



# Aerosol Exposure System for Rabbits: Application to *M. Tuberculosis* Infection

Liana Tsenova, Ryhor Harbacheuski, Evette Ellison, Claudia Manca, and Gilla Kaplan

The Public Health Research Institute, Newark, New Jersey

## Abstract

*The development and testing of new antitubercular vaccines and drugs require the use of experimental animal models that resemble pulmonary tuberculosis in humans. We evaluated the effectiveness of an aerosol exposure system for studying experimental pulmonary tuberculosis in rabbits. The device is a snout-only exposure system with 6-animal capacity that uses an individual facemask for each animal. The system is operated from a single jet Bio Aerosol Nebulizing Generator (BANG). The device is placed inside a laminar flow biosafety enclosure, specifically constructed to contain the exposure system. The entire aerosol unit is located within the Animal BSL-3 facility at the Public Health Research Institute (PHRI). After establishing and validating the technical parameters of operation, rabbits were infected via the respiratory route with the avirulent *M. bovis* strain Bacille Calmette-Guerin (BCG). A suspension of  $5-10 \times 10^6$  CFU/ml resulted in  $3.6 \log_{10}$  organisms deposited in the lungs; these were almost fully cleared by 14 days. Next, rabbits were infected with the same infectious dose of the virulent clinical isolate of *M. tuberculosis* HN878. The bacillary load in the lung at 14 days reached  $5.3 \log_{10}$  CFU and increased progressively. Large granulomatous lesions, distributed evenly in the lung parenchyma developed. We conclude that the aerosol exposure unit is easy to operate, yields a consistent implantation of mycobacteria in the lungs, is safe for the investigators, and induces disease similar to that observed in humans infected with *M. tuberculosis*.*

## Introduction

Tuberculosis (TB) is a leading public health problem worldwide particularly in developing countries. Approximately 2 billion people are infected with *Mycobacterium tuberculosis*, the causative agent of TB, which is responsible for 2.5 million deaths due to this disease annually (WHO, 2004). To better control this epidemic, new drugs and better vaccines are urgently needed. The development and testing of new antitubercular vaccines and drugs require the use of experimental animal models that

resemble pulmonary tuberculosis in humans.

A variety of aerosol systems have been used in the past to induce experimental respiratory infection in mice (Johansson et al., 1991; North, 1995; Oda et al., 1983; Shahin et al., 1990; Sullivan et al., 1976). Most of these systems consist of a chamber in which the whole animal is exposed to the aerosolized pathogen. For safe handling of these animals, the mice must be exposed to UV light to disinfect the fur and skin following aerosol infection. Lower levels of contamination of experimental animals can be achieved by limiting the area of the body exposed to the infectious aerosol. Such systems as snout- or nose-only exposure devices have been used historically mainly for testing of radioactive and toxic substances, including tobacco smoke and asbestos fibers (Bernstein et al., 1994; Dontenwill, 1970; Ferguson et al., 1982; Raabe et al., 1973). We have previously reported on the use of a nose-only exposure device for aerosol infection with *M. tuberculosis* in mice (Party et al., 1997). A number of groups have used nose-only aerosol infections with different pathogens in larger animals such as rabbits and guinea pigs (Converse et al., 1996; Dorman et al., 2004; Niyo et al., 1988; Pitt et al., 2001; Sherman et al., 1994; Williams et al., 2005). However, to date, the technical issues and biosafety considerations of a nose-only aerosol device to infect rabbits have not been fully addressed. This study evaluates the effectiveness of a nose-only aerosol exposure system (CH Technologies, Inc., Westwood, NJ) for studying experimental pulmonary tuberculosis in rabbits.

## Material and Methods

### Aerosol System

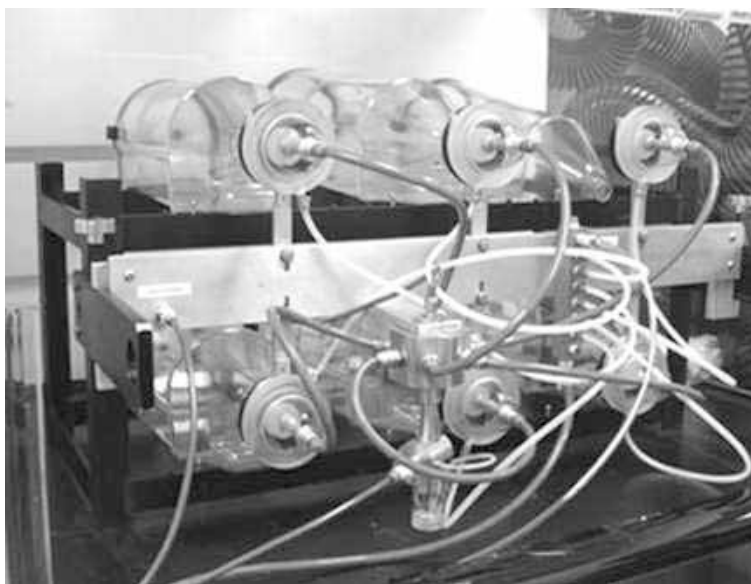
The aerosol device is a snout-only exposure system with 6-animal capacity (2 layers of 3 animals) per run (Figure 1) and a special removable exposure plenum for six masks. The incoming supply air is filtered through a sequence of filters including a coalescing prefilter, a coalescing filter (Arrow Pneumatics; Maximum pressure: 150 psi) (to remove water droplets), and a submicron filter (PALL, Exton, PA) to remove oils and other particles. The filtered dehumidified air is divided into two streams.

One is regulated to 50 lb per square inch gauge (PSIG) and supplies the single jet Bio Aerosol Nebulizing Generator (BANG). The other stream, regulated to 10 PSIG, provides the diluting air that is mixed with the output from the BANG and varied as required (Figure 2). The bacterial suspension is placed in the BANG dose jar. Within the BANG, the liquid inoculum is fed into a high-pressure air stream that enters through a fine-bore channel. The principle is that of a Single Jet Collision with nonrecirculating fluid, in which the impact of the liquid with the air stream and against the device wall

yields a highly respirable aerosol. The aerosol is driven by positive air pressure. Larger droplets condense in the BANG exhaust stack and return to the dose jar. Droplets of 1  $\mu\text{m}$  - 3  $\mu\text{m}$  in diameter enter the aerosol dispenser, where six output jets split the stream for delivery to the six rabbit aerosol masks via static discharge polyurethane tubing (Figure 1). Each rabbit is placed in a separate, specially designed, airtight polyethylene terephthalate restraint tube (Figure 3). The animal tubes are connected to the aerosol nasal masks (In-Tox Products, LCC, Moriarty, NM). When fewer than six animals are being infected,

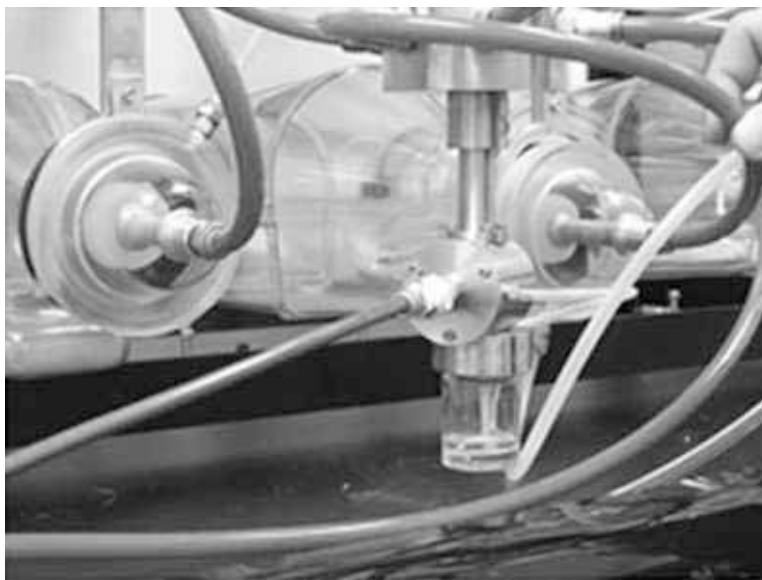
**Figure 1**

The aerosol device is a snout-only exposure system with 6-animal capacity (2 layers of 3 animals) per run.



**Figure 2**

The system is operated from a single jet Bio Aerosol Nebulizing Generator (BANG).



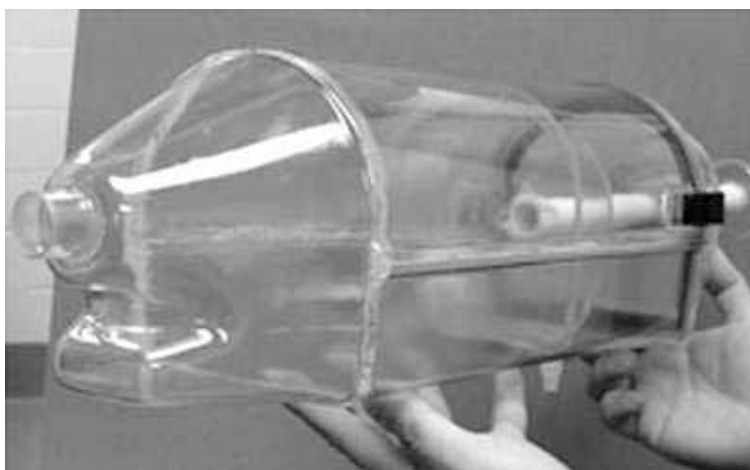
rubber stoppers are used to seal the openings of the unused facemasks. The animals inhale a portion of the delivered aerosol into the lungs, depositing bacilli in the alveoli. Exhaled gasses and the noninhaled aerosol exit the individual masks via an exhaust plenum through a HEPA filter (PALL) connected to the vacuum line.

To ensure that pathogens remain contained in case the integrity of the aerosol system fails, the instrument is placed inside a laminar flow biosafety enclosure (Flow Sciences, Inc., Wilmington, NC), specifically constructed to contain the unit (Figure 4). Flow Sciences, Inc. (FSI) modified one of its existing enclosures to ensure proper fit of the unit and exact placement of ports and cutouts. In the design phase, FSI utilized Computational Fluid

Dynamics (CFD), which allows the user to program in all aspects and designs of the enclosure to produce airflow modeling for a given application. Prior to shipment, an SF6 (tracer gas) test was performed, in accordance with the ANSI/ASHRAE (American Society of Heating, Refrigerating, and Air-Conditioning Engineers) 110-1995 testing standard. (A copy of the original document can be obtained by writing to ASHRAE, Inc. at 1791 Tullie Circle NE, Atlanta, GA 30329.) ASHRAE 110 is part of the Factory Acceptance Test (FAT) that includes small-volume flow visualization, large-volume flow visualization, tracer gas test (released at a rate of 4 liters per minute), and face-opening grid to evaluate flow rates. The system operates at the face velocity between 60 to 100 linear feet

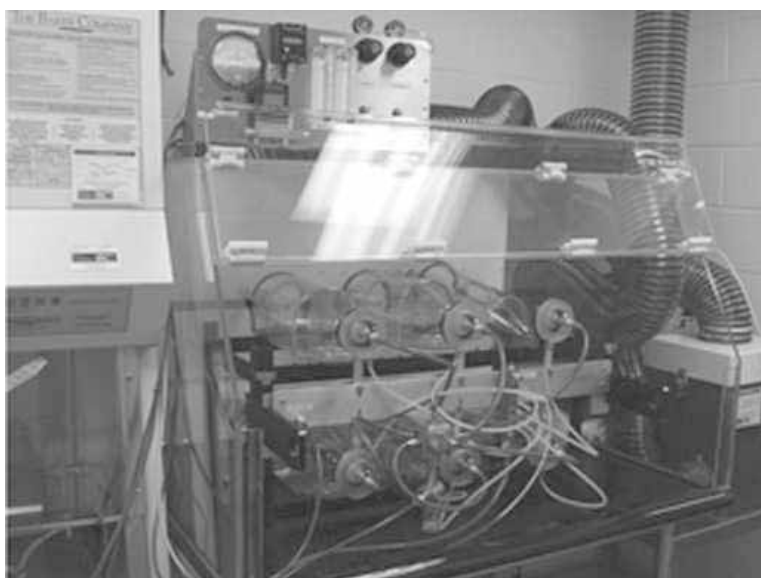
### Figure 3

Each rabbit is inserted into a separate, specially designed, airtight polyethylene terephthalate restraint tube.



### Figure 4

The entire device is placed inside a laminar flow biosafety enclosure, specifically constructed to contain the unit.



per minute (lfpm). The enclosure utilizes two Bag-Out HEPA filters that are 99.97% efficient (supplied by American Air Filters (AAF), Raleigh, NC). Filter lifespan is highly dependent on the particular application. Typically, it is recommended that the filters be changed when the face velocity is reduced to around 60 lfpm. The enclosure is a Class I Type (provides personnel protection only) that is connected to the building exhaust system via a thimble connection. The performance of the cabinet (face velocity and HEPA filter leakage) with the aerosol system in place is evaluated and certified every 6 months, using the following tests: airflow velocity, HEPA filter leak test, and smoke test (flow direction and cross drafts). The smoke test is carefully observed for any disturbance in the smoke pattern and for air losses out of the cabinet.

The rabbit aerosol system is easily operated by an adjacent control panel, placed outside the biosafety enclosure (supplied by CH Technologies Inc.) (Figure 5), with air pressure gauges for the nebulizer and diluted air and a magnehelic gauge for system pressure. The entire device is located within the Animal Biosafety Level 3 (BSL-3) facility at PHRI. A Standard Operating Procedure (SOP) for the infection of rabbits via the respiratory route was prepared and approved by the Institutional Biosafety Protocol Committee at PHRI.

### **Operation of the Aerosol System**

Personnel working on this project wear footed Tyvek suits with an apron on top, Powered Air Purifying Respirators (PAPR) with a full skirt (Fisher Scientific), cover sleeves, and two pair of latex gloves. The events that occur are listed here:

1. To begin, turn on the vacuum pump or initiate the vacuum controlled outflow inside the biosafety cabinet.

Verify that the entire exposure system is negative pressure relative to the pressure in the hood.

2. Turn on the air pump or activate the central air supply inside the biosafety cabinet. On the control panel, set Diluted air to 18 L/min.

3. Sedate the rabbits with Acepromazine, 0.75 mg/kg intramuscularly.

4. Insert each rabbit into a separate, specially designed, airtight polyethylene terephthalate restraint tube.

5. Let the clean diluting air run for 5-10 minutes. This allows the animals to acclimate and gives time to confirm all connections.

6. Prepare the bacterial suspension (10 ml-15 ml) in advance and place it into the nebulizer cup. The density of the suspension may vary and should be chosen based on the desired infectious dose.

7. Turn on the nebulizer. Adjust the total air flow to 18 L/min (12 L/min for diluting air + 6 L/min for the aerosol). Based on preliminary experiments with the above parameters, 4 ml of the suspension are generated into aerosol over 20 minutes.

8. Expose the aerosol for 20 minutes.

9. At the end of exposure time, turn off the nebulizer and run diluting air (18 L/min) for 5-10 minutes to remove any residual aerosol left in the system.

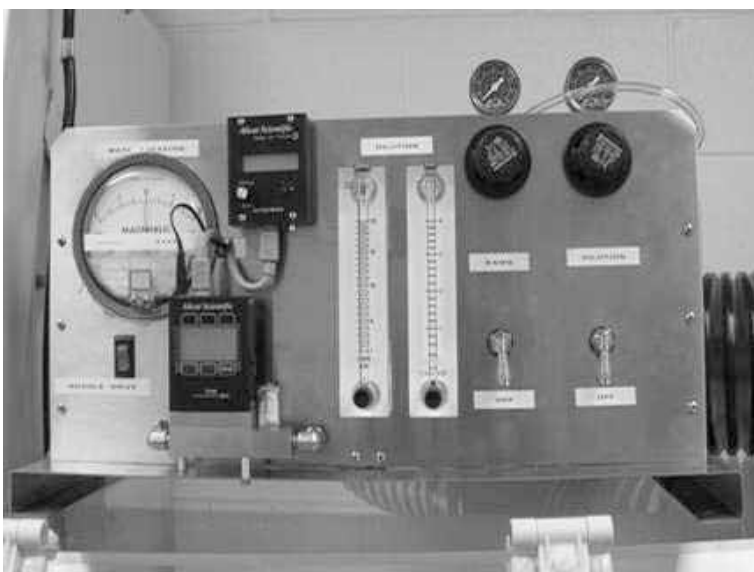
10. Infected rabbits are removed from the animal tubes and placed in filter-isolated individual crates during transport to their individual cages, which are kept in a negative pressure animal hood.

11. Insert rubber stoppers on each mask to seal the system.

12. Disconnect the nebulizer. Discard the remaining bacterial suspension after adding 10% Clorox solution in a screw-cap tube (15 ml) in the biohazard waste, inside

**Figure 5**

The rabbit aerosol system is easily operated by an adjacent control panel, placed outside of the biosafety enclosure.



the biosafety cabinet. Add 10% Clorox in the nebulizer cup and run aerosol to recirculate through the tubing system for 10 minutes. Add dH<sub>2</sub>O and run aerosol for 5 minutes to rinse out the Clorox residual. Add 70% EtOH for 10 minutes. Finally, run diluting air alone to dry the system. This step is to clean and disinfect the unit from the inside.

13. After each exposure, place the animal restraint tubes in a container with a lid (Nalgene Heavy-duty Rectangular HDPE tank, 30 gal, with a cover, Fisher Scientific Co.) and disinfect in Vesphene solution for 2-3 hours; then rinse thoroughly in water.

14. Also disinfect the nebulizer in Vesphene solution for 20 minutes, rinse with clear water, and allow to air dry.

15. Wipe the individual masks on the outside with Vesphene.

To determine the real infectious dose (inoculum) in some experiments, 3 hours after the exposure, one rabbit is sacrificed, the lungs removed, and each lobe is homogenized and plated on appropriate agar medium in the biosafety cabinet.

**Impinger Test.** To determine the approximate number of bacilli aerosolized by the nebulizer during the 20-minute run, a test was performed using All Teflon Impingers (ATI) similar to the All Glass Impinger (AGI). The air sampling devices (purchased from CH Technologies Inc.) were used as in-line arrays of two units connected in series by 316 stainless steel tubing (0.25 in OD). Flow was restricted through the ATI to 500 ml/minute using an in-line flow-limiting orifice. The ATI system was attached to the house vacuum, which was protected by an in-line HEPA filter. Sampling via the ATI with a control flow orifice (CFO) was done from a suspension in the nebulizer containing BCG.

### Aerosol Infection of Rabbits

To establish the working protocol and validate the technical parameters of operation, six separate experiments were performed using the avirulent *M. bovis* strain Bacille Calmette-Guerin (BCG) to infect rabbits. New Zealand white rabbits (2.0 kg) were infected via the respiratory route with BCG as a model for experimental pulmonary tuberculosis. Based on our previous experience with the aerosol infection of mice (Party et al., 1997), the bacterial suspension was prepared at 5-10x10<sup>6</sup> CFU/ml. The aerosol exposure time for each experiment was kept constant at 20 minutes. Three hours after infection, some of the rabbits were euthanized to evaluate the effectiveness of the aerosol delivery. Infected lungs (without the large airways) were homogenized in saline containing 0.05% Tween 80 and 10-fold dilutions plated on Middlebrook 7H11 agar (Difco Laboratories, at [www.vgdusa.com/DIFCO.htm](http://www.vgdusa.com/DIFCO.htm)). Colonies were counted after incubation at 37°C for 15 days. The remaining animals were monitored for up to 14 days. To determine the

kinetics of bacillary survival in the lungs, groups of rabbits were euthanized and evaluated at 24 hours, 7 days, and 14 days postinfection.

In another set of experiments, rabbits were infected by aerosol with the clinical isolate of *M. tuberculosis* HN878 (5-10x10<sup>6</sup> CFU/ml), shown to be highly virulent for the rabbit (Tsenova et al., 2005). Three hours after the infection, some of the rabbits were euthanized, a segment of the lung was collected, and the infected tissue used for the CFU assay as described above for BCG. To determine the bacillary load throughout the infection, groups of animals were euthanized and necropsy was performed on days 14, 28, and 42. Part of each lung was fixed in 10% buffered formalin acetate (v/v) (Fisher Chemical, Fairlawn, NJ) for histopathologic examination. This protocol was approved by the Institutional Animal Care and Use Committee at PHRI in New Jersey.

## Results

### Impinger Test

To determine the approximate number of bacilli aerosolized by the nebulizer and to predict potential deposition of bacilli in the lungs of rabbits exposed to the aerosol, a test was performed using All Teflon Impingers. Sampling was done from a suspension in the nebulizer containing BCG at 5x10<sup>6</sup> CFU/ml. After a 20-minute aerosol run, 10-fold serial dilutions of the impinger fluid (20 ml of saline + Tween 80) were plated on 7H11 agar and cultured for 15 days at 37°C. The number of bacilli in the sample was 3,200 CFU, suggesting that the rabbits would be infected efficiently and that we could predict the dose of infection to be delivered.

### Aerosol Infection of Rabbits with BCG

Using the same experimental conditions, rabbits were exposed to the BCG aerosol and the lungs of infected animals were examined for bacillary seeding and survival of the organisms over a 14-day period. Six rabbits were euthanized at 3 hours postinfection to determine the baseline infectious inoculum. The mean ± SD bacillary load implanted in the lungs of rabbits was 4,068±1,599 CFU (Figure 6A). This number was similar to the number of bacilli predicted by sampling of the aerosol with the impinger. Clearance of BCG from the lungs started by 24 hours postinfection. By 7 days postinfection, most of the rabbits had very low numbers to undetectable mycobacteria in the tissue. BCG clearance continued until day 14, when the experiment was terminated (Figure 6A).

### Aerosol Infection of Rabbits with *M. tuberculosis*

In contrast, when *M. tuberculosis* HN878 was seeded into the lungs of rabbits (inoculum 5.3±2.4x10<sup>3</sup> CFU), the bacillary load at 14 days postinfection had increased

to  $2.4 \pm 0.03 \times 10^5$  CFU. Numbers of bacilli continued to increase with time. By 42 days postinfection, the lungs contained  $4.9 \pm 2.7 \times 10^6$  CFU (Figure 6B). Enhancement in the bacillary numbers was associated with the formation of large, macroscopically visible granulomatous lesions distributed evenly throughout the lung parenchyma. Histologic examination of the lungs of rabbits infected with *M. tuberculosis* HN878 at 6 weeks post-infection revealed large confluent granulomas with lots of lymphocytes and macrophages, and central necrosis (Figure 7). Acid-fast bacilli were seen within cells in the center of the granulomas (not shown).

### Safety Considerations

The aerosol system developed was found to be easy to use and safe for the investigator. Personal protective equipment was utilized for all work with the rabbit aerosol infection system. In accordance with the requirements of the Occupational Safety and Health Administration (OSHA), each person working with PAPR received a physical examination and was approved for its use by the Occupational Medicine Service at New Jersey Medical School, UMDNJ in Newark.

Decontamination and disinfection procedures of the aerosol system were found to be reproducibly efficient. A microbiological wipe test was performed whereby a sterile cotton swab with saline was used to wipe different parts from the surface of the device within the enclosure. After plating on blood agar and 7H11 medium and incubating for a few days at  $37^\circ\text{C}$ , no bacterial growth was detected. Throughout all the experiments, no contamination of the plates with lung homogenates was observed.

To prevent contamination of the vacuum air line, in-line filtration was added as described above. To function properly, the rabbit exposure device must be leak-free. To verify that the instrument did not leak, a microbiological test was performed. Different areas below the tubing and the masks were tested for microbial leaks while running an aerosol from a BCG suspension with the same density ( $5 \times 10^6$  CFU/ml). Standard 7H11 agar plates were placed under potentially leaky connections so that any leaking aerosol could impinge onto the surface of the agar medium. After incubation for 15 days at  $37^\circ\text{C}$ , the plates were mycobacteria free.

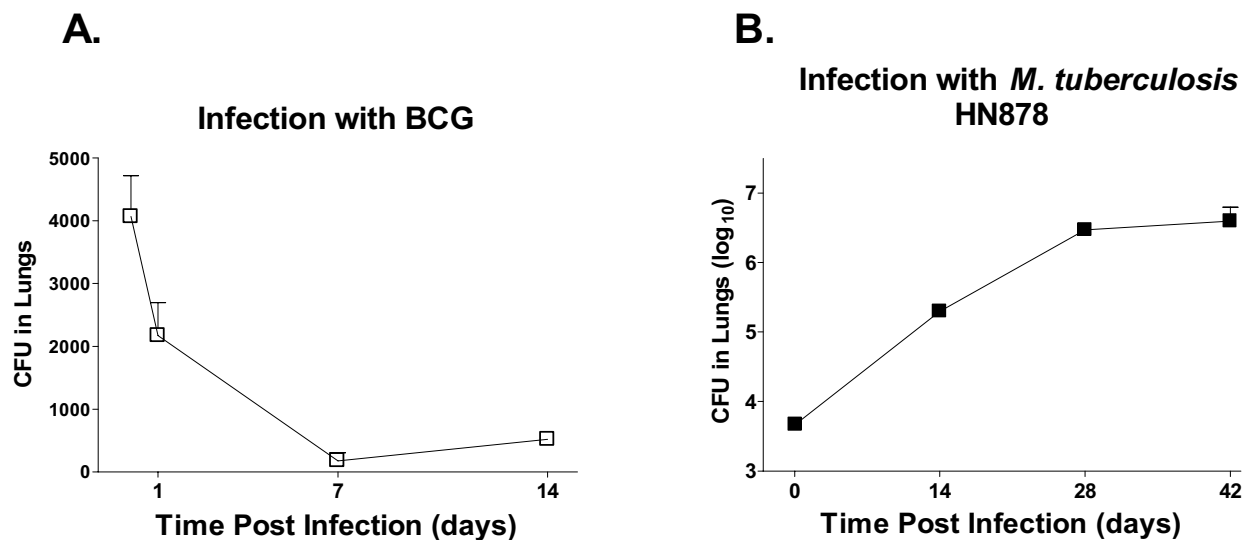
In the event that the system developed positive pressure (observe the pressure gauge on the control panel), it would be shut down and the work stopped. Later, the technical person (manufacturer) in charge of the maintenance of the system will investigate why there has been a reduction in outflow by following all lines (air, chamber exhaust, and vacuum line).

### Conclusions

Previously, nose-only exposure systems have been used mainly for toxicologic studies. Since the aerosol system described here is intended for use with virulent mycobacteria and other pathogens, it was important to establish the effective containment of the organisms within a biosafety enclosure. The aerosol system was shown to maintain a steady air pressure without any air leaks. All rabbits tolerated the aerosol exposure without any signs of pain or distress.

**Figure 6**

Bacillary load in the lungs of rabbits infected by aerosol. Values are means  $\pm$  SEM. 6A. Infection with BCG (white squares); 6 rabbits/time point. 6B. Infection with *M. tuberculosis* HN878 (black squares); 3 rabbits/time point.



SOPs for aerosol infection of rabbits were optimized using *M. bovis* BCG, a nonpathogenic strain of mycobacteria. The aerosol generated by the BANG delivered single microorganisms to the lungs of the rabbits, giving rise to comparable numbers of implanted mycobacteria among rabbits and from experiment to experiment. In contrast to the clearance from the lungs of the avirulent BCG, aerosol infection with the clinical isolate of *M. tuberculosis* HN878 induced progressive, severe, pulmonary disease, associated with high bacillary numbers in the lung and significant pathology, similar to lesions observed in human tuberculosis patients. These results establish the potential usefulness of this system for studies of experimental pulmonary tuberculosis that mimics human disease.

In summary, results showed that the aerosol exposure device is easy to operate, gives reproducible levels of infection, and provides an adequate safety barrier for the investigators.

## Acknowledgements

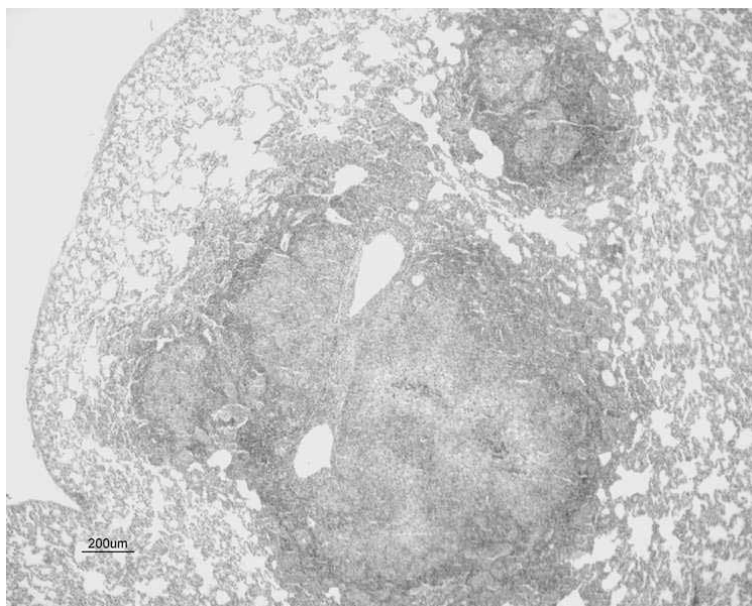
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## Figure 7

Histopathology of the lung of rabbits infected by aerosol with *M. tuberculosis* HN878 (6 weeks postinfection). Big confluent lung granulomas. Magnification, x10.



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### **Fund Donations**

ABSA thanks the many of you have generously contributed to the Richard C. Knudsen Memorial Fund. The proceeds from this fund will be used to establish an award to honor Rich's memory. Those wishing to make donations to this fund should make their checks payable to the American Biological Safety Association. Please add a notation to the memo line that the check is to be used for the Richard C. Knudsen Memorial Fund. Checks should be mailed to ABSA, 1202 Allanson Road, Mundelein, Illinois 60060-3808.