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Keywords:	otitis media, nontypeable Haemophilus influenzae, Junbo mouse, middle ear infection, lung infection
Abstract:	Acute otitis media, inflammation of the middle ear bulla, is the most common bacterial infection in children. For one of the principal otopathogens, non-typeable Haemophilus influenzae (NTHi), animal models allow us to investigate host-microbial interactions relevant to the onset and progression of infection and study treatment of middle ear disease. We have established a robust model of NTHi middle ear infection in the Junbo mouse. Intranasal inoculation with NTHi produces high rates of bulla infection and bacterial titres in bulla fluids; bacteria can also spread down the respiratory tract to the mouse lung. An innate immune response is detected in the bulla of Junbo mice following NTHi infection and bacteria are maintained in some ears at least up to day 56 post-inoculation. The Junbo/NTHi infection model facilitates studies on bacterial pathogenesis and antimicrobial intervention regimens and vaccines for better treatment and prevention of NTHi middle ear infection.

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Non-typeable *Haemophilus influenzae* infection of the *Junbo* mouse

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Running title: NTHi mouse infection

Significance Statement

The *Junbo* mouse/NTHi infection model facilitates investigation of the complex host-microbial interactions that underpin the onset and development of otitis media (middle ear disease). Intranasal inoculation of the mouse with NTHi establishes robust middle ear infection, many aspects of which closely mimic features of this common disease in man. Elements of both the bacteria and host that contribute to disease can be studied and novel treatment regimens investigated.

Abstract

Acute otitis media, inflammation of the middle ear bulla, is the most common bacterial infection in children. For one of the principal otopathogens, non-typeable *Haemophilus influenzae* (NTHi), animal models allow us to investigate host-microbial interactions relevant to the onset and progression of infection and study treatment of middle ear disease. We have established a robust model of NTHi middle ear infection in the *Junbo* mouse. Intranasal inoculation with NTHi produces high rates of bulla infection and bacterial titres in bulla fluids; bacteria can also spread down the respiratory tract to the mouse lung. An innate immune response is detected in the bulla of *Junbo* mice following NTHi infection and bacteria are maintained in some ears at least up to day 56 post-inoculation. The *Junbo*/NTHi infection model facilitates studies on bacterial pathogenesis and antimicrobial intervention regimens and vaccines for better treatment and prevention of NTHi middle ear infection.

Keywords: otitis media, non-typeable *Haemophilus influenzae* (NTHi), *Junbo* mouse, , middle ear infection, lung infection

Acute otitis media (AOM), inflammation of the middle ear bulla, is caused by bacterial infection. It is the most common bacterial infection in children and reason for antibiotic prescription in this age group. Otitis media with effusion (OME), otherwise known as glue ear, can be a sequel to AOM and chronic OME is the most common cause of hearing impairment in children; grommet surgery to alleviate this condition is the most frequent paediatric surgery in Western countries. In the UK, on average, at least one episode of AOM occurs in every child by the age of five. Of the three major otopathogens, non-typeable *Haemophilus influenzae* (NTHi) is emerging as the predominant bacterium associated with AOM. NTHi is part of the normal commensal flora present in the human nasopharynx (NP); no natural animal hosts other than man have been identified for NTHi and there are no inanimate reservoirs. The molecular basis of the processes that lead to contiguous spread of NTHi within the respiratory tract and subsequent disease in the middle ear has been difficult to establish largely because animal models enabling study of all aspects of pathogenesis are not readily available.

Although other animal models have been reported for studies of AOM, mice have been utilized historically to elucidate virulence factors of otopathogens, mechanisms of adherence and invasion, and induction and specificity of immune responses to pathogens such as NTHi. When considering cost, litter size, availability of immunological reagents and control of host genetics through inbred and mutant host lines, the mouse presents substantial potential advantage over other animals for otitis media (OM) studies. Several genes associated with increased susceptibility to OM in the mouse have now been shown to be relevant to human disease through candidate gene studies performed using allelic association analyses in family-based cohorts (Rye et al., 2011). Genetic predisposition can be investigated by studying disease in multiple mouse lines that are each mutated in relevant genes that increase susceptibility to spontaneous or experimental infection of the middle ear. Mouse models can facilitate investigations of the molecular basis and pathophysiology of NTHi infection and provide a means to realise improved treatment and prevention of disease in man. Here we describe a robust model of NTHi infection using the *Junbo* mouse; this focuses upon the study of AOM but bacterial carriage and lung clearance can also be investigated.

Basic Protocol 1 describes the basis for establishing and monitoring middle ear infection in the *Junbo* mouse following intranasal inoculation of NTHi. Basic Protocol 2 describes the adaptation of Basic Protocol 1 to study protection against infection following immunization of the animals and Basic Protocol 3 the use of antibiotic to clear NTHi. Basic Protocol 4 describes a modified infection procedure that favours residence of bacteria in the mouse lung.

NOTE: All protocols using live animals must first be reviewed and approved by the appropriate ethics process such as the Animal Welfare and Ethical Review Body (AWERB) in the UK or the Institutional Animal Care and use Committee (IACUC) in the US, and must conform to local or national governmental regulations regarding the care and use of laboratory animals.

Basic Protocol 1

JUNBO MOUSE NTHI MIDDLE EAR INFECTION

Junbo mouse

Junbo is a mutant mouse line that spontaneously develops chronic middle ear (ME) inflammation under specific pathogen free (SPF) conditions at four to five weeks of age. The heterozygote *Junbo* mouse (*Jbo/+,* hereafter termed *Junbo*) bears an Asn763Ile mutation in the gene encoding the Evi1 transcription factor, otherwise known as Mecom (Parkinson et al., 2006). One mechanism that may underlie the predisposition to OM in *Junbo* mice is that Evi1 is a negative regulator of NFkB; the loss of function Evi1 *Junbo* mutation exacerbates NTHi induced inflammation in the lung (Xu et al., 2012). The pre-existing ME inflammation in *Junbo* mice is critical for bacterial ME infection following intranasal (IN) inoculation, the healthy air-filled ME of wild type mice and the small proportion of *Junbo* mice (5 to 10%) with no fluid present do not sustain infection by NTHi.

NTHi strains

Wild-type NTHi strains used by us for ME infection studies are all isolates from children with OM (Cody et al., 2003) and are phylogenetically distinct (De Chiara et al., 2014). Following infection in the mouse, indigenous commensal bacterial flora, particularly *Proteus* spp., renders a proportion of bacterial count plates unreadable through overgrowth. For this reason, NTHi strains expressing spontaneous resistance to streptomycin, or engineered resistance to kanamycin, were generated (Hood et al., 2016). These strains enable antibiotic selection of NTHi during culture from mouse samples and counter-selection against any indigenous bacteria present; streptomycin selection proved particularly useful at maximizing quantitative NTHi culture data from mouse samples.

Materials

Mice - *Junbo* (*Jbo/+*) mice are congenic on a C3H/HeH background (Parkinson et al., 2006); mice are housed under SPF conditions and are mostly used at between eight to eleven weeks of age. For some studies Germ Free (GF) mice are used, details of the mouse husbandry and microbiological surveillance are given elsewhere (Hood et al., 2016).

NTHi – human OM disease isolates (162, 176, 375, 486, 1124 and 1158) are used for mouse infection studies (Cody et al., 2003).

Autoclaved brain heart infusion (BHI) broth supplemented with haemin and NAD (sBHI) for liquid culture and sBHI agar for plate growth of bacteria.

Sterile PBS/2% gelatine for inoculum preparation, PBS for collecting and diluting *in vivo* samples.

Binocular dissecting microscope with x10 magnification and LED stage lighting.

scissors for removal of the head and fine forceps for puncture of the tympanic membrane, 70% ethanol to sterilize the instruments between the sampling of ears. A <2 µl volume pipette with sterile micro-tip to collect the small volume of ME fluids.

barbiturate solution for intraperitoneal injection or alternatively a rising CO_2 concentration to euthanize mice.

Mouse NTHi inoculum

1. Prepare bacteria for inoculation from -80^{o}C stocks stored in BHI/20% glycerol and grown overnight at 37^{O}C in 5% CO_2 atmosphere on sBHI agar. From this plate, inoculate sBHI broth with a 1 µl loop full of colony growth (to give an initial OD_{490} of ~0.03-0.05) and grow bacteria to log phase (OD_{490} 0.3-0.6) at 37^{O}C in a shaking incubator. The size of inoculum for the mouse is calculated from optical density (OD_{490}) using the conversion factor that an absorbance of 0.4 is equivalent to approximately 1 x 10^9 c.f.u./ml. Pellet bacteria by centrifugation at 13000 x g at room temperature for 3 min and re-suspend to give 10^{10} c.f.u./ml in PBS/2% gelatine. Assess the titre of the inoculum by plating dilutions of each suspension onto sBHI agar plates prior to, and after, inoculation of the mice.

Intranasal challenge

2. Anesthetize mice aged either eight ± 1 week or 11 ± 1 week with isofluorane and inoculate by applying 5 µl of bacterial suspension to each nostril via a pipette tip (Hood et al., 2016).

A typical inoculum is 10^6 c.f.u. but a range of inoculum sizes from 10^3-10^8 c.f.u. NTHi bacteria have been used in individual experiments with cohort sizes of n=11 to 15 mice.

Using bacteria that emit light (expressing lux genes) we have shown that NTHi are distributed along the entire length of the NP within minutes of IN inoculation (Hood et al., 2016).

Terminal sampling of nasopharynx and bulla fluids

3. Euthanize the mouse (typically at 7 days post-inoculation), remove and skin the head, then remove the mandible and sample the NP by washing with 200 µl PBS introduced into the NP opening on the palate and collecting the wash fluid from the nares into an Eppendorf tube. Sampling the NP either before or after the ME has no statistically significant effect on the NP bacterial titres attained.

Avoid euthanasia by cervical dislocation of the mouse as it causes haemorrhage in the upper airway that will compromise sample collection.

A more quantitative approach to determine NP NTHi titres is to dissect out a part (eg soft palate) and homogenise the tissue in PBS prior to dilution and plating. Although this typically yields higher bacterial titres than the wash alone we find that there is a general positive correlation between the data from the two methods.

4. Under x10 binocular magnification, sample fluids from the ME bulla following perforation of the TM and removal of the ME conductive bones using sterile forceps. Collect bulla fluids and estimate the volume using a 0-2 µl filtered pipette tip (average sampled volume ~0.75 µl, range 0.1-1.50 µl). Transfer fluids into 500 µl of PBS in an Eppendorf tube. In a small proportion of *Junbo* mice with clear TM there is no detectable bulla fluid. These bullae can be washed twice with 2 µl of sterile PBS and the washings added back to 500 µl of PBS for culture to monitor infection.

If required for gene or protein expression analysis, the primary bulla fluid preparation (suspended in PBS) is centrifuged at 13000 x g for 3 min then the pellet and supernatant frozen on dry ice prior to storage at -80^{O}C.

Infection rate and bacterial titre

5. Disperse bulla fluid in PBS, or NP wash, by three 10 second bursts on a vortex mixer then make 10-fold dilutions (10^{-1}, 10^{-2}) in PBS. Spread 50 µl of each ME dilution or undiluted NP wash on a sBHI agar plate. The detection limit is 10 c.f.u./µl for the initial bulla fluid suspension and 100 c.f.u./µl for the 10^{-1} dilution. In experiments using antibiotic resistant NTHi, samples are plated on sBHI agar plates supplemented with the appropriate antibiotic (e.g., 300 µg/ml streptomycin or 20 µg/ml kanamycin). If required, non-selective agar plates can be used in parallel to monitor the presence of commensal bacteria. Incubate agar plates overnight at 37^{O}C.

Calculate an infection rate for the middle ear:

Infection rate = number of NTHi positive bulla fluids/number of bulla fluids sampled

Bacterial titres given as c.f.u./µl fluid are calculated from the number of colonies obtained on culture plates, adjusted for sample dilution and bulla fluid volume.

NTHi has also been shown to infect the bulla of other mutant mouse lines that exhibit ME inflammation and OM (Hood et al., 2016); these include the Jeff (Hardisty-Hughes et al., 2006) and tgif (Tateossian et al., 2013) mice. The protocol will similarly work for Streptococcus pneumoniae *(pneumococcus) infection although this has been tested by us only in the* Junbo *mouse. We note that* Streptococcus pneumoniae *strain D39 is highly virulent in* Junbo *mice. All new infection models should be established with less virulent bacterial strains, and pilot studies with small numbers of mice should be conducted first to establish whether there are unexpected adverse effects.*

Histology, immunohistochemistry and in situ hybridization of ME

The histology of NTHi infected ME bullae can be examined and enhanced by immunohistochemistry or by *in situ* hybridization and lesion profiling. Typically this is carried out using 11–week-old *Junbo* mice inoculated IN with 10^6 c.f.u. NTHi and heads collected seven days post-inoculation. Heads from non-NTHi challenged *Junbo* mice collected at the same time serve as negative controls.

Histology

1. Assess ME histology on infected and non-infected mice using 4 μm wax sections (Parkinson et al., 2006). Fix skinned mouse heads for 48 hrs in 10% neutral buffered formalin. Decalcify bone using EDTA and embed decalcified tissues in paraffin wax then cut 4 μm dorsal plane sections before staining with Haematoxylin and Eosin, or special stains. Carry out morphometric analysis and lesion profiling on standard 1000 µm lengths of ME mucosa as described below.

We note that Gram-staining failed to identify NTHi in tissue sections and IHC and ISH may be the best approach to identify and localise bacteria.

Immunohistochemistry and In situ hybridization

1. Decalcify formalin fixed tissues using EDTA. Dissected mouse heads can be decalcified in 5 to 7 days. To maximize RNA integrity for any *in situ* work, a band saw can be used to isolate the bullae from heads fixed for 24 hours and EDTA decalcification can be achieved in 48 hours.

2. Cut 4 μm thick wax sections onto electrostatically charged slides and dry overnight at 37°C before a final drying at 60°C for 25 minutes. De-wax sections in xylene then hydrate through ethanol and washing three times in Tris buffered saline (TBS). Block endogenous

peroxidase using Dako REAL peroxidase blocker (S2023) for 10 minutes following antibody incubations.

3. Detect target cells with antigen markers in the mouse ME using appropriate antibodies. An illustrative example is using rat monoclonal anti-F4/80 (Serotec MCA497G) to detect mouse macrophages. Perform antigen retrieval using Dako proteinase K (S3020) for 20 minutes at room temperature. Incubate the primary antibody (diluted 1/400) with sections for 30 minutes at room temperature; carry out secondary antibody detection using the Vector Labs ImmPress HRP anti-rat kit (MP-744-15). Dilute antibodies in Dako antibody diluent (S0809) and carry out negative controls using the antibody diluent alone. Visualize the target using the Dako (K3468) liquid DAB+ substrate chromogen system. Counterstain using Harris haematoxylin prior to dehydration through ethanol, clearing in xylene and mounting in Clearview mountant (Thermo Fisher Scientific).

4. Perform *in situ* hybridization on representative NTHi-challenged and non-challenged *Junbo* mice. As an illustrative example we use a probe that targets the 16sRNA of the bacteria. Hybridize 4 μm wax sections with probe B-HInfluenzae-NTHi375-16SrRNA as per the manufacturer's instructions (Advanced Cell Diagnostic) using the HRP visualization kit. Use a positive control for RNA integrity (e.g. PpiB) and a negative hybridization control (e.g. DapB) (Advanced Cell Diagnostic) in experiments.

ISH is a useful approach when antibodies against bacteria produce high background or suitable ones are not commercially available. We found that mouse antisera from NTHi immunization and protection studies did not make useful reagents to localise bacteria in bulla sections. Another advantage of ISH is that there are a wide range of mouse probes available making it potentially possible to obtain hybridization signals for bacteria and host cells in the same section.

Lesion profiling

For lesion profiling, acquire bright field images of Haematoxylin and Eosin stained sections using a slide scanner such as a Hamamatsu NanoZoomer or equivalent and make the morphometric measurements using suitable software. Assess the mucosal thickness and the numbers and size of capillary and lymphatic vessels present as required (Cheeseman et al., 2011). Calculate the average thickness of the mucosa lining the medial surface of the bulla (avoiding the cochlea and the region close to the Eustachian tube) by dividing the area of mucosa overlying a delineated ~1000 μm length of supporting bulla bone. Calculate the proportion of bulla space occupied by exudate by dividing the exudate area by the area bounded by bulla mucosa surface and the TM.

If comparing infected and non-infected ME then blind slides for the analysis.

Immune response measured by real time quantitative PCR (RT-qPCR) of bulla fluids

The degree of the host immune response dependent upon microbial infection in the ME can be estimated by RT-qPCR determination of cytokine and chemokine mRNA levels.

1. Inoculate SPF *Junbo* mice with 10^6 c.f.u. NTHi and sample cohorts terminally at day 1, 3, 7, 10 or 14 post-inoculation, collect and process ME fluids as described in Protocol 1.

2. Obtain cell pellets by centrifugation (13000 x g for 5 mins) of bulla fluids (*n*=4 to 6) collected in PBS that yielded NTHi monocultures from the 10^{-1} dilution when cultured; this is equivalent to <100 c.f.u./µl commensal bacterial present. Place pellets on dry ice and store at -80°C prior to RNA isolation. Obtain at least three biological replicate pools for each time point.

3. As a baseline control, collect bulla fluids (*n*=4 to 6) from eight to ten week old non-infected germ free (GF) *Junbo* mice into 20 µl of RNase free water for RNA isolation. Each GF pool of bulla fluids comprises at least 3 biological replicates as controls for each time point. As an alternative to GF mice, measure the baseline control expression level using bulla fluids obtained from age-matched non-NTHi infected mice.

4. Extract RNA from the re-suspended bulla fluid cell pellets and carry out cDNA synthesis and RT-qPCR TaqMan as described by Cheeseman et al. (Cheeseman et al., 2011). Typical immune molecule genes to be monitored are those activated by bacterial ligands binding to TLR-receptors that are expressed by macrophages, neutrophils and monocytes and are known to be relevant to OM and AOM in man (Juhn et al., 2008; Kaur et al., 2015). Genes that we routinely monitor include Ccl3, Ccl4, Ccl5, IL-1b, IL-6, IL-12a, IL-17a, Tnfa, Vegfa (Hood et al., 2016). Perform RT-qPCR in triplicate technical assays. Normalize data using Hrpt1 and beta Actin as the endogenous control and calculate fold changes of expression (ddCts) of NTHi-infected bulla fluid cells over non-infected bulla fluid cells using AB 7500 software v2.0.1 and express as mean relative quantification (RQ) ± min/max error bars representing 95% CI.

Imaging of bacteria

1. NTHi strains expressing the *lux* genes emit bioluminescent signals that can be used to monitor bacterial distribution *in situ* in the skinned heads of infected Junbo mice; image bacteria using an IVIS Lumina II system (Perkin Elmer) (Hood et al., 2016).

2. Detect NTHi strains expressing a GFP protein by confocal microscopy on mouse bulla fluid and soft palate samples.

3. Detect NTHi by ISH in tissue sections (see above).

Basic Protocol 2

MOUSE PROTECTION STUDIES

Mice are subcutaneously immunized with either whole killed bacteria or with derived and purified cell wall fractions or selected antigens (Ercoli et al., 2015). Following a three step immunization procedure, the mice are inoculated IN with live NTHi bacteria to assess if antibody raised in the mouse elicits protection against infection in the ME or carriage in the NP. Significant levels of protection when found compared to control animals can act as a surrogate that predicts the efficacy of an antigen combination for use as a vaccine in man.

Materials

five week old SPF *Junbo* mice

killed NTHi bacteria for immunization; live NTHi bacteria for infection post immunization

Adjuvant

PBS/0.08% paraformaldehyde

materials for terminal sampling of animals and fluids as described in Protocol 1

Immunization with NTHi bacteria

1. Obtain pre-immune serum as a base-line control for antibody levels prior to the first immunization being carried out. Prepare small volumes of sera from blood obtained from mice by tail vein bleed (see step 6).

2. When using whole NTHi bacteria for mouse immunization, culture NTHi to mid-log phase (OD_{490} 0.2 to 0.6) in sBHI broth. Pellet 1 ml of culture at 13000 x g for 3 mins, resuspend the pellet and wash in PBS, then pellet the bacteria at 13000 x g before re-suspending in 500 µl of PBS/0.08% paraformaldehyde (PFA). Kill bacteria by incubating the suspension for 1 hour at 37^{o}C then overnight at 4^{o}C; confirm loss of viability by culturing 20 µl of the neat bacterial suspension on sBHI agar. Store killed NTHi in PBS/ PFA for up to 8 weeks at 4^{o}C, assess bacterial integrity by phase contrast microscopy.

3. Prior to Immunization, pellet the killed bacteria by centrifugation at 13000 x g for 3 mins then re-suspend in PBS to give 10^{10} c.f.u./ml before mixing with adjuvant according to the

suppliers instructions. We typically use Adjuplex (Sigma) adjuvant at a 1:4 ratio with the bacterial suspension.

4. Immunize each *Junbo* mouse with three subcutaneous injections in intra-scapular skin of a mixture of 10^8 c.f.u. killed bacteria and adjuvant in a 50 µl final volume. The first immunizations are at the age of 5 weeks, then subsequently at 8 weeks and 10 weeks of age. Take a second intermediate blood sample by tail vein bleed prior to the second immunization when the mice are at 8 weeks of age.

5. Inoculate immunized mice IN at 12 weeks with 10^6 c.f.u. NTHi bacteria. Determine the ME infection rate and bacterial titre by terminal sampling at 7 days post-inoculation (mice at 13 weeks of age), as described above in Protocol 1.

6. Obtain terminal blood samples at the end of the experiment by retro-orbital bleed immediately after euthanasia of the mouse. Incubate blood samples at room temperature for two hours then obtain serum using 1.1 ml Z-Gel spin columns (Sarstedt) following centrifugation according to the supplier's instructions. Store serum at -80ºC until required.

In a typical protection experiment cohorts of 12 to 15 mice are immunized with alternative NTHi strains then are infected with either the homologous or heterologous bacteria. By comparing with the infection rate in control mice immunized with PBS, the degree of protection (reduced ME bulla infection and bulla NTHi titres) can be ascertained.

Basic Protocol 3

ANTIMICROBIAL TREATMENT OF NTHI INFECTION

Antibiotic resistance is on the increase for otopathogens such as NTHi and in the clinic this could soon have a have a major impact on treatment regimens for disease. The *Junbo*/NTHi infection model can be used to study antimicrobial treatment for AOM; as an example we describe the procedure for oral administration and systemic treatment of NTHi-infected mice with the clinically relevant antibiotic, Azithromycin.

1. Inoculate eight week old *Junbo* mice IN with 10^6 c.f.u. of NTHi bacteria.

2. At day four post IN-inoculation, give mice a three day course of an antimicrobial; for example, 100 mg/kg of the antibiotic Azithromycin in a 2% methoxycellulose solution delivered once a day by oral gavage.

3. As a control, use mice inoculated with NTHi at the same time as the treatment group of animals but gavaged with 2% methoxycellulose solution alone for three days.

4. Sample bulla fluids terminally on day seven post IN-inoculation and ascertain the number of bacteria by dilution and plating as described above in Protocol 1. If required, also determine the number of bacteria in the NP by sampling through a terminal NP wash (Protocol 1).

5. Determine the efficacy of antibiotic treatment by the difference in ME bulla infection rates and bulla NTHi titres between the antimicrobial treated and control mice.

Importantly, as well as its use for oral administration of antimicrobials, the Junbo*/NTHi infection model has added value through its utility for investigating potential new application strategies specifically targeted at the ME; antimicrobials can be applied directly on the TM and subsequently transferred into the ME bulla.*

The outcome of the antimicrobial treatment can be ascertained for NTHi alone by plating mouse samples on the appropriate selective growth plate for the respective NTHi strain or can include an indication of the effect on other general bacterial flora present by also plating samples on non-selective growth medium.

Basic Protocol 4

NTHi MOUSE PULMONARY INFECTION MODEL

NTHi pulmonary infection has been used to investigate the host immune response in the *Junbo* mouse (Xu et al., 2012). To achieve lung infection the mouse can be inoculated via the intratracheal route but here we describe the more straightforward IN method adapted from that of Morey et al. (Morey et al., 2013).

1. Prior to infection, grow NTHi overnight on sBHI agar then use to inoculate sBHI broth. Grow bacteria to log phase (OD_{490} 0.3-0.6), pellet by centrifugation at 13000 x g for 3 min then re-suspend to give 5 x 10^9 c.f.u./ml in PBS/2% gelatine.

2. Anesthetize mice aged eight weeks with isofluorane and inoculate IN by applying 10 µl of bacterial suspension to each nostril (10^8 c.f.u. in 20 µl total volume).

3. Euthanize the mouse (described in Protocol 1) typically at time points either 24 or 48 hours post-inoculation. Remove lungs aseptically, weigh individually then homogenize to release bacteria from the tissue.

4. Plate serial tenfold dilutions of the lung homogenate in PBS on sBHI agar plates, incubate overnight at 37ºC then determine the number of bacteria in the lung from the colony counts on plates, the dilution factor, and the lung homogenate volume (detection limit <10 c.f.u./ml).

5. These experiments are typically used to compare bacterial counts between different wild type or paired isogenic wild type and mutant NTHi strains; the difference in bacterial count serves as an indicator of altered propensity for bacterial clearance in the lung.

6. For histopathology and lesion score, fix trachea and lungs overnight in 10% buffered formalin and embed in paraffin. Obtain four to six µm thick sections and stain with Hematoxylin and Eosin prior to examination by microscope.

Basic Protocol Material List

Culture media

Brain Heart Infusion broth (Merck); 37g powder made up to 1 l with distilled water. Autoclave in glass bottles and store at room temperature for two to three months. Before use supplement BHI by adding 2 µg/ml nicotinamide adenine dinucleotide (NAD) (1 mg/ml stock solution) and 10 µg/ml haemin (10 mg/ml stock solution) (sBHI). NAD and haemin are both growth requirements for NTHi. For antibiotic resistant NTHi strains, antibiotics (e.g. streptomycin (300 µg/ml) or kanamycin (20 µg/ml)) are added to the growth medium as appropriate. Use sBHI within 24 hrs.

For plate growth make up BHI liquid medium then add agar (Oxoid) to 1% before autoclaving. Cool media to 50ºC, add supplements then pour into sterile petri dishes and allow to set. Store plates at 4ºC for up to two weeks.

Buffers

PBS; made up as per suppliers instructions (a typical solution contains 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4). Sterilize and store at room temperature for up to 6 months.

TBS; 50 mM Tris base, 150 mM sodium chloride, pH 7.6; store at room temperature for up to 6 months.

COMMENTARY

Background Information on Bacteria

Haemophilus influenzae is a Gram-negative bacterium that is part of the normal flora present in the human NP but is also a frequent etiological agent of disease in man. *H. influenzae* is divided into six typeable (a through f) or non-typeable (NTHi) forms based on the presence or absence of a polysaccharide capsule respectively. A majority of healthy adults have upper airway colonization with *H. influenzae* and the predominant strains (>98%) are NTHi. Residence in the NP enables *H. influenzae* to colonize and initiate infections in both the upper and lower respiratory tracts through contiguous spread; diseases caused by NT*Hi* are of significant public health importance and include AOM and acute pneumonia in young children, and bronchopneumonia in patients with chronic pulmonary diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD) (Murphy, 2003). In the UK, on average, at least one episode of AOM occurs in every child by the age of five, making it one of the commonest reasons for antibiotic prescription in general practice. In the developing world, acute lower respiratory tract infections top the list of causes of death in young infants of which about 20% are attributable to NTHi. (Shann et al., 1984). For adults, in 2013 COPD was the third leading cause of death in the USA and considered to be the third leading cause worldwide. Following the implementation of type b capsular conjugate vaccines, invasive (bacteraemic) *H. influenzae* infections have declined in frequency although NTHi is an emerging and significant cause of bacteraemia and meningitis.

The three major human otopathogens, NTHi, *Streptococcus pneumoniae* (pneumococcus) and *Moraxella catarrhalis* are each commensal bacteria commonly found in the human NP. The NP serves as a reservoir for respiratory tract infection. Each bacterium can spread contiguously and as well as being responsible for OM, are also commonly found in the sputum of COPD patients with acute and recurrent exacerbations (Sethi and Murphy, 2001). Thus, the increasing use of pneumococcal vaccines in children is resulting in NTHi becoming the predominant cause of bacterial respiratory infections, including AOM.

Some Modifications Used For NTHi Infection in Other Mouse Models

The presence of fluid in the *Junbo* mouse ME facilitates translocation of NTHi to the ear via a natural route (Eustachian tube) following IN inoculation. In other NTHi mouse AOM models, live or heat killed NTHi bacteria are introduced directly into the ME bulla; this can be achieved via direct injection through the tympanic membrane (Woo et al., 2014) or an incision made in the mouse neck to expose the bulla bone through which the inoculum is injected (Yao et al., 2014). The size of the NTHi inoculum can be adjusted depending upon the mouse line used and the nature of the investigation undertaken. Direct inoculation mouse OM models have been used to investigate potential treatment for the disease; an example is human β-defensin 2, expressed in the ME following introduction via an

adenoviral vector (Woo et al., 2015). In a modified co-infection AOM model, mice can be inoculated IN with influenza A virus then three days later challenged IN with NTHi; this results in significant bacterial infection of both the mouse ME and NP (Langereis et al., 2012).

Following IN inoculation, the distribution of NTHi in the mouse NP can be monitored; in a typical mouse line this can only be achieved reliably for up to the first 24 hours before bacteria are cleared. The carriage of NTHi in the mouse NP following IN inoculation has been used to investigate the competitive index for colonization between two NTHi strains and has also been used to study the efficacy of mouse immunization procedures.

NTHi are commonly introduced into the mouse lung to investigate host and bacterial factors relevant to the pathogenesis associated with COPD; this model is an important resource to study *in vivo* the immune mechanisms and regulation that respond to NTHi infection. Several methods can be used to alter the pathophysiology of the mouse lung to be more like that found in COPD patients. These include pre-exposure of mice to cigarette smoke (Roos et al., 2015), this treatment is carried out for four to 30 weeks prior to introduction of NTHi, and treatment of the lung with modifying molecules such as the enzyme elastase (Pang et al., 2008). Intra-tracheal inoculation is a less commonly used route to deliver NTHi than IN, but can provide higher bacterial doses more directly to the target tissue. Bacterial numbers, lung immunopathology and the host response over time (typically up to 48 hrs post-inoculation) can be studied by utilising mutant mouse lines that are altered in specific immune genes; this allows host-microbial interactions important for the progression and persistence of lung disease to be teased out (Roos et al., 2015). The expression pattern of both bacterial and host genes in response to NTHi infection of the lung can be studied from broncho-alveolar lavage (BAL) fluids and homogenised lung material. The lung infection model can also be used to test potential antimicrobial regimens to alleviate disease (Euba et al., 2015a) and the effectiveness of vaccination to prevent it (Lugade et al., 2014), again the focus is usually upon treatment of NTHi associated exacerbations of COPD. NTHi clearance in the mouse lung can be delayed when mice are first infected by rhinovirus then subsequently are superinfected with NTHi (Unger et al., 2012). A modified mouse model has also been used to investigate the relationship between NTHi infection, COPD and lung cancer (Chang et al., 2014)

NTHi Infection in Other Animal OM Models

Other than the mouse, several animal models have been reported for studies on OM including the chinchilla (*Chinchilla lanigera*) (Bakaletz, 2009) and the rat (Clark et al., 2000). The chinchilla has taken a lead in studies of NTHi pathogenesis because of the ease of

access to the middle ear bullae for infection and sampling. However, translocation to the ear from the NP is difficult to achieve in this model unless barotrauma or concomitant viral infection procedures are employed. When considering cost, litter size, availability of immunological reagents and control of host genetics through inbred and mutant host lines, the mouse presents substantial potential advantages for OM studies.

Critical parameters and Troubleshooting

Middle Ear Infection

Following IN inoculation, NTHi bacteria transfer rapidly along the NP and can access the ME space of the Junbo mouse within 1 hour (Hood et al., 2016). After day 4 post-inoculation there is a strong positive correlation between the presence of NTHi in both the ME and the NP of the *Junbo* mouse (Hood et al., 2016); this suggests that the ME can act as a reservoir for NP re-infection, or vice versa. The hypoxic inflamed ME in *Junbo* mice (Cheeseman et al., 2011) may favour the growth of microaerophilic bacteria such as NTHi.

Normal mouse microbial flora is also present in bulla fluid of the *Junbo* mouse after 5 weeks of age. In experiments using non-antibiotic resistant NTHi strains, bulla cultures giving *Proteus* overgrowth on the culture plate can prevent detection of NTHi colonies; these are not included in the calculations for infection rates and titres.

Protection experiments

Mice can be immunized with whole bacteria, bacterial lysates, outer membrane vesicles (OMVs), single purified antigens, or any combination thereof. To test the effectiveness of the antibody response in the mouse following immunization, serum obtained from immunized mice can be utilised in *in vitro* assays of bacterial killing to ascertain and compare the effectiveness of immunization between individual animals. The serum bactericidal (Ercoli et al., 2015) and opsonophagocytosis assays measure the effectiveness of the antibody raised in bacterial killing by complement and phagocytes respectively. These assays are key for determining the effectiveness and functional outcome of an immunization regime in the mouse and provide useful data that can be extrapolated to predict the effectiveness of vaccination in man with the same antigens.

Pulmonary infection

Intra-tracheal rather than IN inoculation of NTHi can be used to maximise the lung infection dose achieved. Intra-tracheal inoculation has been used to establish chronic NTHi lung infection by repeated dosing (e.g. twice a week for 8 weeks) (Lugade et al., 2014). As an alternative to homogenizing lung tissue, BAL fluids collected from the NTHi infected mouse

lungs can be used to investigate bacterial numbers and the mouse lung inflammatory response to infection. Immune cells present in the BAL can be isolated and examined, and RNA prepared for differential gene expression analysis.

Anticipated Results

Junbo mouse infection

Typical infection rates for NTHi OM strains in the *Junbo* mouse range from 40 to 90% ME infection with titres achieved of 10^4 to 10^5 c.f.u./µl at 7 days post-inoculation. At the same time point, semi-quantitative recovery of NTHi in NP washes typically yields bacterial titres of 10^1 to 10^2 c.f.u. in a 200 µl wash volume. During longitudinal studies with our most tested NTHi strain, 162sr, ME infection rates peak to 80-90% at day 7 to 14 post-inoculation and decrease to around 20% by day 35, then are maintained at this rate up to the maximum length of time tested of 56 days (Hood et al., 2016). The ME infection rates and titres are not significantly different when inoculum doses in the range of 10^4 to10^8 c.f.u. are used for NTHi strain 162 (Hood et al., 2016).

Bulla fluids and dissected soft palate tissue obtained from the NP of mice 7 days post-inoculation with NTHI 375gfp reveal the presence of individual and small aggregates of bacteria but no evidence of significant microcolony growth or large bacterial aggregates consistent with a mature NTHi biofilm being present in the ME of these mice (at this time point).

The histology of the ME in 12-week-old *Junbo* mice is overall similar between NTHi-challenged and non-challenged animals at day 7 post IN inoculation (Hood et al., 2016). 60% of the bulla is occupied by neutrophils and foamy macrophages and the average thickness of middle ear mucosa is 100 to 111 µm in NTHi-challenged and non-challenged mice respectively. Typically in the ME bulla, a necrotic caseous core of neutrophils is surrounded by viable and apoptotic neutrophils (cleaved caspase 3 positive) and an outer, variably thick, band of foamy macrophages (F 4/80 positive). Variable amounts of amorphous extracellular chromatin exist within the caseous areas.

When localizing NTHi bacteria in the bulla using *in situ* hybridization targeting the 16S rRNA of NTHi we found strong signals in the bulla exudate but not elsewhere in head tissues from *Junbo* mice challenged with NTHi, but not in non-challenged mice . NTHi hybridization signals comprise punctate or larger aggregates scattered throughout the ME bulla exudate but less frequent in the caseous core (Hood et al., 2016). Again, evidence consistent with the presence of significant or mature biofilm was not found.

Following infection IN with 10^6 c.f.u. NTHi bacteria we found that chemokine/cytokine levels were generally enhanced in the *Junbo* mouse ME over the period tested of one to 14 days post-inoculation, when compared to GF control animals; IL-17a, Tnfa, Ccl3 and Ccl14 demonstrated the highest relative upregulation (Hood et al., 2016).

Using the *Junbo* infection model we have shown by comparing isogenic wild type and mutant bacterial strains that the major NTHi membrane lipoprotein P4 is important for maintaining high bacterial loads during middle ear infection of the mouse (Su et al., 2016).

Protection experiments

When *Junbo* mice are immunized with one of three NTHi strains (162, 176, 375) then are each infected with NTHi 162, significant protection was found (15% infection rate for immunized versus 81% infection rate for control) for mice immunized with the homologous, but not mice immunized with the heterologous, NTHi strains (Hood et al., 2016). For mice that were immunized with heterologous NTHi strains (176, 375), bacterial titres attained in the middle ear were approximately one $\log_{10}$ lower than those found in control (PBS) immunized animals. Thus, there is discrimination in the model against homologous and heterologous NTHi challenge.

Antibiotic treatment

NTHi infected mice treated with a three day course of Azithromycin starting at day 4 post-inoculation had eliminated all NTHi from their middle ears when sampled post treatment (0% ME infection rate and NTHi titre of beneath detection limit of ($<10^2$ c.f.u./µl) in treated mice, whereas >80% of non-antibiotic control treated ears were infected at titres of 10^4 to 10^5 c.f.u./µl bulla fluid).

Lung infection

Typical lung infection titres achieved for NTHi by this method are ~10^4 c.f.u./lung at 24 hrs and 10^2 c.f.u./lung at 48 hrs post-inoculation. When the lung of the NTHi-infected *Junbo* mouse is monitored at up to 24 hours post-inoculation, mRNA levels of NF-κB regulated pro-inflammatory cytokines, such as TNF-α, IL-1β and MIP-2, are markedly increased compared to the lung of the wild type littermate mouse (Xu et al., 2012). Correspondingly, the histopathology of the NTHi-infected *Junbo* mouse lung shows enhanced leukocyte infiltration and neutrophil activity when compared to the wild type mouse. Lesions in the lungs can be scored subjectively depending upon the observed percentage of tissue affected, the epithelial changes noted, the degree of inflammatory cell infiltration and the nature of the

exudate present; for example a score of 0 to 3 where 0=absent, 1=mild, 2=moderate and 3=severe (Morey et al., 2013).

A mouse pulmonary infection model has been used to demonstrate *in vivo* efficacy of host-directed antimicrobial drugs against NTHi lung infection (Euba et al., 2015) and the role of NTHi membrane proteins P5 and Hap in NTHi virulence (Euba et al., 2015b).

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Conflicts of Interest

The authors have no conflict of interest to report.

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Current Protocols in Mouse Biology

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Non-typeable *Haemophilus influenzae* infection of the *Junbo* mouse

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Running title: NTHi mouse infection

Significance Statement

The *Junbo* mouse/NTHi infection model facilitates investigation of the complex host-microbial interactions that underpin the onset and development of otitis media (middle ear disease). Intranasal inoculation of the mouse with NTHi establishes robust middle ear infection, many aspects of which closely mimic features of this common disease in humans. Elements of both the bacteria and host that contribute to disease can be studied and novel treatment regimens investigated.

Abstract

Acute otitis media, inflammation of the middle ear bulla, is the most common bacterial infection in children. For one of the principal otopathogens, non-typeable *Haemophilus influenzae* (NTHi), animal models allow us to investigate host-microbial interactions relevant to the onset and progression of infection and to study treatment of middle ear disease. We have established a robust model of NTHi middle ear infection in the *Junbo* mouse. Intranasal inoculation with NTHi produces high rates of bulla infection and bacterial titres in bulla fluids; bacteria can also spread down the respiratory tract to the mouse lung. An innate immune response is detected in the bulla of *Junbo* mice following NTHi infection and bacteria are maintained in some ears at least up to day 56 post-inoculation. The *Junbo*/NTHi infection model facilitates studies on bacterial pathogenesis and antimicrobial intervention regimens and vaccines for better treatment and prevention of NTHi middle ear infection.

Keywords: otitis media, non-typeable *Haemophilus influenzae* (NTHi), *Junbo* mouse, middle ear infection, lung infection

INTRODUCTION

Acute otitis media (AOM), inflammation of the middle ear bulla, is caused by bacterial infection. It is the most common bacterial infection in children and the major reason for antibiotic prescription in this age group. Otitis media with effusion (OME), otherwise known as glue ear, can be a sequel to AOM and chronic OME is the most common cause of hearing impairment in children; grommet surgery to alleviate this condition is the most frequent paediatric surgery in Western countries. In the UK, on average, at least one episode of AOM occurs in every child by the age of five. Of the three major otopathogens, non-typeable *Haemophilus influenzae* (NTHi) is emerging as the predominant bacterium associated with AOM. NTHi is part of the normal commensal flora present in the human nasopharynx (NP); no natural animal hosts other than man have been identified for NTHi and there are no inanimate reservoirs. The molecular basis of the processes that lead to contiguous spread of NTHi within the respiratory tract and subsequent disease in the middle ear has been difficult to establish largely because animal models enabling study of all aspects of pathogenesis are not readily available.

Although other animal models have been reported for studies of AOM, mice have been utilized historically to elucidate virulence factors of otopathogens, mechanisms of adherence and invasion, and induction and specificity of immune responses to pathogens such as NTHi. When considering cost, litter size, availability of immunological reagents and control of host genetics through inbred and mutant host lines, the mouse presents substantial potential advantage over other animals for otitis media (OM) studies. Several genes associated with increased susceptibility to OM in the mouse have now been shown to be relevant to human disease through candidate gene studies performed using allelic association analyses in family-based cohorts (Rye et al., 2011). Genetic predisposition can be investigated by studying disease in multiple mouse lines that are each mutated in relevant genes that increase susceptibility to spontaneous or experimental infection of the middle ear. Mouse models can facilitate

investigations of the molecular basis and pathophysiology of NTHi infection and provide a means to realise improved treatment and prevention of disease in humans. Here we describe a robust model of NTHi infection using the *Junbo* mouse; this focuses upon the study of AOM but bacterial carriage and lung clearance can also be investigated.

Basic Protocol 1 describes the basis for establishing and monitoring middle ear infection in the *Junbo* mouse following intranasal inoculation of NTHi. Basic Protocol 2 describes the adaptation of Basic Protocol 1 to study protection against infection following immunization of the animals and Basic Protocol 3 the use of antibiotic to clear NTHi. Basic Protocol 4 describes a modified infection procedure that favours residence of bacteria in the mouse lung.

NOTE: All protocols using live animals must first be reviewed and approved by the appropriate ethics process such as the Animal Welfare and Ethical Review Body (AWERB) in the UK or the Institutional Animal Care and use Committee (IACUC) in the US, and must conform to local or national governmental regulations regarding the care and use of laboratory animals.

Basic Protocol 1

JUNBO MOUSE NTHI MIDDLE EAR INFECTION

Junbo mouse

Junbo is a mutant mouse line that spontaneously develops chronic middle ear (ME) inflammation under specific pathogen free (SPF) conditions at four to five weeks of age. The heterozygote *Junbo* mouse (*Jbo/+,* hereafter termed *Junbo*) bears an Asn763Ile mutation in the gene encoding the Evi1 transcription factor, otherwise known as Mecom (Parkinson et al., 2006). One mechanism that may underlie the predisposition to OM in *Junbo* mice is that Evi1 is a negative regulator of NFkB; the loss of function Evi1 *Junbo* mutation exacerbates NTHi induced inflammation in the lung (Xu et al., 2012). The pre-existing ME inflammation in *Junbo*

mice is critical for bacterial ME infection following intranasal (IN) inoculation, the healthy air-filled ME of wild type mice and the small proportion of *Junbo* mice (5 to 10%) with no fluid present do not sustain infection by NTHi.

NTHi strains

Wild-type NTHi strains used by us for ME infection studies are all isolates from children with OM (Cody et al., 2003) and are phylogenetically distinct (De Chiara et al., 2014). Following infection in the mouse, indigenous commensal bacterial flora, particularly *Proteus* spp., renders a proportion of bacterial count plates unreadable through overgrowth. For this reason, NTHi strains expressing spontaneous resistance to streptomycin (sr), or engineered resistance to kanamycin (kan), were generated (Hood et al., 2016). These strains enable antibiotic selection of NTHi during culture from mouse samples and counter-selection against any indigenous bacteria present; streptomycin selection proved particularly useful at maximizing quantitative NTHi culture data from mouse samples.

Materials

Mice - *Junbo* mice are congenic on a C3H/HeH background (Parkinson et al., 2006); mice are housed under SPF conditions and are mostly used at between eight to eleven weeks of age. The *Junbo* mouse is available from the European Mouse Mutant Archive (EM:00091) via MRC Harwell; for non-academic groups the *Junbo* mouse is available through MRC Technology, UK. For some studies Germ Free (GF) mice are used, details of the mouse husbandry and microbiological surveillance are given elsewhere (Hood et al., 2016).

NTHi – human OM disease isolates (162, 176, 375, 486, 1124 and 1158) (available from MRC Harwell) are used by us for mouse infection studies (Cody et al., 2003). Streptomycin resistant NTHi strains are designated sr, e.g. strain 162sr.

Autoclaved brain heart infusion (BHI) broth supplemented with haemin and NAD (sBHI) for liquid culture and sBHI agar for plate growth of bacteria (see recipe).

Sterile PBS/2% gelatine for inoculum preparation.

PBS for collecting and diluting *in vivo* samples.

Binocular dissecting microscope with x10 magnification and LED stage lighting.

scissors for removal of the head.

fine forceps for puncture of the tympanic membrane.

70% ethanol to sterilize the instruments between the sampling of ears.

A <2 µl volume pipette with sterile micro-tip to collect the small volume of ME fluids.

barbiturate solution (50% Euthatal) for intraperitoneal injection (delivered at 3.3 ml/kg body weight), or alternatively a rising CO_2 concentration to euthanize mice.

Mouse NTHi inoculum

1. Prepare bacteria for inoculation from -80^{o}C stocks stored in BHI/20% glycerol and grown overnight at 37^{O}C in 5% CO_2 atmosphere on sBHI agar. From this plate, inoculate sBHI broth with a 1 µl loop full of colony growth (to give an initial OD_{490} of ~0.03-0.05) and grow bacteria to log phase (OD_{490} 0.3-0.6) at 37^{O}C in a shaking incubator.
2. Calculate the size of inoculum for the mouse from optical density (OD_{490}) using the conversion factor that an absorbance of 0.4 is equivalent to approximately 1 x 10^9 c.f.u./ml.
3. Pellet bacteria by centrifugation at 13000 x g at room temperature for 3 min, remove supernatant and re-suspend pellet to achieve 10^{10} c.f.u./ml in PBS/2% gelatine.

4. Assess the titre of the inoculum by plating dilutions of each suspension onto sBHI agar plates prior to, and after, inoculation of the mice.

Intranasal challenge

5. Anesthetize mice aged either 8 ± 1 week or 11 ± 1 week with isofluorane (mixed with O_2 in anaesthetic chamber) and inoculate by applying 5 µl of bacterial suspension to each nostril via a pipette tip (Hood et al., 2016).

A typical inoculum is 10^6 c.f.u. but a range of inoculum sizes from 10^3-10^8 c.f.u. NTHi bacteria have been used in individual experiments with cohort sizes of n=11 to 15 mice.

Using bacteria that emit light (expressing lux genes) we have shown that NTHi are distributed along the entire length of the NP within minutes of IN inoculation (Hood et al., 2016).

Terminal sampling of nasopharynx and bulla fluids

6. Euthanize the mouse (typically at 7 days post-inoculation), use scissors to remove and skin the head (Fig. 1), then remove the mandible and sample the NP by washing with 200 µl PBS introduced into the NP opening on the palate and collecting the wash fluid from the nares into an Eppendorf tube (Fig. 2). Sampling the NP either before or after the ME has no statistically significant effect on the NP bacterial titres attained.

Avoid euthanasia by cervical dislocation of the mouse as it causes haemorrhage in the upper airway that will compromise sample collection.

A more quantitative approach to determine NP NTHi titres is to dissect out a part (e.g., soft palate) and homogenise the tissue in PBS prior to dilution and plating. Although this typically yields higher bacterial titres than the wash alone we find that there is a general positive correlation between the data from the two methods.

7. Under x10 binocular magnification, sample fluids from the ME bulla following perforation of the TM and removal of the ME conductive bones using sterile forceps (Fig. 2). Collect bulla fluids and estimate the volume using a 0-2 µl filtered pipette tip (average sampled volume ~0.75 µl, range 0.1-1.50 µl) (Fig. 2). Transfer fluids into 500 µl of PBS in an Eppendorf tube. In a small proportion of *Junbo* mice with clear TM there is no detectable bulla fluid. These bullae can be washed twice with 2 µl of sterile PBS and the washings added back to 500 µl of PBS for culture to monitor infection.

If required for gene or protein expression analysis, the primary bulla fluid preparation (suspended in PBS) is centrifuged at 13000 x g for 3 min then the pellet and supernatant frozen on dry ice prior to storage at -80^{O}C.

Infection rate and bacterial titre

8. Disperse bulla fluid in PBS, or NP wash, by three 10 second bursts on a vortex mixer then make 10-fold dilutions (10^{-1}, 10^{-2}) in PBS. Spread 50 µl of each ME dilution or undiluted NP wash on a sBHI agar plate. Incubate agar plates overnight at 37^{O}C.

The detection limit is 10 c.f.u./µl for the initial bulla fluid suspension and 100 c.f.u./µl for the 10^{-1} dilution. In experiments using antibiotic-resistant NTHi, samples are plated on sBHI agar plates supplemented with the appropriate antibiotic (e.g., 300 µg/ml streptomycin or 20 µg/ml kanamycin). If required, non-selective agar plates can be used in parallel to monitor the presence of commensal bacteria.

9. Calculate an infection rate for the middle ear:

Infection rate = number of NTHi positive bulla fluids/number of bulla fluids sampled.

As an example, if from a cohort of 12 mice (24 ears) there are 2 ears with no fluid and 22 bulla fluids are sampled, 18 of which give a positive NTHi culture on plates, then the ME infection rate is calculated as 18/22 or 81.8%.

10. Calculate a bacterial titre (c.f.u./µl ME fluid) from the number of colonies obtained on culture plates, adjusted for sample dilution and bulla fluid volume.

As an example, if 85 bacterial colonies were obtained on a culture plate spread with 50 µl of a 10^{-2} dilution made from 1 µl ME fluid dispersed into 500 µl PBS, this would correspond to a ME bacterial titre of 8.5 x 10^4 c.f.u./µl fluid.

NTHi has also been shown to infect the bulla of other mutant mouse lines that exhibit ME inflammation and OM (Hood et al., 2016); these include the Jeff (Hardisty-Hughes et al., 2006) and Tgif (Tateossian et al., 2013) mouse mutants. The protocol will similarly work for Streptococcus pneumoniae *(pneumococcus) infection although this has been tested by us only in the* Junbo *mouse. We note that* Streptococcus pneumoniae *strain D39 is highly virulent in* Junbo *mice. All new infection models should be established with less virulent bacterial strains, and pilot studies with small numbers of mice should be conducted first to establish whether there are unexpected adverse effects.*

Basic Protocol 1 - Support Protocol 1

JUNBO MOUSE NTHI MIDDLE EAR INFECTION – Histology and Distribution of NTHi

Following infection of the *Junbo* mouse, the histology and the relative distribution of NTHi of the infected ME bullae can be examined by a number of techniques that include histology, immunohistochemistry (IHC), *in situ* hybridization (ISH) and lesion profiling. Typically this is carried out using 11–week-old *Junbo* mice inoculated IN with 10^6 c.f.u. NTHi and heads collected seven days post-inoculation as described above in Basic Protocol 1. Heads from non-NTHi challenged *Junbo* mice collected at the same time serve as negative controls.

Histology

Haematoxylin and Eosin stained sections

1. ME histology on infected and non-infected mice is assessed using 4 μm wax sections (Parkinson et al., 2006). To do this, fix skinned mouse heads for 48 hrs in 10% neutral buffered formalin. Decalcify bone using EDTA and embed decalcified tissues in paraffin wax then cut 4 μm dorsal plane sections before staining with Haematoxylin and Eosin, or special stains. Carry out morphometric analysis and lesion profiling on standard 1000 μm lengths of ME mucosa as described below.

We note that Gram staining failed to identify NTHi in tissue sections so IHC and ISH may be the best approaches to identify and localise bacteria.

Immunohistochemistry and In situ hybridization

2. Decalcify formalin fixed tissues using EDTA. Dissected mouse heads can be decalcified in 5 to 7 days. To maximize RNA integrity for any *in situ* work, a band saw can be used to isolate the bullae from heads fixed for 24 hours and EDTA decalcification can be achieved in 48 hours.

3. Cut 4 μm thick wax sections onto electrostatically charged slides and dry overnight at 37°C before a final drying at 60°C for 25 minutes. De-wax sections in xylene then hydrate through ethanol and washing three times in Tris buffered saline (TBS). Block endogenous peroxidase using Dako REAL peroxidase blocker (S2023) for 10 minutes following antibody incubations.

4. Detect target cells in the mouse ME fluid using antibodies against appropriate antigen markers. An illustrative example is using rat monoclonal anti-F4/80 (Serotec MCA497G) to detect mouse macrophages. Perform antigen retrieval using Dako proteinase K (S3020) for 20 minutes at room temperature. Incubate the primary antibody (diluted 1/400) with sections for 30 minutes at room temperature; carry out secondary antibody detection using the Vector Labs ImmPress HRP anti-rat kit (MP-744-15). Dilute

antibodies in Dako antibody diluent (S0809) and carry out negative controls using the antibody diluent alone. Visualize the target using the Dako (K3468) liquid DAB+ substrate chromogen system. Counterstain using Harris haematoxylin prior to dehydration through ethanol, clearing in xylene and mounting in Clearview mountant (Thermo Fisher Scientific).

5. Perform *in situ* hybridization on representative NTHi-challenged and non-challenged *Junbo* mice. As an illustrative example we use a probe that targets the 16sRNA of the bacteria. Hybridize 4 μm wax sections with probe B-HInfluenzae-NTHi375-16SrRNA as per the manufacturer's instructions (Advanced Cell Diagnostic) using the HRP visualization kit. Use a positive control for RNA integrity (e.g. PpiB) and a negative hybridization control (e.g. DapB) (Advanced Cell Diagnostic) in experiments.

ISH is a useful approach when antibodies against bacteria produce high background or suitable ones are not commercially available. We found that mouse antisera from NTHi immunization and protection studies did not make useful reagents to localise bacteria in bulla sections. Another advantage of ISH is that there are a wide range of mouse probes available making it potentially possible to obtain hybridization signals for bacteria and host cells in the same section.

NTHi bacteria can also be visualized directly following infection of the Junbo mouse. NTHi strains expressing the lux genes emit bioluminescent signals that can be used to monitor bacterial distribution in situ in the skinned heads of infected Junbo mice; bacteria are imaged using an IVIS Lumina II system (Perkin Elmer) (Hood et al., 2016). NTHi strains expressing a green fluorescent protein (GFP) can be detected following infection by confocal microscopy on mouse bulla fluid and soft palate samples.

Lesion profiling

6. Acquire bright field images of Haematoxylin and Eosin stained sections using a slide scanner such as a Hamamatsu NanoZoomer or equivalent and make the morphometric measurements using suitable software.
7. Assess the mucosal thickness and the numbers and size of capillary and lymphatic vessels present as required (Cheeseman et al., 2011).
8. Calculate the average thickness of the mucosa lining the medial surface of the bulla (avoiding the cochlea and the region close to the Eustachian tube) by dividing the area of mucosa overlying a delineated ~1000 μm length of supporting bulla bone.
9. Calculate the proportion of bulla space occupied by exudate by dividing the exudate area by the area bounded by bulla mucosa surface and the TM.

If comparing infected and non-infected ME then blind slides for the analysis.

Basic Protocol 1 - Support Protocol 2

IMMUNE RESPONSE MEASURED BY REAL TIME QUANTITATIVE PCR (RT-QPCR) OF BULLA FLUIDS

Following NTHi infection of the *Junbo* mouse as described above in Basic Protocol 1, the degree of the host immune response dependent upon microbial infection in the ME can be estimated by RT-qPCR determination of cytokine and chemokine mRNA levels.

1. Inoculate SPF *Junbo* mice with 10^6 c.f.u. NTHi and sample cohorts terminally at day 1, 3, 7, 10 or 14 post-inoculation, collect and process ME fluids as described in Basic Protocol 1.
2. Obtain cell pellets by centrifugation (13000 x g for 5 mins) of bulla fluids (*n*=4 to 6) collected in PBS that yielded NTHi monocultures from the 10^{-1} dilution when cultured; this is equivalent to <100 c.f.u./μl commensal bacteria present. Place pellets on dry ice

and store at -80°C prior to RNA isolation. Obtain at least three biological replicate pools for each time point.

3. As a baseline control, collect bulla fluids (*n*=4 to 6) from eight to ten week old non-infected germ free (GF) *Junbo* mice into 20 µl of RNase free water for RNA isolation. Each GF pool of bulla fluids comprises at least 3 biological replicates as controls for each time point. As an alternative to GF mice, measure the baseline control expression level using bulla fluids obtained from age-matched non-NTHi infected SPF *Junbo* mice.
4. Extract RNA from the re-suspended bulla fluid cell pellets and carry out cDNA synthesis and RT-qPCR TaqMan as described by Cheeseman et al. (Cheeseman et al., 2011). Typical immune molecule genes to be monitored are those activated by bacterial ligands binding to TLR-receptors that are expressed by macrophages, neutrophils and monocytes and are known to be relevant to OM and AOM in man (Juhn et al., 2008; Kaur et al., 2015). Genes that we routinely monitor include Ccl3, Ccl4, Ccl5, IL-1b, IL-6, IL-12a, IL-17a, Tnfa, Vegfa (Hood et al., 2016). Perform RT-qPCR in triplicate technical assays. Normalize data using Hrpt1 and beta Actin as the endogenous control and calculate fold changes of expression (ddCts) of NTHi-infected bulla fluid cells over non-infected bulla fluid cells using AB 7500 software v2.0.1 and express as mean relative quantification (RQ) ± min/max error bars representing 95% CI.

Basic Protocol 2

MOUSE IMMUNIZATION AND PROTECTION AGAINST NTHi INFECTION

In this procedure, mice are subcutaneously immunized with either whole killed bacteria or with derived and purified cell wall fractions or selected antigens (Ercoli et al., 2015). Following a three step immunization procedure, the mice are inoculated IN with live NTHi bacteria to assess if antibody raised in the mouse elicits protection against infection in the ME or carriage in the

NP. Significant levels of protection when found compared to control animals can act as a surrogate that predicts the efficacy of an antigen combination for use as a vaccine in humans.

Materials

five week old SPF *Junbo* mice (MRC Harwell)

killed NTHi bacteria for immunization

live NTHi bacteria (Basic Protocol 1 steps 1 to 5) for intranasal challenge post-immunization

Adjuvant

PBS/0.08% paraformaldehyde

materials for terminal sampling of animals and fluids as described in Basic Protocol 1

Immunization with NTHi bacteria

1. Obtain pre-immune serum as a base-line control for antibody levels prior to the first immunization being carried out. Prepare small volumes of sera from blood obtained from mice by tail vein bleed (see step 8).

2. When using whole NTHi bacteria for mouse immunization, culture NTHi to mid-log phase (OD_{490} 0.2 to 0.6) in sBHI broth. Pellet 1 ml of culture at 13000 x g for 3 mins then resuspend the pellet and wash in PBS.

3. To kill the bacteria, pellet the washed suspension at 13000 x g before re-suspending in 500 µl of PBS/0.08% paraformaldehyde (PFA). Incubate the suspension for 1 hour at 37^{o}C then overnight at 4^{o}C before culturing 20 µl of the neat bacterial suspension on sBHI agar to confirm loss of viability of bacteria.

4. Store killed NTHi in PBS/ PFA for up to 8 weeks at 4^{o}C, confirm bacterial integrity before each use by spreading 5 µl of the bacterial suspension on a microscope slide and examining by phase contrast microscopy.

5. Prior to immunization, pellet the killed bacteria by centrifugation at 13000 x g for 3 mins, remove the supernatant and then re-suspend in PBS to achieve 10^{10} c.f.u./ml before mixing with adjuvant according to the suppliers instructions. We typically use Adjuplex (Sigma) adjuvant at a 1:4 ratio with the bacterial suspension.

6. Immunize each *Junbo* mouse with three subcutaneous injections in intra-scapular skin of a mixture of 10^8 c.f.u. killed bacteria and adjuvant in a 50 µl final volume. The first immunizations are at the age of 5 weeks, then subsequently at 8 weeks and 10 weeks of age.

7. Take a second intermediate blood sample by tail vein bleed prior to the second immunization when the mice are at 8 weeks of age.

8. Inoculate immunized mice IN at 12 weeks with 10^6 c.f.u. NTHi bacteria. Determine the ME infection rate and bacterial titre by terminal sampling at 7 days post-inoculation (mice at 13 weeks of age), as described above in Basic Protocol 1.

9. Obtain terminal blood samples at the end of the experiment by retro-orbital bleed immediately after euthanasia of the mouse. Incubate blood samples at room temperature for two hours then obtain serum using 1.1 ml Z-Gel spin columns (Sarstedt) following centrifugation according to the supplier's instructions. Store serum at -80ºC until required.

In a typical protection experiment cohorts of 12 to 15 mice are immunized with alternative NTHi strains then are infected with either the homologous or heterologous bacteria. Control mice are immunized with PBS and adjuvant. When comparing the bulla infection rate and bacterial titres in PBS-immunized control mice with those of NTHi-immunized mice, any significant reduction in NTHi ME infection rate or titre in the NTHi-immunized mice will indicate protection dependent upon antibody produced in those vaccinated mice (Hood et al., 2016).

Basic Protocol 3

ANTIMICROBIAL TREATMENT OF NTHI INFECTION

Antibiotic resistance is on the increase for otopathogens such as NTHi and in the clinic this could soon have a have a major impact on treatment regimens for disease. The *Junbo*/NTHi infection model can be used to study antimicrobial treatment for AOM; as an example we describe the procedure for oral administration and systemic treatment of NTHi-infected mice with the clinically relevant antibiotic, Azithromycin.

Materials

Eight week old SPF *Junbo* mice

Azithromycin in 2% methoxycellulose (or other antibiotic in solution as appropriate)

NTHi bacteria for intranasal challenge as described in Basic Protocol 1

materials for terminal sampling of animals and fluids as described in Basic Protocol 1

1. Inoculate eight week old *Junbo* mice IN with 10^6 c.f.u. of NTHi bacteria (Basic Protocol 1 steps 1 to 5).
2. At day four post IN-inoculation, give mice a three day course of an antimicrobial; for example, 100 mg/kg of the antibiotic Azithromycin in a 2% methoxycellulose solution delivered once a day by oral gavage.
3. As a control, use mice inoculated with NTHi at the same time as the treatment group of animals but gavaged with 2% methoxycellulose solution alone for three days.
4. Sample bulla fluids terminally on day seven post IN-inoculation and ascertain the number of bacteria by dilution and plating as described in Basic Protocol 1. If required, also determine the number of bacteria in the NP by sampling through a terminal NP wash (Basic Protocol 1).

5. Determine the efficacy of antibiotic treatment by the difference in ME bulla infection rates and bulla NTHi titres between the antimicrobial treated and control mice.

Importantly, as well as its use for oral administration of antimicrobials, the Junbo*/NTHi infection model has added value through its utility for investigating potential new application strategies specifically targeted at the ME; antimicrobials can potentially be applied directly on the TM and subsequently transferred into the ME bulla.*

The outcome of the antimicrobial treatment can be ascertained for NTHi alone by plating mouse samples on the appropriate selective growth plate for the respective NTHi strain or can include an indication of the effect on other general bacterial flora present by also plating samples on non-selective growth medium.

Basic Protocol 4

NTHI MOUSE PULMONARY INFECTION MODEL

NTHi pulmonary infection has been used to investigate the host immune response in the *Junbo* mouse (Xu et al., 2012). To achieve lung infection the mouse can be inoculated via the intratracheal route but here we describe the more straightforward IN method adapted from that of Morey et al. (Morey et al., 2013).

Materials

Eight week old SPF Junbo mice

NTHi bacteria for intranasal challenge (as described in Basic Protocol 1)

Materials for terminal sampling of animals and fluids (as described in Basic Protocol 1)

Homogeniser (we use an IKA Ultra-Turax T25 operated in a Class II Microbiological safety cabinet)

1. Prior to infection, grow NTHi overnight on sBHI agar then use to inoculate sBHI broth. Grow bacteria to log phase (OD_{490} 0.3-0.6), pellet by centrifugation at 13000 x g for 3 min, remove the supernatant and then re-suspend to achieve 5 x 10^9 c.f.u./ml in PBS/2% gelatine.
2. Anesthetize mice aged eight weeks with isofluorane and inoculate IN by applying 10 µl of bacterial suspension to each nostril (10^8 c.f.u. in 20 µl total volume).
3. Euthanize the mouse (described in Basic Protocol 1) typically at time points either 24 or 48 hours post-inoculation. Remove lungs aseptically, weigh individually then homogenize to release bacteria from the tissue.
4. Plate serial tenfold dilutions of the lung homogenate in PBS on sBHI agar plates, incubate overnight at 37ºC then determine the number of bacteria in the lung from the colony counts on plates, the dilution factor, and the lung homogenate volume (detection limit <10 c.f.u./ml) (as described in Basic Protocol 1 steps 8 and 10).
5. To obtain a histopathology and lesion score, fix trachea and lungs overnight in 10% buffered formalin and embed in paraffin. Obtain four to six µm thick sections and stain with Hematoxylin and Eosin prior to examination by microscope.

These experiments are typically used to compare bacterial counts following infection between different wild type strains or paired isogenic wild type and mutant NTHi strains; the difference in bacterial count serves as an indicator of altered propensity for bacterial clearance in the lung.

Basic Protocol Material List

Culture media

Brain Heart Infusion broth (Merck); 37g powder made up to 1 l with distilled water. Autoclave in glass bottles and store at room temperature for two to three months. Before use supplement BHI by adding 2 µg/ml nicotinamide adenine dinucleotide (NAD) (1 mg/ml stock solution) and 10

μg/ml haemin (10 mg/ml stock solution) (sBHI). NAD and haemin are both growth requirements for NTHi. For antibiotic resistant NTHi strains, antibiotics (e.g. streptomycin (300 μg/ml) or kanamycin (20 μg/ml)) are added to the growth medium as appropriate. Use sBHI within 24 hrs.

For plate growth make up BHI liquid medium then add agar (Oxoid) to 1% before autoclaving. Cool media to 50ºC, add supplements then pour into sterile petri dishes and allow to set; store plates at 4ºC for up to two weeks.

Buffers

PBS; made up as per suppliers instructions (a typical solution contains 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4). Sterilize and store at room temperature for up to 6 months.

TBS; 50 mM Tris base, 150 mM sodium chloride, pH 7.6; store at room temperature for up to 6 months.

COMMENTARY

Background Information on Bacteria

Haemophilus influenzae is a Gram-negative bacterium that is part of the normal flora present in the human NP but is also a frequent etiological agent of disease in humans. *H. influenzae* is divided into six typeable (a through f) or non-typeable (NTHi) forms based on the presence or absence of a polysaccharide capsule, respectively. A majority of healthy adults have upper airway colonization with *H. influenzae* and the predominant strains (>98%) are NTHi. Residence in the NP enables *H. influenzae* to colonize and initiate infections in both the upper and lower respiratory tracts through contiguous spread; diseases caused by NT*Hi* are of significant public health importance and include AOM and acute pneumonia in young children, and bronchopneumonia in patients with chronic pulmonary diseases such as cystic fibrosis and

chronic obstructive pulmonary disease (COPD) (Murphy, 2003). In the UK, on average, at least one episode of AOM occurs in every child by the age of five, making it one of the commonest reasons for antibiotic prescription in general practice. In the developing world, acute lower respiratory tract infections top the list of causes of death in young infants of which about 20% are attributable to NTHi (Shann et al., 1984). For adults, in 2013 COPD was the third leading cause of death in the USA and considered to be the third leading cause worldwide. Following the implementation of type b capsular conjugate vaccines, invasive (bacteraemic) *H. influenzae* infections have declined in frequency although NTHi is an emerging and significant cause of bacteraemia and meningitis.

The three major human otopathogens, NTHi, *Streptococcus pneumoniae* (pneumococcus) and *Moraxella catarrhalis* are each commensal bacteria commonly found in the human NP. The NP serves as a reservoir for respiratory tract infection. Each bacterium can spread contiguously and as well as being responsible for OM, are also commonly found in the sputum of COPD patients with acute and recurrent exacerbations (Sethi and Murphy, 2001). Thus, the increasing use of pneumococcal vaccines in children is resulting in NTHi becoming the predominant cause of bacterial respiratory infections, including AOM.

Some Modifications Used For NTHi Infection in Other Mouse Models

The presence of fluid in the *Junbo* mouse ME facilitates translocation of NTHi to the ear via a natural route (Eustachian tube) following IN inoculation. In other NTHi mouse AOM models, live or heat killed NTHi bacteria are introduced directly into the ME bulla; this can be achieved via direct injection through the tympanic membrane (Woo et al., 2014) or an incision made in the mouse neck to expose the bulla bone through which the inoculum is injected (Yao et al., 2014). The size of the NTHi inoculum can be adjusted depending upon the mouse line used and the nature of the investigation undertaken. Direct inoculation mouse OM models have been used to

investigate potential treatment for the disease; an example is human β-defensin 2, expressed in the ME following introduction via an adenoviral vector (Woo et al., 2015). In a modified co-infection AOM model, mice can be inoculated IN with influenza A virus then three days later challenged IN with NTHi; this results in significant bacterial infection of both the mouse ME and NP (Langereis et al., 2012).

Following IN inoculation, the distribution of NTHi in the mouse NP can be monitored; in a typical mouse line this can only be achieved reliably for up to the first 24 hours before bacteria are cleared. The carriage of NTHi in the mouse NP following IN inoculation has been used to investigate the competitive index for colonization between two NTHi strains and has also been used to study the efficacy of mouse immunization procedures.

NTHi are commonly introduced into the mouse lung to investigate host and bacterial factors relevant to the pathogenesis associated with COPD; this model is an important resource to study *in vivo* the immune mechanisms and regulation that respond to NTHi infection. Several methods can be used to alter the pathophysiology of the mouse lung to be more like that found in COPD patients. These include pre-exposure of mice to cigarette smoke (Roos et al., 2015), a treatment carried out for four to 30 weeks prior to introduction of NTHi, and treatment of the lung with modifying molecules such as the enzyme elastase (Pang et al., 2008). Intra-tracheal inoculation is a less commonly used route to deliver NTHi than IN, but can provide higher bacterial doses more directly to the target tissue. Bacterial numbers, lung immunopathology and the host response over time (typically up to 48 hrs post-inoculation) can be studied by utilising mutant mouse lines that are altered in specific immune genes; this allows host-microbial interactions important for the progression and persistence of lung disease to be teased out (Roos et al., 2015). The expression pattern of both bacterial and host genes in response to NTHi infection of the lung can be studied from broncho-alveolar lavage (BAL) fluids and homogenised lung material. The lung infection model can also be used to test potential

antimicrobial regimens to alleviate disease (Euba et al., 2015a) and the effectiveness of vaccination to prevent it (Lugade et al., 2014), again the focus is usually upon treatment of NTHi associated exacerbations of COPD. NTHi clearance in the mouse lung can be delayed when mice are first infected by rhinovirus then subsequently are superinfected with NTHi (Unger et al., 2012). A modified mouse model has also been used to investigate the relationship between NTHi infection, COPD and lung cancer (Chang et al., 2014)

NTHi Infection in Other Animal OM Models

Other than the mouse, several animal models have been reported for studies on OM including the chinchilla (*Chinchilla lanigera*) (Bakaletz, 2009) and the rat (Clark et al., 2000). The chinchilla has taken a lead in studies of NTHi pathogenesis because of the ease of access to the middle ear bullae for infection and sampling. However, translocation to the ear from the NP is difficult to achieve in this model unless barotrauma or concomitant viral infection procedures are employed. When considering cost, litter size, availability of immunological reagents and control of host genetics through inbred and mutant host lines, the mouse presents substantial potential advantages for OM studies.

Critical parameters and Troubleshooting

Middle Ear Infection

Following IN inoculation, NTHi bacteria transfer rapidly along the NP and can access the ME space of the Junbo mouse within 1 hour (Hood et al., 2016). After day 4 post-inoculation there is a strong positive correlation between the presence of NTHi in both the ME and the NP of the *Junbo* mouse (Hood et al., 2016); this suggests that the ME can act as a reservoir for NP re-infection, or vice versa. The hypoxic inflamed ME in *Junbo* mice (Cheeseman et al., 2011) may favour the growth of microaerophilic bacteria such as NTHi.

Normal mouse microbial flora is also present in bulla fluid of the *Junbo* mouse after 5 weeks of age. In experiments using non-antibiotic resistant NTHi strains, bulla cultures giving *Proteus* overgrowth on the culture plate can prevent detection of NTHi colonies; these are not included in the calculations for infection rates and titres.

Protection experiments

Mice can be immunized with whole bacteria, bacterial lysates, outer membrane vesicles (OMVs), single purified antigens, or any combination thereof. To test the effectiveness of the antibody response in the mouse following immunization, serum obtained from immunized mice can be utilised in *in vitro* assays of bacterial killing to ascertain and compare the effectiveness of immunization between individual animals. The serum bactericidal (Ercoli et al., 2015) and opsonophagocytosis assays measure the effectiveness of the antibody raised in bacterial killing by complement and phagocytes respectively. These assays are key for determining the effectiveness and functional outcome of an immunization regime in the mouse and provide useful data that can be extrapolated to predict the effectiveness of vaccination in man with the same antigens.

Pulmonary infection

Intra-tracheal rather than IN inoculation of NTHi can be used to maximise the lung infection dose achieved. Intra-tracheal inoculation has been used to establish chronic NTHi lung infection by repeated dosing (e.g. twice a week for 8 weeks) (Lugade et al., 2014). As an alternative to homogenizing lung tissue, BAL fluids collected from the NTHi infected mouse lungs can be used to investigate bacterial numbers and the mouse lung inflammatory response to infection. Immune cells present in the BAL can be isolated and examined, and RNA prepared for differential gene expression analysis.

Anticipated Results

Junbo mouse infection

Typical infection rates for NTHi OM strains in the *Junbo* mouse range from 40 to 90% ME infection with titres achieved of 10^4 to 10^5 c.f.u./µl at 7 days post-inoculation. At the same time point, semi-quantitative recovery of NTHi in NP washes typically yields bacterial titres of 10^1 to 10^2 c.f.u. in a 200 µl wash volume. During longitudinal studies with our most tested NTHi strain, 162sr, ME infection rates peak to 80-90% at day 7 to 14 post-inoculation and decrease to around 20% by day 35, then are maintained at this rate up to the maximum length of time tested of 56 days (Hood et al., 2016). The ME infection rates and titres are not significantly different when inoculum doses in the range of 10^4 to10^8 c.f.u. are used for NTHi strain 162 (Hood et al., 2016).

Bulla fluids and dissected soft palate tissue obtained from the NP of mice 7 days post-inoculation with NTHI 375gfp reveal the presence of individual and small aggregates of bacteria but no evidence of significant microcolony growth or large bacterial aggregates consistent with a mature NTHi biofilm being present in the ME of these mice (at this time point).

The histology of the ME in 12-week-old *Junbo* mice is overall similar between NTHi-challenged and non-challenged animals at day 7 post IN inoculation (Hood et al., 2016). 60% of the bulla is occupied by neutrophils and foamy macrophages and the average thickness of middle ear mucosa is 100 to 111 µm in NTHi-challenged and non-challenged mice respectively. Typically in the ME bulla, a necrotic caseous core of neutrophils is surrounded by viable and apoptotic neutrophils (cleaved caspase 3 positive) and an outer, variably thick, band of foamy macrophages (F 4/80 positive). Variable amounts of amorphous extracellular chromatin exist within the caseous areas.

When localizing NTHi bacteria in the bulla using *in situ* hybridization targeting the 16S rRNA of NTHi we found strong signals in the bulla exudate but not elsewhere in head tissues from *Junbo* mice challenged with NTHi, but not in non-challenged mice . NTHi hybridization signals

comprise punctate or larger aggregates scattered throughout the ME bulla exudate but less frequent in the caseous core (Hood et al., 2016). Again, evidence consistent with the presence of significant or mature biofilm was not found.

Following infection IN with 10^6 c.f.u. NTHi bacteria we found that chemokine/cytokine levels were generally enhanced in the *Junbo* mouse ME over the period tested of one to 14 days post-inoculation, when compared to GF control animals; IL-17a, Tnfa, Ccl3 and Ccl14 demonstrated the highest relative upregulation (Hood et al., 2016).

Using the *Junbo* infection model we have shown by comparing isogenic wild type and mutant bacterial strains that the major NTHi membrane lipoprotein P4 is important for maintaining high bacterial loads during middle ear infection of the mouse (Su et al., 2016).

Protection experiments

When *Junbo* mice are immunized with one of three NTHi strains (162, 176, 375) then are each infected with NTHi 162sr, significant protection was found (15% infection rate for immunized versus 81% infection rate for control) for mice immunized with the homologous, but not mice immunized with the heterologous, NTHi strains (Hood et al., 2016). For mice that were immunized with heterologous NTHi strains (176, 375), bacterial titres attained in the middle ear were approximately one $\log_{10}$ lower than those found in control (PBS) immunized animals. Thus, there is discrimination in the model against homologous and heterologous NTHi challenge.

Antibiotic treatment

NTHi infected mice treated with a three day course of Azithromycin starting at day 4 post-inoculation had eliminated all NTHi from their middle ears when sampled post treatment (0% ME infection rate and NTHi titre of beneath detection limit of ($<10^2$ c.f.u./µl) in treated mice, whereas >80% of non-antibiotic control treated ears were infected at titres of 10^4 to 10^5 c.f.u./µl bulla fluid).

Lung infection

Typical lung infection titres achieved for NTHi by this method are ~10^4 c.f.u./lung at 24 hrs and 10^2 c.f.u./lung at 48 hrs post-inoculation. When the lung of the NTHi-infected *Junbo* mouse is monitored at up to 24 hours post-inoculation, mRNA levels of NF-κB regulated pro-inflammatory cytokines, such as TNF-α, IL-1β and MIP-2, are markedly increased compared to the lung of the wild type littermate mouse (Xu et al., 2012). Correspondingly, the histopathology of the NTHi-infected *Junbo* mouse lung shows enhanced leukocyte infiltration and neutrophil activity when compared to the wild type mouse. Lesions in the lungs can be scored subjectively depending upon the observed percentage of tissue affected, the epithelial changes noted, the degree of inflammatory cell infiltration and the nature of the exudate present; for example a score of 0 to 3 where 0=absent, 1=mild, 2=moderate and 3=severe (Morey et al., 2013).

A mouse pulmonary infection model has been used to demonstrate *in vivo* efficacy of host-directed antimicrobial drugs against NTHi lung infection (Euba et al., 2015) and the role of NTHi membrane proteins P5 and Hap in NTHi virulence (Euba et al., 2015b).

Time Considerations

NTHi infection of *Junbo* mouse

The time for the combined bacterial preparation and inoculation procedures, relevant to a cohort of 12 mice being utilised in Basic Protocols 1 to 4, is between 4 to 5 hours, having previously cultured the NTHi strain overnight on plates. Typical times taken are two and a half to three hours for bacterial culture in liquid, 30 minutes to prepare the inoculum, and 60 minutes to inoculate the mice. Our standard infection period for NTHi in the *Junbo* mouse is seven days post-inoculation. Terminal sampling of the mouse NP and ME and plating of bacteria from a cohort of 12 mice takes around three hours; culture plates must then be incubated overnight before counting colonies to ascertain bacterial titres.

Mouse immunization and protection experiment

A typical three step immunization procedure covers a seven week period, followed immediately by a seven day infection period with the test bacteria, making a total of eight weeks for each experiment. At each step, immunization of a cohort of 12 mice takes up to 30 minutes depending upon experience. Obtaining blood samples by retro-orbital bleed will add up to 30 minutes to the time taken for terminal sampling following bacterial infection. The processing of blood to obtain serum samples takes a further two and a half hours.

Antibiotic treatment of NTHi infection

The protocol for NTHi infection of the *Junbo* mouse, followed by a three dose oral administration of antibiotic, spans a seven day time period. Oral gavage takes around 30 minutes for a cohort of 12 mice.

NTHi mouse pulmonary infection

Following the standard NTHi intranasal inoculation procedure, the infection experiment is typically run for a 24 or 48 hour time period prior to terminal sampling of bacteria from the animals. Terminal sampling of bacteria from mouse lung homogenate takes about three hours.

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Conflicts of Interest

The authors have no conflict of interest to report.

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Figure 1

(**A**) The dissected *Junbo* mouse head with hair on. (**B**) Making the initial incision with scissors under the skin on the top of the head moving forwards from the back towards the snout. (**C**) Pulling cut skin forward to reveal skinned head underneath.

Figure 2

(**A**) Skinned head of *Junbo* mouse (top view). (**B**) Skinned head of *Junbo* mouse (side view), the access to the tympanic membrane from the outer ear is indicated by the arrow. (**C**) Removing mandible by cutting through each side of the jaw with scissors. (**D**) View of *Junbo* mouse head with mandible removed (underside view), the rectangle indicates the area enlarged in panel E and the arrow the position of the nasopharyngeal opening on the palate. (**E**) Inserting the pipette tip into nasopharyngeal opening to wash the nasopharynx with PBS, eluate is collected through the nares into a sterile Eppendorf tube. (**F**) Clear tympanic membrane with no underlying middle ear fluid (top), inflamed cloudy tympanic membrane with underlying fluid (bottom). (**G**) Puncturing the tympanic membrane and removing the middle ear conductive bones using sterile fine forceps. (**H**) Inserting filtered pipette tip into middle ear bulla. (**I**) Removing the middle ear fluid with pipette tip; if requiring all accessible bulla fluid this is obtained typically in between 5 and 8 aliquots by repeated sampling.

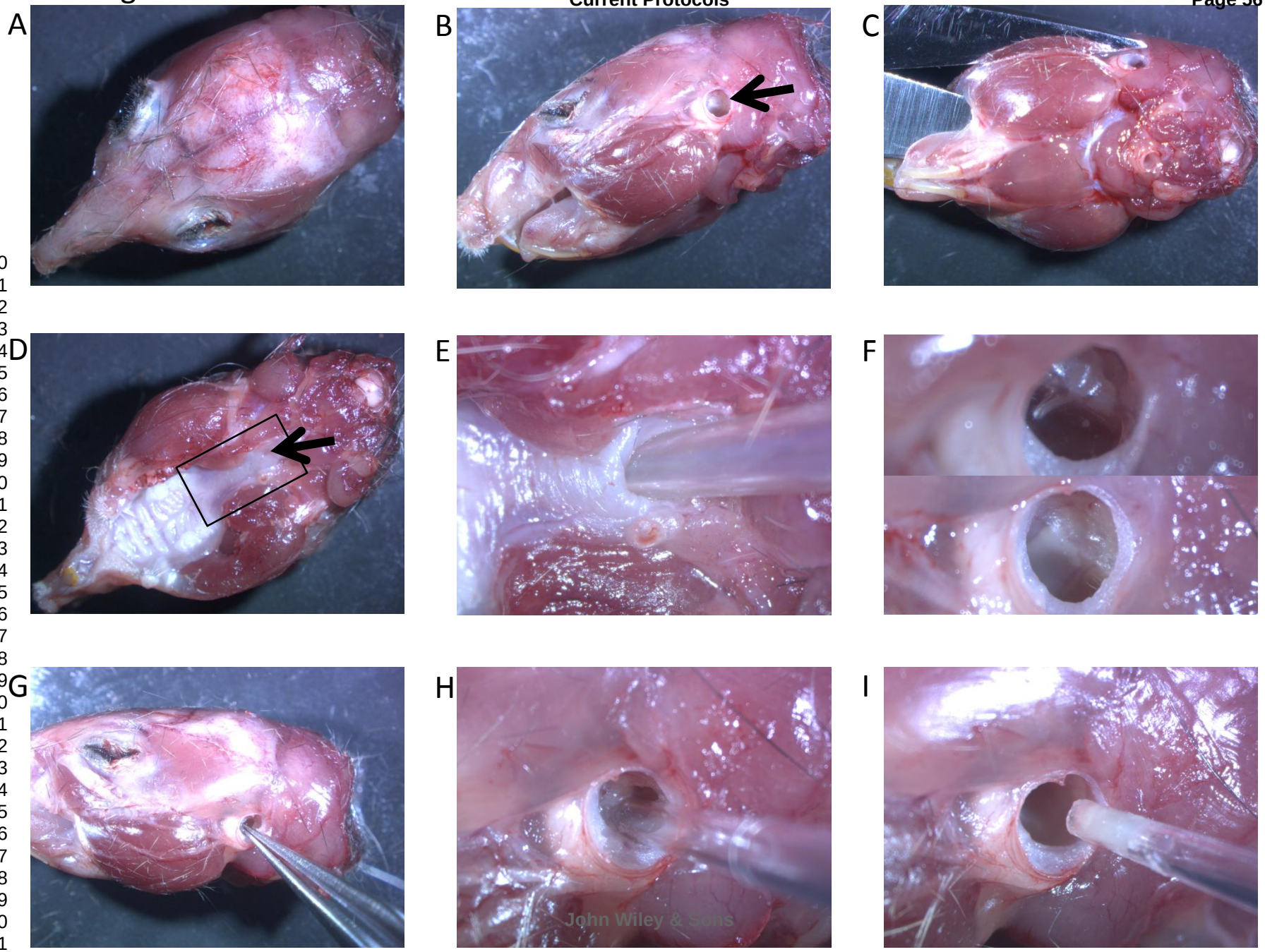
Figure 1

A

B

C

Figure 2



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