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(54) Title: ULTRA LOW DOSE OF AEROSOLIZED MYCOBACTERIUM TUBERCULOSIS: MODEL OF PARTIAL INFECTION

(57) Abstract: The invention provides an animal model and methods for producing an animal model of partial *Mycobacterium tuberculosis* (MTB) infection. Methods for ultra-low dose administration of MTB or other bacterial pathogens for generating an experimental animal model of partial infection are disclosed. This animal model is useful for measuring vaccine efficacy and/or animal susceptibility or resistance following administration or exposure to vaccines, treatments or environmental agents.



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Ultra Low Dose of Aerosolized *Mycobacterium Tuberculosis*: Model of Partial Infection

Priority

This application claims priority to U.S. Provisional Patent Application Serial No. 61/470,256 filed March 31, 2011, which is incorporated herein by reference in its entirety.

Field of the Invention

The invention provides an animal model and methods for producing an experimental animal model used for determining a host's susceptibility or resistance to *Mycobacterium tuberculosis* (MTB) and other pathogens. More specifically, the invention provides methods for generating an animal model of partial bacterial infection. The invention further provides methods for accurately measuring a host's susceptibility or resistance to MTB infection. Such methods are useful for identifying host factors that provide resistance to infection, for testing vaccine and treatment efficacy, or assessing the impact of environmental agents on infection.

Background of the Invention

A number of murine models have been developed to study tuberculosis (Beamer et al., 2005, *Arch. Immunol. Ther. Exp.* 53(6): 469-483; Cardona et al., 2004, *Infect. Immun.* 72(2): 1065-1071; Hovav et al., 2006, *Microbes Infect.* 8(7): 1750-1757; Steenwinkel et al., 2009, *Int. J. Immunopathol. Pharmacol.* 22(3): 723-24). In these models, mice were challenged with a low dose (LD) of aerosolized *Mycobacterium tuberculosis* (MTB), typically in a dose range between 100-1400 colony forming units (CFU)/lung (Orme et al.,

2007, *Curr. Protoc. Microbiol.* 7:10A.15.11-10A.15.29; Rhoades et al., 1997, *Tuber. Lung Dis.* 78(1): 57-66). The low dose exposure causes disseminated MTB infection in the lungs that eventually plateaued at around 10^5 - 10^7 CFU at 4 weeks. The infection can also disseminate from the lungs to other organs such as the liver and spleen during the early phase of infection (Chackerian et al., 2002, *Infec. Immun.* 70(8): 4501-09). This progressive chronic disease model results in an early cell-mediated immune response, tissue destruction and eventually death of the infected mice.

The low dose (LD) model is used to study host susceptibility or resistance to disease progression. However, there exists a need in the art for an animal model of partial infection. Such models allows for a more accurate assessment of host susceptibility or resistance to initial MTB infection. Furthermore, such models would aid in identification of host factors that influence MTB infection. Additionally, such models would facilitate selection of appropriately tailored vaccines, and in turn aid in prevention of pulmonary tuberculosis.

Summary of the Invention

This invention provides an animal model and methods for producing experimental animal models to assess susceptibility or resistance to MTB infection. In the practice of the methods of this invention, animals are exposed to an ultra-low dose of MTB aerosol, and as an example the MTB dose is about 1 CFU/lung to about 11 CFU/lung. Following MTB exposure, the methods include determining if an animal is infected by using the presence or absence of MTB in the animal's lungs, other organs, or bodily fluids as a measurement.

In one embodiment, the invention provides an animal model of attenuated MTB infection. Such a model can be used to assess the effectiveness of vaccines and MTB treatments. For example, the susceptibility of an animal to MTB infection following vaccine administration can be more accurately assessed by practicing the claimed methods.

In an alternative embodiment, experimental animal models can be used to identify and evaluate host factors of an animal that contribute to an animal's susceptibility or resistance to MTB infection. This can be achieved through statistical analysis to identify mechanisms or factors that convey susceptibility or resistance to MTB infection.

The use of the methods of this invention is beneficial for measuring and determining vaccine or treatment efficacy. This in turn permits the clinical selection of vaccines and treatments better suited for inhibiting MTB infection.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of the Drawings

This invention can be further appreciated and understood from the following detailed description taken in conjunction with the drawings wherein:

Figure 1 is a photograph of the multiplex sampling port added to a Madison chamber used to monitor chamber conditions and bioaerosol characteristics. The APS, BioSampler, RH/Temperature probe, and chamber pressure sensor are attached to the port. The port penetrates the door of the Madison chamber to sample the aerosol as delivered to the animals.

Figure 2 is a diagram of a whole body exposure system. The modified Madison chamber is connected to a class III biological safety cabinet (BSC, The Baker Company IsoGARD® series Glovebox). The Collison nebulizer which is attached to the front door of the Madison chamber is located inside the class III BSC. This setup enhances the safety of operation. A 1" diameter stainless steel port was installed through the door and the internal baffle of the Madison Chamber to allow sampling of the aerosol at the location of animal exposure. The AeroMP (Aerosol Management Platform, Biaera Technologies, LLC) included in the system receives real-time data from the chamber pressure sensor,

RH/temperature probe, and Aerodynamic Particle Sizer (APS, TSI Inc., Model 3321). It also controls the nebulizer flow, dilution air flow, humidifier, BioSampler, and APS.

Figure 3 is a graph demonstrating particle concentration measured during aerosol delivery. Polystyrene latex spheres of 1.0 μm diameter were aerosolized in the chamber for three different days. Each aerosol exposure was run for 20 minute with one minute sampling interval. The mean concentrations of beads in the chamber measured on each of the three days were 2.95 e2, 2.81e2 and 2.78e2 particles/mL.

Figure 4 is a graph demonstrating the particle size distribution by count. Bacteria or polystyrene latex spheres were aerosolized in the Madison chamber using a Collison nebulizer. Aerosols were sampled through the multisampling port and analyzed using the APS. The graph includes actual counts normalized to a peak of 1.0.

Figure 5 is a graph demonstrating the particle size distribution by mass. Bacteria or polystyrene latex spheres were aerosolized using a Collison nebulizer. The APS sampled and analyzed the aerosol inside the Madison chamber through the multisampling port. The APS measured aerodynamic particle sizes, and this data was used to estimate particle mass. This graph includes mass distributions normalized to a peak of 1.0.

Figures 6A-6C are graphs illustrating MTB colony forming units in mice lungs (Fig. 6A), livers (Fig. 6B) and spleens (Fig. 6C) at various time points after exposure to MTB aerosol. Four groups of mice were exposed to MTB aerosol with a dose ranging from 1 to 395 CFU/mouse as summarized in Table 4. At 24 hours, 4 weeks, 10 weeks and 18 weeks post infection, CFU values in the lung, liver and spleen were determined by necropsy. The limit of detection (LOD) is represented for each group at each time point as a dashed line. In instances where no bacteria were grown, the results are plotted on the X-axis.

Figure 7 is a graph illustrating MTB CFU in the lungs of mice in groups E-G at 3-5 weeks after ultra-low dose exposure. Three ultra-low dose exposures, 1.1, 1.6 and 11

CFU/mouse were carried out (Table 5) and lung CFU was determined by necropsy at 3-5 weeks after exposure. The LOD is represented for each group at each time point as a dashed line. In instances where no bacteria were grown, the results are plotted on the X-axis.

Figure 8 is a graph illustrating the proportion of mice infected based on presented dose. The Aerosol exposure groups A-G are described in Tables 4 and 5. The area of each data point is proportional to the total number of mice in the group. Curves were plotted based on a sigmoid dose-response (solid line) and a Poisson distribution (dashed line) as described in Methods. The best-fit curves were determined by weighted least squares.

Figure 9 is a graph showing the lung burden in naïve and bacille Calmette-Guerin BCG vaccinated mice 3-5 weeks after infection. P-value was calculated by unpaired *t*-test.

Detailed Description of the Preferred Embodiments

The invention is more specifically described below and particularly in the Examples set forth herein, which are intended as illustrative only, as numerous modifications and variations therein will be apparent to those skilled in the art.

As used in the description herein and throughout the claims that follow, the meaning of “a”, “an”, and “the” includes plural reference unless the context clearly dictates otherwise. The terms used in the specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Some terms have been more specifically defined below to provide additional guidance to the practitioner regarding the description of the invention.

This invention involves methodology for generating an experimental animal model to assess susceptibility or resistance of an animal to MTB infection. These animal models provide a system for investigating the process of MTB infection as well as a means for infection prevention.

In contrast with other prior art models and methods of production, which administer MTB at a dose of 100-400 CFU aerosol, the methods of the present invention use an ultra-low dose (ULD) of 1-11 CFU aerosol. By exposing animals to an ULD aerosol, the median infectious dose will be about 1.4 CFU/mouse to about 1.9 CFU/mouse. The ultra-low dose aerosol causes only a portion of animals exposed to the MTB aerosol to develop infection whereas the standard dose of 100-400 CFU results in all exposed animals to develop diffuse pulmonary infection. Thus the practice of the claimed methods provides an attenuated animal model of MTB infection. Because a reduced number of animals develop MTB following the practice of the inventive methods, the methods allow for a more accurate assessment of vaccine efficacy. Such a model permits assessment of infection rates for an experimental population, not merely absolute MTB infection for an exposed animal. Furthermore, use of the ULD model allows for assessment of susceptibility in murine models 4 weeks post ULD exposure, which is a faster method of determination compared to the currently used end point of median survival time (Medina et al., 1998, *Immunology*. 93(2): 270-274).

The term “animal” as used herein refers to a non-human mammal, including without limitation laboratory animals such as mice, rats, hamsters, gerbils, rabbits, and guinea pigs. In one embodiment the animals used in this invention are preferably mice although of the MTB dosage can be adjusted to account for inter-species differences in development. Thus, similar effects may be obtained in mammals other than mice.

The term “resistance” as used herein refers to the capacity of an animal to fail to develop infection following exposure to MTB or other pathogens.

The term “susceptibility” as used herein refers to the capacity of an animal to develop infection following exposure to MTB or other pathogens.

As used herein, “partial” infection refers to infection of less than all (*e.g.*, 100%) of the animals in an experimental population that is exposed to aerosolized MTB or other

pathogens. Similarly, the term “attenuated” as used herein refers to infection of a population of animals wherein less than all of the animals develop infection. In a preferred embodiment, the infection rate is 50%.

The phrase, “measuring the rate of infection” as used herein refers to determining the number of animals that become infected with MTB, or other pathogen, in an experimental population following exposure to aerosolized MTB. In certain embodiments, infection can be diagnosed by the presence of MTB in the lungs or other organs such as, for example, spleen or liver. In specific embodiments, infection can be determined by routine analysis of blood, saliva, other body fluids, and/or cell or tissue samples.

The bacteria dose delivered to the animals can be varied by using different concentrations of bacterial inoculum. For example, the inoculum can be diluted in a buffer to bring the final inoculum concentration to the desired level. Preferably the inocula is prepared from frozen stocks of MTB and diluted to a final concentration using phosphate buffer saline (PBS) containing 0.05% Tween 80. The dose can also be varied by manipulation of the aerosol delivery system. Preferably the desired levels of bacteria dose are between about 1 CFU to about 11 CFU/mouse. More preferably, the desired levels of bacteria are between about 1 colony forming unit/lung (CFU) to about 1.6 CFU/lung. More preferably the desired level of bacteria is about 1 CFU/lung.

The aerosolized MTB can be delivered to the animals for example using a whole-body Madison chamber. The chamber may be equipped with a sampling device that samples the air in the chamber to determine the viable aerosol concentration. The sampling device can be used to measure a number of aerobiology parameters for example, viable aerosol concentration, spray factor, dose presented, VARR, and particle size characteristics can be measured. VARR is herein defined as the rate at which the viable aerosol volume is retained in the animal's lungs compared to the viable aerosol concentration in the chamber. VARR

quantifies the ability of a microbe to deposit and survive in the lungs and is based directly on the measure values without including adjustments based on predicted minute ventilation.

The spray factor measures the viable efficiency of the aerosol that is aerosolized compared to the initial inocula. In some examples, VARR can be used in conjunction with spray factor to measure the efficiency of aerosol that was generated and delivered to the animals. These measurements provide the user with many advantages such as allowing the user to optimize the viable aerosol concentration without the need for experimental animals. Also the sampling device is advantageous in that it allows the effect of humidity, flow rates, and inoculum preparation methods on the viable aerosol concentration to be directly measured.

The presence or absence of bacteria in the lungs of an animal exposed to MTB aerosol is used as a primary endpoint indicative of infection. In particular, the presence or absence of bacteria is measured by using lung homogenates of the animals exposed to MTB aerosol and determining the number of CFU/lung. These measurements are conducted at various time points including but not limited to 24 hours, 4 weeks, 10 weeks, and 18 weeks post exposure. In addition, the CFU burden in the liver and spleen of an exposed animal is measured to determine the level of infection in exposed animals.

The presented dose and the retained dose is calculated to determine appropriate levels of aerosol delivery required for partial infection of exposed animals. The presented dose represents the number of viable bacterial CFU inhaled by an animal during exposure and the retained dose in the lungs is determined by lung necropsy from a sample of the exposed animal taken 24 hours post-exposure.

The dose presented (D_p) is be calculated using Guyton's formula (Guyton 1947).

$$V_m = 2.10 \times BW^{0.75} \quad (1)$$

where BW is weight of mice in grams and V_m is minute volume.

$$V_E = V_m \times t_d \quad (2)$$

where V_E is the exposure volume and t_d is exposure time

$$D_p = V_E \times C_a \quad (3)$$

where D_p is dose presented and C_a is the viable aerosol concentration

Viable aerosol concentration in the Madison chamber used in dose presented formula (Eq. 3) can be calculated as

$$C_a = \frac{C_{sam} \times (V_{Sampler} - (E_c \times t_d))}{Q_{Sampler} \times t_d} \quad (4)$$

where C_{sam} is concentration in sampler, $V_{Sampler}$ is sampler initial volume, E_c is evaporation rate, t_d is exposure time duration, and $Q_{Sampler}$ is sampler airflow rate

Spray factor (Swearengen, 2005, *Biodefense: Research Methodology and Animal Models*: CRC Press) can be calculated as

$$F_s = \frac{C_a}{C_s} \quad (5)$$

where F_s is spray factor, C_a is concentration of bioaerosol (CFU/mL), C_s is concentration of liquid (CFU/ mL).

The dose retained in the lungs (D_R) can be determined by titring the lung homogenate. The dose retention efficiency can be calculated as D_R divided by D_p .

The viable aerosol retention rate (VARR) can be calculated as

$$VARR = \frac{D_R}{C_a \times t_d}$$

After the animal model is generated according to the methods herein described, the animal model is used to study the infection rates of different host genetic backgrounds. For instance, partial infection of exposed animals will facilitate the identification of genes associated with susceptibility to TB infection. As previously described, “attenuated” or “partial” infection refers to infection of less than all of the animals exposed to aerosolized MTB or other pathogens. The identification of genes associated with susceptibility to TB infection can be achieved by comparing SCID mice to wild-type controls exposed to ULD MTB aerosol to determine the role of acquired immune response in protection from infection. The portion of mice infected with MTB is compared using Fisher Exact Test and lung log₁₀ CFUs can be compared by *t*-test. A positive result can be used to identify which component of acquired immunity is most important in susceptibility to MTB infection. As used herein a positive result includes a p-value of 0.05.

Further studies to evaluate mechanisms conveying susceptibility or resistance to MTB infection include studies of the early kinetics of MTB infection of alveolar macrophages and other host cells in the animal. This is accomplished by infecting animals with a high number of aerosolized MTB bacteria (1000 CFU dose retained), then analyzing cell preparations from whole lungs to determine the numbers of intracellular and extracellular bacteria, and determining subsets of lung cells containing viable intracellular MTB at multiple early time points including 4 hours, 1 day, 3 days, 7 days. By tracking the numbers of intercellular and extracellular bacteria, it can be determined whether protection from infection is mediated by interference with the infection of the first mammalian cell, or by clearance of the bacteria after MTB has entered mammalian cells.

The experimental animal model is also used to evaluate the effects on infection rates of vaccine-induced immunity. Many vaccines, including BCG, reduce the organ CFU at 4 or 8 weeks by 1-2 logs, and prolong survival time in the standard mouse model. Human BCG trials have demonstrated variable effects in reducing MTB disease. (Sunderland et al., 1979, *Tubercle*. 60(4): 225). There are no human or animal studies evaluating the effect of BCG on the rate of MTB infection. The standard method for detecting MTB infection in humans, the tuberculin skin test, often becomes positive after BCG vaccination, complicating analysis of MTB infection rates after BCG vaccination. To assess the effect of BCG vaccine on MTB infection, a vaccine is delivered by the standard subcutaneous route and by aerosol to animals and then exposing the animals to ULD MTB aerosol. The animals are then evaluated to determine CFU counts in the animal's lungs. Demonstrating vaccine-induced protection from infection in the experimental animal model will lead to a new efficacy goal for TB vaccine candidates, both in preclinical animal studies and in human clinical trials, namely reducing the rate of TB infection.

Furthermore, the experimental animal model can be used to evaluate the effect of environmental agents, and in particular, particulate materials (PMs) on MTB infection. Human populations encounter PMs through air pollution or industrial exposure, which leads to lung damage. Silicosis, a chronic lung disease caused by exposure to silica, is known to increase the risk of active TB disease in humans. The experimental animal models are used to evaluate the effect of PMs including carbon black, silica, and carbon nanotubes on susceptibility to MTB infection. As a mechanistic control for the PM studies, liposomes containing dichloromethylene bisphosphonate (CL₂MBP) to induce depletion of alveolar macrophages can be used. CL₂MBP liposomes are instilled at various days relative to TB aerosol challenge. These liposomes leads to depletion of alveolar macrophages in mice. Bronchio-alveolar lavage (BAL) total and differential cell populations and cytokine are

utilized after exposure to PMs to determine pulmonary inflammation in the animal. Cytokines including TNF- α , IL-1 β , and TGF- β 1 are analyzed to determine inflammation. Acute lung injury has the potential to reduce or increase susceptibility to MTB infection. If lung injury is shown to enhance MTB infection, this could have implications for human populations in the developing world where high rates of MTB exposure co-exist with high levels of air pollution.

Embodiments of the methods of this invention comprising the above-mentioned features are intended to fall within the scope of this invention.

Examples

The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They set forth for explanatory purposes only, and are not to be taken as limiting the invention.

Example 1

Bioaerosol in Whole Body Exposure System

Multiple factors influence viability of aerosolized bacteria. For instance, the conditions of aerosol delivery using a whole body exposure system will effect the viability of aerosolized bacteria. The current disclosure provides analysis of conditions of bioaerosol delivery using a whole body exposure system for determining optimum bioaersol dosage.

Aerobiology setup

A modified Madison chamber was used for aerosol exposure and assessment of conditions within the chamber during exposure (Fig. 1). The aerosol exposure was conducted with the turnkey system AeroMP (Aerosol Management Platform, Biaera Technologies, LLC) (Hartings et al., 2004, *J. Pharma and Toxicol. Meth.*, 49(1), 39-55). The AeroMP controlled, monitored, and recorded the aerobiology parameters. All the aerobiology

parameters of nebulizer, dilution, and sampler air flows were programmed into the AeroMP software before the start of the aerosol exposure. The total airflow (aerosol and dilution) entering the Madison chamber was set to 50 liters per minute (lpm) by the AeroMP. After the animals were loaded into the chamber, the exposure was initiated by executing the software. Ambient conditions of the chamber were monitored using an RH and temperature probe attached to the multiplex sampling port. Humidified air was generated using an Air Bubbler connected to the AeroMP which regulated flowrate to set the chamber humidity to 50% RH (Fig. 2).

Exhaust of the Madison chamber was connected to a vacuum pump which generated exhaust flow while maintaining negative pressure inside the chamber. The AeroMP was used to control the flow rate, and a control valve located between the two exhaust filters and the vacuum pump (Baker Manual 2006) was used to maintain chamber pressure at -13 ± 1 ”WC.

To measure the chamber pressure one of the outlets of the multiplex sampling port was connected to the pressure sensor in the AeroMP. The chamber pressure was displayed in real time during the exposure run on AeroMP software. The software also recorded all the exposure parameters (airflow, chamber pressure, RH, and temperature) every five seconds.

Aerosol concentration and spray factor in the chamber could be reduced if there were any leaks in the sampling port or in the chamber itself. This issue was resolved by doing a positive pressure decay test on the chamber before generating any aerosol. A positive pressure decay test was performed on the chamber connected to the multiplex sampling port and Collison nebulizer before every aerosol exposure. The end connections of the port were sealed with a sanitary fitting. The chamber was pressurized to 3” of water column with air flow supplied with the AeroMP at 10 lpm. The chamber was considered to be in an operational condition for aerosol exposure experiments if the positive pressure in the chamber

did not drop more than 20% in 30 minutes. Aerosol exposures were conducted once the setup had passed the pressure decay test.

To generate the aerosols, a 6-jet Collision nebulizer (CN25, BGI Incorporation) was operated at 13 ± 1 lpm and 19 ± 1 pounds per square inch (PSI). For *M. tuberculosis* aerosols the nebulizer was operated at a higher flow 19 ± 1 lpm corresponding to 35 ± 1 PSI (Baker Manual 2006).

To determine the viability of the aerosol in the chamber, an all-glass impinger (AGI-4) (Ace Glass Inc., Model 7541-10) or BioSampler (SKC Inc., Model 225-9595) was connected to the multiplex sampling port. The AGI-4 was used to capture *Y. pestis* aerosol operating at a flowrate of 6 lpm while the BioSampler was used for *M. tuberculosis* aerosol operating at 12.5 lpm. A 20 mL volume of Heart Infusion Broth with 0.05% antifoam (Sigma-Aldrich Co., A5758) was used as the capture media solution in the impinge and 15 mL of phosphate-buffered saline (PBS) 1X was used in the BioSampler. Operation of both the devices was controlled by AeroMP and they were operated full time during the 20 or 60 minute aerosol exposure cycle. The samplers were turned off during the 20-minute purge cycle after the aerosol exposure.

To measure the particle size distributions of various aerosols, the APS (aerodynamic particle sizer) was connected to the multiplex sampling port (Fig. 2). No diluter was used along with the APS. The APS operation is controlled by AeroMP which set the APS flowrate at 5 lpm and sample for 30 seconds every 5 minutes, starting at 1 minute into the aerosol exposure. The vacuum pump of the APS was kept running during the whole exposure to maintain a constant airflow and stable negative pressure in the Madison chamber. AeroMP software was used to gather and analyze particle diameter and count data. The particle size distribution plots were generated using Microsoft Excel.

APS 3321 was calibrated for both size and concentration using the TSI SMPS 3080 classifier and TSI Condensation Particle Counter 3772. Similar, the AeroMP was yearly calibrated for accurate flow, pressure, temperature and RH (%) readings.

PSL bead sample preparation

Polystyrene latex (PSL) spheres of 1.0 μm size (5100A, Duke Scientific Corp.) were aerosolized in the chamber using the 6-jet Collison nebulizer at 13 ± 1 lpm and 19 ± 1 PSI. PSL beads were diluted 200 times in distilled water for a total volume of 15 mL. For each aerosol run a new bead solution was prepared and sonicated for 1 minute prior to aerosolization. The PSL aerosol was sampled at every one minute interval during the 20 minute aerosol.

The mean concentrations of beads in the chamber were measured on three different days (Fig. 3). The coefficient of variance of the mean concentration aerosolized on three different days was 3.19%. The particle size distribution for aerosolized beads is shown in Fig. 4 by count and in Fig. 5 by mass. The count mean aerodynamic diameter (CMAD) and mass mean aerodynamic diameter (MMAD) of the beads aerosolized on three different days were both 0.96 μm with a geometric standard deviation (GSD) of 1.06. The GSD was calculated using the particle size distribution by mass (Hinds, 1999, *Aerosol Technology: Properties, Behavior, and Measurement of Airborne Particles*: Wiley Interscience).

***B. anthracis* spore inocula preparation**

To prepare the *B. anthracis* spores fresh 34F2 was streaked on a blood agar plate and incubated overnight at 37°C. A single colony of *B. anthracis* was placed into 6 mL Brain Heart Infusion broth, and then shaken at 300 RPM at 37°C until turbid. A 1-mL volume of the culture was added to 200 mL Minimal Growth Medium in a 1 L baffled flask. The solution was shaken at 300 RPM at 37°C for 2 days to induce sporulation. The spore preparation was washed three times in sterile water, and incubated for 30 min in a 65°C water

bath for 30 min to kill any vegetative anthrax. The spore preparations were pelleted once more, then resuspended in 10 mL sterile water, and stored at room temperature.

For this study mice were challenged with aerosol of *B. anthracis*. Six-eight old C57BL/6 mice were used for the aerosol exposures in the whole body exposure system. Animals were acclimatized in an animal holding area set to a 12-hour ON/OFF light cycle, 21°C room temperature and 50% relative humidity for one week prior to exposure.

Starting and ending inocula concentrations were measured before and after aerosolization. Table 1 shows aerosol parameters for *B. anthracis* spores including the dose presented and the dose retained in the lung homogenate obtained one hour after exposure. The CMAD and MMAD of anthrax spores were 0.98 and 1.25 microns with a GSD of 1.6 (Figs. 4 and 5). The vegetative *B. anthrax* CMAD and MMAD were measured to be 1.29 and 1.35 microns with a GSD of 1.33 (Figs. 4 and 5). The dose presented was theoretically calculated using the Guyton's formula and was close to the dose retained in the mouse lungs after 1 hr necropsy. The spray factor for the *B. anthracis* spores was higher than *Y. pestis* or *M. tuberculosis* and was close to that of the PSL test particles. This could be a result of the *B. anthracis* spores ability to survive for prolonged periods under harsh environmental conditions. Dose retention efficiency and VARR was also highest for *B. anthracis* spores. These results could be because the spores evade the lung defense mechanisms thereby surviving in the mouse lungs longer (Welkos et al., 1986, *Infect. Immun.*, 51(3), 795-800).

Table 1: Aerosol parameters for *B. anthracis* spores and polystyrene latex beads

Material	Concen- tration in chamber	Spray Factor	Dose Presented (mean CFU±SD)	Dose Retained (mean CFU±SD)	Dose Retention Efficiency (%)	Viable Aerosol Retention Rate (mL/min)

<i>B.anthraxis</i>	2.11e2 CFU/mL	2.13e-7	2.50e5± 1.12e4	2.14e5± 6.7e4	84	16.9
PSL	1.60e2 particles/mL	3.07e-7	-	-	-	-

***Y. pestis* inocula preparation**

Frozen stocks of *Y. pestis* stored at -80° C were used to prepare the culture medium. One mL of thawed stock solution was added to 100 mL of Heart Infusion Broth containing 100 µM FeCl₂ and 1 mL of 50% glycerol. The solution was incubated at 27°C in a shaker incubator at 250 rpm for 24 hours. *Y. pestis* was grown in log phase overnight; its optical density (OD) was measured using a spectrophotometer at 600 nm. Once the OD was between 0.4-0.7, an 8 fold dilution of the solution was done. Bacteria were collected by centrifugation for 10 min at 2000 rpm (863g) and washed three times, first with HI media, second with 1:1 mix of HI media and diH₂O, last with just diH₂O. Washed bacteria were resuspended in diH₂O at concentration of OD = 2.0 and sonicated using Misonix 2000 (output level 30%; three 10-second pulses with 20-second pause between pulses). Final inoculum preparation of 20 mL was supplemented with 0.2% xylose (Sigma-Aldrich Co, X2126-25G). 0.002% of anti-foam Y-30 (Sigma-Aldrich Co., A5758) was added immediately prior to nebulization. 100 µl of sonicated inoculum was used for determining starting inocula concentrations. The starting, ending inocula, and sampler concentrations were done within 1 hour of aerosol exposure.

For this study C57BL/6 mice (n = 3) were challenged with aerosol of *Y. pestis* KIM5 strain on three different days. Starting and ending inocula concentrations were measured before and after aerosolization showed slight viability loss in the nebulizer. Dose retained in the mouse lungs at 1 hr post exposure had similar counts. The theoretically calculated dose

presented using Guyton's formula was higher. Table 2 shows aerosol parameters for *Y. pestis* spores including the dose presented and the dose retained in the lung homogenate obtained one hour after exposure. The CMAD, MMAD measured by the APS were 0.78, 1.04 microns, respectively and GSD was 1.90 (Fig. 4). The Particle size distribution of *Y. pestis* was measured in real-time during the aerosol exposure. (Figs. 4 and 5).

The ability of microorganism to undergo the process of nebulization and aerosolize in the Madison chamber was estimated based on the viable aerosol concentration and spray factor (Swearengen, 2005, *Biodefense: Research Methodology and Animal Models*: CRC Press)). Three different runs of *Y. pestis* showed similar spray factor values, however, they were less than that of test particles. *Y. pestis* aerosol gave the lowest dose retention efficiency and VARR values.

Table 2: Aerosol parameters for *Y. pestis* bacteria.

Starting Inoculum Concentration (CFU/mL)	Ending Inoculum Concentration (CFU/mL)	Viable Chamber Concentration (CFU/mL)	Spray Factor	Dose Presented (mean CFU±SD)	Dose Retained (mean CFU±SD)	Dose Retention Efficiency (%)	Viable Aerosol Retention Rate (mL/min)
5.75e9	5.10e9	6.52e1	1.13e-8	25500±200	4280±335	17	3.3
7.90e9	7.00e9	6.09e1	0.83e-8	23433±666	4270±568	18	3.5
5.70e9	3.20e9	4.59e1	0.80e-8	17233±493	3570±230	21	3.9

***M. tuberculosis* inocula preparation**

Frozen stocks of *M. tuberculosis* were used for preparing inocula for aerosolization studies. Frozen stock was thawed, and calculated volume of stock was diluted in 2 mL solution of PBS and tyloxapol. It was then sonicated twice for 15 seconds with two minute interval. The solution was diluted in PBS (containing 0.05% Tween 80) to bring the final

volume to 15 mL. 0.002% of antifoam Y-30 was added to the final volume. The solution was poured into the precious liquid jar of the Collison nebulizer. The starting, ending inocula, and sampler concentrations were done within 1 hour of aerosol exposure.

B6 mice (n = 4) were challenged with four different *M. tuberculosis* aerosols each for 20 minutes. The particle size distribution (PSD) of *M. tuberculosis* was measured by the APS, CMAD and MMAD were 0.77 μm and 1.98 μm , respectively and GSD was 2.37 (Figs. 4 and 5). Results of the exposures are shown in Table 3. There was a 100 fold loss between the starting and ending inocula concentrations. The dose retained in mice at 24 hours was in a range of 119-210 CFU/lung. *M. tuberculosis* aerosol had the lowest viable aerosol concentration and its spray factor was 40 times lower than that of PSL beads. Theoretically calculated dose retained using the Guyton's formula was higher than the dose retained in the mouse lungs. *M. tuberculosis* aerosol gave dose retention efficiency and VARR intermediate between *B. anthracis* and *Y. pestis* aerosols.

Table 3: Aerosol parameters for *M. tuberculosis* bacteria.

Starting Inoculum Concentration (CFU/mL)	Ending Inoculum Concentration (CFU/mL)	Viable Chamber Concentration (CFU/mL)	Spray Factor	Dose Presented (mean CFU $\pm$ SD)	Dose Retained (mean CFU $\pm$ SD)	Dose Retention Efficiency (%)	Viable Aerosol Retention Rate (ml/min)
1.02e8	1.86e6	0.40e0	0.40e-8	172 $\pm$ 9	119 $\pm$ 42	69	14.9
3.30e7	2.48e6	1.22e0	3.71e-8	526 $\pm$ 10	210 $\pm$ 24	40	8.75
1.08e8	3.20e6	1.33e0	1.24e-8	574 $\pm$ 23	168 $\pm$ 12	29	6.45
2.13e8	--	1.37e0	0.64e-8	584 $\pm$ 25	171 $\pm$ 18	29	6.25

Lung Homogenates

To isolate lungs for measurement following MTB exposure, lungs were harvested by necropsy within 1 hour of *Y. pestis* or *B. anthracis* aerosol exposure and 24 hours of *M. tuberculosis* aerosol exposure. Lungs were removed from the mice using sterile techniques into WhirlPak bags filled with 2 mL of PBST. Lungs were then homogenized in the bags using Stomacher, with two 120 second cycles, with the bags flipped in between. Homogenized lungs were plated in an appropriate bacterial medium to determine titer, and to a blood agar plate to screen for contamination. Titer plates were incubated at 37°C (*B. anthracis* and *M. tuberculosis*), or 27°C (*Y. pestis*) until distinct colonies were visualized.

Example 2

Ultra-low Dose (ULD) of Aerosolized Mycobacterium Tuberculosis in Mice

This study was conducted to determine the amount of MTB aerosol required to only impart partial infection on the experimental set of animals.

Bacterial strains and growth conditions.

MTB H37Rv (MTB) was used for ultra-low dose aerosol challenge. Frozen stock of MTB with an 0.72 optical density was used to prepare the inoculum. After thawing the frozen stock a calculated volume of stock was diluted in 2 mL solution of PBS containing 0.05% tyloxapol. The solution was then sonicated twice for 15 seconds with two minute interval using an ultrasonicator (Misonix S-4000, Qsonica, LLC) and diluted in PBS containing 0.05% Tween 80 to bring the final volume of the starting inoculum to 15 mL. Antifoam Y-30 (Sigma-Aldrich Co., A5758) at a concentration of 0.002% was added to the inoculum prior to the aerosol exposure. Bacterial titers were performed to determine the viable concentration of the inoculum before and after aerosolization, and to determine the viable aerosol concentration in the chamber.

Aerosol exposures

To establish the ultra-low dose infection model various groups of mice were exposed to different concentrations of MTB aerosol. The aerosol dose was varied by using differing concentrations of bacterium inoculum. A biosampler was used to sample the air in the chamber throughout the exposure to determine the viable aerosol concentration and Guyton's formula was used to calculate the minute ventilatory volume, and the dose presented to the mice (Swaim et al., 2006, *Infect. Immun.* 74(11): 6108-6117; Swearengen, 2005, *Biodefense: Research Methodology and Animal Models*: CRC Press)).

Four groups of mice were exposed to the varied concentrations of MTB aerosol with a dose presented ranging from 1 to 395 CFU/lung using a whole-body system Madison chamber (Table 4). The Madison chamber was connected to a BioSafety Level-3 cabinet (Saini et al., 2010, *J. Pharmacol Toxicol. Meth.* doi:10.1016/j.vascn.2010.09.002). Aerosol was generated using a 6-jet Collision nebulizer operated at 19 ± 1 lpm and 35 ± 1 PSI (Baker Manual, 2006). The aerosol exposure was controlled by a turnkey system, Aerosol Management platform (AeroMP, Biaera Technologies, LLC) (Hartings et al., 2004, *J. Pharmacol Toxicol. Meth.* 49(1): 39-55). The total airflow through the Madison chamber was set to 50 lpm by the AeroMP. The mice were loaded into the chamber with the air flow running through the system. Time duration of aerosol exposures was 20 minutes, initiated by executing the software.

Groups A through D received presented doses ranging from 1 to 395 CFU/ mouse, corresponding to a retained dose of <2 to 53. The presented dose represents the dose inhaled by the mouse, while the retained dose was determined by lung necropsy at 24 hours. Group D is an example of a LD aerosol exposure, and Groups A-C are examples of ULD exposures.

All mice in groups B, C, and D were infected based on detection of MTB in lung, liver, and spleen at 4, 10, and 18 weeks (Figs. 6A-C). Infection was detected in only one mouse in group A at each time point. There was a positive dose-response relationship

between the presented dose and the lung CFU at each time point. Based on linear regression between log-transformed presented dose and log-transformed lung CFU, the p-values for the correlation were <0.001 , <0.001 , 0.021, and 0.078 at 24 hours, 4 weeks, 10 weeks, and 18 weeks, respectively.

The lung burden of mice in all the four groups increased rapidly over the first 4 weeks, then slowed between weeks 4 and 18. This pattern has been observed in previous reports using the LD aerosol infection model. ULD exposure led to a persistently lower lung CFU than the LD exposure. The lung CFU was lower in group B compared to D at 24 hours, 4 weeks, and 18 weeks by 0.8, 1.4, and 1.4 logs respectively ($p = <0.001$, <0.001 , and 0.01). Dissemination to liver and spleen was observed at 4, 10, and 18 weeks. There was greater variation in CFU at these sites. The dose-response relationship was statistically significant in both liver and spleen at 10 weeks, but not at 4 and 18 weeks.

Based on the dose presented, the results demonstrate that mice can be infected with a very low dose of MTB aerosol and suggest that some mice were infected by a single bacterium delivered to the lung. These results further provide a method for delivery of ultra-low dose of TB to mice. This partial infection model can be advantageous in testing vaccines and host factors that can influence TB infection, by exposing treated and untreated groups of mice to the same ultra-low dose aerosol. The two groups can then be analyzed for differences in rates of proportion infected with MTB.

Table 4: Results from four aerosol exposures with varied doses of MTB

Aerosol Exposure Group	Starting inoculum OD	Dose (CFU/mouse)		Infected/Total Mice (%)			Combined Infected/Total Mice
		Presented	Retained	4 wk	10 wk	18	

			(24 h)			wk	(%)
A	3.33e-4	1.0	<0.5 ⁺	1/4 (25)	1/4 (25)	1/3 (33)	03/11 (27)
B	3.33e-3	5.4	7.5±3.42	4/4 (100)	ND	3/3 (100)	7/7 (100)
C	3.33e-2	106	17.5±6.61	4/4 (100)	4/4 (100)	3/3 (100)	11/11 (100)
D	3.33e-1	395	53.0±7.02	4/4 (100)	4/4 (100)	3/3 (100)	11/11 (100)

The primary endpoint and secondary endpoints were the proportion of mice infected and the organ CFU counts. The ID₅₀ was estimated by the method of Reed and Muench et al., 1938. *Am. J. Epidemiol.* 27(3): 493-497) and by fitting curves for a sigmoid dose-response and for a Poisson distribution. The sigmoid curve was represented by $P = K / (1 + e^{-(a + (b \times \log D_p))})$, where P is the proportion of animals infected, and D_p is the presented dose. The constant K represents the maximal response, while constants a and b together generate the slope and intercept. The Poisson curve was represented by $P = 1 - e^{-(D_p/D_I)}$, where the constant D_I represents the presented dose corresponding to an infection event. Constants were varied to generate the best fit, defined by least squares, weighted by group size. Log-transformed organ CFUs were compared by t-test. The correlation between the log-transformed presented dose and the log-transformed organ CFU was assessed by linear regression.

Establishing Reproducibility of Partial Infection in Mice

To achieve partial infection after ultra-low dose exposure, the dose presented should be highly consistent from day to day. To establish reproducibility of the partial infection method three ultra-low dose exposures on different days were carried out (Table 5). Groups E, F, and G received presented doses of 1.1, 1.6, and 11 CFU/ mouse, resulting in infection of 36%, 36%, and 95% of mice, respectively (Table 5). The retained dose was not measured in these groups, as it was predicted to be too low for accurate quantification.

Lung CFUs for each group are displayed in Fig 7. There was a clear separation between infected and uninfected mice in groups E and G, since the CFU in all infected mice were at least one log over the limit of detection. This separation was less definite in group F as several mice were near the limit of detection. Group F also showed a large variation in lung CFU with SD of 1.85 logs. This is explained by combination of the ULD and the early necropsy at 3 weeks. The SD in lung CFU in groups E and G was 0.51 and 0.39 logs respectively. This is higher than the SD typically observed in the standard LD aerosol model of around 0.2 logs at the 4-week time point. As an example of LD aerosol exposure, the SD in group D was 0.17 logs. The ULD aerosol leads to a great stochastic variation in the actual dose delivered to each mouse as compared to the LD aerosol.

The proportion of mice infected is plotted in Fig. 8 as a function of the log presented dose. The area of each data point is proportional to the number of mice in the group. Best fit curves are shown based on a sigmoid dose response relationship and a Poisson distribution. The sigmoid curve is based on a biological dose-response assumption, while the Poisson curve assumes that infection is a stochastic (all-or-none) event. The sigmoid curves provided a good fit to the data (weighed $R^2 = 0.970$) with an ID50 estimate of 1.7 CFU/ mouse presented dose, and an estimated maximal response of 100% infected. The Poisson curve also provided a good fit ($R^2 = 0.973$) with an ID50 estimate of 1.9 CFU/ mouse, and an estimate

of 2.8 CFU as the presented dose corresponding to an infection event. The traditional method of Reed and Muench provided an ID50 estimate of 1.4 CFU/ mouse presented dose.

Table 5: Results from three aerosol exposures with low doses of MTB

Aerosol Exposure Group	Starting inoculum OD	Dose Presented (CFU/mouse)	Time of necropsy	Infected/Total Mice	Mice Infected (%)
E	2.5e-4	1.1	5 weeks	10/28	36
F	8.0e-5	1.6	3 weeks	5/14	36
G	1.0e-3	11.0	4 weeks	19/20	95

Example 3

BCG Vaccination in ULD Mice

To test the effect of BCG vaccination on infection rates, the infection rates in the ULD MTB model and the standard low dose (LD) model were compared following vaccination. In each experiment, age and gender matched naïve and vaccinated mice were exposed simultaneously to LD or ULD aerosols. Mice were exposed to MTB aerosol in the whole body chamber using a Collision nebulizer. The dose delivered to the lungs was 58 CFU in the LD exposure. The dose presented to the mice was 1.6 and 1.1 CFU in ULD-A and ULD-B exposures, respectively. Infection was defined by a positive lung homogenate culture 3-5 weeks after infection.

All mice exposed in the standard LD model were infected, while a partial infection was established in the ULD-A and B groups (Table 6). A trend towards protection was observed in both the ULD groups, there was a reduction in the rate of MTB infections in BCG vaccinated mice.

Table 6: Infection rates in naïve and vaccinated mice after MTB aerosol challenge.

Aerosol Challenge	Naive Infected/total (%)	Vaccinated Infected/total (%)	p-value (Fisher Exact)
LD	6/6 (100%)	5/5 (100%)	1.00
ULD-A	5/14 (36%)	3/9 (33%)	>0.1
ULD-B	10/28 (36%)	2/20 (10%)	0.054

This study showed that there was no significant reduction in the lung burden of the BCG vaccinated mice compared to the naïve group (Figure 9). In previous published literature using LD aerosol, BCG vaccination leads to a reduction of 1-2 log₁₀ CFU in lungs.

In addition, the invention is not intended to be limited to the disclosed embodiments of the invention. It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

We claim:

1. A method of determining resistance of an animal to MTB infection comprising:
 - (i) delivering a vaccine to an animal,
 - (ii) exposing the animal to an ultra-low dose of *Mycobacterium tuberculosis* (MTB) aerosol,
 - (iii) measuring the rate of MTB infection.
2. The method of claim 1, wherein the ultra-low dose of MTB aerosol is about 1 colony forming unit/lung (CFU) to about 11 CFU/lung.
3. The method of claim 2, wherein the ultra-low dose of MTB aerosol is about 1 colony forming unit/lung (CFU) to about 1.6 CFU/lung.
4. The method of claim 3 wherein the ultra-low dose of MTB aerosol is 1 CFU/lung.
5. The method of claim 1, wherein the presence or absence of MTB in the animal's lungs determines infection.
6. The method of claim 1 wherein the vaccine is bacilli calmette-guerin vaccine.
7. A method of determining susceptibility of an animal to MTB infection comprising:
 - (i) administering a particulate material to an animal,
 - (ii) exposing the animal to an ultra-low dose of *Mycobacterium tuberculosis* (MTB) aerosol,
 - (iii) measuring the rate of infection following exposure.
8. The method of claim 7, wherein the particulate material is administered by pharyngeal aspiration.
9. The method of claim 8, wherein the particulate material is carbon black, silica, or

carbon nanotubes.

10. The method of claim 7, wherein the ultra-low dose of MTB aerosol is about 1 colony forming unit/lung (CFU) to about 11 CFU/lung.

11. The method of claim 10, wherein the ultra-low dose of MTB aerosol is about 1 colony forming unit/lung (CFU) to about 1.6 CFU/lung.

12. The method of claim 11 wherein the ultra-low dose of MTB aerosol is 1 CFU/lung.

13. The method of claim 7, wherein the presence or absence of MTB in the animal's lungs determines infection.

14. A method of producing an animal model for determining susceptibility or resistance to *Mycobacterium tuberculosis* (MTB) comprising:

- (i) exposing an animal to an ultra-low dose of MTB aerosol,
- (ii) measuring the rate of infection following exposure.

15. The method of claim 14, wherein the ultra-low dose of MTB aerosol is about from about 1 colony forming unit/lung (CFU) to about 11 CFU/lung.

16. The method of claim 15, wherein the ultra-low dose of MTB aerosol is from about 1 colony forming unit/lung (CFU) to about 1.6 CFU/lung.

17. The method of claim 16, wherein the ultra-low dose of MTB aerosol is about 1 CFU/lung.

18. The animal of claim 14 wherein the animal is a mammal.

19. The animal of claim 18, wherein the mammal is a mouse.

20. The method of claim 14, wherein the presence or absence of MTB in the animal's lungs determines infection.

21. An animal model to determine susceptibility or resistance to MTB, wherein

the animal has been exposed to an ultra-low dose of MTB aerosol.

22. The animal model of claim 21, wherein the ultra-low dose of MTB aerosol is about 1 colony forming unit/lung (CFU) to about 11 CFU/lung.

23. The animal model of claim 22, wherein the ultra-low dose of MTB aerosol is about 1 colony forming unit/lung (CFU) to about 1.6 CFU/lung.

24. The animal model of claim 23, wherein the ultra-low dose of MTB aerosol is 1 CFU/lung.

25. An animal model produced by the method of claim 14.

26. The animal model of claim 25 wherein the animal is a mammal.

27. The animal of claim 26, wherein the mammal is a mouse.

FIGURE 1

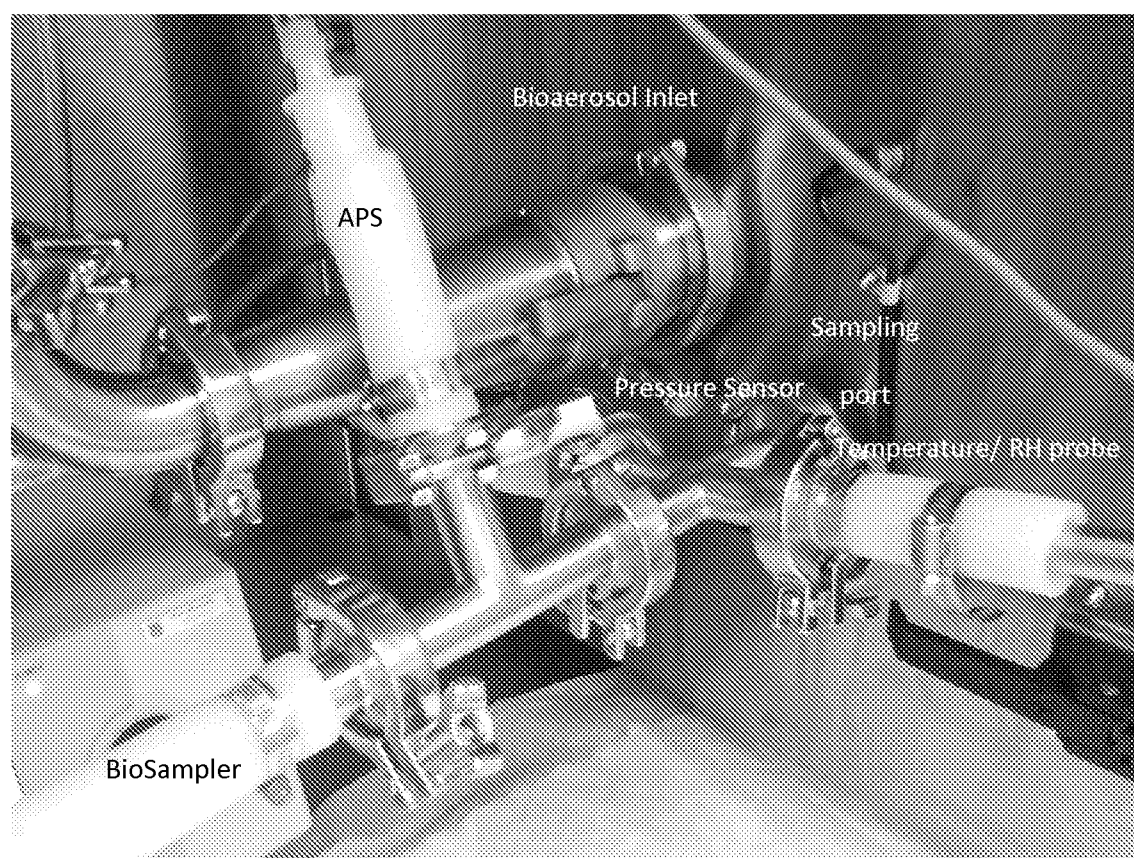


FIGURE 2

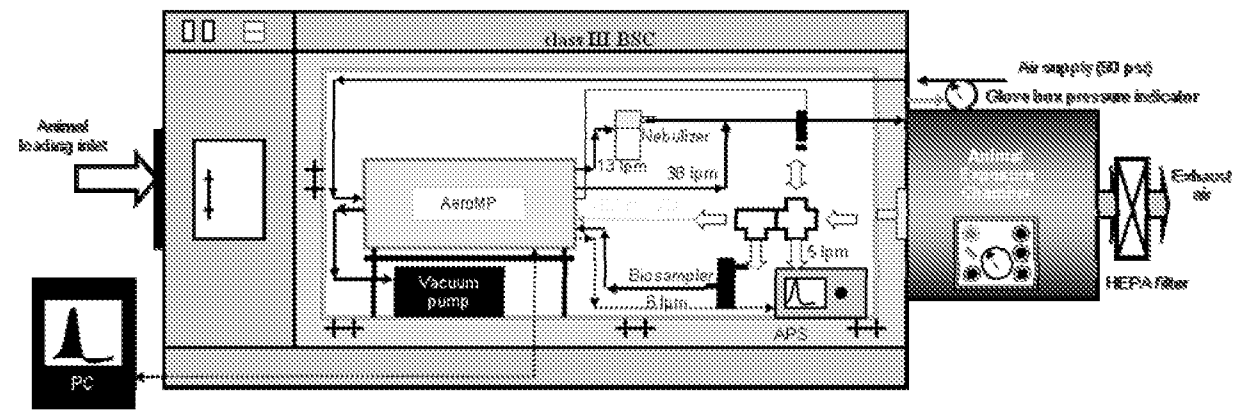


FIGURE 3

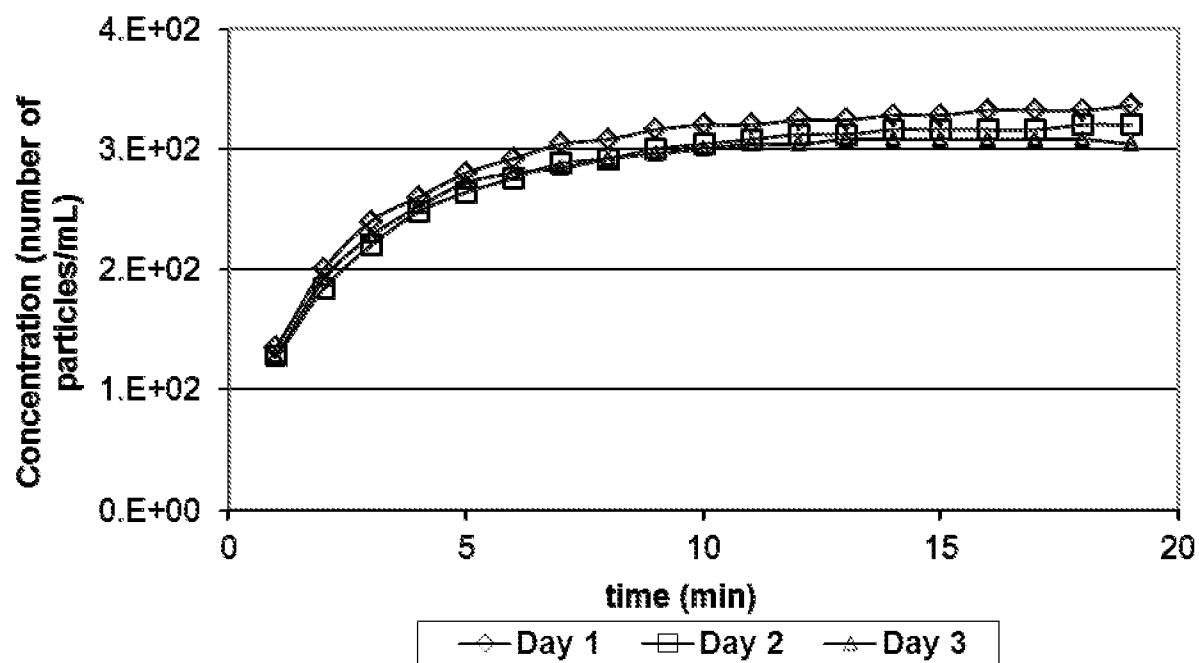
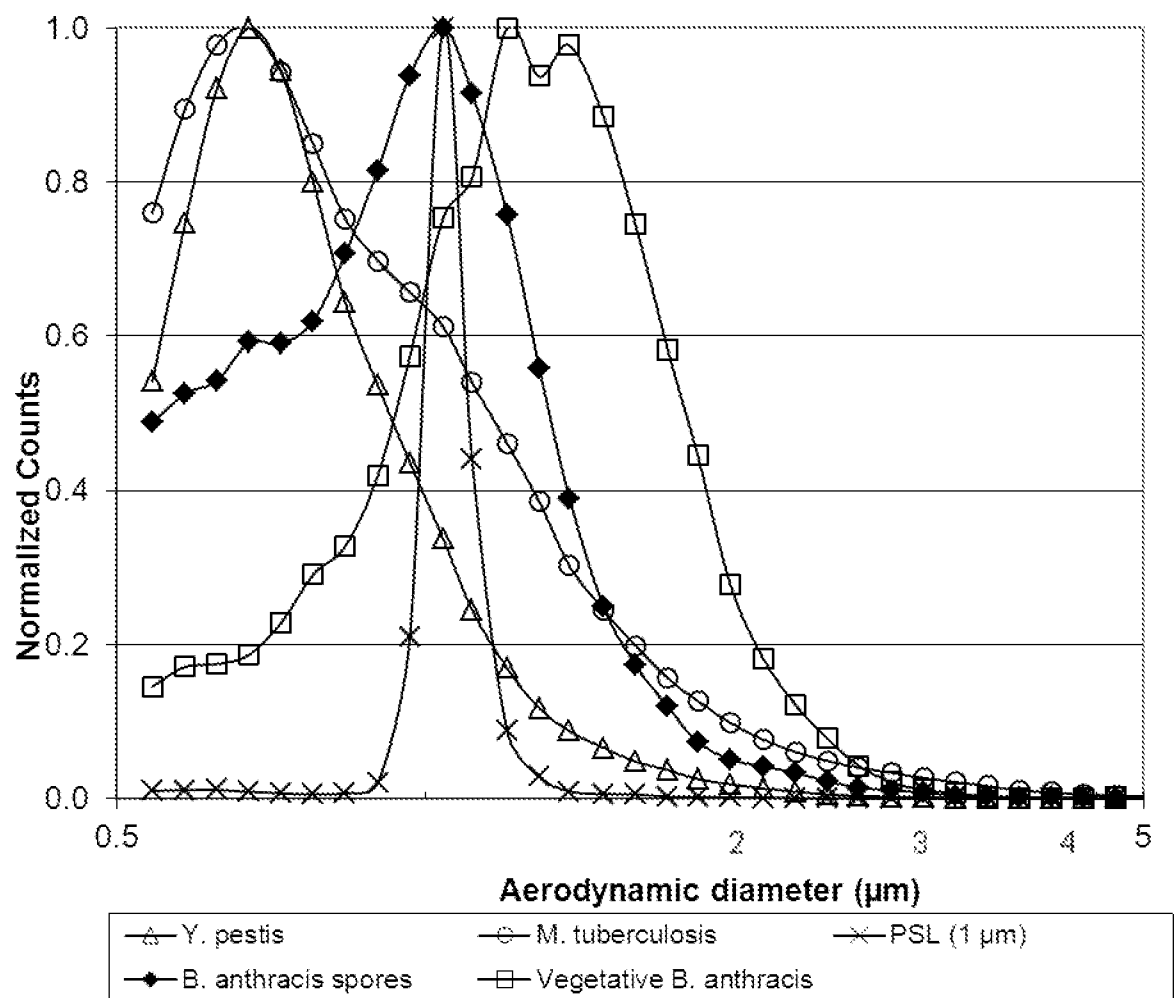


FIGURE 4



5/11

FIGURE 5

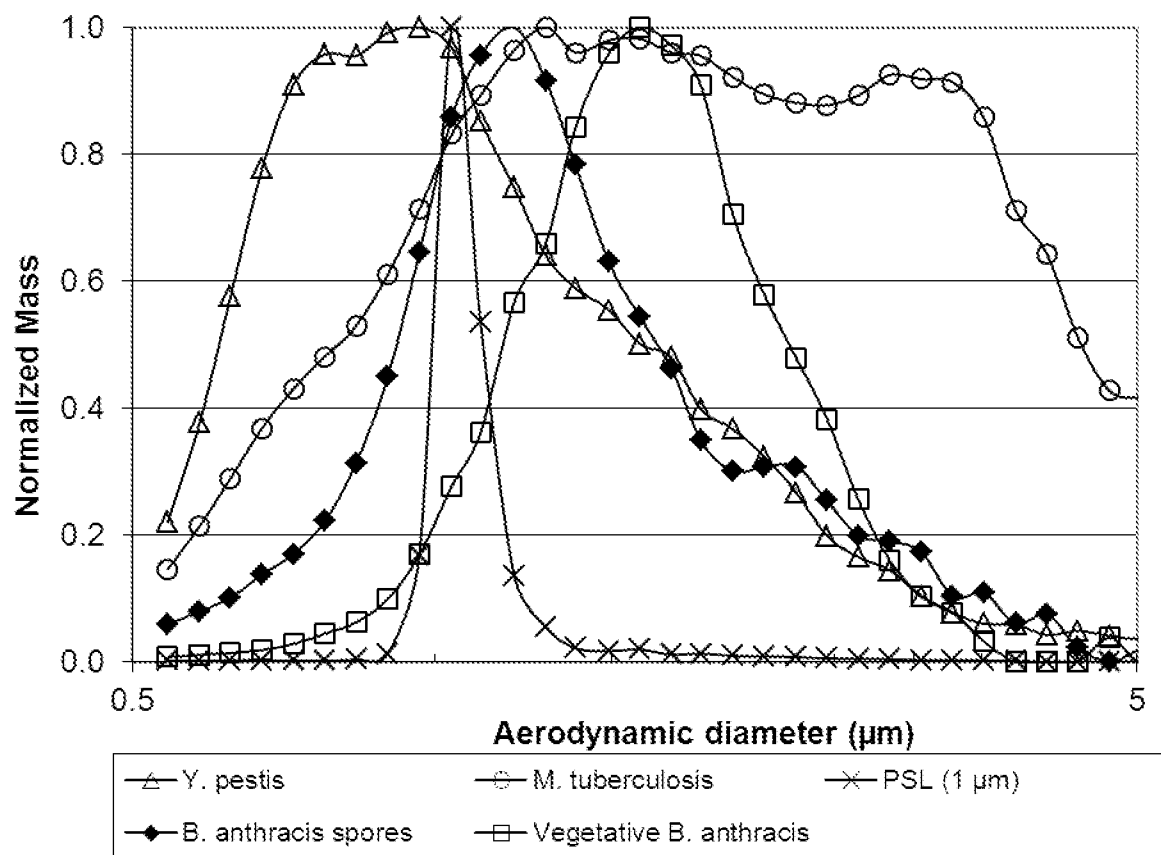


FIGURE 6

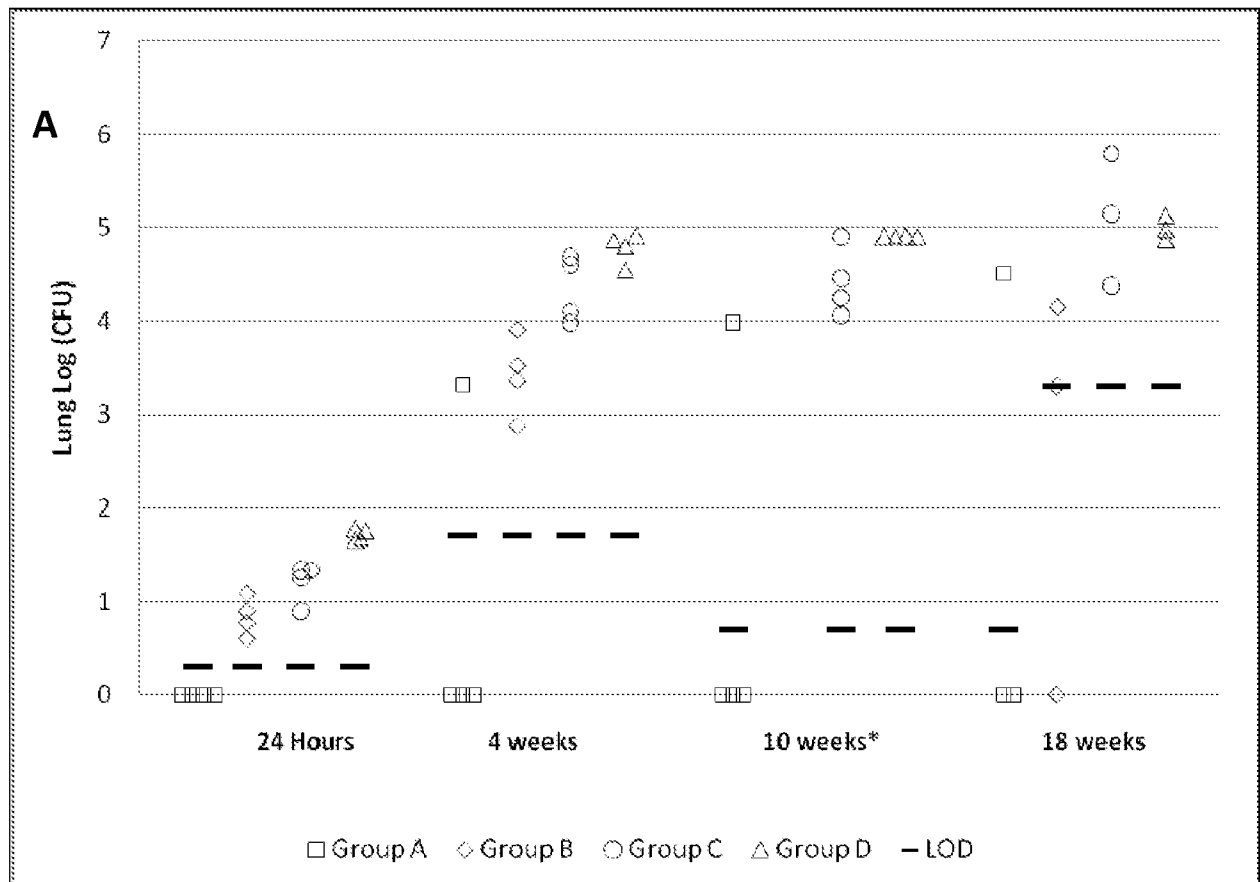


FIGURE 6

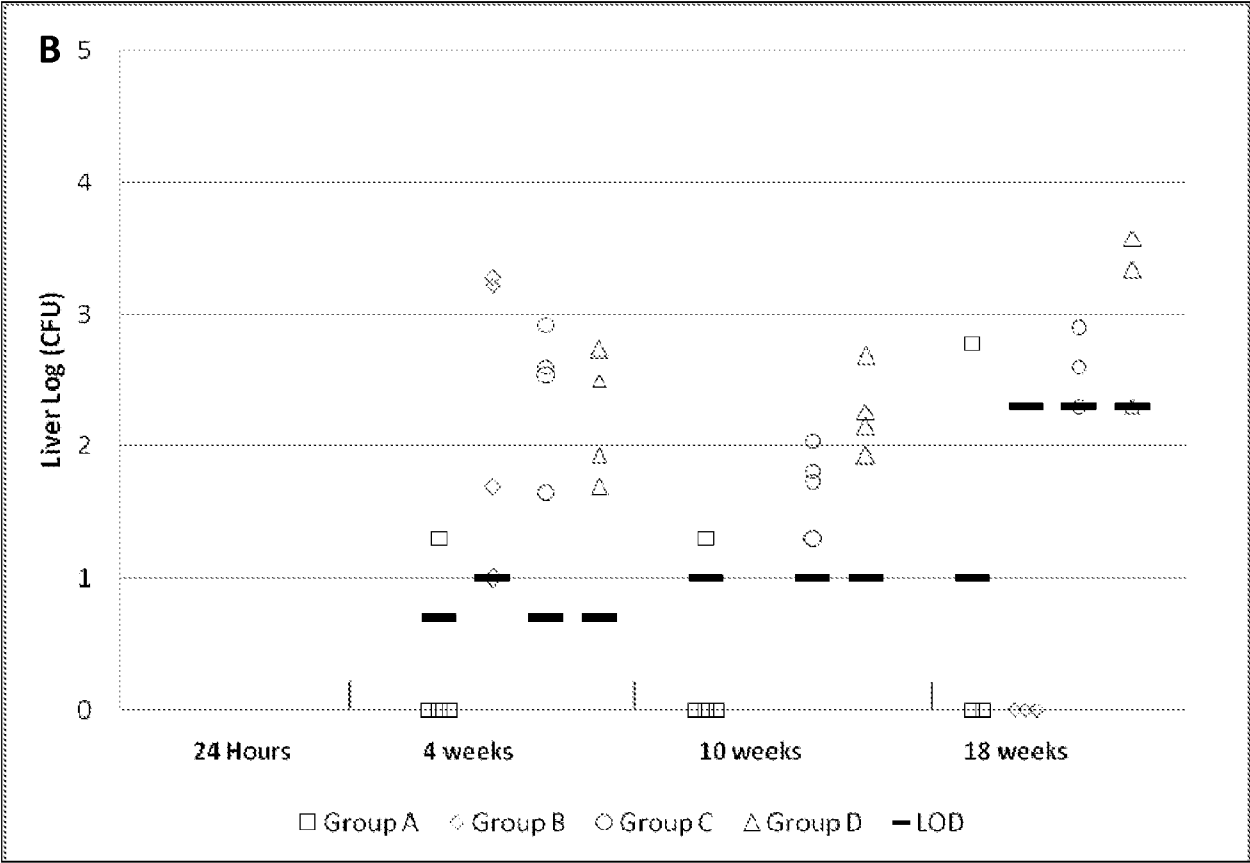


FIGURE 6

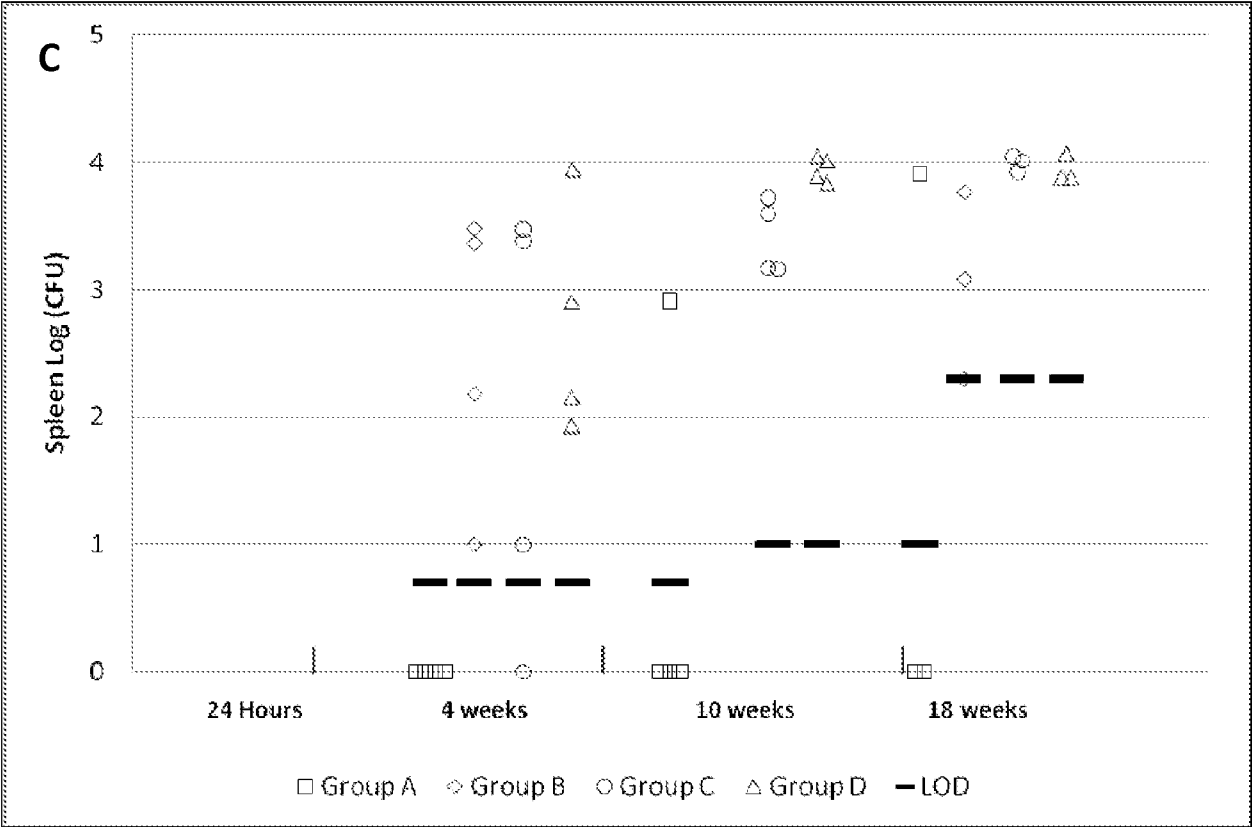


FIGURE 7

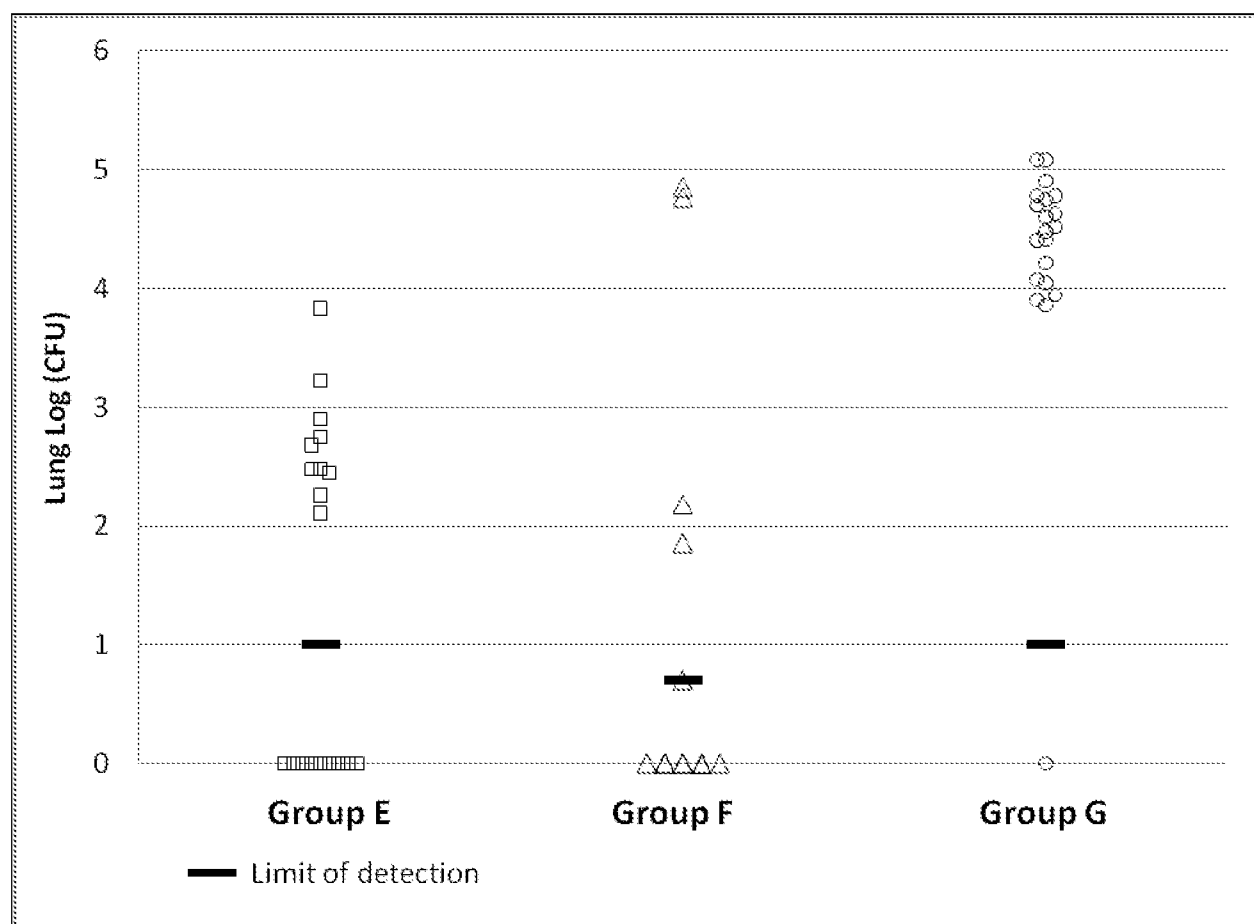


FIGURE 8

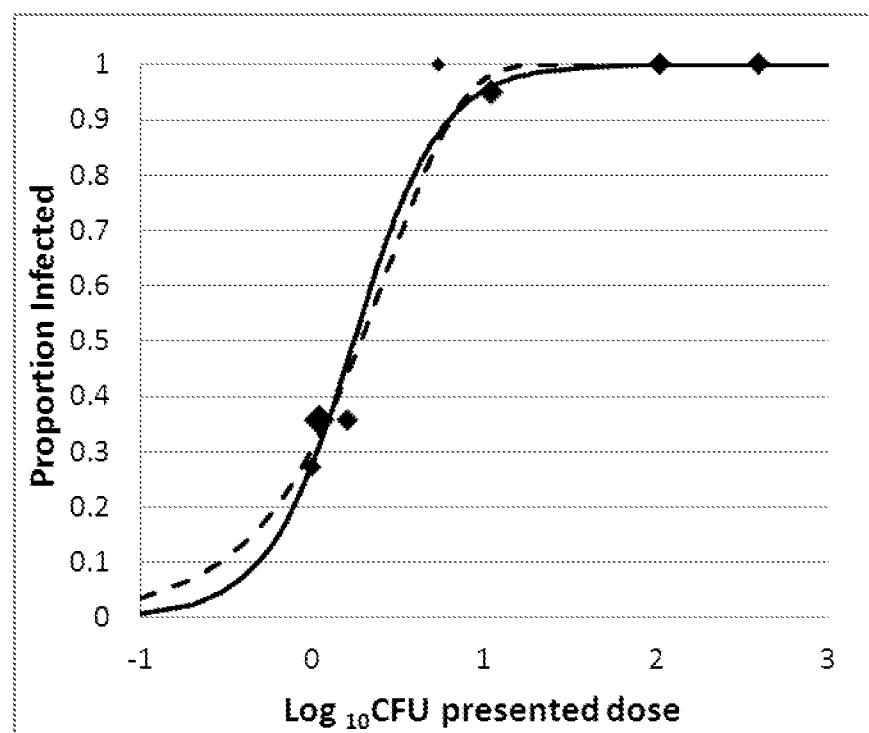
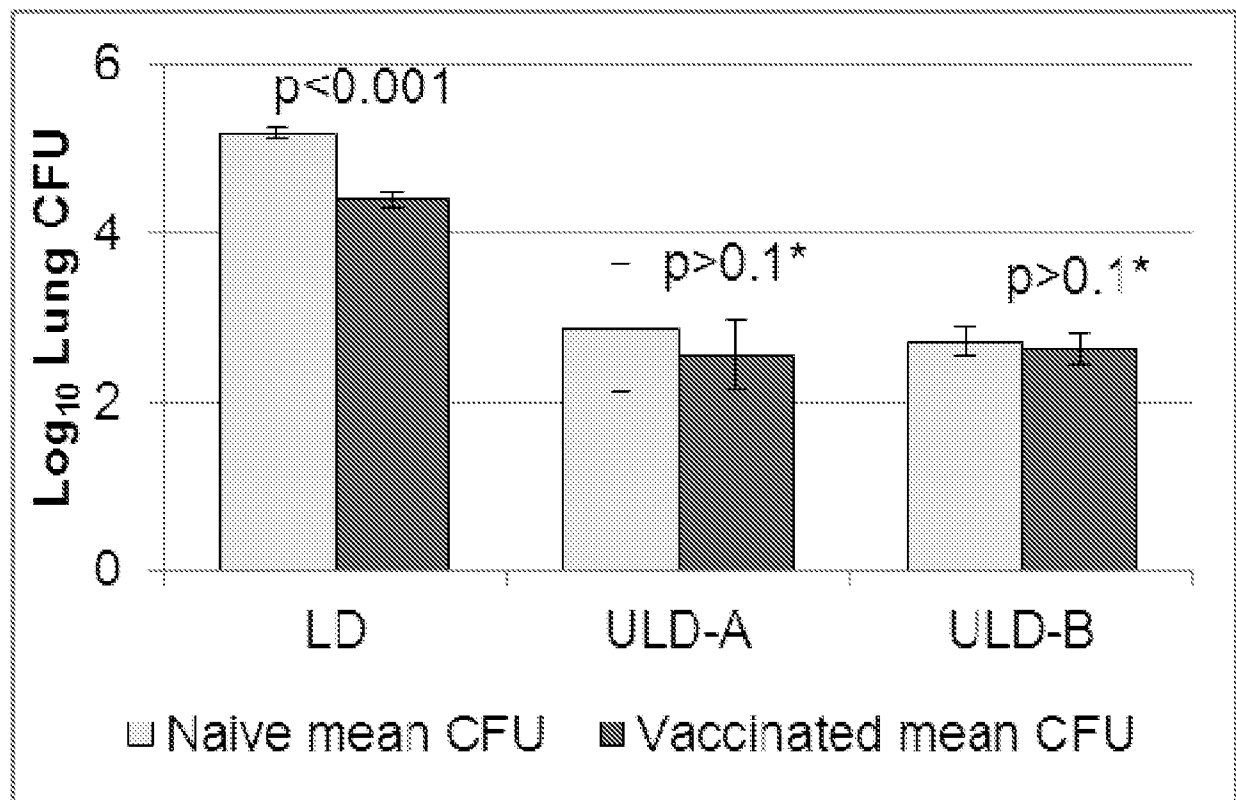


FIGURE 9



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/029871

A. CLASSIFICATION OF SUBJECT MATTER

INV. A01K67/027

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	DIVEY SAINI ET AL: "Ultra-low dose of Mycobacterium tuberculosis aerosol creates partial infection in mice", TUBERCULOSIS, vol. 92, no. 2, 1 March 2012 (2012-03-01), pages 160-165, XP55027080, ISSN: 1472-9792, DOI: 10.1016/j.tube.2011.11.007 available online 21 December 2011; the whole document ----- -/--	1-27



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 May 2012

Date of mailing of the international search report

01/06/2012

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Authorized officer

Renggli-Zulliger, N

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/029871

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GUPTA ET AL: "Animal models of tuberculosis", TUBERCULOSIS, ELSEVIER, GB, vol. 85, no. 5-6, 1 September 2005 (2005-09-01), pages 277-293, XP005156229, ISSN: 1472-9792, DOI: 10.1016/J.TUBE.2005.08.008 page 283, column 1, paragraph 3 - column 2, paragraph 3 -----	1-6, 14-18, 20-26
X	TJARNLUND A ET AL: "Determinant role for Toll-like receptor signalling in acute mycobacterial infection in the respiratory tract", MICROBES AND INFECTION, ELSEVIER, PARIS, FR, vol. 8, no. 7, 1 June 2006 (2006-06-01), pages 1790-1800, XP025243528, ISSN: 1286-4579, DOI: 10.1016/J.MICINF.2006.02.017 [retrieved on 2006-06-01] Abstract; page 1792, column 1, paragraph 1 -----	14,15, 18-22, 25-27
X	BASARABA ET AL: "Lymphadenitis as a major element of disease in the guinea pig model of tuberculosis", TUBERCULOSIS, ELSEVIER, GB, vol. 86, no. 5, 1 September 2006 (2006-09-01), pages 386-394, XP005596696, ISSN: 1472-9792, DOI: 10.1016/J.TUBE.2005.11.003 abstract; pages 387,388; figure 2 -----	1,2,5,6, 14,15, 18, 20-22, 25,26
Y		3,4, 7-13,16, 17,23,24
X	SANGEETA BHASKAR ET AL: "Design and evaluation of an aerosol infection chamber for small animals", INTERNATIONAL JOURNAL OF PHARMACEUTICS, vol. 255, no. 1-2, 1 April 2003 (2003-04-01), pages 43-48, XP55027932, ISSN: 0378-5173, DOI: 10.1016/S0378-5173(03)00023-1 abstract ----- -/--	14,15, 18-22, 25-27

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/029871

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	G. S. DEAN ET AL: "Minimum Infective Dose of Mycobacterium bovis in Cattle", INFECTION AND IMMUNITY, vol. 73, no. 10, 1 October 2005 (2005-10-01), pages 6467-6471, XP55027453, ISSN: 0019-9567, DOI: 10.1128/IAI.73.10.6467-6471.2005 the whole document in particular page 6467 -----	3,4, 7-13,16, 17,23,24
Y	DIVEY SAINI ET AL: "Sampling port for real-time analysis of bioaerosol in whole body exposure system for animal aerosol model development", JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS, ELSEVIER, NEW YORK, NY, US, vol. 63, no. 2, 2 September 2010 (2010-09-02), pages 143-149, XP028148452, ISSN: 1056-8719, DOI: 10.1016/J.VASCN.2010.09.002 [retrieved on 2010-09-16] the whole document -----	3,4, 7-13,16, 17,23,24