission within a cage, while longer periods between cage cleaning can be tolerated in modern rodent facilities due to desiccation of worms and feces by the low-humidity HEPA-filtered air flowing through the cages.

MAINTENANCE AND PROPAGATION OF *NIPPOSTRONGYLUS BRASILIENSIS*

Maintenance of the *Nippostrongylus* life cycle in rats is a reliable and robust method. Large quantities of third-stage (L3) infective larvae can be produced with minimum time and effort. The most convenient laboratory host for the maintenance of *N. brasiliensis* is the rat. It allows for easy production and preparation of a large number ($>1 \times 10^6$) of parasites, sufficient to provide a good number of reproducible infections over several weeks. Although the natural route of infection is by skin penetration, the maintenance of the parasite and experimental infection is more efficient using subcutaneous injection. Other routes of infection such as intraperitoneal injection and oral administration can be used, but the productivity of the infection is significantly reduced. Intracutaneous injection of third-stage (L3) infective larvae in the ear has specialized application for measuring the local response in the draining cervical lymph nodes.

All procedures outlined for passage and storage of third-stage (L3) infective larvae from the rat can be adapted to the mouse as the supporting host for the life cycle (see Basic Protocol 5). Swiss Webster mice are a relatively inexpensive strain of mice that can be easily used to maintain the life cycle in the laboratory; many other strains are also suitable.

Inoculation of Rats with *Nippostrongylus brasiliensis* Third-Stage (L3) Infective Larvae

BASIC PROTOCOL 1

Materials

Nippostrongylus brasiliensis third-stage (L3) infective larvae (see Basic Protocol 3 and Alternate Protocol 1)

PBS (APPENDIX 2A), sterile

Female Lewis rats, 4 months old, or female Sprague-Dawley rats with a minimum weight of 150 g

5-ml sterile plastic bottles

1-ml disposable syringes and 21- or 23-G needles

Additional reagents and equipment for rat restraint (UNIT 1.3) and injection (UNIT 1.6)

1. Prepare a suspension of 10,000 L3/ml in PBS.
2. Draw 0.5 ml of the suspension into a 1-ml syringe with a 21- or 23-G needle and prepare for injection (this gives a dose of 5000 L3/rat).

Take special care to mix worm suspension immediately before each drawing of the syringe, as the larvae settle very quickly.

For maintenance in rats, the typical dose used is 5000 L3/rat; >8000 L3 per rat may kill the animal or make it very sick. Higher inocula of L3 larvae result in a reduced production of eggs due to the morbidity of the host and possible nutrient competition between worms.

3. Restrain the rat (UNIT 1.3) and inject the worm suspension subcutaneously (UNIT 1.6) in the left flank of the rat.

For convenience, someone can assist in restraining the rat or one can lightly anesthetize it using CO₂ gas from a piece of dry ice.

4. Allow 6 days before collecting eggs (see Basic Protocol 2).

Collection, Counting, and Culturing of *Nippostrongylus brasiliensis* Eggs and Larvae from Infected Rats

In this protocol, infected rats are placed on wire-grid cages so fecal pellets fall through the grid onto paper towels which have been wetted sufficiently to remain moist (but not soaking) overnight. Collection starts on day 7 post-inoculation, with daily collections through to day 10; eggs on day 6 can be omitted for routine purposes, as egg numbers are low and variable.

The timing of the *Nippostrongylus brasiliensis* life cycle is robust and very reliable, but as a precaution daily counts are performed to assess number of eggs present; this number should be recorded in a log book. This procedure ensures confidence in the fecal culture system.

The culture method here describes the hatching and growth of L1 through to third-stage (L3) infective larvae that eventually migrate to the edge of the moist filter paper and accumulate as a thick ring of stationary L3 infective larvae around the edge of the filter paper and the perimeter of the plate.

Materials

Nippostrongylus brasiliensis–infected rats (see Basic Protocol 1)
Saturated NaCl solution
Coarsely granulated charcoal, autoclaved
Sphagnum moss (available from garden supply stores), cut into small pieces and autoclaved

Cage with wire-grid floor (Tecniplast USA)
Paper towels
100- and 250-ml sterile containers
Spoon or spatula
McMaster worm egg counting chamber (J.A. Whitlock & Co.)
Gridded coverslip or glass sheet scored with a diamond pen
Stereoscopic microscope
140-mm sterile petri dishes
Sponge-cut into 4 × 4-cm pieces (dishwashing cloth or cotton wool can also be used), autoclaved
110-mm Whatman filter paper circles
Plastic lidded container large enough to hold culture plates, lined with moistened paper towels or dishcloth
Incubator set at 26°C, without CO₂ (room temperature benchtop incubation is adequate)

Collect eggs and larvae from fecal material

1. On day 6 post-inoculation, prepare wire-grid cage by lining the bottom of the cage with a layer of dampened paper towels.
2. Place rats on wire grid overnight. On the following day, collect fecal pellets in a 250-ml sterile container with a spoon or spatula.
3. Discard the used paper towels from the cage and replace with fresh dampened paper towels ready for the next collection.

IMPORTANT NOTE: *Urine, as well as residual detergents on the cage surface, can adversely affect the eggs, so ensure that fecal collection is performed with thoroughly rinsed cages.*

4. Cull rats on day 11 post-inoculation, as they are fully protected from reinfection.

An alternative procedure for collection of fecal material for culture is to euthanize the rats at 7 or 8 days after inoculation, remove all of the gut contents from the cecum to the rectum, and use this as a source of fecal culture material.

Enumerate eggs

5. Place ~1 g feces (typically 4 pellets) into a 100-ml container and add 30 ml distilled water and soak for ~1 hr.
6. Vortex vigorously, add 30 ml saturated NaCl solution, and vortex again.
7. Immediately remove an aliquot using a plastic transfer pipet and transfer to the McMaster counting chamber.

It is important not to allow the mixture to settle before taking an aliquot, to allow for uniform distribution of eggs in suspension. Keep the pipet at an angle to prevent eggs from rising to the top of the pipet.

8. Slide a gridded cover slip (grid-side down) across the counting chamber; try to prevent any air bubbles from being trapped inside the chamber.
9. Place the chamber on to the stage of the stereoscopic microscope. Focus on the top plane by focusing on the grid lines of the coverslip.

Fecal debris will settle to the bottom of the chamber and eggs should come into focus.

10. Count the number of eggs present within the marked field.
11. Calculate the total number of eggs per g feces by multiplying the slide egg count by 400.

Egg counts can also be adjusted to numbers per fecal pellet by dividing the total number by the number of pellets per g (typically 4 pellet/g).

The McMaster counting chamber is 150 μ l in volume: $10 \times 10 \times 1.5$ mm.

12. Perform three separate counts for an accurate calculation.

Once egg number has been recorded, proceed with culturing fecal samples.

Culture fecal pellets

13. Soften the remaining fecal pellets by adding enough distilled water to cover the fecal pellets.
14. Mix equal volumes of granulated charcoal and sphagnum moss to form a coarse paste, using a spoon or spatula.

The paste should not be too dry or crumbly. If the paste is too wet, mix in some additional charcoal to absorb excess moisture.

15. Place a 4×4 -cm piece of sponge in the middle of a 140-mm petri dish and dampen it with distilled water.

The dampened sponge acts as a reservoir. Figure 19.12.2 shows the assembled setup for fecal culture.

16. Place filter paper on top of the sponge and moisten. Allow some water to run along the edges of the Petri dish.
17. Evenly distribute fecal paste on top of the filter paper in the prepared petri dishes and label with date of collection.

Typically 3 to 4 dishes of fecal cultures can be prepared per day.

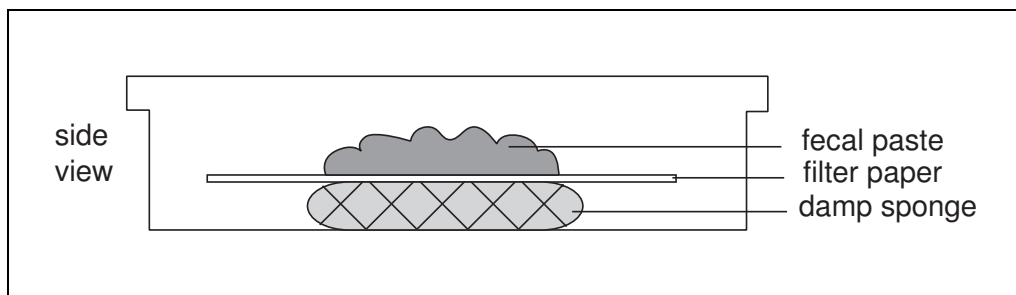


Figure 19.12.2 Diagram of the fecal culture system. 140-mm sterile petri dishes are used.

18. Place prepared dishes into a large plastic lidded container to ensure that sufficient humidity is maintained.

19. Incubate at 26°C in the dark for ≥ 7 days.

Aeration of the culture dishes is necessary. This is done by periodically taking the plates out of the plastic box, removing the lids, and leaving for ~1 min. The lids are then replaced and the plates put back in the box, which is returned to the 26°C incubator. It is also crucial to maintain the level of moisture in each plate. Plates should be routinely checked every few days and the fecal paste moistened using a spray bottle containing distilled water, should it be required.

Over the next week, eggs will hatch and larvae will move, feed, grow, and molt twice in the fecal paste. The L3 larvae form will migrate as described above and remain infective for at least 4 weeks with up to 50% still remaining infective at 6 weeks. After six weeks of culture, L3 infectivity is significantly reduced.

Fecal cultures can also be set up without sphagnum moss, using charcoal alone in this system.

If the fecal paste is in contact with the lid of the dish, larvae will also accumulate on the lid.

20. Store fecal cultures containing viable L3 larvae at 26°C or room temperature for up to several weeks, maintaining hydration of the culture dish.

*Fungal contamination of the cultures becomes an increasing problem with time. It can also adversely affect the development of the *N. brasiliensis* larvae; the use of fungicidal substances can help combat this problem and Nystatin can be added at 240U/ml when culturing fecal pellets. Alternatively, the mold growth can be saturated with sterile water to prevent new spores from developing. Also dispose immediately of any cultures carrying heavy loads of sporulating fungi.*

BASIC PROTOCOL 3

Harvesting of *Nippostrongylus brasiliensis* Third-Stage (L3) Infective Larvae from Fecal Cultures

N. brasiliensis third-stage (L3) infective larvae can be harvested from the culture dishes 8 days after the start of the fecal culture.

Materials

Nippostrongylus brasiliensis fecal culture dish (see Basic Protocol 2)
25-ml sterile LabServ plastic container with conical bottom or other sterile universal container
Gridded petri dish or glass sheet scored with a diamond pen
Stereoscopic microscope

Transfer and wash L3 larvae

1. Using a transfer pipet, add ~1 ml distilled water to the periphery of the fecal culture dish and swirl it around the edge of the dish and edges of the filter paper, carefully avoiding the fecal culture material in the center of the filter paper.
2. Transfer the larval suspension to a 25-ml sterile container using a transfer pipet and wash larvae by adding 10 ml distilled water and allowing larvae to settle to bottom of the container.
3. Aspirate the wash water and repeat the washing procedure.
4. Add 5 ml distilled water and count larvae as described in steps 5 to 9.

Count larvae

5. Take a 50- μ l aliquot of the larval suspension.

Ensure the larval suspension is mixed well as larvae settle very quickly.

6. Place the 50- μ l aliquot on a gridded petri dish or glass sheet, and, using a stereoscopic microscope, count the number of larvae present.

Check viability of the larvae; straight larvae with no movement are assumed to be dead.

7. Repeat procedure for an accurate count.
8. Multiply the number of L3 by 20 to give the number of L3 larvae/ml and adjust the concentration to the desired number.

Alternatively, yield of L3 can be estimated by packed volume after centrifuging 10 min at 400 $\times$ g. Using this procedure, 1 ml packed larvae is equal to $\sim 1 \times 10^6$ L3.

9. Rehydrate the harvested plate and continue incubating for future use (see Basic Protocol 2).

Modified Baermann Methods for Harvesting *Nippostrongylus brasiliensis* Third-Stage (L3) Infective Larvae from Fecal Cultures

This is a good method for harvesting an entire culture dish if large amounts of third-stage (L3) infective larvae are required.

Materials

Phosphate-buffered saline (PBS; APPENDIX 2A; optional)

Nippostrongylus brasiliensis fecal culture dishes (see Basic Protocol 2)

10 $\times$ 13-cm glass filter funnel

1.5 $\times$ 10-cm plastic tubing

5 ml (1.2 $\times$ 7.5-cm) plastic collection tube

Stand and clamp

Cheesecloth cut into 8 $\times$ 8-cm squares

Additional reagents and equipment for counting larvae (see Basic Protocol 3)

1. Set up the adapted Baermann apparatus using a glass filter funnel, plastic tubing, and collection tube, as shown in Figure 19.12.3.
2. Add tap water or PBS at 37°C to the apparatus until it reaches approximately 1 cm below the rim of the funnel
3. Place two Kimwipes between two pieces of cheesecloth and place on top of the filter funnel.

ALTERNATE PROTOCOL 1

Animal Models for Infectious Diseases

19.12.7

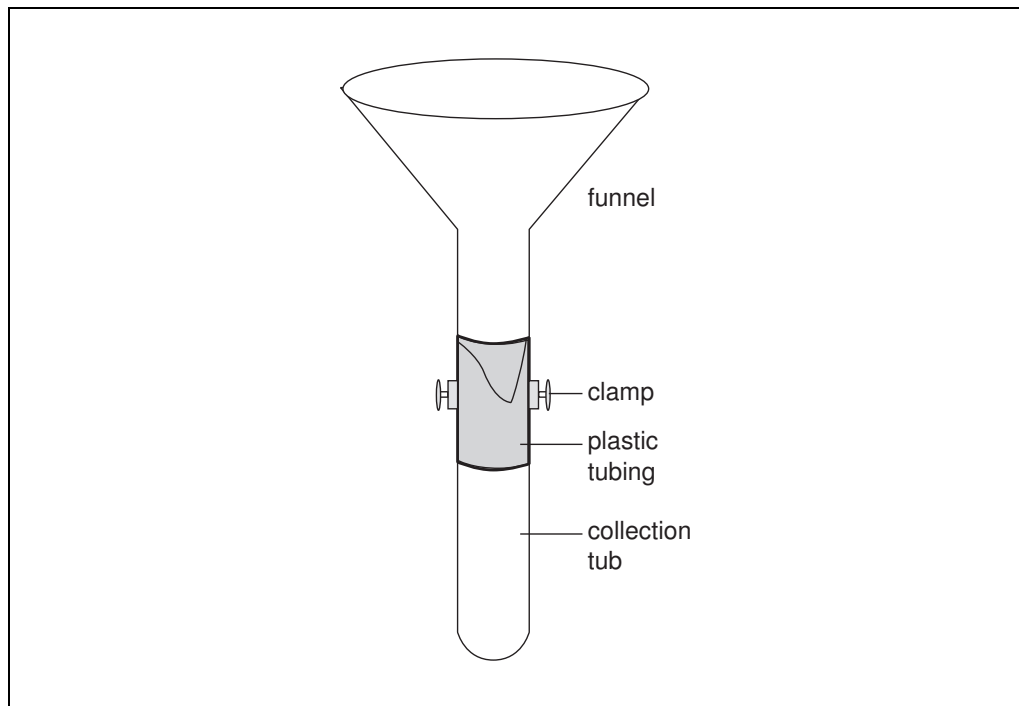


Figure 19.12.3 Diagram of the adapted Baermann apparatus for the collection of L3 infective larvae from fecal cultures.

4. Add the contents of two culture dishes on to the cheesecloth.

If the water is not in contact with the fecal paste, add more water. Alternatively, “spoon” out the areas of the culture where larvae are visible to reduce the level of material added to the top of the funnel.

5. Let stand 60 min.

Larvae will collect in the bottom tube

6. Disconnect the collection tube and wash larvae by adding 4 ml water and letting the larvae settle by gravity. Repeat process if a cleaner preparation is required.

A brief (1-min) centrifugation at $150 \times g$ can alternatively be used to quickly pellet the larvae

7. Count larvae (see Basic Protocol 3, steps 5 to 8).

Larvae not used for injection of rats can be added back to the culture for future use.

BASIC PROTOCOL 4

Storage of *Nippostrongylus brasiliensis* Third-Stage (L3) Infective Larvae

Freezing L3 larvae using various methods such as cryopreservation with various freezing solutions, for example, DMSO/glucose has given minimal success. Fecal culture dishes have been frozen whole, but this method has also given inconsistent results. In the authors' laboratory, the method described here has allowed larvae to be stored for several weeks at room temperature.

Materials

Nippostrongylus brasiliensis L3 larvae harvested in 5 ml water (see Basic Protocol 3)
 100-mm Whatman filter paper circles
 25-ml sterile LabServ plastic container with conical bottom or other universal container with lid

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*Heligmosomoides
 polygyrus***

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1. Fold filter paper in half and then fold in half again to form a cone.
2. Sit the cone on the rim of the 25-ml sterile universal container so that the top of the cone aligns with the rim.
3. Draw the larval suspension up in a transfer pipet and slowly add the larvae to the filter paper.

The larvae will stay on the filter paper while the water will settle to the bottom of the container; this will allow moisture to be maintained.

4. Place lid on the container and store at room temperature in the dark.

Routinely check the container and moisten the filter paper with a spray bottle containing distilled water if required

Mouse Model of *Nippostrongylus brasiliensis* Infection: Inoculation of Mice with *Nippostrongylus Brasiliensis* Third-Stage (L3) Infective Larvae

The mouse model of *Nippostrongylus brasiliensis* is commonly used for studies of the acute Th2 immune response. Typically the mouse host clears itself of intestinal worms by day 11 post-inoculation. Mice previously infected with *N. brasiliensis* are fully protected from subsequent infection by a profound Th2 immune response. The strains of mice routinely used are the C56Bl/6 or Balb/c, although other common mouse strains are similarly suitable.

BASIC PROTOCOL 5

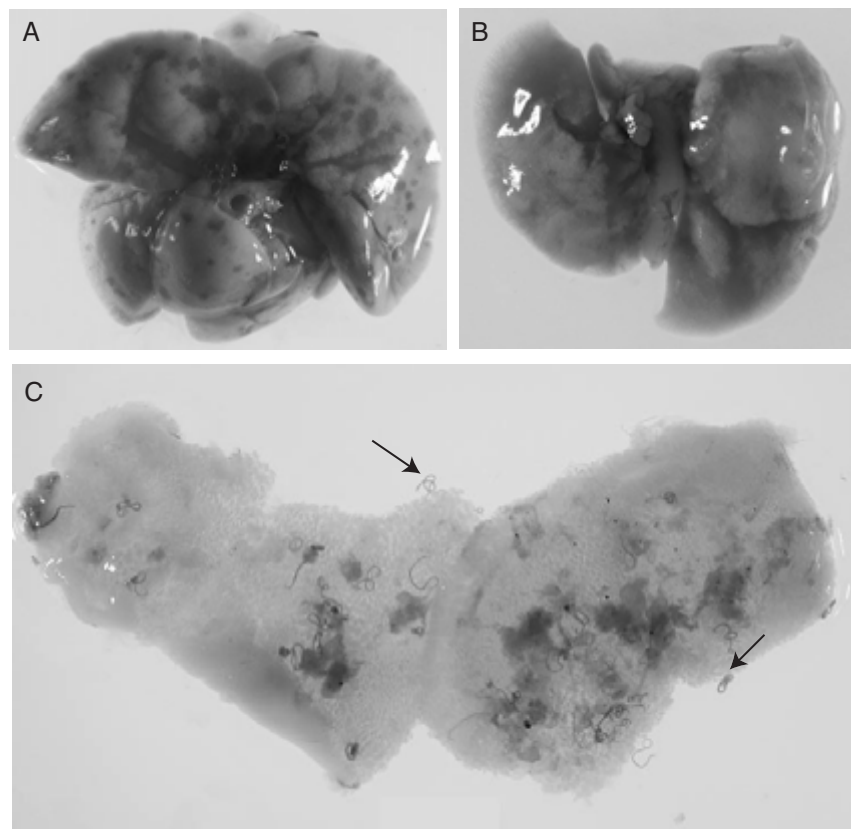


Figure 19.12.4 (A) Photograph of lung from *Nippostrongylus brasiliensis* infected mouse at 24 hr post-inoculation. Note hemorrhagic spots on surface of lungs. (B) Photograph of lung from naïve mouse. (C) Photograph of adult worms in intestine of *Nippostrongylus brasiliensis*-infected mouse, day 7 post-inoculation. Note adult worms (thread-like spirals) in the intestine.

Animal Models for Infectious Diseases

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Both male and female mice at 6 to 8 weeks of age are used for experimental work. Younger mice as well as females are more susceptible to infection than males and older mice. Mice are normally infected with 500 (mouse-adapted) or 750 (rat-adapted) L3 larvae using subcutaneous injection as the inoculation route, administered in 0.2 ml. Infection with inocula of greater than 1000 L3 larvae in a primary infection usually results in significant mouse morbidity, immune suppression, and possible death. Mice infected with secondary infections of *Nippostrongylus brasiliensis* can cope with infections of greater than 1000 L3. Figure 19.12.4 shows photographs of tissues from some of the key collection points of the life cycle.

Materials

Nippostrongylus brasiliensis infective L3 larvae (see Basic Protocol 3)

Phosphate-buffered saline (PBS; APPENDIX 2A)

6- to 8-week-old C57Bl/6 or Balb/c mice

25-ml sterile LabServ plastic containers with conical bottoms or other universal plastic containers

1-ml syringes with 21- or 23-G needles

Additional reagents and equipment for counting larvae (see Basic Protocol 3), mouse restraint (UNIT 1.3), and injection of mice (UNIT 1.6)

1. Prepare a suspension of 3750 L3 larvae/ml in a clean 25-ml sterile plastic container (see Basic Protocol 3 for counting of larvae)
2. Draw 0.2 ml of the suspension into each of the 1-ml syringes (with 21- or 23-G needles) that will be used for injecting the mice.

This gives a dose of 750 L3/mouse.

3. Place syringes in an upright, needle-down position before injecting to allow worms to settle towards the tip of the needle and not be trapped in the back of the syringe barrel.
4. Restrain the mouse (UNIT 1.3) and inject subcutaneously (UNIT 1.6) into the scruff of the neck causing a bleb to form.

Other routes of infection such as intraperitoneal injection and oral administration can be used, but the productivity of the infection is much reduced.

Natural infection through the skin can also be performed after the site of application on the skin has been suitably cleansed of fur and oils by shaving and washing.

5. Allow 5 days before collecting eggs (see Basic Protocol 6).

Enumeration of *Nippostrongylus brasiliensis* Egg Production in Infected Mice

Nippostrongylus brasiliensis eggs can be counted to provide a measure of the level of infection intensity and also a measure of the viability and fecundity of the infecting worms.

For materials, see Basic Protocol 2

1. On day 5 post-inoculation, place mice in wire-grid-bottom cages (prepared as in Basic Protocol 2, step 1). Perform daily collections of fecal pellets as in Basic Protocol 2, steps 2 and 3. Cull mice on day 10 post-inoculation (see Basic Protocol 2, step 4).

The time point for egg production is from day 5 to 10 post-inoculation.

BASIC PROTOCOL 6

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2. Enumerate eggs as in Basic Protocol 2, steps 5 to 12, but use 50 fecal pellets, 25 ml of distilled water (in step 5), and 25 ml of saturated NaCl solution (in step 6). To obtain the number of eggs per g of feces, divide egg number by 0.15 to give eggs/ml (with a chamber volume of 150 μ l), then multiply by 25.

For the mouse 25 fecal pellets are normally equivalent to 1 g.

Quick Method for Determining Egg Counts in Individual Mice

There is a statistical advantage in obtaining individual egg counts to test the significance of treatment group differences.

Materials

N. brasiliensis–infected mice (See Basic Protocol 5)

1.5-ml microcentrifuge tubes

Analytical balance

Additional reagents and equipment for mouse restraint (*UNIT 1.3*) and enumeration of worm eggs (see Basic Protocol 2)

1. Restrain an individual mouse (*UNIT 1.3*) over a paper towel for enough time to collect 3 to 4 fecal pellets. Place the feces into a preweighed 1.5-ml tube. Repeat procedure for other mice in treatment group.
2. Weigh tube with the feces and subtract the tare weight to determine the weight of feces in each tube.
3. Enumerate the eggs in each tube (see Basic Protocol 2, steps 5 to 12).

Collection of *Nippostrongylus brasiliensis* Fourth-Stage (L4) Lung Larvae

Determining the number of fourth-stage (L4) larvae in the lung provides a measure of successful infection by the worm in the mouse host. The protocol described here is a very simple but effective method to obtain viable L4 larvae from infected mice. The time-point for collection is day 2 post-inoculation

Materials

N. brasiliensis–infected mice (see Basic Protocol 5)

70% ethanol

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

1% (w/v) agarose

Dissecting instruments including iris scissors and forceps

6-well plastic tissue culture dishes

Cheesecloth cut into 8 $\times$ 8–cm squares

8 $\times$ 8–cm plastic disposable weigh boats

50-ml centrifuge tubes and corresponding racks

40-mm petri dishes

Dissecting microscope

Additional reagents and equipment for euthanasia of the mouse (*UNIT 1.8*)

1. On day 2 post-inoculation, euthanize the mouse by cervical dislocation (*UNIT 1.8*).
2. Place mouse on its back and wet with 70% ethanol.
3. Expose the thoracic cavity and remove lungs using dissecting scissors. Place lungs into one well of a 6-well dish containing 1 ml PBS.

**ALTERNATE
PROTOCOL 2**

**BASIC
PROTOCOL 7**

**Animal Models
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4. Place an 8 × 8–cm piece of cheesecloth onto an 8 × 8–cm weigh boat with one edge of the cloth hanging over the side of the vessel.
5. Prepare submerging vessel by adding 45 ml PBS to a 50-ml centrifuge tube. Keep warm in 37°C water bath until ready to use.
6. Transfer the lungs into a clean 40-mm petri dish and mince the lungs into fine tissue sections using iris scissors and forceps.
7. Warm the 1% agarose solution to 37°C and add 5 ml to the diced organ.
8. Take up the agarose/organ suspension mixture and pour into the prepared weigh boat on top of the cheesecloth. Allow a few minutes for the agarose to set.
More agarose can be added to any remnants and transferred to the weigh boat.
9. Take the exposed edge of the cheesecloth, roll the cheesecloth, and place the whole piece of cheesecloth along with the tissue/agarose mixture into the submerging vessel prepared in step 5, in such a way that the exposed cheesecloth edge hangs over the edge of the 50-ml tube. Seal the tube so as to trap the edge of the cloth under the cap.
10. Place in water bath and incubate overnight at 37°C.
11. After incubation, discard cheesecloth/agarose mixture.
Worms will have migrated through the tissue and agarose and collected at the bottom of the centrifuge tube.
12. Discard all but 2 to 3 ml of the PBS by aspirating with a 25-ml pipet.
13. Place the final 2 to 3 ml in a petri dish and use a dissecting microscope to count the L4 larvae.

ALTERNATE PROTOCOL 3

Bronchoalveolar Lavage as an Alternative Method for the Recovery of *Nippostrongylus brasiliensis* Fourth-Stage (L4) Lung Larvae

This method describes the recovery of fourth-stage (L4) larvae from the lung by rinsing the contents from the alveolar spaces. It is a quick and effective method that does not involve dissecting and mincing of the lungs, as in Basic Protocol 7. This procedure is carried out at day 2 post-inoculation.

Materials

N. brasiliensis–infected mice (see Basic Protocol 5)
70% ethanol

Dissecting instruments including iris scissors and forceps
18-G Insite catheter (Becton Dickinson)
1-ml syringes
90-mm petri dish
Dissecting microscope

Additional reagents and equipment for euthanasia of the mouse (UNIT 1.8)

1. On day 2 post-inoculation, euthanize the mouse by cervical dislocation (UNIT 1.8).
2. Attach an 18-G Insite catheter to a 1-ml syringe and fill with 1 ml distilled water.
3. Place mouse on its back and wet with 70% ethanol.

4. Make a midline incision and pull back the skin and expose the throat by carefully separating the muscle tissue above the trachea. Make a horizontal cut across the exposed trachea.
5. Insert the catheter into the trachea and inject contents of the syringe into the lungs.
6. Draw fluid in and out five times, then place contents of syringe into a 90-mm petri dish and use a dissecting microscope to count the larvae.

Collection of *Nippostrongylus brasiliensis* Adult Worms (L5)

Freshly collected worms are bright red in color due to the hemoglobin in the cuticle, body wall, and pseudocoelomic fluid of the parasite. This protocol describes a clean and effective method of obtaining viable adult worms from the gut of infected animals to measure “development take.” The time point for collection of the L5 worms is day 7 post-inoculation

Materials

N. brasiliensis–infected mice (see Basic Protocol 5)

70% ethanol

Phosphate-buffered saline (PBS; APPENDIX 2A)

Dissecting instruments including iris scissors and forceps

50-ml centrifuge tubes and corresponding racks

Cheesecloth cut into 8 × 8–cm squares

40-mm petri dishes

Dissecting microscope

Additional reagents and equipment for euthanasia of the mouse (UNIT 1.8)

1. On day 7 post-inoculation, euthanize the mouse by cervical dislocation (UNIT 1.8).

For optimal recovery, it is important to withhold food from the animals for 24 hr prior to harvesting, although recovery is still possible when this is omitted.

2. Place mouse on its back and wet with 70% ethanol.
3. Open the abdominal cavity and remove the proximal half of the small intestine with dissecting scissors; place the tissue into a dish containing 1 ml PBS.
4. Prepare a submerging vessel by adding 45 ml PBS to a 50-ml centrifuge tube and placing an 8 × 8–cm square of cheesecloth on top of the tube.
5. Place the intestines into a clean 40-mm petri dish and slice longitudinally, then cut into small pieces.
6. Place the intestine pieces in the cheesecloth on top of the prepared tube and submerge in the PBS. Seal the tube so as to trap the excess cheesecloth under the cap at the edge of the tube.
7. Place in water bath and incubate for 2 hr at 37°C.

Worms will migrate out and settle at the bottom of the tube.

8. After incubation, examine the intestines for any worms that may have remained attached to the intestinal mucosa.
9. Using a transfer pipet, draw up worms from the bottom of the collection tube and place in a petri dish. Count the worms in the entire dish using a dissecting microscope.

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10. Calculate “development take” by dividing the number of worms counted by the number of infective larvae that were inoculated, and multiplying by 100%.

Large numbers of adult worms can be collected from several rats or mice as a source of starting material for in vitro culture by modifying the Baermann apparatus (see Alternate Protocol 1). A large funnel (20-cm in diameter) filled with PBS can be fitted with a screen insert with 1 to 2 layers of cheesecloth secured to the bottom. The intestines from several rats or mice are placed above the screen, which supports the intestines, and the adult worms move through the screen and cloth, and settle to the neck of the funnel while debris from the intestinal contents of the host is retained. The unit can be sealed in foil and sterilized before use to minimize the initial level of microbial contamination

Recovery of *Nippostrongylus brasiliensis* Third-Stage (L3) Infective Larvae from the Site of Infection

Parasitic L3 can be retrieved from the skin site of infection. Mice should be shaved in the area they are to be injected for ease of dissection. The time points for collection are 2, 24, and 48 hr post-inoculation.

Materials

N. brasiliensis infected mice (see Basic Protocol 5)

70% ethanol

Phosphate-buffered saline (PBS; APPENDIX 2A)

1% (w/v) agarose

Dissecting instruments including iris scissors and forceps

6-well plastic tissue culture dishes

Cheesecloth cut into 8 × 8-cm squares

8 × 8-cm plastic disposable weigh boats

50-ml centrifuge tubes and corresponding racks

40-mm petri dishes

Dissecting microscope

Additional reagents and equipment for euthanasia of the mouse (UNIT 1.8)

1. At 2, 24, or 48 hr post-inoculation, euthanize the mouse by cervical dislocation (UNIT 1.8).
2. Place mouse on its back and wet with 70% ethanol.
3. Excise the skin injection site including the tissue under the skin with dissecting scissors. Place the tissue into a well of 6-well dish containing 1 ml PBS.
4. Place an 8 × 8-cm piece of cheesecloth onto an 8 × 8-cm weigh boat with a one edge of the cloth hanging over the side of the vessel.
5. Prepare submerging vessel by adding 45 ml PBS to a 50-ml centrifuge tube. Keep warm in 37°C water bath until ready to use.
6. Place skin excision into a clean 40-mm petri dish and mince into fine sections.
7. Place the minced skin onto the cheesecloth in the vessel prepared in step 4.
8. Warm the 1% agarose solution to 37°C and pour 3 ml over the minced skin on the cheesecloth in the prepared weigh boat. Allow a few minutes for the agarose to set.
9. Take the exposed edge of the cheesecloth, roll the cheesecloth, and place the whole piece of cheesecloth along with the tissue/agarose mixture into into the submerging vessel prepared in step 5, in such a way that the exposed cheesecloth edge hands over the edge of the 50-ml tube. Seal the tube so as to trap the edge of the cloth under the cap.
10. Place in water-bath and incubate overnight at 37°C.

11. After incubation, discard cheesecloth/agarose mixture.

Worms will have collected at the bottom of the centrifuge tube.

12. Discard all but 2 to 3 ml of the PBS by aspirating with a 25-ml pipet.
13. Place the final 2 to 3 ml in a petri dish and use a dissecting microscope to count the L3 larvae.

Preparation of *Nippostrongylus brasiliensis* Antigen (NES)

In the course of the infection, the host is exposed to a large number of parasite antigens of secreted, somatic, and metabolic origin. This method describes the preparation of excretory/secretory antigens from three stages of the life cycle of *Nippostrongylus brasiliensis*: infective L3, L4 lung larvae, and L5 adult worms. The culturing method is the same for all three stages, but due to the nature of the L3 cultures, more extensive washing is required to eliminate any possibility of contamination.

Materials

N. brasiliensis: L3 larvae from 4 culture plates (see Basic Protocol 3), L4 larvae from 10 mice (see Basic Protocol 7 or Alternate Protocol 3), or L5 adult worms from 10 mice (see Basic Protocol 8)

NES wash solution I (see recipe)

NES wash solution II (see recipe)

NES culturing solution (see recipe)

15% SDS-PAGE gel (UNIT 8.4)

50-ml sterile glass test tubes

50 ml sterile tissue culture flasks

37°C, 5% CO₂ incubator

0.2-µm filter units

Ultrafiltration concentrator unit, 5000 MWCO (APPENDIX 3H)

Additional reagents and equipment for counting worms (see Basic Protocol 3), concentration via ultrafiltration (APPENDIX 3H), protein assay (e.g., UNIT 2.11), and SDS-PAGE (UNIT 8.4)

1. Wash larvae or worms in 25 ml NES wash solution I, allowing worms to settle by gravity between washes, then aspirating the wash solution. For L3, wash larvae five times; for L4 and L5 worms, wash three times.

Settling of worms can take up to 30 min at each wash.

2. Transfer worms to clean, sterile 50-ml glass test tubes and wash three times, each time with 25 ml NES wash solution II, allowing worms to settle by gravity and then aspirating the wash solution between washes.

Viability of worms is excellent. Glass tubes reduce the number of worms sticking to the sides of the tubes.

3. Transfer worms to fresh sterile 50-ml glass test tubes and incubate worms in 25 ml NES wash solution II for 1 hr at room temperature.
4. Count worms (see Basic Protocol 3) and make up to following concentrations in NES culturing solution:

For L3 10,000 organisms/ml

For L4 500 organisms/ml

For L5, 100 organisms/ml

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5. Place 15 ml of the worm suspension in a 50-ml tissue culture flask.
6. Incubate at 37°C with 5% CO₂ for 2 days.
7. Collect all of the supernatant from the flask. Filter through 0.2-µm filter and concentrate NES by ultrafiltration (APPENDIX 3H) using an MWCO 5000 membrane. Filter-sterilize the resulting NES and divide into working aliquots. Store at -70°C.
8. Determine protein concentration (e.g., UNIT 2.11) and analyze the antigen on a 15% SDS-PAGE gel (UNIT 8.4).

Note that larvae and adult worms that are viable before or after collection for in vitro cultivation can be homogenized in cold buffer (Tris, PBS, saline) to provide a protein extract of somatic tissue that can be used as a large source of antigen for ELISA testing for serum antibodies or for stimulation of lymphocyte proliferation. The NES is generally better for these tests because it provides “functional” antigens that are thought to be released and recognized by the host as the worm develops. In addition, adult worms shed fertilized eggs into the culture media that can be isolated as a starting material for cultivation of the free-living stage of the parasite.

MAINTENANCE AND PROPAGATION OF *HELIGMOSOMOIDES POLYGYRUS*

Inoculation of Mice with *Heligmosomoides polygyrus* Infective Larvae

The gastrointestinal nematode *Heligmosomoides polygyrus* (synonym, *Nematospiroides dubius* Baylis) belongs to the phylum Nematoda, the superfamily Trichostrongyloidea and the family Heligmosomidae, with the wild house mouse (*Mus musculus*) being its natural host. However, *Heligmosomids* are common in rodents, with a wide geographical range. The adult worms have a coiled and cork-screw appearance with complex cuticular ridges known collectively as synlophe that anchor the worm to intestinal villi as they coil these structures and feed on host tissue in the crypt regions.

H. polygyrus, like *N. brasiliensis*, is a potent activator of both systemic and local Th2 immune responses. However, its route of infection in the mouse host is strictly enteral, and once established in the gut the infection lasts weeks to months in genetically distinct strains of laboratory mice. *H. polygyrus* provides a natural model of efficient parasitism that is subclinical and chronic, and usefully models the situation observed in most nematode parasite infections of humans and livestock.

The elliptical eggs are passed in the feces as an 8- to 16-cell stage, and are 70 to 84 by 37 to 53 µm in size. Eggs appear in the feces of infected mice starting day 9 post-inoculation, and continue to pass for several months. There is a free-living stage where the first-stage larvae hatch from the egg after 36 hr at 20°C, with a first molt 28 hr later, followed by a second molt within 24 hr. The infective L3 is 300 to 600 µm in length and is infectious by oral ingestion but not through the skin. Parasitic larvae move into the gastric mucosa of the fundic region of the stomach as early as 4 hr after inoculation, emerge 36 hr later, and enter the duodenum to lie close to the longitudinal muscle layer of the gut. There is no evidence of a true cyst, but a deformation of muscle tissue and a largely eosinophilic cellular infiltration surround the larvae; the lesion can be seen grossly from the serosal side of the duodenum. The third molt is 2 days post-inoculation, the fourth molt 4 to 6 days later, and adults emerge at 8 days into the lumen, mate, and produce eggs, starting at 9 days post-inoculation, for several months.

Maintenance of *H. polygyrus* requires a different approach to that of *N. brasiliensis* due to its different life cycle; however the method is as robust and reliable as that used for *N.*

brasiliensis. Once chronic *H. polygyrus* infection is established in mice, eggs can be collected daily for many weeks, and the infective L3 derived from fecal cultures can be stored in a water or PBS suspension at 4°C for months with little loss of infectivity. Inoculation of mice with doses of L3 >400 can be lethal. Rats are not a suitable choice of host for *H. polygyrus* maintenance because worm development is delayed and infection is not as efficient as it is in mice.

The natural route of *H. polygyrus* infection is by mouth, and experimental inoculations are done with a rounded-end feeding needle. Other routes of infection such as intraperitoneal and subcutaneous injection are significantly less productive.

Materials

Heligmosomoides polygyrus infective L3 at 2000 L3/ml (see Basic Protocol 12)

Swiss Webster mice or relevant inbred strains

1-ml plastic syringe fitted with 18-G rounded-end feeding needle

Additional reagents and equipment for mouse restraint (UNIT 1.3)

1. Mix the suspension of *H. polygyrus* L3, then remove 100 µl into a 1-ml syringe fitted with the feeding needle.
2. Restrain the mouse (UNIT 1.3).
3. Slide the rounded-end needle down the throat of the mouse and dispense the suspension.
4. Collect eggs starting on day 12 post-inoculation (see Basic Protocol 11).

Collection, Counting, and Culturing of *Heligmosomoides polygyrus* Eggs from Fecal Pellets of Infected Mice

Start collection of fecal pellets on day 12 post-inoculation, with daily collections for up to several weeks. For collection and enumeration of *H. polygyrus* eggs, see Basic Protocol 2 and apply the same procedures to mice. The culturing method described herein is best used with the modified Baermann method (see Alternate Protocol 1) for harvesting L3 larvae.

Materials

H. polygyrus infected mice (see Basic Protocol 11)

Granulated charcoal/sphagnum moss

Spoon or spatula

90-mm sterile petri dishes

Incubator set at 26°C, without CO₂ (room temperature benchtop incubation is adequate)

Additional reagents and equipment for collection and counting of worm eggs from rodent fecal pellets (as for *N. brasiliensis*; see Basic Protocol 2)

1. Starting on day 12 post-inoculation, collect fecal pellets and enumerate eggs as for *N. brasiliensis* (see Basic Protocol 2).
2. Soften fecal pellets by soaking in enough distilled water to just cover pellets.
3. Add equal amount of granulated charcoal and sphagnum moss to the fecal pellets to form a paste (see Basic Protocol 2, step 14 for more detail).
4. Distribute fecal paste to completely fill 90-mm petri dishes.

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5. Label dishes with date of collection.
6. Incubate at 26°C or at room temperature for ≥ 7 days.

*This method can also be applied to *N. brasiliensis*, although harvesting of L3 larvae is usually faster, cleaner, and simpler using the technique described in Basic Protocol 2.*

BASIC PROTOCOL 12

Harvesting and Storage of *Heligmosomoides polygyrus* Infective L3 from Fecal Cultures

The method of choice for isolating infective L3 larvae for experimental use and storage at 4°C in water or PBS is the modified Baermann method (see Alternate Protocol 1). The L3 larvae of *H. polygyrus* do not aggregate in culture like *N. brasiliensis*, but are more dispersed and at different depths in the culture. Therefore, the contents of the entire culture should be placed in the Baermann apparatus to collect L3 larvae. After harvesting, adjust suspension of larvae to 2000 L3/ml in PBS; store at 4°C as a ready source of inoculum. Periodically check the viability of the inoculum and readjust the inoculation dose based on moving L3; prepare new inoculum when the viability begins to decline.

BASIC PROTOCOL 13

Obtaining *Heligmosomoides polygyrus* Adult Worms and Antigen

Collection of *Heligmosomoides polygyrus* (L5) adult worms

Apply the same procedure as for *N. brasiliensis* (see Basic Protocol 8). Size range of male and female worms is distorted because of the coiled-appearance. The male has spread bursa rays, while the caudal end of the female is rounded and hooked.

Adult *Heligmosomoides polygyrus* can be cultured at 8 days after inoculation as they emerge into the lumen as young L5, or can be obtained at 12 days after inoculation or later as they mate and begin to produce eggs. Several reports have indicated that fecund adults produce regulatory substances that can down-regulate different aspects of the host's immune system.

Preparation of *Heligmosomoides polygyrus* antigen (HES)

Apply the same procedure as for *N. brasiliensis* (see Support Protocol 1).

SUPPORT PROTOCOL 2

Measurement of CD4⁺ T Cell–Dependent Primary and Secondary Immune Responses to *Heligmosomoides Polygyrus*

Although a primary infection of mice with *Heligmosomoides polygyrus* is characterized as chronic, it is subject to immune regulation. Balb/c or C57Bl/6 mice treated with anti-CD4 mAb develop higher numbers of established adults than nontreated control mice, and the worms have a greater fecundity. In addition, the persistence of an infection is strain-dependent, indicating a genetic component to resistance. Protective immunity to *H. polygyrus* is typically demonstrated as an increase in resistance to a secondary infection. This can be most easily observed by antihelminthic drug cure of the primary infection, followed by a second infection with reduced fecundity and reduced numbers of adult worms that establish being a measure of maintained CD4 T cell–dependent immunity. The success of the secondary infection is compared with that of a primary infection in naïve mice with the same inocula of larvae.

Immunity during a primary infection is related to IL-4 and/or IL-13 binding to the IL-4R and is Stat6-dependent. Protective immunity to a secondary infection is also CD4⁺ and Stat6-dependent, and neutralization of IL-4 or IL-13 reduces the level of protective immunity to a secondary infection.

**Animal Model of
Nippostrongylus
brasiliensis and
Heligmosomoides
*polygyrus***

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Materials

H. polygyrus infective L3 at 2000 L3/ml (see Basic Protocol 12)

Mice of relevant in-bred strain

Anti-CD4 monoclonal antibody

50 mg/ml liquid suspension of pyrantel pamoate (Pfizer)

1-ml plastic syringe fitted with 18-G rounded-end feeding needle

Additional reagents and equipment for mouse restraint (*UNIT 1.3*) injection of mice (*UNIT 1.6*), determining egg counts in individual mice (see Alternate Protocol 2)

1. Mix the suspension of *H. polygyrus* L3, then remove 100 μ l into the 1-ml syringe fitted with the feeding needle
2. Restrain the mouse (*UNIT 1.3*). Slide the rounded-end needle down the throat of the mouse and dispense the suspension.

To determine effect of CD4⁺ T cell–dependent immune response on primary infection

- 3a. Inject mice intravenously (*UNIT 1.6*) with 1 mg anti-CD4 monoclonal antibody at weekly intervals starting on the day of infection or later; maintain an untreated infected group.
- 4a. On day 12 post-inoculation, compare fecundity of the parasite in infected versus anti-CD4 mAb-treated and infected mice by evaluating eggs per g of feces in individual mice in each treatment group (see Alternate Protocol 2).

Total adult worm numbers can also be determined (see Basic Protocol 13).

To determine effect of CD4⁺ T cell–dependent immune response on secondary infection (using anthelmintic drug cure)

- 3b. At a point 2 weeks or more after inoculation, check for infectivity by determination the levels of egg production in infected mice (see Alternate Protocol 2).
- 4b. Using 1-ml syringe fitted with feeding needle, administer an oral suspension of pyrantel pamoate at 1 mg/mouse.
- 5b. Re-examine levels of egg production 3 or more days later to confirm that the worm fecundity has been reduced to zero (see Alternate Protocol 2).

Allow 2 weeks before beginning anti-CD4 treatment

- 6b. Administer challenge infection as in steps 1 and 2, above. Administer 1 mg of anti-CD4 mAb intravenously (*UNIT 1.6*) at weekly intervals starting on the day of the challenge infection; inject a similar challenge-infected group with an isotype-control mAb.

Intervals between primary infection, anthelmintic drug treatment to cure the infection, and the challenge infection can be changed to evaluate the induction and persistence of a protective memory response.

- 7b. Compare fecundity of infected versus anti-CD4 mAb-treated and infected mice by evaluating eggs per g of feces in individual mice (see Alternate Protocol 2) in each treatment group.

A primary infection group can be included as a positive control for the inoculum. Total adult worm numbers can also be determined as described in section 4.1

IMMUNOLOGICAL ASSAYS OF CYTOKINES, PARASITE-SPECIFIC ANTIBODY PRODUCTION, EOSINOPHILIA, MUCOSAL MASTOCYTOSIS, GLOBLET CELL HYPERPLASIA, AND MUCUS PRODUCTION

Nippostrongylus brasiliensis and *Heligmosomoides polygyrus* act as potent activators of Th2 immune responses that lead to the production of the cytokines IL-3, IL-4, IL-5, IL-6, IL-9, IL-13 and IL-10, which, in turn, directly mediate IgG1 and IgE antibody production, eosinophilia, mucosal mast cell (MMC) hyperplasia, goblet cell hyperplasia, and mucus production. There are numerous procedures for evaluating components of a Th2 type immune response that go beyond the scope of this unit. However, a few widely used or easily applied procedures will be mentioned as a point of reference.

Cytokine measurements

Gene expression analysis by quantitative and semiquantitative RT-PCR provide a measure of changing patterns of cytokine RNA synthesis during the infection. Tissues of interest are rapidly excised from the body of an infected mouse and immediately frozen in liquid nitrogen or homogenized in a nucleic acid extraction buffer and then frozen, or, can be put in RNeasy (Qiagen) and stored at room temperature or 4°C. *UNIT 10.23* describes protocols for detecting cytokine mRNA expression by PCR.

Antibody-based cytokine detection assays for synthesis of proteins generally are used to support gene-expression data. Cells isolated from lymphoid tissues of infected mice can be stimulated in vitro with parasite antigens, or mitogens, and cytokines released in the media can be measured by ELISA methods (see Chapter 6). Preparation of *N. brasiliensis* excretory/secretory antigen (NES) is described in Support Protocol 1; preparation of *H. polygyrus* excretory/secretory antigen (HES) is described in Basic Protocol 13. The use of ELISPOT techniques (*UNIT 6.19*) provides a measure of explanted lymphoid cells that continue to secrete cytokines induced in situ or following in vitro stimulation with parasite antigen or mitogen.

A novel assay is the Cincinnati Cytokine Capture Assay (CCCA), which captures the level of circulating cytokines for IL-2, IL-4, IL-6, IL-10, IFN- γ , and TNF- β using injection of biotinylated anti-cytokine antibodies that are then detected in vitro using a modified ELISA method and an avidin-labeled readout (see *UNIT 6.28*).

Intracutaneous injection and recovery of cervical lymph nodes

Most assays that follow immunological aspects of the infection focus on gut-associated lymphoid tissues (GALT), including the mesenteric lymph nodes draining the site of adult worm infection, the Peyer's patch in the duodenum and jejunum, and cells that can be released from the intestinal epithelium and lamina propria using EDTA and enzymatic dissociation methods. The tracheal/bronchial lymph nodes have also been isolated to characterize responses from the draining lymph nodes of the lung following migration of developing L4. Early development of infective to parasitic L3 in the skin can be assessed immunologically by removal of cervical lymph nodes that drain the ear when L3 are injected intracutaneously.

Parasite specific and total levels of serum antibody

Parasite excretory/secretory antigens or extracts from the worm can be used as a source of antigen to measure parasite antigen specific antibody response during an infection. Preparation of *N. brasiliensis* excretory/secretory antigen (NES) is described in Support Protocol 1 and preparation of *H. polygyrus* excretory/secretory antigen is described in Basic Protocol 13.

Total isotype patterns of serum antibody levels can be detected by the use of anti-isotype specific reagents in an ELISA-based assay (see Chapter 6). Typically a shift towards IgE and IgG1 and a diminution of the IgG2a and IgG2b responses are characteristic of a strong Th2 immune response.

Eosinophilia

Classical Giemsa staining of blood smears (*APPENDIX 3C*) from infected mice can be used to measure peripheral blood eosinophilia. This method is preferable when eosinophils cannot be evaluated within a short time because slides can be fixed for future evaluation.

Commercial metachromatic staining solutions such as the Unopette system (Becton Dickinson), which employs a phloxine B stain, are available for rapid quantitation of peripheral eosinophils using a standard hemocytometer.

Detection of mucosal mast cell (MMC), goblet cell hyperplasia, and mucus secretion

The “Swiss roll” technique is used to evaluate a 3- to 5-mm length of intestinal tissue with a single tangential microtome cut. The intestinal section is opened and rolled around an applicator stick (glass or wooden) with the serosal side in contact with the stick surface. The roll is pushed off the stick into a fixative and then processed for histology.

Tissues placed in Carnoy’s fixative preserve the sulfated glycoproteins within MMC that are visible with a metachromatic stain using toluidine blue or Astra Blue dye. Tissues fixed in buffered formalin can be processed for Giemsa staining to reveal tissue eosinophils and goblet cells that contain mucus.

Changes in mucus glycosylation can be detected by using a series of terminal sugar-specific lectins that are biotinylated and can be detected with avidin-bound detection systems.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.

NES culturing solution

- 1000 ml RPMI 1640 medium (e.g., Life Technologies)
- 10 ml of 10 mg/ml gentamicin sulfate
- 10 ml 100× (10,000 U/ml) penicillin-streptomycin
- 10 ml 100× L-glutamine
- 1% (w/v) glucose
- Filter sterilize through 0.2-μm filter
- Store up to 3 weeks at 4°C

NES Wash solution I

- 1000 ml phosphate-buffered saline (PBS; *APPENDIX 2A*)
- 20 ml of 10 mg/ml gentamicin sulfate
- 20 ml 100× (10,000 U/ml) penicillin-streptomycin
- Filter sterilize through 0.2-μm filter
- Store up to 3 weeks at 4°C

NES Wash solution II

- 1000 ml RPMI 1640 medium (e.g., Life Technologies)
- 20 ml of 10 mg/ml gentamicin sulfate
- 20 ml 100× (10,000 U/ml) penicillin-streptomycin
- Filter sterilize through 0.2-μm filter
- Store up to 3 weeks at 4°C

COMMENTARY

Background Information

Nippostrongylus brasiliensis

The study of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* infection in rodents has given the discipline of immunology profound insights into the cellular and hormonal basis of the induction of Th2 immune responses and the role of these response in mediating protective immunity (Ishizaka et al., 1976; Ogilvie and Jones, 1971; Urban et al., 1992; Yokohama, 1922). The major advantages of the *N. brasiliensis* model are its safety and its ability to rapidly and reliably induce acute Th2 mediated immune responses in the skin, lung, and gut. The lymph nodes draining these tissue sites become directly involved in the ensuing immune response and become an easily obtainable source of cytokine and immunoglobulin-producing lymphocytes during the peak of the infection (Conrad et al., 1990; Finkelman et al., 1997; Svetic et al., 1993).

The *N. brasiliensis* infection model in mice is without parallel as a selective inducer of the complete range of CD4 Th2 lymphocyte-dependent immune responses. The *N. brasiliensis*-induced responses include a rapid (7 to 13 day post infection) IL-4 dependent induction of IgE with serum levels commonly measured in the range 2 to 30 µg/ml (Le Gros et al., 1996). There is also a profound IL-5 dependent eosinophilia induced in both the blood (5% to 10% eosinophils) and lung (70% to 80% eosinophils), appearing 7 to 9 days after infection (Coffman et al., 1989; Kopf et al., 1993). At the final phase of the infection cycle, a profound IL-3/IL-4/IL-9 dependent mastocytosis (Madden et al., 1991; Nawa et al., 1994; Townsend et al., 2000) is induced in the mucosal wall of the gut, which is followed by a dramatic IL-4/IL-13 mediated expulsion of worms from the small intestine (Urban et al., 1998).

Infection with *N. brasiliensis* has also been used as a "Th2 type" adjuvant to stimulate Th2 or IgE immune responses to antigens unrelated to *N. brasiliensis* but of interest to the investigator (Holland et al., 2000). In recent times, the *N. brasiliensis* infection model has been used to modify other types of immune responses or immune inflammatory conditions in an attempt to suppress or regulate activity (Erb et al., 1998; Rocken et al., 1992; Urban et al., 1993). These studies are an ongoing and exciting area of research activity.

The *N. brasiliensis* infection model is superior to alternative methods for inducing Th2 immune responses which involve either chronic exposure to low levels of antigen in adjuvant over a period of several weeks or infection with other pathogenic parasites (Le Gros et al., 1996). The biggest disadvantage of these alternative methods compared to *N. brasiliensis*-stimulated responses is that they often do not stimulate all the features of a Th2 immune response. Furthermore, many of the alternative pathogens only target selected tissue sites and are dangerous or time-consuming to work with.

The infection model has proven especially useful for the phenotypic analysis of mutant strains of mice missing key cytokines, signaling molecules, and lymphocyte subsets that are of immunological interest (Finkelman et al., 2000; Hook et al., 2000; Kopf et al., 1993). It should be noted that severely immune-deficient or immune-suppressed mice do not expel the adult L5 worms, and a chronic state of infection can be maintained for several weeks. An additional advantage is that it is relatively easy to generate sufficient worms to infect groups of 30 to 50 mice with very little individual variation occurring with respect to the immune responses being induced.

The induction of a productive life cycle in mice requires infection with (500 mouse-adapted or 750 rat-adapted) fully viable L3 worms (see below). If the scientific intent is to only stimulate an immune response in the mouse, significantly fewer worms can be used for the infection (Lawrence et al., 1996). It should be noted that *N. brasiliensis* worms adapted to a life cycle in mice are more efficient in infecting the mouse host than worms maintained in rats (Wescott and Todd, 1966). The major consequence of this is that only 500 mouse-adapted L3 larvae are required for a full infection while up to 750 rat-adapted worms are needed to achieve a productive infection with good numbers of eggs. It is important to accept that a little effort is required at first to set up the infection life cycle in rats or mice in a new laboratory and ensure that a regular collection of feces and passage occurs as detailed in this unit.

Although the infection life cycle of *N. brasiliensis* in rodents does not model the full life cycle of any significant human or livestock pathogen, it has proven very useful for modeling the specific aspects of immunopathology

arising from nematode biology including migration, feeding, and excretion of immunomodulatory and enzymatically active secretory products (Ehigiator et al., 2000; Grigg et al., 1997; Holland et al., 2000). It is anticipated that the great improvement in molecular cloning technologies should ensure that investigators working with *N. brasiliensis* will soon have available the relevant molecular tools for understanding in more detail the biology of this fascinating pathogen.

Heligmosomoides polygyrus

The biology of *H. polygyrus* in mice is of research interest because it is a natural model of nematode infection that can be examined from several aspects of the mammalian host-parasite interface including afferent activation of adaptive immunity, induction of efferent resistance mechanisms, and the interplay between parasite avoidance and host response. Chronic gastrointestinal nematode infections are of particular importance in livestock and humans because there is an enormous economic cost in terms of management of the infection, poor growth and development, and the inappropriate consequences of immune pathology and/or reduced responses to bacterial, protozoan, and viral infections. The difference between transient versus chronic intestinal nematode infection in mice has long been debated without clear resolution vis-à-vis the difference between host responses to *N. brasiliensis* and *H. polygyrus* (Day et al., 1979), but there is renewed optimism for clarity as molecular biological techniques are applied to the study of these infections.

A feature of high *H. polygyrus* worm burden is the ability to persist in the host for periods much longer than low-level infections (Dobson et al., 1985). This observation supports a role for adult worms acting locally to down-regulate immune effector mechanisms in the intestine (Dehlawi and Wakelin, 1988, reviewed by Behnke, 1987), and can even affect protective responses to heterologous worm infections (Behnke, et al., 1978). Both sex and genetic composition of the host are factors that contribute to dose-dependent persistence of *H. polygyrus* adult infection (Robinson et al., 1989). Nevertheless, resistance to *H. polygyrus* is immune-mediated and CD4⁺ T cell-dependent (Urban et al., 1991a). The level of infection is limited by expression of IL-4 (Urban et al., 1991b, Urban et al., 1995) and is dependent on IL-4R α chain receptor activation of Stat6 (Finkelman et al., 1997). In addition, the early

activation and maintenance of a robust type 2 immune response that includes expression of IL-3 and IL-9 is critical for controlling the level of infection in strains of mice with different resistant phenotypes (Svetic et al., 1993; Wahid et al., 1994).

Studies of afferent activation of adaptive immunity to a primary infection with *H. polygyrus* have recently revealed that induction of IL-4 expression by CD4⁺ T cells in vivo is B7-dependent (Lu et al., 1994; Greenwald et al., 1997; Ekkens et al., 2002). In contrast, in vivo activation of CD4⁺ T cell-dependent IL-4 expression during an infection with *N. brasiliensis* is B7-independent (Liu et al., 2002). More striking is that the development of non-parasite antigen specific Th2 cells (in vivo development of adoptively transferred D011.10 TCR transgenic T cells to Th2 cells specific for OVA peptide) during an infection with *N. brasiliensis*, but not *H. polygyrus*, is B7-independent (Liu et al., 2002). This suggests that either components of the biology of these infections (parenteral followed by enteral migration, Nb, versus strictly enteral, Hp, infection), or the nature of parasite products released during the infection, can provide an adjuvant effect that enhances or circumvents B7-dependent development of Th2 cells to both parasite and nonparasite antigens. How these costimulatory molecule requirements relate to acquired resistance to infection remains to be determined, but the more stringent conditions of antigen presentation with B7-dependent activation of host protective type 2 responses to *H. polygyrus* might favor a persistent worm infection with low fecundity, while products released by *N. brasiliensis* that activate a vigorous self-cure response may be inconsequential to the biology of an infection adapted to high fecundity and prolonged survival of infective stages ex vivo.

Mechanisms of resistance to *H. polygyrus* in the intestine are clearly compromised during both a primary infection (Wahid et al., 1994) and following a challenge infection with or without an intervening drug-induced cure of adult worms (Urban et al., 1991b). Host resistance is CD4⁺ T cell- and type 2-dependent, but it is unclear if immunity is expressed by reducing invasion of the intestinal mucosa by parasitic larvae (Chaicumpa et al., 1977), by destruction within the tissue, or by rapid clearance as adults emerge from the region of the intestinal submucosa/smooth muscle (Cypess et al., 1988). Some recent studies have addressed the issue of intestinal resistance to *H. polygyrus* infection by examining the interac-

tion of local immunity and changes in intestinal physiology. Neural-mediated IL-4-dependent increases in intestinal smooth muscle contractility have been observed during the response to a challenge infection with *H. polygyrus* (Goldhill et al., 1997). In addition, a primary *H. polygyrus* infection induces modest changes in intestinal epithelial cell function, including increased intestinal tissue permeability, reduced sodium-linked glucose absorption, and increased Cl⁻ ion secretion when exposed to selected secretagogues, but these responses are significantly enhanced when infected mice are also given exogenous IL-4 or challenged with a secondary infection. It has been proposed that the IL-4/IL-13-generated augmentation of both epithelial cell secretion, decreased glucose absorption, decreased resistance, and increased smooth muscle contractility contribute to a “weep and sweep” response that favors elimination of adult worms from the intestine (Shea-Donohue et al., 2001). This is supported by recent studies that demonstrated that IL-4-induced increases in intestinal tissue permeability and decreased glucose absorption are Stat6-dependent, while IL-4 but not IL-13 enhances a Stat6-dependent tissue response to PGE₂ and histamine that is also affected by the presence of mucosal mast cells (Madden et al., 2002). These results suggest unique activities for IL-4 and IL-13 downstream of the interaction with the IL-4 receptor that may explain relative differences in the prominence of these cytokines during protective immunity to any particular gastrointestinal nematode parasite species (Finkelman et al., 1998).

Neutralization of host anti-parasite resistance mechanisms is a component of successful parasitism, and infection with *H. polygyrus* provides a fertile area to examine this type of host-parasite interaction (Behnke et al., 1992). Ben-Smith and colleagues (2002) have linked the differential expression of catalase and superoxide dismutase by adult *H. polygyrus* to evasion of host-generated free radical-mediated worm damage in strains of mice with different resistance phenotypes. This is particularly interesting when compared to infections with *N. brasiliensis*, which were found to have lower levels of superoxide dismutase, catalase, and glutathione peroxidase than *H. polygyrus* adults (Smith and Bryant, 1986). Another strategy for inactivation of host effector mechanisms by the parasite is upstream inhibition of lymphocyte activation, which blocks or modifies local accumulation of inflammatory cells that contribute to the expression of resistance

(Dehlawi and Wakelin, 1988; Telford et al., 1998).

There are numerous other aspects of this infection that are amenable to study including modulation of inflammatory interactions (Fox et al., 2000), nutritional interventions (Kristan and Hammond, 2001; Ing et al., 2000), and behavioral interactions that can be measured internally (Kavaliers et al., 1998), socially (Bernard et al., 1998; Hernandez and Sukhdeo, 1995), and ecologically (Scott, 1987; Berding et al., 1987). The mouse-*H. polygyrus* infection system is a robust model of parasitism that can be conveniently adapted and maintained in the laboratory.

Critical Parameters and Troubleshooting

Nippostrongylus brasiliensis

The critical aspects to inducing immune responses in mice with *N. brasiliensis* is to ensure that infection is performed promptly after harvesting with accurately counted, viable worms. When counting the collected L3 larvae and when preparing an aliquot for injection it is critical to ensure the worms are fully resuspended, since they rapidly sink to the bottom of the holding vessel or syringe. The productivity of the fecal cultures is sensitive to dryness, overheating, or fungal contamination. The fecal cultures of L3 larvae can be readily transported, with viable infective larvae being obtainable from cultures for up to 2 to 3 months. The most viable L3 larvae are obtained from 2- to 3-week-old cultures. When analyzing various immune parameters in infected mice, it is important to ensure that the critical timing of the developing immune response be considered, as it is quite specific as to where and when the selected inflammatory responses are induced. The size and maturity of the mice also can determine to some degree the strength of the immune response, with too heavy infections in small mice resulting in cachexia and immunological unresponsiveness.

The critical aspects to setting up productive cultures of L3 larvae include ensuring that the starting fecal material has a high concentration of eggs and that all materials involved in the preparation and culture of the worms are free of detergents and antihelminthic agents. The source of food used for the rats and mice is often overlooked for its potential to greatly affect *N. brasiliensis* egg production.

The animal facility source of *N. brasiliensis* should also be considered, as rodent feces are

a potential source of other pathogens which could be introduced into the colony. Fecal material from *N. brasiliensis* infected SPF mice (UNIT 1.1) may be more appropriate.

Heligmosomoides polygyrus

A welcome feature of the *H. polygyrus* L3 form of the nematode is its ability to remain viable and infective for several months when maintained at 4°C. As with *N. brasiliensis*, it is important to ensure that mice infected with *H. polygyrus* are not exposed to anthelmintic therapy unless it is part of the experimental protocol, and that the diet of the mice does not contain agents which compromise the infection.

Anticipated Results

Nippostrongylus brasiliensis

The establishment of the *N. brasiliensis* life cycle is within the scope of any standard laboratory. It normally takes 3 to 6 months to establish the maintenance infection cycle of *N. brasiliensis* in a laboratory. Once established, the *N. brasiliensis* infection model provides a robust system for inducing “natural” Th2 immune responses, which have the potential to enable investigators to understand the basic biology of the immune system. The only limitation on the model is the need to have some degree of forward planning to ensure sufficient quantities of worms are produced during the maintenance cycle for the proposed experimental infections.

The *N. brasiliensis* infection model offers many advantages to the research immunologist over the more highly evolved pathogenic parasites, being less fastidious and complex while still offering for investigation all the fundamental aspects of immunoparasitology needing to be studied.

Heligmosomoides polygyrus

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The *H. polygyrus* infection model offers many advantages to the research immunologist over the more highly evolved pathogenic parasites while still allowing the fundamental aspects of immunoparasitology to be studied.

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- Thorough review of the basic techniques involved in preparation, handling and use of Nippostrongylus brasiliensis.*

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**Animal Models
for Infectious
Diseases**

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