



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

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

Citation for published version:

Hood, D & Cheeseman, M 2017, 'Non-typeable *Haemophilus influenzae* infection of the Junbo mouse', *Current Protocols in Mouse Biology*, vol. 7, no. 1, pp. 29-46. <https://doi.org/10.1002/cpmo.24>

Digital Object Identifier (DOI):

[10.1002/cpmo.24](https://doi.org/10.1002/cpmo.24)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Current Protocols in Mouse Biology

Publisher Rights Statement:

This is the peer reviewed version of the following article: Cheeseman, M.T. and Hood, D.W. 2017. Non-typeable *Haemophilus influenzae* infection of the Junbo mouse. *Curr. Protoc. Mouse Biol.* 7:29-46., which has been published in final form at <http://dx.doi.org/10.1002/cpmo.24>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.





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

Journal:	<i>Current Protocols</i>
Manuscript ID	CP-16-0090.R1
Wiley - Manuscript type:	Protocol
Date Submitted by the Author:	12-Aug-2016
Complete List of Authors:	Cheeseman, Michael; The Roslin Institute and Royal (Dick) School of Veterinary Studies, Developmental Biology Hood, Derek; MRC Harwell, Molecular Genetics Unit
Keywords:	otitis media, nontypeable <i>Haemophilus influenzae</i> , 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 <i>Haemophilus influenzae</i> (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.

SCHOLARONE™
Manuscripts

1
2
3 **Non-typeable *Haemophilus influenzae* infection of the *Junbo* mouse**
4
5

6
7 Michael T. Cheeseman¹ and Derek W. Hood^{2*}
8

9
10 ¹ The Roslin Institute and Royal (Dick) School of Veterinary Studies
11 University of Edinburgh, Easter Bush, EH25 9RG, UK.
12

13
14 ² Molecular Genetics Unit, MRC Harwell, Harwell Science and Innovation Campus,
15 Oxfordshire, OX11 0RD, UK.
16

17 (phone +44 1235841177; email d.hood@har.mrc.ac.uk)
18
19

20
21
22 * Corresponding author
23
24
25
26
27
28
29
30
31
32
33
34

35 Running title: NTHi mouse infection
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 Acute otitis media (AOM), inflammation of the middle ear bulla, is caused by bacterial
4 infection. It is the most common bacterial infection in children and reason for antibiotic
5 prescription in this age group. Otitis media with effusion (OME), otherwise known as glue
6 ear, can be a sequel to AOM and chronic OME is the most common cause of hearing
7 impairment in children; grommet surgery to alleviate this condition is the most frequent
8 paediatric surgery in Western countries. In the UK, on average, at least one episode of AOM
9 occurs in every child by the age of five. Of the three major otopathogens, non-typeable
10 *Haemophilus influenzae* (NTHi) is emerging as the predominant bacterium associated with
11 AOM. NTHi is part of the normal commensal flora present in the human nasopharynx (NP);
12 no natural animal hosts other than man have been identified for NTHi and there are no
13 inanimate reservoirs. The molecular basis of the processes that lead to contiguous spread of
14 NTHi within the respiratory tract and subsequent disease in the middle ear has been difficult
15 to establish largely because animal models enabling study of all aspects of pathogenesis are
16 not readily available.

17
18
19
20
21
22
23
24
25 Although other animal models have been reported for studies of AOM, mice have been
26 utilized historically to elucidate virulence factors of otopathogens, mechanisms of adherence
27 and invasion, and induction and specificity of immune responses to pathogens such as
28 NTHi. When considering cost, litter size, availability of immunological reagents and control of
29 host genetics through inbred and mutant host lines, the mouse presents substantial potential
30 advantage over other animals for otitis media (OM) studies. Several genes associated with
31 increased susceptibility to OM in the mouse have now been shown to be relevant to human
32 disease through candidate gene studies performed using allelic association analyses in
33 family-based cohorts (Rye et al., 2011). Genetic predisposition can be investigated by
34 studying disease in multiple mouse lines that are each mutated in relevant genes that
35 increase susceptibility to spontaneous or experimental infection of the middle ear. Mouse
36 models can facilitate investigations of the molecular basis and pathophysiology of NTHi
37 infection and provide a means to realise improved treatment and prevention of disease in
38 man. Here we describe a robust model of NTHi infection using the *Junbo* mouse; this
39 focuses upon the study of AOM but bacterial carriage and lung clearance can also be
40 investigated.

41
42
43
44
45
46
47
48
49
50 Basic Protocol 1 describes the basis for establishing and monitoring middle ear infection in
51 the *Junbo* mouse following intranasal inoculation of NTHi. Basic Protocol 2 describes the
52 adaptation of Basic Protocol 1 to study protection against infection following immunization of
53 the animals and Basic Protocol 3 the use of antibiotic to clear NTHi. Basic Protocol 4
54 describes a modified infection procedure that favours residence of bacteria in the mouse
55 lung.

1
2
3 NOTE: All protocols using live animals must first be reviewed and approved by the
4 appropriate ethics process such as the Animal Welfare and Ethical Review Body (AWERB)
5 in the UK or the Institutional Animal Care and Use Committee (IACUC) in the US, and must
6 conform to local or national governmental regulations regarding the care and use of
7 laboratory animals.
8
9

10 11 **Basic Protocol 1**

12 13 ***JUNBO* MOUSE NTHI MIDDLE EAR INFECTION**

14 15 ***Junbo* mouse**

16
17
18 *Junbo* is a mutant mouse line that spontaneously develops chronic middle ear (ME)
19 inflammation under specific pathogen free (SPF) conditions at four to five weeks of age. The
20 heterozygote *Junbo* mouse (*Jbo*+/+, hereafter termed *Junbo*) bears an Asn763Ile mutation in
21 the gene encoding the Evi1 transcription factor, otherwise known as Mecom (Parkinson et
22 al., 2006). One mechanism that may underlie the predisposition to OM in *Junbo* mice is that
23 Evi1 is a negative regulator of NFκB; the loss of function Evi1 *Junbo* mutation exacerbates
24 NTHi induced inflammation in the lung (Xu et al., 2012). The pre-existing ME inflammation in
25 *Junbo* mice is critical for bacterial ME infection following intranasal (IN) inoculation, the
26 healthy air-filled ME of wild type mice and the small proportion of *Junbo* mice (5 to 10%) with
27 no fluid present do not sustain infection by NTHi.
28
29

30 31 ***NTHi* strains**

32
33
34 Wild-type NTHi strains used by us for ME infection studies are all isolates from children with
35 OM (Cody et al., 2003) and are phylogenetically distinct (De Chiara et al., 2014). Following
36 infection in the mouse, indigenous commensal bacterial flora, particularly *Proteus* spp.,
37 renders a proportion of bacterial count plates unreadable through overgrowth. For this
38 reason, NTHi strains expressing spontaneous resistance to streptomycin, or engineered
39 resistance to kanamycin, were generated (Hood et al., 2016). These strains enable antibiotic
40 selection of NTHi during culture from mouse samples and counter-selection against any
41 indigenous bacteria present; streptomycin selection proved particularly useful at maximizing
42 quantitative NTHi culture data from mouse samples.
43
44

45 46 ***Materials***

47
48
49 Mice - *Junbo* (*Jbo*+/+) mice are congenic on a C3H/HeH background (Parkinson et al., 2006);
50 mice are housed under SPF conditions and are mostly used at between eight to eleven
51 weeks of age. For some studies Germ Free (GF) mice are used, details of the mouse
52 husbandry and microbiological surveillance are given elsewhere (Hood et al., 2016).
53
54
55
56
57
58
59
60

1
2
3 NTHi – human OM disease isolates (162, 176, 375, 486, 1124 and 1158) are used for
4 mouse infection studies (Cody et al., 2003).

5
6 Autoclaved brain heart infusion (BHI) broth supplemented with haemin and NAD (sBHI) for
7 liquid culture and sBHI agar for plate growth of bacteria.

8
9
10 Sterile PBS/2% gelatine for inoculum preparation, PBS for collecting and diluting *in vivo*
11 samples.

12
13
14 Binocular dissecting microscope with x10 magnification and LED stage lighting.

15
16
17 scissors for removal of the head and fine forceps for puncture of the tympanic membrane,
18 70% ethanol to sterilize the instruments between the sampling of ears. A <2 µl volume
19 pipette with sterile micro-tip to collect the small volume of ME fluids.

20
21
22 barbiturate solution for intraperitoneal injection or alternatively a rising CO₂ concentration to
23 euthanize mice.
24

25 26 **Mouse NTHi inoculum**

27
28 1. Prepare bacteria for inoculation from -80°C stocks stored in BHI/20% glycerol and grown
29 overnight at 37°C in 5% CO₂ atmosphere on sBHI agar. From this plate, inoculate sBHI
30 broth with a 1 µl loop full of colony growth (to give an initial OD₄₉₀ of ~0.03-0.05) and grow
31 bacteria to log phase (OD₄₉₀ 0.3-0.6) at 37°C in a shaking incubator. The size of inoculum
32 for the mouse is calculated from optical density (OD₄₉₀) using the conversion factor that an
33 absorbance of 0.4 is equivalent to approximately 1 x 10⁹ c.f.u./ml. Pellet bacteria by
34 centrifugation at 13000 x g at room temperature for 3 min and re-suspend to give 10¹⁰
35 c.f.u./ml in PBS/2% gelatine. Assess the titre of the inoculum by plating dilutions of each
36 suspension onto sBHI agar plates prior to, and after, inoculation of the mice.
37
38
39
40
41
42

43 **Intranasal challenge**

44
45 2. Anesthetize mice aged either eight ± 1 week or 11 ± 1 week with isoflurane and inoculate
46 by applying 5 µl of bacterial suspension to each nostril via a pipette tip (Hood et al., 2016).

47
48 *A typical inoculum is 10⁶ c.f.u. but a range of inoculum sizes from 10³ -10⁸ c.f.u. NTHi*
49 *bacteria have been used in individual experiments with cohort sizes of n=11 to 15 mice.*

50
51
52
53 *Using bacteria that emit light (expressing lux genes) we have shown that NTHi are*
54 *distributed along the entire length of the NP within minutes of IN inoculation (Hood et al.,*
55 *2016).*
56
57
58
59
60

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 μ l 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 μ l filtered pipette tip (average sampled volume \sim 0.75 μ l, range 0.1-1.50 μ l). Transfer fluids into 500 μ l 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 μ l of sterile PBS and the washings added back to 500 μ l 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°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 μ l of each ME dilution or undiluted NP wash on a sBHI agar plate. The detection limit is 10 c.f.u./ μ l for the initial bulla fluid suspension and 100 c.f.u./ μ l 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 μ g/ml streptomycin or 20 μ g/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°C.

Calculate an infection rate for the middle ear:

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

1
2
3 Bacterial titres given as c.f.u./ μ l fluid are calculated from the number of colonies obtained on
4 culture plates, adjusted for sample dilution and bulla fluid volume.

5
6 *NTHi* has also been shown to infect the bulla of other mutant mouse lines that exhibit ME
7 inflammation and OM (Hood et al., 2016); these include the Jeff (Hardisty-Hughes et al.,
8 2006) and *tgif* (Tateossian et al., 2013) mice. The protocol will similarly work for
9 Streptococcus pneumoniae (*pneumococcus*) infection although this has been tested by us
10 only in the Junbo mouse. We note that Streptococcus pneumoniae strain D39 is highly
11 virulent in Junbo mice. All new infection models should be established with less virulent
12 bacterial strains, and pilot studies with small numbers of mice should be conducted first to
13 establish whether there are unexpected adverse effects.

14 **Histology, immunohistochemistry and in situ hybridization of ME**

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

20 **Histology**

21 1. Assess ME histology on infected and non-infected mice using 4 μ m wax sections
22 (Parkinson et al., 2006). Fix skinned mouse heads for 48 hrs in 10% neutral buffered
23 formalin. Decalcify bone using EDTA and embed decalcified tissues in paraffin wax then cut
24 4 μ m dorsal plane sections before staining with Haematoxylin and Eosin, or special stains.
25 Carry out morphometric analysis and lesion profiling on standard 1000 μ m lengths of ME
26 mucosa as described below.

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

29 **Immunohistochemistry and In situ hybridization**

30 1. Decalcify formalin fixed tissues using EDTA. Dissected mouse heads can be decalcified in
31 5 to 7 days. To maximize RNA integrity for any *in situ* work, a band saw can be used to
32 isolate the bullae from heads fixed for 24 hours and EDTA decalcification can be achieved in
33 48 hours.

34 2. Cut 4 μ m thick wax sections onto electrostatically charged slides and dry overnight at
35 37°C before a final drying at 60°C for 25 minutes. De-wax sections in xylene then hydrate
36 through ethanol and washing three times in Tris buffered saline (TBS). Block endogenous
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 peroxidase using Dako REAL peroxidase blocker (S2023) for 10 minutes following antibody
4 incubations.
5

6
7 3. Detect target cells with antigen markers in the mouse ME using appropriate antibodies. An
8 illustrative example is using rat monoclonal anti-F4/80 (Serotec MCA497G) to detect mouse
9 macrophages. Perform antigen retrieval using Dako proteinase K (S3020) for 20 minutes at
10 room temperature. Incubate the primary antibody (diluted 1/400) with sections for 30 minutes
11 at room temperature; carry out secondary antibody detection using the Vector Labs
12 ImmPress HRP anti-rat kit (MP-744-15). Dilute antibodies in Dako antibody diluent (S0809)
13 and carry out negative controls using the antibody diluent alone. Visualize the target using
14 the Dako (K3468) liquid DAB+ substrate chromogen system. Counterstain using Harris
15 haematoxylin prior to dehydration through ethanol, clearing in xylene and mounting in
16 Clearview mountant (Thermo Fisher Scientific).
17
18
19
20

21
22 4. Perform *in situ* hybridization on representative NTHi-challenged and non-challenged
23 *Junbo* mice. As an illustrative example we use a probe that targets the 16sRNA of the
24 bacteria. Hybridize 4 μm wax sections with probe B-HInfluenzae-NTHi375-16SrRNA as per
25 the manufacturer's instructions (Advanced Cell Diagnostic) using the HRP visualization kit.
26 Use a positive control for RNA integrity (e.g. PpiB) and a negative hybridization control (e.g.
27 DapB) (Advanced Cell Diagnostic) in experiments.
28
29
30
31

32 *ISH is a useful approach when antibodies against bacteria produce high background or*
33 *suitable ones are not commercially available. We found that mouse antisera from NTHi*
34 *immunization and protection studies did not make useful reagents to localise bacteria in*
35 *bulla sections. Another advantage of ISH is that there are a wide range of mouse probes*
36 *available making it potentially possible to obtain hybridization signals for bacteria and host*
37 *cells in the same section.*
38
39
40

41 **Lesion profiling**

42
43 For lesion profiling, acquire bright field images of Haematoxylin and Eosin stained sections
44 using a slide scanner such as a Hamamatsu NanoZoomer or equivalent and make the
45 morphometric measurements using suitable software. Assess the mucosal thickness and the
46 numbers and size of capillary and lymphatic vessels present as required (Cheeseman et al.,
47 2011). Calculate the average thickness of the mucosa lining the medial surface of the bulla
48 (avoiding the cochlea and the region close to the Eustachian tube) by dividing the area of
49 mucosa overlying a delineated ~ 1000 μm length of supporting bulla bone. Calculate the
50 proportion of bulla space occupied by exudate by dividing the exudate area by the area
51 bounded by bulla mucosa surface and the TM.
52
53
54
55
56

57 *If comparing infected and non-infected ME then blind slides for the analysis.*
58
59
60

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./ μ l 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 μ l 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) \pm 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).

1
2
3 2. Detect NTHi strains expressing a GFP protein by confocal microscopy on mouse bulla
4 fluid and soft palate samples.
5

6 3. Detect NTHi by ISH in tissue sections (see above).
7
8
9

10 **Basic Protocol 2**

11 **MOUSE PROTECTION STUDIES**

12
13
14 Mice are subcutaneously immunized with either whole killed bacteria or with derived and
15 purified cell wall fractions or selected antigens (Ercoli et al., 2015). Following a three step
16 immunization procedure, the mice are inoculated IN with live NTHi bacteria to assess if
17 antibody raised in the mouse elicits protection against infection in the ME or carriage in the
18 NP. Significant levels of protection when found compared to control animals can act as a
19 surrogate that predicts the efficacy of an antigen combination for use as a vaccine in man.
20
21
22
23

24 **Materials**

25
26 five week old SPF *Junbo* mice

27
28 killed NTHi bacteria for immunization; live NTHi bacteria for infection post immunization

29
30 Adjuvant

31
32 PBS/0.08% paraformaldehyde

33
34 materials for terminal sampling of animals and fluids as described in Protocol 1
35

36 **Immunization with NTHi bacteria**

37
38 1. Obtain pre-immune serum as a base-line control for antibody levels prior to the first
39 immunization being carried out. Prepare small volumes of sera from blood obtained from
40 mice by tail vein bleed (see step 6).
41
42

43 2. When using whole NTHi bacteria for mouse immunization, culture NTHi to mid-log phase
44 (OD₄₉₀ 0.2 to 0.6) in sBHI broth. Pellet 1 ml of culture at 13000 x g for 3 mins, resuspend the
45 pellet and wash in PBS, then pellet the bacteria at 13000 x g before re-suspending in 500 µl
46 of PBS/0.08% paraformaldehyde (PFA). Kill bacteria by incubating the suspension for 1 hour
47 at 37°C then overnight at 4°C; confirm loss of viability by culturing 20 µl of the neat bacterial
48 suspension on sBHI agar. Store killed NTHi in PBS/ PFA for up to 8 weeks at 4°C, assess
49 bacterial integrity by phase contrast microscopy.
50
51
52
53

54 3. Prior to Immunization, pellet the killed bacteria by centrifugation at 13000 x g for 3 mins
55 then re-suspend in PBS to give 10¹⁰ c.f.u./ml before mixing with adjuvant according to the
56
57
58
59
60

1
2
3 suppliers instructions. We typically use Adjuvax (Sigma) adjuvant at a 1:4 ratio with the
4 bacterial suspension.
5

6
7 4. Immunize each *Junbo* mouse with three subcutaneous injections in intra-scapular skin of
8 a mixture of 10^8 c.f.u. killed bacteria and adjuvant in a 50 μ l final volume. The first
9 immunizations are at the age of 5 weeks, then subsequently at 8 weeks and 10 weeks of
10 age. Take a second intermediate blood sample by tail vein bleed prior to the second
11 immunization when the mice are at 8 weeks of age.
12

13
14 5. Inoculate immunized mice IN at 12 weeks with 10^6 c.f.u. NTHi bacteria. Determine the ME
15 infection rate and bacterial titre by terminal sampling at 7 days post-inoculation (mice at 13
16 weeks of age), as described above in Protocol 1.
17

18
19 6. Obtain terminal blood samples at the end of the experiment by retro-orbital bleed
20 immediately after euthanasia of the mouse. Incubate blood samples at room temperature for
21 two hours then obtain serum using 1.1 ml Z-Gel spin columns (Sarstedt) following
22 centrifugation according to the supplier's instructions. Store serum at -80°C until required.
23

24
25 *In a typical protection experiment cohorts of 12 to 15 mice are immunized with alternative*
26 *NTHi strains then are infected with either the homologous or heterologous bacteria. By*
27 *comparing with the infection rate in control mice immunized with PBS, the degree of*
28 *protection (reduced ME bulla infection and bulla NTHi titres) can be ascertained.*
29
30
31
32
33

34 35 36 **Basic Protocol 3**

37 38 **ANTIMICROBIAL TREATMENT OF NTHI INFECTION**

39
40 Antibiotic resistance is on the increase for otopathogens such as NTHi and in the clinic this
41 could soon have a have a major impact on treatment regimens for disease. The *Junbo*/NTHi
42 infection model can be used to study antimicrobial treatment for AOM; as an example we
43 describe the procedure for oral administration and systemic treatment of NTHi-infected mice
44 with the clinically relevant antibiotic, Azithromycin.
45
46
47
48

49
50
51 1. Inoculate eight week old *Junbo* mice IN with 10^6 c.f.u. of NTHi bacteria.
52

53
54 2. At day four post IN-inoculation, give mice a three day course of an antimicrobial; for
55 example, 100 mg/kg of the antibiotic Azithromycin in a 2% methoxycellulose solution
56 delivered once a day by oral gavage.
57
58
59
60

1
2
3 3. As a control, use mice inoculated with NTHi at the same time as the treatment group of
4 animals but gavaged with 2% methoxycellulose solution alone for three days.
5

6
7 4. Sample bulla fluids terminally on day seven post IN-inoculation and ascertain the number
8 of bacteria by dilution and plating as described above in Protocol 1. If required, also
9 determine the number of bacteria in the NP by sampling through a terminal NP wash
10 (Protocol 1).
11

12
13 5. Determine the efficacy of antibiotic treatment by the difference in ME bulla infection rates
14 and bulla NTHi titres between the antimicrobial treated and control mice.
15

16
17 *Importantly, as well as its use for oral administration of antimicrobials, the Junbo/NTHi*
18 *infection model has added value through its utility for investigating potential new application*
19 *strategies specifically targeted at the ME; antimicrobials can be applied directly on the TM*
20 *and subsequently transferred into the ME bulla.*
21

22
23 *The outcome of the antimicrobial treatment can be ascertained for NTHi alone by plating*
24 *mouse samples on the appropriate selective growth plate for the respective NTHi strain or*
25 *can include an indication of the effect on other general bacterial flora present by also plating*
26 *samples on non-selective growth medium.*
27
28
29

30 31 **Basic Protocol 4**

32 33 **NTHi MOUSE PULMONARY INFECTION MODEL**

34
35 NTHi pulmonary infection has been used to investigate the host immune response in the
36 *Junbo* mouse (Xu et al., 2012). To achieve lung infection the mouse can be inoculated via
37 the intratracheal route but here we describe the more straightforward IN method adapted
38 from that of Morey et al. (Morey et al., 2013).
39

40
41 1. Prior to infection, grow NTHi overnight on sBHI agar then use to inoculate sBHI broth.
42 Grow bacteria to log phase (OD_{490} 0.3-0.6), pellet by centrifugation at 13000 x g for 3 min
43 then re-suspend to give 5×10^9 c.f.u./ml in PBS/2% gelatine.
44

45
46 2. Anesthetize mice aged eight weeks with isoflurane and inoculate IN by applying 10 μ l of
47 bacterial suspension to each nostril (10^8 c.f.u. in 20 μ l total volume).
48

49
50 3. Euthanize the mouse (described in Protocol 1) typically at time points either 24 or 48
51 hours post-inoculation. Remove lungs aseptically, weigh individually then homogenize to
52 release bacteria from the tissue.
53
54
55
56
57
58
59
60

1
2
3 4. Plate serial tenfold dilutions of the lung homogenate in PBS on sBHI agar plates, incubate
4 overnight at 37°C then determine the number of bacteria in the lung from the colony counts
5 on plates, the dilution factor, and the lung homogenate volume (detection limit <10 c.f.u./ml).
6
7

8
9 5. These experiments are typically used to compare bacterial counts between different wild
10 type or paired isogenic wild type and mutant NTHi strains; the difference in bacterial count
11 serves as an indicator of altered propensity for bacterial clearance in the lung.
12

13
14 6. For histopathology and lesion score, fix trachea and lungs overnight in 10% buffered
15 formalin and embed in paraffin. Obtain four to six µm thick sections and stain with
16 Hematoxylin and Eosin prior to examination by microscope.
17
18

19 **Basic Protocol Material List**

20 **Culture media**

21
22 Brain Heart Infusion broth (Merck); 37g powder made up to 1 l with distilled water. Autoclave
23 in glass bottles and store at room temperature for two to three months. Before use
24 supplement BHI by adding 2 µg/ml nicotinamide adenine dinucleotide (NAD) (1 mg/ml stock
25 solution) and 10 µg/ml haemin (10 mg/ml stock solution) (sBHI). NAD and haemin are both
26 growth requirements for NTHi. For antibiotic resistant NTHi strains, antibiotics (e.g.
27 streptomycin (300 µg/ml) or kanamycin (20 µg/ml)) are added to the growth medium as
28 appropriate. Use sBHI within 24 hrs.
29
30
31
32
33
34

35 For plate growth make up BHI liquid medium then add agar (Oxoid) to 1% before
36 autoclaving. Cool media to 50°C, add supplements then pour into sterile petri dishes and
37 allow to set. Store plates at 4°C for up to two weeks.
38
39

40 **Buffers**

41
42 PBS; made up as per suppliers instructions (a typical solution contains 0.01 M phosphate
43 buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4). Sterilize and store at
44 room temperature for up to 6 months.
45
46
47

48 TBS; 50 mM Tris base, 150 mM sodium chloride, pH 7.6; store at room temperature for up to
49 6 months.
50
51

52 **COMMENTARY**

53
54 *Background Information on Bacteria*
55
56
57
58
59
60

1
2
3 *Haemophilus influenzae* is a Gram-negative bacterium that is part of the normal flora present
4 in the human NP but is also a frequent etiological agent of disease in man. *H. influenzae* is
5 divided into six typeable (a through f) or non-typeable (NTHi) forms based on the presence
6 or absence of a polysaccharide capsule respectively. A majority of healthy adults have upper
7 airway colonization with *H. influenzae* and the predominant strains (>98%) are NTHi.
8
9 Residence in the NP enables *H. influenzae* to colonize and initiate infections in both the
10 upper and lower respiratory tracts through contiguous spread; diseases caused by NTHi are
11 of significant public health importance and include AOM and acute pneumonia in young
12 children, and bronchopneumonia in patients with chronic pulmonary diseases such as cystic
13 fibrosis and chronic obstructive pulmonary disease (COPD) (Murphy, 2003). In the UK, on
14 average, at least one episode of AOM occurs in every child by the age of five, making it one
15 of the commonest reasons for antibiotic prescription in general practice. In the developing
16 world, acute lower respiratory tract infections top the list of causes of death in young infants
17 of which about 20% are attributable to NTHi. (Shann et al., 1984). For adults, in 2013 COPD
18 was the third leading cause of death in the USA and considered to be the third leading
19 cause worldwide. Following the implementation of type b capsular conjugate vaccines,
20 invasive (bacteraemic) *H. influenzae* infections have declined in frequency although NTHi is
21 an emerging and significant cause of bacteraemia and meningitis.
22
23
24
25
26
27
28
29
30

31 The three major human otopathogens, NTHi, *Streptococcus pneumoniae* (pneumococcus)
32 and *Moraxella catarrhalis* are each commensal bacteria commonly found in the human NP.
33 The NP serves as a reservoir for respiratory tract infection. Each bacterium can spread
34 contiguously and as well as being responsible for OM, are also commonly found in the
35 sputum of COPD patients with acute and recurrent exacerbations (Sethi and Murphy, 2001).
36 Thus, the increasing use of pneumococcal vaccines in children is resulting in NTHi becoming
37 the predominant cause of bacterial respiratory infections, including AOM.
38
39
40
41
42

43 *Some Modifications Used For NTHi Infection in Other Mouse Models*

44
45 The presence of fluid in the *Junbo* mouse ME facilitates translocation of NTHi to the ear via
46 a natural route (Eustachian tube) following IN inoculation. In other NTHi mouse AOM
47 models, live or heat killed NTHi bacteria are introduced directly into the ME bulla; this can be
48 achieved via direct injection through the tympanic membrane (Woo et al., 2014) or an
49 incision made in the mouse neck to expose the bulla bone through which the inoculum is
50 injected (Yao et al., 2014). The size of the NTHi inoculum can be adjusted depending upon
51 the mouse line used and the nature of the investigation undertaken. Direct inoculation
52 mouse OM models have been used to investigate potential treatment for the disease; an
53 example is human β -defensin 2, expressed in the ME following introduction via an
54
55
56
57
58
59
60

1
2
3 adenoviral vector (Woo et al., 2015). In a modified co-infection AOM model, mice can be
4 inoculated IN with influenza A virus then three days later challenged IN with NTHi; this
5 results in significant bacterial infection of both the mouse ME and NP (Langereis et al.,
6 2012).
7
8

9
10 Following IN inoculation, the distribution of NTHi in the mouse NP can be monitored; in a
11 typical mouse line this can only be achieved reliably for up to the first 24 hours before
12 bacteria are cleared. The carriage of NTHi in the mouse NP following IN inoculation has
13 been used to investigate the competitive index for colonization between two NTHi strains
14 and has also been used to study the efficacy of mouse immunization procedures.
15
16

17
18 NTHi are commonly introduced into the mouse lung to investigate host and bacterial factors
19 relevant to the pathogenesis associated with COPD; this model is an important resource to
20 study *in vivo* the immune mechanisms and regulation that respond to NTHi infection. Several
21 methods can be used to alter the pathophysiology of the mouse lung to be more like that
22 found in COPD patients. These include pre-exposure of mice to cigarette smoke (Roos et
23 al., 2015), this treatment is carried out for four to 30 weeks prior to introduction of NTHi, and
24 treatment of the lung with modifying molecules such as the enzyme elastase (Pang et al.,
25 2008). Intra-tracheal inoculation is a less commonly used route to deliver NTHi than IN, but
26 can provide higher bacterial doses more directly to the target tissue. Bacterial numbers, lung
27 immunopathology and the host response over time (typically up to 48 hrs post-inoculation)
28 can be studied by utilising mutant mouse lines that are altered in specific immune genes; this
29 allows host-microbial interactions important for the progression and persistence of lung
30 disease to be teased out (Roos et al., 2015). The expression pattern of both bacterial and
31 host genes in response to NTHi infection of the lung can be studied from broncho-alveolar
32 lavage (BAL) fluids and homogenised lung material. The lung infection model can also be
33 used to test potential antimicrobial regimens to alleviate disease (Euba et al., 2015a) and the
34 effectiveness of vaccination to prevent it (Lugade et al., 2014), again the focus is usually
35 upon treatment of NTHi associated exacerbations of COPD. NTHi clearance in the mouse
36 lung can be delayed when mice are first infected by rhinovirus then subsequently are
37 superinfected with NTHi (Unger et al., 2012). A modified mouse model has also been used
38 to investigate the relationship between NTHi infection, COPD and lung cancer (Chang et al.,
39 2014)
40
41
42
43
44
45
46
47
48
49
50

51 52 *NTHi Infection in Other Animal OM Models*

53

54
55 Other than the mouse, several animal models have been reported for studies on OM
56 including the chinchilla (*Chinchilla lanigera*) (Bakaletz, 2009) and the rat (Clark et al., 2000).
57 The chinchilla has taken a lead in studies of NTHi pathogenesis because of the ease of
58
59
60

1
2
3 access to the middle ear bullae for infection and sampling. However, translocation to the ear
4 from the NP is difficult to achieve in this model unless barotrauma or concomitant viral
5 infection procedures are employed. When considering cost, litter size, availability of
6 immunological reagents and control of host genetics through inbred and mutant host lines,
7 the mouse presents substantial potential advantages for OM studies.
8
9

10 11 **Critical parameters and Troubleshooting**

12 *Middle Ear Infection*

13
14
15
16 Following IN inoculation, NTHi bacteria transfer rapidly along the NP and can access the ME
17 space of the *Junbo* mouse within 1 hour (Hood et al., 2016). After day 4 post-inoculation
18 there is a strong positive correlation between the presence of NTHi in both the ME and the
19 NP of the *Junbo* mouse (Hood et al., 2016); this suggests that the ME can act as a reservoir
20 for NP re-infection, or vice versa. The hypoxic inflamed ME in *Junbo* mice (Cheeseman et
21 al., 2011) may favour the growth of microaerophilic bacteria such as NTHi.
22
23
24

25
26 Normal mouse microbial flora is also present in bulla fluid of the *Junbo* mouse after 5 weeks
27 of age. In experiments using non-antibiotic resistant NTHi strains, bulla cultures giving
28 *Proteus* overgrowth on the culture plate can prevent detection of NTHi colonies; these are
29 not included in the calculations for infection rates and titres.
30
31

32 *Protection experiments*

33
34 Mice can be immunized with whole bacteria, bacterial lysates, outer membrane vesicles
35 (OMVs), single purified antigens, or any combination thereof. To test the effectiveness of the
36 antibody response in the mouse following immunization, serum obtained from immunized
37 mice can be utilised in *in vitro* assays of bacterial killing to ascertain and compare the
38 effectiveness of immunization between individual animals. The serum bactericidal (Ercoli et
39 al., 2015) and opsonophagocytosis assays measure the effectiveness of the antibody raised
40 in bacterial killing by complement and phagocytes respectively. These assays are key for
41 determining the effectiveness and functional outcome of an immunization regime in the
42 mouse and provide useful data that can be extrapolated to predict the effectiveness of
43 vaccination in man with the same antigens.
44
45
46
47
48
49

50 *Pulmonary infection*

51
52
53 Intra-tracheal rather than IN inoculation of NTHi can be used to maximise the lung infection
54 dose achieved. Intra-tracheal inoculation has been used to establish chronic NTHi lung
55 infection by repeated dosing (e.g. twice a week for 8 weeks) (Lugade et al., 2014). As an
56 alternative to homogenizing lung tissue, BAL fluids collected from the NTHi infected mouse
57
58
59
60

1
2
3 lungs can be used to investigate bacterial numbers and the mouse lung inflammatory
4 response to infection. Immune cells present in the BAL can be isolated and examined, and
5 RNA prepared for differential gene expression analysis.
6
7

8 **Anticipated Results**

9 *Junbo mouse infection*

10
11
12 Typical infection rates for NTHi OM strains in the *Junbo* mouse range from 40 to 90% ME
13 infection with titres achieved of 10^4 to 10^5 c.f.u./ μ l at 7 days post-inoculation. At the same
14 time point, semi-quantitative recovery of NTHi in NP washes typically yields bacterial titres of
15 10^1 to 10^2 c.f.u. in a 200 μ l wash volume. During longitudinal studies with our most tested
16 NTHi strain, 162sr, ME infection rates peak to 80-90% at day 7 to 14 post-inoculation and
17 decrease to around 20% by day 35, then are maintained at this rate up to the maximum
18 length of time tested of 56 days (Hood et al., 2016). The ME infection rates and titres are not
19 significantly different when inoculum doses in the range of 10^4 to 10^8 c.f.u. are used for NTHi
20 strain 162 (Hood et al., 2016).
21
22

23
24
25
26
27 Bulla fluids and dissected soft palate tissue obtained from the NP of mice 7 days post-
28 inoculation with NTHi 375gfp reveal the presence of individual and small aggregates of
29 bacteria but no evidence of significant microcolony growth or large bacterial aggregates
30 consistent with a mature NTHi biofilm being present in the ME of these mice (at this time
31 point).
32
33

34
35
36 The histology of the ME in 12-week-old *Junbo* mice is overall similar between NTHi-
37 challenged and non-challenged animals at day 7 post IN inoculation (Hood et al., 2016).
38 60% of the bulla is occupied by neutrophils and foamy macrophages and the average
39 thickness of middle ear mucosa is 100 to 111 μ m in NTHi-challenged and non-challenged
40 mice respectively. Typically in the ME bulla, a necrotic caseous core of neutrophils is
41 surrounded by viable and apoptotic neutrophils (cleaved caspase 3 positive) and an outer,
42 variably thick, band of foamy macrophages (F 4/80 positive). Variable amounts of
43 amorphous extracellular chromatin exist within the caseous areas.
44
45
46

47
48
49 When localizing NTHi bacteria in the bulla using *in situ* hybridization targeting the 16S rRNA
50 of NTHi we found strong signals in the bulla exudate but not elsewhere in head tissues from
51 *Junbo* mice challenged with NTHi, but not in non-challenged mice. NTHi hybridization
52 signals comprise punctate or larger aggregates scattered throughout the ME bulla exudate
53 but less frequent in the caseous core (Hood et al., 2016). Again, evidence consistent with
54 the presence of significant or mature biofilm was not found.
55
56
57
58
59
60

1
2
3 Following infection IN with 10^6 c.f.u. NTHi bacteria we found that chemokine/cytokine levels
4 were generally enhanced in the *Junbo* mouse ME over the period tested of one to 14 days
5 post-inoculation, when compared to GF control animals; IL-17a, Tnfa, Ccl3 and Ccl14
6 demonstrated the highest relative upregulation (Hood et al., 2016).
7
8

9
10 Using the *Junbo* infection model we have shown by comparing isogenic wild type and
11 mutant bacterial strains that the major NTHi membrane lipoprotein P4 is important for
12 maintaining high bacterial loads during middle ear infection of the mouse (Su et al., 2016).
13
14

15 *Protection experiments*

16
17 When *Junbo* mice are immunized with one of three NTHi strains (162, 176, 375) then are
18 each infected with NTHi 162, significant protection was found (15% infection rate for
19 immunized versus 81% infection rate for control) for mice immunized with the homologous,
20 but not mice immunized with the heterologous, NTHi strains (Hood et al., 2016). For mice
21 that were immunized with heterologous NTHi strains (176, 375), bacterial titres attained in
22 the middle ear were approximately one \log_{10} lower than those found in control (PBS)
23 immunized animals. Thus, there is discrimination in the model against homologous and
24 heterologous NTHi challenge.
25
26
27
28

29 *Antibiotic treatment*

30
31
32 NTHi infected mice treated with a three day course of Azithromycin starting at day 4 post-
33 inoculation had eliminated all NTHi from their middle ears when sampled post treatment (0%
34 ME infection rate and NTHi titre of beneath detection limit of ($<10^2$ c.f.u./ μ l) in treated mice,
35 whereas $>80\%$ of non-antibiotic control treated ears were infected at titres of 10^4 to 10^5
36 c.f.u./ μ l bulla fluid).
37
38
39
40

41 *Lung infection*

42
43 Typical lung infection titres achieved for NTHi by this method are $\sim 10^4$ c.f.u./lung at 24 hrs
44 and 10^2 c.f.u./lung at 48 hrs post-inoculation. When the lung of the NTHi-infected *Junbo*
45 mouse is monitored at up to 24 hours post-inoculation, mRNA levels of NF- κ B regulated pro-
46 inflammatory cytokines, such as TNF- α , IL-1 β and MIP-2, are markedly increased compared
47 to the lung of the wild type littermate mouse (Xu et al., 2012). Correspondingly, the
48 histopathology of the NTHi-infected *Junbo* mouse lung shows enhanced leukocyte infiltration
49 and neutrophil activity when compared to the wild type mouse. Lesions in the lungs can be
50 scored subjectively depending upon the observed percentage of tissue affected, the
51 epithelial changes noted, the degree of inflammatory cell infiltration and the nature of the
52
53
54
55
56
57
58
59
60

1
2
3 exudate present; for example a score of 0 to 3 where 0=absent, 1=mild, 2=moderate and
4 3=severe (Morey et al., 2013).
5

6
7 A mouse pulmonary infection model has been used to demonstrate *in vivo* efficacy of host-
8 directed antimicrobial drugs against NTHi lung infection (Euba et al., 2015) and the role of
9 NTHi membrane proteins P5 and Hap in NTHi virulence (Euba et al., 2015b).
10

11 **Acknowledgements**

12
13 This work was supported by the Medical Research Council UK (MC_EX_MR/K014986/1 and
14 MC_U142684175). MTC is supported by a BBSRC Institute Strategic Programme Grant to
15 the Roslin Institute.
16
17
18

19 **Conflicts of Interest**

20
21 The authors have no conflict of interest to report.
22
23

24 **Literature Cited**

- 25
26
27
28 Bakaletz, L.O. 2009. Chinchilla as a robust, reproducible and polymicrobial model of otitis media and
29 its prevention. *Expert review of vaccines* 8:1063-1082.
30
31
32 Chang, S.H., Mirabolfathinejad, S.G., Katta, H., Cumpian, A.M., Gong, L., Caetano, M.S., Moghaddam,
33 S.J., and Dong, C. 2014. T helper 17 cells play a critical pathogenic role in lung cancer. *Proc*
34 *Natl Acad Sci U S A* 111:5664-5669.
35
36
37 Cheeseman, M.T., Tyrer, H.E., Williams, D., Hough, T.A., Pathak, P., Romero, M.R., Hilton, H., Bali, S.,
38 Parker, A., Vizor, L., Purnell, T., Vowell, K., Wells, S., Bhutta, M.F., Potter, P.K., and Brown,
39 S.D. 2011. HIF-VEGF pathways are critical for chronic otitis media in Junbo and Jeff mouse
40 mutants. *PLoS Genet* 7:e1002336.
41
42
43 Clark, J.M., Brinson, G., Newman, M.K., Jewett, B.S., Sartor, B.R., Prazma, J., and Pillsbury, H.C., 3rd.
44 2000. An animal model for the study of genetic predisposition in the pathogenesis of middle
45 ear inflammation. *The Laryngoscope* 110:1511-1515.
46
47
48 Cody, A.J., Field, D., Feil, E.J., Stringer, S., Deadman, M.E., Tsolaki, A.G., Gratz, B., Bouchet, V.,
49 Goldstein, R., Hood, D.W., and Moxon, E.R. 2003. High rates of recombination in otitis media
50 isolates of non-typeable Haemophilus influenzae. *Infect Genet Evol* 3:57-66.
51
52
53 De Chiara, M., Hood, D., Muzzi, A., Pickard, D.J., Perkins, T., Pizza, M., Dougan, G., Rappuoli, R.,
54 Moxon, E.R., Soriani, M., and Donati, C. 2014. Genome sequencing of disease and carriage
55 isolates of nontypeable Haemophilus influenzae identifies discrete population structure.
56 *Proc Natl Acad Sci U S A* 111:5439-5444.
57
58
59
60

- 1
2
3
4
5 Ercoli, G., Baddal, B., Alessandra, G., Marchi, S., Petracca, R., Arico, B., Pizza, M., Soriani, M., and
6 Rossi-Paccani, S. 2015. Development of a serological assay to predict antibody bactericidal
7 activity against non-typeable *Haemophilus influenzae*. *BMC Microbiol* 15:87.
8
9
10 Euba, B., Molerres, J., Segura, V., Viadas, C., Morey, P., Moranta, D., Leiva, J., de-Torres, J.P.,
11 Bengoechea, J.A., and Garmendia, J. 2015a. Genome Expression Profiling-Based
12 Identification and Administration Efficacy of Host-Directed Antimicrobial Drugs against
13 Respiratory Infection by Nontypeable *Haemophilus influenzae*. *Antimicrob Agents*
14 *Chemother* 59:7581-7592.
15
16
17 Euba, B., Molerres, J., Viadas, C., Ruiz de los Mozos, I., Valle, J., Bengoechea, J.A., and Garmendia, J.
18 2015b. Relative Contribution of P5 and Hap Surface Proteins to Nontypable *Haemophilus*
19 *influenzae* Interplay with the Host Upper and Lower Airways. *PLoS ONE* 10:e0123154.
20
21
22 Hardisty-Hughes, R.E., Tateossian, H., Morse, S.A., Romero, M.R., Middleton, A., Tymowska-Lalanne,
23 Z., Hunter, A.J., Cheeseman, M., and Brown, S.D. 2006. A mutation in the F-box gene,
24 *Fbxo11*, causes otitis media in the Jeff mouse. *Hum Mol Genet* 15:3273-3279.
25
26
27 Hood, D., Moxon, R., Purnell, T., Richter, C., Williams, D., Azar, A., Crompton, M., Wells, S., Fray, M.,
28 Brown, S.D., and Cheeseman, M.T. 2016. A new model for non-typeable *Haemophilus*
29 *influenzae* middle ear infection in the Junbo mutant mouse. *Disease models & mechanisms*
30 9:69-79.
31
32
33 Juhn, S.K., Jung, M.K., Hoffman, M.D., Drew, B.R., Preciado, D.A., Sausen, N.J., Jung, T.T., Kim, B.H.,
34 Park, S.Y., Lin, J., Ondrey, F.G., Mains, D.R., and Huang, T. 2008. The role of inflammatory
35 mediators in the pathogenesis of otitis media and sequelae. *Clinical and experimental*
36 *otorhinolaryngology* 1:117-138.
37
38
39 Kaur, R., Casey, J., and Pichichero, M. 2015. Cytokine, chemokine, and Toll-like receptor expression
40 in middle ear fluids of children with acute otitis media. *The Laryngoscope* 125:E39-44.
41
42
43 Langereis, J.D., Stol, K., Schweda, E.K., Twelkmeyer, B., Bootsma, H.J., de Vries, S.P., Burghout, P.,
44 Diavatopoulos, D.A., and Hermans, P.W. 2012. Modified lipooligosaccharide structure
45 protects nontypeable *Haemophilus influenzae* from IgM-mediated complement killing in
46 experimental otitis media. *mBio* 3:e00079-00012.
47
48
49 Lugade, A.A., Bogner, P.N., Thatcher, T.H., Sime, P.J., Phipps, R.P., and Thanavala, Y. 2014. Cigarette
50 smoke exposure exacerbates lung inflammation and compromises immunity to bacterial
51 infection. *J Immunol* 192:5226-5235.
52
53
54
55 Morey, P., Viadas, C., Euba, B., Hood, D.W., Barberan, M., Gil, C., Grillo, M.J., Bengoechea, J.A., and
56 Garmendia, J. 2013. Relative contributions of lipooligosaccharide inner and outer core
57
58
59
60

- 1
2
3 modifications to nontypeable Haemophilus influenzae pathogenesis. *Infect Immun* 81:4100-
4 4111.
5
6
7 Murphy, T.F. 2003. Respiratory infections caused by non-typeable Haemophilus influenzae. *Curr*
8 *Opin Infect Dis* 16:129-134.
9
10
11 Pang, B., Hong, W., West-Barnette, S.L., Kock, N.D., and Swords, W.E. 2008. Diminished ICAM-1
12 expression and impaired pulmonary clearance of nontypeable Haemophilus influenzae in a
13 mouse model of chronic obstructive pulmonary disease/emphysema. *Infect Immun* 76:4959-
14 4967.
15
16
17 Parkinson, N., Hardisty-Hughes, R.E., Tateossian, H., Tsai, H.T., Brooker, D., Morse, S., Lalane, Z.,
18 MacKenzie, F., Fray, M., Glenister, P., Woodward, A.M., Polley, S., Barbaric, I., Dear, N.,
19 Hough, T.A., Hunter, A.J., Cheeseman, M.T., and Brown, S.D. 2006. Mutation at the Evi1
20 locus in Junbo mice causes susceptibility to otitis media. *PLoS Genet* 2:e149.
21
22
23
24 Roos, A.B., Sethi, S., Nikota, J., Wrona, C.T., Dorrington, M.G., Sanden, C., Bauer, C.M., Shen, P.,
25 Bowdish, D., Stevenson, C.S., Erjefalt, J.S., and Stampfli, M.R. 2015. IL-17A and the
26 Promotion of Neutrophilia in Acute Exacerbation of Chronic Obstructive Pulmonary Disease.
27 *Am J Respir Crit Care Med* 192:428-437.
28
29
30 Rye, M.S., Bhutta, M.F., Cheeseman, M.T., Burgner, D., Blackwell, J.M., Brown, S.D., and Jamieson,
31 S.E. 2011. Unraveling the genetics of otitis media: from mouse to human and back again.
32 *Mammalian genome : official journal of the International Mammalian Genome Society*
33 22:66-82.
34
35
36 Sethi, S. and Murphy, T.F. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000:
37 a state-of-the-art review. *Clin Microbiol Rev* 14:336-363.
38
39
40 Shann, F., Hart, K., and Thomas, D. 1984. Acute lower respiratory tract infections in children: possible
41 criteria for selection of patients for antibiotic therapy and hospital admission. *Bull World*
42 *Health Organ* 62:749-753.
43
44
45 Su, Y.C., Mukherjee, O., Singh, B., Hallgren, O., Westergren-Thorsson, G., Hood, D., and Riesbeck, K.
46 2016. Haemophilus influenzae P4 Interacts With Extracellular Matrix Proteins Promoting
47 Adhesion and Serum Resistance. *J Infect Dis* 213:314-323.
48
49
50 Tateossian, H., Morse, S., Parker, A., Mburu, P., Warr, N., Acevedo-Arozena, A., Cheeseman, M.,
51 Wells, S., and Brown, S.D. 2013. Otitis media in the Tgif knockout mouse implicates TGFbeta
52 signalling in chronic middle ear inflammatory disease. *Hum Mol Genet* 22:2553-2565.
53
54
55 Unger, B.L., Faris, A.N., Ganesan, S., Comstock, A.T., Hershenson, M.B., and Sajjan, U.S. 2012.
56 Rhinovirus attenuates non-typeable Hemophilus influenzae-stimulated IL-8 responses via
57 TLR2-dependent degradation of IRAK-1. *PLoS Pathog* 8:e1002969.
58
59
60

- 1
2
3
4
5 Woo, J.I., Kil, S.H., Brough, D.E., Lee, Y.J., Lim, D.J., and Moon, S.K. 2015. Therapeutic potential of
6 adenovirus-mediated delivery of beta-defensin 2 for experimental otitis media. *Innate*
7 *Immun* 21:215-224.
8
9
10 Woo, J.I., Oh, S., Webster, P., Lee, Y.J., Lim, D.J., and Moon, S.K. 2014. NOD2/RICK-dependent beta-
11 defensin 2 regulation is protective for nontypeable *Haemophilus influenzae*-induced middle
12 ear infection. *PLoS ONE* 9:e90933.
13
14
15 Xu, X., Woo, C.H., Steere, R.R., Lee, B.C., Huang, Y., Wu, J., Pang, J., Lim, J.H., Xu, H., Zhang, W.,
16 Konduru, A.S., Yan, C., Cheeseman, M.T., Brown, S.D., and Li, J.D. 2012. EVI1 acts as an
17 inducible negative-feedback regulator of NF-kappaB by inhibiting p65 acetylation. *J Immunol*
18 188:6371-6380.
19
20
21 Yao, W., Frie, M., Pan, J., Pak, K., Webster, N., Wasserman, S.I., and Ryan, A.F. 2014. C-Jun N-
22 terminal kinase (JNK) isoforms play differing roles in otitis media. *BMC immunology* 15:46.
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 **Current Protocols in Mouse Biology**
4

5
6 **Ms#CP-16-0090**
7

8
9 **Non-typeable *Haemophilus influenzae* infection of the *Junbo* mouse**
10

11
12 Michael T. Cheeseman¹ and Derek W. Hood^{2*}
13

14
15
16
17 ¹ Developmental Biology Division, The Roslin Institute and Royal (Dick) School of Veterinary
18 Studies
19

20
21 University of Edinburgh, Easter Bush, EH25 9RG, UK.
22

23
24
25 ² Molecular Genetics Unit, MRC Harwell, Harwell Science and Innovation Campus, Oxfordshire,
26 OX11 0RD, UK.
27

28
29 (phone +44 1235841177; email d.hood@har.mrc.ac.uk)
30
31

32
33
34 * Corresponding author
35

36 Running title: NTHi mouse infection
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 investigations of the molecular basis and pathophysiology of NTHi infection and provide a
4 means to realise improved treatment and prevention of disease in humans. Here we describe a
5 robust model of NTHi infection using the *Junbo* mouse; this focuses upon the study of AOM but
6 bacterial carriage and lung clearance can also be investigated.
7
8
9
10

11
12 Basic Protocol 1 describes the basis for establishing and monitoring middle ear infection in the
13 *Junbo* mouse following intranasal inoculation of NTHi. Basic Protocol 2 describes the adaptation
14 of Basic Protocol 1 to study protection against infection following immunization of the animals
15 and Basic Protocol 3 the use of antibiotic to clear NTHi. Basic Protocol 4 describes a modified
16 infection procedure that favours residence of bacteria in the mouse lung.
17
18
19
20
21
22
23

24 NOTE: All protocols using live animals must first be reviewed and approved by the appropriate
25 ethics process such as the Animal Welfare and Ethical Review Body (AWERB) in the UK or the
26 Institutional Animal Care and use Committee (IACUC) in the US, and must conform to local or
27 national governmental regulations regarding the care and use of laboratory animals.
28
29
30
31
32
33

34 **Basic Protocol 1**

35 ***JUNBO* MOUSE NTHI MIDDLE EAR INFECTION**

36 ***Junbo* mouse**

37
38 *Junbo* is a mutant mouse line that spontaneously develops chronic middle ear (ME)
39 inflammation under specific pathogen free (SPF) conditions at four to five weeks of age. The
40 heterozygote *Junbo* mouse (*Jbo*+, hereafter termed *Junbo*) bears an Asn763Ile mutation in the
41 gene encoding the Evi1 transcription factor, otherwise known as Mecom (Parkinson et al.,
42 2006). One mechanism that may underlie the predisposition to OM in *Junbo* mice is that Evi1 is
43 a negative regulator of NFkB; the loss of function Evi1 *Junbo* mutation exacerbates NTHi
44 induced inflammation in the lung (Xu et al., 2012). The pre-existing ME inflammation in *Junbo*
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 mice is critical for bacterial ME infection following intranasal (IN) inoculation, the healthy air-
4 filled ME of wild type mice and the small proportion of *Junbo* mice (5 to 10%) with no fluid
5 present do not sustain infection by NTHi.
6
7
8

9 10 ***NTHi strains***

11
12
13 Wild-type NTHi strains used by us for ME infection studies are all isolates from children with OM
14 (Cody et al., 2003) and are phylogenetically distinct (De Chiara et al., 2014). Following infection
15 in the mouse, indigenous commensal bacterial flora, particularly *Proteus* spp., renders a
16 proportion of bacterial count plates unreadable through overgrowth. For this reason, NTHi
17 strains expressing spontaneous resistance to streptomycin (sr), or engineered resistance to
18 kanamycin (kan), were generated (Hood et al., 2016). These strains enable antibiotic selection
19 of NTHi during culture from mouse samples and counter-selection against any indigenous
20 bacteria present; streptomycin selection proved particularly useful at maximizing quantitative
21 NTHi culture data from mouse samples.
22
23
24
25
26
27
28
29
30
31
32

33 ***Materials***

34
35
36 Mice - *Junbo* mice are congenic on a C3H/HeH background (Parkinson et al., 2006); mice are
37 housed under SPF conditions and are mostly used at between eight to eleven weeks of age.
38
39 The *Junbo* mouse is available from the European Mouse Mutant Archive (EM:00091) via MRC
40 Harwell; for non-academic groups the *Junbo* mouse is available through MRC Technology, UK.
41
42 For some studies Germ Free (GF) mice are used, details of the mouse husbandry and
43 microbiological surveillance are given elsewhere (Hood et al., 2016).
44
45
46
47
48
49

50 NTHi – human OM disease isolates (162, 176, 375, 486, 1124 and 1158) (available from MRC
51 Harwell) are used by us for mouse infection studies (Cody et al., 2003). Streptomycin resistant
52 NTHi strains are designated sr, e.g. strain 162sr.
53
54
55
56
57
58
59
60

1
2
3 Autoclaved brain heart infusion (BHI) broth supplemented with haemin and NAD (sBHI) for
4 liquid culture and sBHI agar for plate growth of bacteria (see recipe).
5
6

7
8 Sterile PBS/2% gelatine for inoculum preparation.
9

10
11 PBS for collecting and diluting *in vivo* samples.
12

13
14 Binocular dissecting microscope with x10 magnification and LED stage lighting.
15

16
17 scissors for removal of the head.
18

19
20 fine forceps for puncture of the tympanic membrane.
21

22
23 70% ethanol to sterilize the instruments between the sampling of ears.
24

25
26 A <2 µl volume pipette with sterile micro-tip to collect the small volume of ME fluids.
27

28
29 barbiturate solution (50% Euthatal) for intraperitoneal injection (delivered at 3.3 ml/kg body
30 weight), or alternatively a rising CO₂ concentration to euthanize mice.
31
32

33 34 **Mouse NTHi inoculum** 35

- 36
37
- 38 1. Prepare bacteria for inoculation from -80°C stocks stored in BHI/20% glycerol and grown
39 overnight at 37°C in 5% CO₂ atmosphere on sBHI agar. From this plate, inoculate sBHI
40 broth with a 1 µl loop full of colony growth (to give an initial OD₄₉₀ of ~0.03-0.05) and
41 grow bacteria to log phase (OD₄₉₀ 0.3-0.6) at 37°C in a shaking incubator.
42
43
 - 44 2. Calculate the size of inoculum for the mouse from optical density (OD₄₉₀) using the
45 conversion factor that an absorbance of 0.4 is equivalent to approximately 1 x 10⁹
46 c.f.u./ml.
47
48
 - 49 3. Pellet bacteria by centrifugation at 13000 x g at room temperature for 3 min, remove
50 supernatant and re-suspend pellet to achieve 10¹⁰ c.f.u./ml in PBS/2% gelatine.
51
52
53
54
55
56
57
58
59
60

- 1
2
3 4. Assess the titre of the inoculum by plating dilutions of each suspension onto sBHI agar
4
5 plates prior to, and after, inoculation of the mice.
6
7

8 ***Intranasal challenge***

- 9
10
11 5. Anesthetize mice aged either 8 ± 1 week or 11 ± 1 week with isofluorane (mixed with O₂
12 in anaesthetic chamber) and inoculate by applying 5 µl of bacterial suspension to each
13 nostril via a pipette tip (Hood et al., 2016).
14
15
16
17

18
19 *A typical inoculum is 10⁶ c.f.u. but a range of inoculum sizes from 10³-10⁸ c.f.u. NTHi bacteria*
20 *have been used in individual experiments with cohort sizes of n=11 to 15 mice.*
21

22
23
24 *Using bacteria that emit light (expressing lux genes) we have shown that NTHi are distributed*
25 *along the entire length of the NP within minutes of IN inoculation (Hood et al., 2016).*
26
27

28 ***Terminal sampling of nasopharynx and bulla fluids***

- 29
30
31
32 6. Euthanize the mouse (typically at 7 days post-inoculation), use scissors to remove and
33 skin the head (Fig. 1), then remove the mandible and sample the NP by washing with
34 200 µl PBS introduced into the NP opening on the palate and collecting the wash fluid
35 from the nares into an Eppendorf tube (Fig. 2). Sampling the NP either before or after
36 the ME has no statistically significant effect on the NP bacterial titres attained.
37
38
39
40
41
42

43
44 *Avoid euthanasia by cervical dislocation of the mouse as it causes haemorrhage in the upper*
45 *airway that will compromise sample collection.*
46
47

48
49 *A more quantitative approach to determine NP NTHi titres is to dissect out a part (e.g., soft*
50 *palate) and homogenise the tissue in PBS prior to dilution and plating. Although this typically*
51 *yields higher bacterial titres than the wash alone we find that there is a general positive*
52 *correlation between the data from the two methods.*
53
54
55
56
57
58
59
60

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
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 $\sim 0.75 \mu\text{l}$, 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.

19
20
21
22
23
24
25

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°C .

26 27 **Infection rate and bacterial titre**

- 28
29
30
31
32
33
34
35
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°C .

36
37
38
39
40
41
42
43
44
45
46

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 $\mu\text{g}/\text{ml}$ streptomycin or 20 $\mu\text{g}/\text{ml}$ kanamycin). If required, non-selective agar plates can be used in parallel to monitor the presence of commensal bacteria.

- 47
48
49
9. Calculate an infection rate for the middle ear:

50
51
52

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

53
54
55
56
57
58
59
60

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%.

1
2
3 10. Calculate a bacterial titre (c.f.u./ μ l ME fluid) from the number of colonies obtained on
4
5 culture plates, adjusted for sample dilution and bulla fluid volume.
6
7

8 *As an example, if 85 bacterial colonies were obtained on a culture plate spread with 50 μ l of a*
9
10 *10^{-2} dilution made from 1 μ l ME fluid dispersed into 500 μ l PBS, this would correspond to a ME*
11
12 *bacterial titre of 8.5×10^4 c.f.u./ μ l fluid.*
13

14
15 *NTHi has also been shown to infect the bulla of other mutant mouse lines that exhibit ME*
16
17 *inflammation and OM (Hood et al., 2016); these include the Jeff (Hardisty-Hughes et al., 2006)*
18
19 *and Tgif (Tateossian et al., 2013) mouse mutants. The protocol will similarly work for*
20
21 *Streptococcus pneumoniae (pneumococcus) infection although this has been tested by us only*
22
23 *in the Junbo mouse. We note that Streptococcus pneumoniae strain D39 is highly virulent in*
24
25 *Junbo mice. All new infection models should be established with less virulent bacterial strains,*
26
27 *and pilot studies with small numbers of mice should be conducted first to establish whether*
28
29 *there are unexpected adverse effects.*
30
31

32 33 **Basic Protocol 1 - Support Protocol 1**

34 35 **JUNBO MOUSE NTHI MIDDLE EAR INFECTION – Histology and Distribution of NTHi**

36
37
38
39 Following infection of the *Junbo* mouse, the histology and the relative distribution of NTHi of the
40
41 infected ME bullae can be examined by a number of techniques that include histology,
42
43 immunohistochemistry (IHC), *in situ* hybridization (ISH) and lesion profiling. Typically this is
44
45 carried out using 11–week-old *Junbo* mice inoculated IN with 10^6 c.f.u. NTHi and heads
46
47 collected seven days post-inoculation as described above in Basic Protocol 1. Heads from non-
48
49 NTHi challenged *Junbo* mice collected at the same time serve as negative controls.
50
51

52 53 **Histology**

54 55 **Haematoxylin and Eosin stained sections**

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
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

1
2
3 antibodies in Dako antibody diluent (S0809) and carry out negative controls using the
4 antibody diluent alone. Visualize the target using the Dako (K3468) liquid DAB+
5 substrate chromogen system. Counterstain using Harris haematoxylin prior to
6 dehydration through ethanol, clearing in xylene and mounting in Clearview mountant
7 (Thermo Fisher Scientific).
8
9

- 10
11
12
13
14 5. Perform *in situ* hybridization on representative NTHi-challenged and non-challenged
15 *Junbo* mice. As an illustrative example we use a probe that targets the 16sRNA of the
16 bacteria. Hybridize 4 μm wax sections with probe B-HInfluenzae-NTHi375-16SrRNA as
17 per the manufacturer's instructions (Advanced Cell Diagnostic) using the HRP
18 visualization kit. Use a positive control for RNA integrity (e.g. PpiB) and a negative
19 hybridization control (e.g. DapB) (Advanced Cell Diagnostic) in experiments.
20
21
22
23
24
25
26
27

28 *ISH is a useful approach when antibodies against bacteria produce high background or suitable*
29 *ones are not commercially available. We found that mouse antisera from NTHi immunization*
30 *and protection studies did not make useful reagents to localise bacteria in bulla sections.*
31
32 *Another advantage of ISH is that there are a wide range of mouse probes available making it*
33 *potentially possible to obtain hybridization signals for bacteria and host cells in the same*
34 *section.*
35
36
37
38
39
40
41

42 *NTHi bacteria can also be visualized directly following infection of the Junbo mouse. NTHi*
43 *strains expressing the lux genes emit bioluminescent signals that can be used to monitor*
44 *bacterial distribution in situ in the skinned heads of infected Junbo mice; bacteria are imaged*
45 *using an IVIS Lumina II system (Perkin Elmer) (Hood et al., 2016). NTHi strains expressing a*
46 *green fluorescent protein (GFP) can be detected following infection by confocal microscopy on*
47 *mouse bulla fluid and soft palate samples.*
48
49
50
51
52
53
54

55 **Lesion profiling**

56
57
58
59
60

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

1
2
3 and store at -80°C prior to RNA isolation. Obtain at least three biological replicate pools
4
5 for each time point.
6

- 7
8 3. As a baseline control, collect bulla fluids ($n=4$ to 6) from eight to ten week old non-
9
10 infected germ free (GF) *Junbo* mice into 20 μ l of RNase free water for RNA isolation.
11
12 Each GF pool of bulla fluids comprises at least 3 biological replicates as controls for
13
14 each time point. As an alternative to GF mice, measure the baseline control expression
15
16 level using bulla fluids obtained from age-matched non-NTHi infected SPF *Junbo* mice.
17
18 4. Extract RNA from the re-suspended bulla fluid cell pellets and carry out cDNA synthesis
19
20 and RT-qPCR TaqMan as described by Cheeseman et al. (Cheeseman et al., 2011).
21
22 Typical immune molecule genes to be monitored are those activated by bacterial ligands
23
24 binding to TLR-receptors that are expressed by macrophages, neutrophils and
25
26 monocytes and are known to be relevant to OM and AOM in man (Juhn et al., 2008;
27
28 Kaur et al., 2015). Genes that we routinely monitor include *Ccl3*, *Ccl4*, *Ccl5*, *IL-1b*, *IL-6*,
29
30 *IL-12a*, *IL-17a*, *Tnfa*, *Vegfa* (Hood et al., 2016). Perform RT-qPCR in triplicate technical
31
32 assays. Normalize data using *Hrpt1* and beta Actin as the endogenous control and
33
34 calculate fold changes of expression (ddCts) of NTHi-infected bulla fluid cells over non-
35
36 infected bulla fluid cells using AB 7500 software v2.0.1 and express as mean relative
37
38 quantification (RQ) \pm min/max error bars representing 95% CI.
39
40
41
42

43 **Basic Protocol 2**

44 **MOUSE IMMUNIZATION AND PROTECTION AGAINST NTHi INFECTION**

45
46
47 In this procedure, mice are subcutaneously immunized with either whole killed bacteria or with
48
49 derived and purified cell wall fractions or selected antigens (Ercoli et al., 2015). Following a
50
51 three step immunization procedure, the mice are inoculated IN with live NTHi bacteria to assess
52
53 if antibody raised in the mouse elicits protection against infection in the ME or carriage in the
54
55
56
57
58
59
60

1
2
3 NP. Significant levels of protection when found compared to control animals can act as a
4 surrogate that predicts the efficacy of an antigen combination for use as a vaccine in humans.
5
6
7

8 **Materials**

9
10 five week old SPF *Junbo* mice (MRC Harwell)

11
12 killed NTHi bacteria for immunization

13
14 live NTHi bacteria (Basic Protocol 1 steps 1 to 5) for intranasal challenge post-immunization

15
16 Adjuvant

17
18 PBS/0.08% paraformaldehyde

19
20 materials for terminal sampling of animals and fluids as described in Basic Protocol 1

21 **Immunization with NTHi bacteria**

22
23 1. Obtain pre-immune serum as a base-line control for antibody levels prior to the first
24 immunization being carried out. Prepare small volumes of sera from blood obtained from mice
25 by tail vein bleed (see step 8).
26

27
28 2. When using whole NTHi bacteria for mouse immunization, culture NTHi to mid-log phase
29 (OD₄₉₀ 0.2 to 0.6) in sBHI broth. Pellet 1 ml of culture at 13000 x g for 3 mins then resuspend
30 the pellet and wash in PBS.
31

32
33 3. To kill the bacteria, pellet the washed suspension at 13000 x g before re-suspending in 500 µl
34 of PBS/0.08% paraformaldehyde (PFA). Incubate the suspension for 1 hour at 37°C then
35 overnight at 4°C before culturing 20 µl of the neat bacterial suspension on sBHI agar to confirm
36 loss of viability of bacteria.
37

38
39 4. Store killed NTHi in PBS/ PFA for up to 8 weeks at 4°C, confirm bacterial integrity before
40 each use by spreading 5 µl of the bacterial suspension on a microscope slide and examining by
41 phase contrast microscopy.
42
43
44
45
46
47
48
49
50
51
52

1
2
3 5. Prior to immunization, pellet the killed bacteria by centrifugation at 13000 x g for 3 mins,
4
5 remove the supernatant and then re-suspend in PBS to achieve 10^{10} c.f.u./ml before mixing with
6
7 adjuvant according to the suppliers instructions. We typically use Adjuplex (Sigma) adjuvant at a
8
9 1:4 ratio with the bacterial suspension.
10

11
12 6. Immunize each *Junbo* mouse with three subcutaneous injections in intra-scapular skin of a
13
14 mixture of 10^8 c.f.u. killed bacteria and adjuvant in a 50 μ l final volume. The first immunizations
15
16 are at the age of 5 weeks, then subsequently at 8 weeks and 10 weeks of age.
17

18
19
20 7. Take a second intermediate blood sample by tail vein bleed prior to the second immunization
21
22 when the mice are at 8 weeks of age.
23

24
25 8. Inoculate immunized mice IN at 12 weeks with 10^6 c.f.u. NTHi bacteria. Determine the ME
26
27 infection rate and bacterial titre by terminal sampling at 7 days post-inoculation (mice at 13
28
29 weeks of age), as described above in Basic Protocol 1.
30

31
32 9. Obtain terminal blood samples at the end of the experiment by retro-orbital bleed immediately
33
34 after euthanasia of the mouse. Incubate blood samples at room temperature for two hours then
35
36 obtain serum using 1.1 ml Z-Gel spin columns (Sarstedt) following centrifugation according to
37
38 the supplier's instructions. Store serum at -80°C until required.
39

40
41
42 *In a typical protection experiment cohorts of 12 to 15 mice are immunized with alternative NTHi*
43
44 *strains then are infected with either the homologous or heterologous bacteria. Control mice are*
45
46 *immunized with PBS and adjuvant. When comparing the bulla infection rate and bacterial titres*
47
48 *in PBS-immunized control mice with those of NTHi-immunized mice, any significant reduction in*
49
50 *NTHi ME infection rate or titre in the NTHi-immunized mice will indicate protection dependent*
51
52 *upon antibody produced in those vaccinated mice (Hood et al., 2016).*
53
54
55
56
57
58
59
60

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).

- 1
2
3 5. Determine the efficacy of antibiotic treatment by the difference in ME bulla infection rates
4 and bulla NTHi titres between the antimicrobial treated and control mice.
5
6
7

8 *Importantly, as well as its use for oral administration of antimicrobials, the Junbo/NTHi infection*
9 *model has added value through its utility for investigating potential new application strategies*
10 *specifically targeted at the ME; antimicrobials can potentially be applied directly on the TM and*
11 *subsequently transferred into the ME bulla.*
12
13
14
15

16 *The outcome of the antimicrobial treatment can be ascertained for NTHi alone by plating mouse*
17 *samples on the appropriate selective growth plate for the respective NTHi strain or can include*
18 *an indication of the effect on other general bacterial flora present by also plating samples on*
19 *non-selective growth medium.*
20
21
22
23
24
25
26

27 **Basic Protocol 4**

28 **NTHI MOUSE PULMONARY INFECTION MODEL**

29
30
31
32
33 NTHi pulmonary infection has been used to investigate the host immune response in the *Junbo*
34 mouse (Xu et al., 2012). To achieve lung infection the mouse can be inoculated via the
35 intratracheal route but here we describe the more straightforward IN method adapted from that
36 of Morey et al. (Morey et al., 2013).
37
38
39
40
41
42

43 **Materials**

44 Eight week old SPF Junbo mice
45
46
47

48 NTHi bacteria for intranasal challenge (as described in Basic Protocol 1)
49
50
51

52 Materials for terminal sampling of animals and fluids (as described in Basic Protocol 1)
53
54

55 Homogeniser (we use an IKA Ultra-Turax T25 operated in a Class II Microbiological safety
56 cabinet)
57
58
59
60

- 1
2
3 1. Prior to infection, grow NTHi overnight on sBHI agar then use to inoculate sBHI broth.
4
5 Grow bacteria to log phase (OD_{490} 0.3-0.6), pellet by centrifugation at 13000 x g for 3
6
7 min, remove the supernatant and then re-suspend to achieve 5×10^9 c.f.u./ml in
8
9 PBS/2% gelatine.
10
- 11
12 2. Anesthetize mice aged eight weeks with isofluorane and inoculate IN by applying 10 μ l
13
14 of bacterial suspension to each nostril (10^8 c.f.u. in 20 μ l total volume).
15
- 16
17 3. Euthanize the mouse (described in Basic Protocol 1) typically at time points either 24
18
19 or 48 hours post-inoculation. Remove lungs aseptically, weigh individually then
20
21 homogenize to release bacteria from the tissue.
22
- 23
24 4. Plate serial tenfold dilutions of the lung homogenate in PBS on sBHI agar plates,
25
26 incubate overnight at 37°C then determine the number of bacteria in the lung from the
27
28 colony counts on plates, the dilution factor, and the lung homogenate volume
29
30 (detection limit <10 c.f.u./ml) (as described in Basic Protocol 1 steps 8 and 10).
31
- 32
33 5. To obtain a histopathology and lesion score, fix trachea and lungs overnight in 10%
34
35 buffered formalin and embed in paraffin. Obtain four to six μ m thick sections and stain
36
37 with Hematoxylin and Eosin prior to examination by microscope.
38

39 *These experiments are typically used to compare bacterial counts following infection between*
40 *different wild type strains or paired isogenic wild type and mutant NTHi strains; the difference in*
41 *bacterial count serves as an indicator of altered propensity for bacterial clearance in the lung.*
42
43
44

45 **Basic Protocol Material List**

46 **Culture media**

47
48
49 Brain Heart Infusion broth (Merck); 37g powder made up to 1 l with distilled water. Autoclave in
50
51 glass bottles and store at room temperature for two to three months. Before use supplement
52
53 BHI by adding 2 μ g/ml nicotinamide adenine dinucleotide (NAD) (1 mg/ml stock solution) and 10
54
55
56
57
58
59
60

1
2
3 µg/ml haemin (10 mg/ml stock solution) (sBHI). NAD and haemin are both growth requirements
4
5 for NTHi. For antibiotic resistant NTHi strains, antibiotics (e.g. streptomycin (300 µg/ml) or
6
7 kanamycin (20 µg/ml)) are added to the growth medium as appropriate. Use sBHI within 24 hrs.
8
9

10 For plate growth make up BHI liquid medium then add agar (Oxoid) to 1% before autoclaving.
11
12 Cool media to 50°C, add supplements then pour into sterile petri dishes and allow to set; store
13
14 plates at 4°C for up to two weeks.
15
16

17 **Buffers**

18
19
20
21 PBS; made up as per suppliers instructions (a typical solution contains 0.01 M phosphate buffer,
22
23 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4). Sterilize and store at room
24
25 temperature for up to 6 months.
26
27

28 TBS; 50 mM Tris base, 150 mM sodium chloride, pH 7.6; store at room temperature for up to 6
29
30 months.
31
32

33 **COMMENTARY**

34 *Background Information on Bacteria*

35
36
37
38
39 *Haemophilus influenzae* is a Gram-negative bacterium that is part of the normal flora present in
40
41 the human NP but is also a frequent etiological agent of disease in humans. *H. influenzae* is
42
43 divided into six typeable (a through f) or non-typeable (NTHi) forms based on the presence or
44
45 absence of a polysaccharide capsule, respectively. A majority of healthy adults have upper
46
47 airway colonization with *H. influenzae* and the predominant strains (>98%) are NTHi. Residence
48
49 in the NP enables *H. influenzae* to colonize and initiate infections in both the upper and lower
50
51 respiratory tracts through contiguous spread; diseases caused by NTHi are of significant public
52
53 health importance and include AOM and acute pneumonia in young children, and
54
55 bronchopneumonia in patients with chronic pulmonary diseases such as cystic fibrosis and
56
57
58
59
60

1
2
3 chronic obstructive pulmonary disease (COPD) (Murphy, 2003). In the UK, on average, at least
4 one episode of AOM occurs in every child by the age of five, making it one of the commonest
5 reasons for antibiotic prescription in general practice. In the developing world, acute lower
6 respiratory tract infections top the list of causes of death in young infants of which about 20%
7 are attributable to NTHi (Shann et al., 1984). For adults, in 2013 COPD was the third leading
8 cause of death in the USA and considered to be the third leading cause worldwide. Following
9 the implementation of type b capsular conjugate vaccines, invasive (bacteraemic) *H. influenzae*
10 infections have declined in frequency although NTHi is an emerging and significant cause of
11 bacteraemia and meningitis.
12
13
14
15
16
17
18
19
20
21
22

23 The three major human otopathogens, NTHi, *Streptococcus pneumoniae* (pneumococcus) and
24 *Moraxella catarrhalis* are each commensal bacteria commonly found in the human NP. The NP
25 serves as a reservoir for respiratory tract infection. Each bacterium can spread contiguously and
26 as well as being responsible for OM, are also commonly found in the sputum of COPD patients
27 with acute and recurrent exacerbations (Sethi and Murphy, 2001). Thus, the increasing use of
28 pneumococcal vaccines in children is resulting in NTHi becoming the predominant cause of
29 bacterial respiratory infections, including AOM.
30
31
32
33
34
35
36
37
38
39

40 *Some Modifications Used For NTHi Infection in Other Mouse Models*

41
42 The presence of fluid in the *Junbo* mouse ME facilitates translocation of NTHi to the ear via a
43 natural route (Eustachian tube) following IN inoculation. In other NTHi mouse AOM models, live
44 or heat killed NTHi bacteria are introduced directly into the ME bulla; this can be achieved via
45 direct injection through the tympanic membrane (Woo et al., 2014) or an incision made in the
46 mouse neck to expose the bulla bone through which the inoculum is injected (Yao et al., 2014).
47
48 The size of the NTHi inoculum can be adjusted depending upon the mouse line used and the
49 nature of the investigation undertaken. Direct inoculation mouse OM models have been used to
50
51
52
53
54
55
56
57
58
59
60

1
2
3 investigate potential treatment for the disease; an example is human β -defensin 2, expressed in
4 the ME following introduction via an adenoviral vector (Woo et al., 2015). In a modified co-
5 infection AOM model, mice can be inoculated IN with influenza A virus then three days later
6 challenged IN with NTHi; this results in significant bacterial infection of both the mouse ME and
7 NP (Langereis et al., 2012).

8
9
10 Following IN inoculation, the distribution of NTHi in the mouse NP can be monitored; in a typical
11 mouse line this can only be achieved reliably for up to the first 24 hours before bacteria are
12 cleared. The carriage of NTHi in the mouse NP following IN inoculation has been used to
13 investigate the competitive index for colonization between two NTHi strains and has also been
14 used to study the efficacy of mouse immunization procedures.

15
16
17 NTHi are commonly introduced into the mouse lung to investigate host and bacterial factors
18 relevant to the pathogenesis associated with COPD; this model is an important resource to
19 study *in vivo* the immune mechanisms and regulation that respond to NTHi infection. Several
20 methods can be used to alter the pathophysiology of the mouse lung to be more like that found
21 in COPD patients. These include pre-exposure of mice to cigarette smoke (Roos et al., 2015), a
22 treatment carried out for four to 30 weeks prior to introduction of NTHi, and treatment of the lung
23 with modifying molecules such as the enzyme elastase (Pang et al., 2008). Intra-tracheal
24 inoculation is a less commonly used route to deliver NTHi than IN, but can provide higher
25 bacterial doses more directly to the target tissue. Bacterial numbers, lung immunopathology and
26 the host response over time (typically up to 48 hrs post-inoculation) can be studied by utilising
27 mutant mouse lines that are altered in specific immune genes; this allows host-microbial
28 interactions important for the progression and persistence of lung disease to be teased out
29 (Roos et al., 2015). The expression pattern of both bacterial and host genes in response to
30 NTHi infection of the lung can be studied from broncho-alveolar lavage (BAL) fluids and
31 homogenised lung material. The lung infection model can also be used to test potential

1
2
3 antimicrobial regimens to alleviate disease (Euba et al., 2015a) and the effectiveness of
4 vaccination to prevent it (Lugade et al., 2014), again the focus is usually upon treatment of NTHi
5 associated exacerbations of COPD. NTHi clearance in the mouse lung can be delayed when
6 mice are first infected by rhinovirus then subsequently are superinfected with NTHi (Unger et al.,
7 2012). A modified mouse model has also been used to investigate the relationship between
8 NTHi infection, COPD and lung cancer (Chang et al., 2014)

16 *NTHi Infection in Other Animal OM Models*

17
18
19
20 Other than the mouse, several animal models have been reported for studies on OM including
21 the chinchilla (*Chinchilla lanigera*) (Bakaletz, 2009) and the rat (Clark et al., 2000). The
22 chinchilla has taken a lead in studies of NTHi pathogenesis because of the ease of access to
23 the middle ear bullae for infection and sampling. However, translocation to the ear from the NP
24 is difficult to achieve in this model unless barotrauma or concomitant viral infection procedures
25 are employed. When considering cost, litter size, availability of immunological reagents and
26 control of host genetics through inbred and mutant host lines, the mouse presents substantial
27 potential advantages for OM studies.
28
29
30
31
32
33
34
35
36
37

38 **Critical parameters and Troubleshooting**

39 *Middle Ear Infection*

40
41
42 Following IN inoculation, NTHi bacteria transfer rapidly along the NP and can access the ME
43 space of the *Junbo* mouse within 1 hour (Hood et al., 2016). After day 4 post-inoculation there is
44 a strong positive correlation between the presence of NTHi in both the ME and the NP of the
45 *Junbo* mouse (Hood et al., 2016); this suggests that the ME can act as a reservoir for NP re-
46 infection, or vice versa. The hypoxic inflamed ME in *Junbo* mice (Cheeseman et al., 2011) may
47 favour the growth of microaerophilic bacteria such as NTHi.
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Normal mouse microbial flora is also present in bulla fluid of the *Junbo* mouse after 5 weeks of
4
5 age. In experiments using non-antibiotic resistant NTHi strains, bulla cultures giving *Proteus*
6
7 overgrowth on the culture plate can prevent detection of NTHi colonies; these are not included
8
9 in the calculations for infection rates and titres.
10

11 *Protection experiments*

12
13
14 Mice can be immunized with whole bacteria, bacterial lysates, outer membrane vesicles
15
16 (OMVs), single purified antigens, or any combination thereof. To test the effectiveness of the
17
18 antibody response in the mouse following immunization, serum obtained from immunized mice
19
20 can be utilised in *in vitro* assays of bacterial killing to ascertain and compare the effectiveness of
21
22 immunization between individual animals. The serum bactericidal (Ercoli et al., 2015) and
23
24 opsonophagocytosis assays measure the effectiveness of the antibody raised in bacterial killing
25
26 by complement and phagocytes respectively. These assays are key for determining the
27
28 effectiveness and functional outcome of an immunization regime in the mouse and provide
29
30 useful data that can be extrapolated to predict the effectiveness of vaccination in man with the
31
32 same antigens.
33
34
35
36

37 *Pulmonary infection*

38
39
40 Intra-tracheal rather than IN inoculation of NTHi can be used to maximise the lung infection
41
42 dose achieved. Intra-tracheal inoculation has been used to establish chronic NTHi lung infection
43
44 by repeated dosing (e.g. twice a week for 8 weeks) (Lugade et al., 2014). As an alternative to
45
46 homogenizing lung tissue, BAL fluids collected from the NTHi infected mouse lungs can be used
47
48 to investigate bacterial numbers and the mouse lung inflammatory response to infection.
49
50 Immune cells present in the BAL can be isolated and examined, and RNA prepared for
51
52 differential gene expression analysis.
53
54
55

56 **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./ μ l 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 μ l 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 to 10^8 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 μ m 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

1
2
3 comprise punctate or larger aggregates scattered throughout the ME bulla exudate but less
4 frequent in the caseous core (Hood et al., 2016). Again, evidence consistent with the presence
5 of significant or mature biofilm was not found.
6
7
8

9
10 Following infection IN with 10^6 c.f.u. NTHi bacteria we found that chemokine/cytokine levels
11 were generally enhanced in the *Junbo* mouse ME over the period tested of one to 14 days post-
12 inoculation, when compared to GF control animals; IL-17a, Tnfa, Ccl3 and Ccl14 demonstrated
13 the highest relative upregulation (Hood et al., 2016).
14
15
16
17

18
19 Using the *Junbo* infection model we have shown by comparing isogenic wild type and mutant
20 bacterial strains that the major NTHi membrane lipoprotein P4 is important for maintaining high
21 bacterial loads during middle ear infection of the mouse (Su et al., 2016).
22
23
24
25

26 *Protection experiments*

27
28 When *Junbo* mice are immunized with one of three NTHi strains (162, 176, 375) then are each
29 infected with NTHi 162sr, significant protection was found (15% infection rate for immunized
30 versus 81% infection rate for control) for mice immunized with the homologous, but not mice
31 immunized with the heterologous, NTHi strains (Hood et al., 2016). For mice that were
32 immunized with heterologous NTHi strains (176, 375), bacterial titres attained in the middle ear
33 were approximately one \log_{10} lower than those found in control (PBS) immunized animals. Thus,
34 there is discrimination in the model against homologous and heterologous NTHi challenge.
35
36
37
38
39
40
41
42
43
44

45 *Antibiotic treatment*

46
47 NTHi infected mice treated with a three day course of Azithromycin starting at day 4 post-
48 inoculation had eliminated all NTHi from their middle ears when sampled post treatment (0%
49 ME infection rate and NTHi titre of beneath detection limit of ($<10^2$ c.f.u./ μ l) in treated mice,
50 whereas $>80\%$ of non-antibiotic control treated ears were infected at titres of 10^4 to 10^5 c.f.u./ μ l
51 bulla fluid).
52
53
54
55
56
57
58
59
60

Lung infection

Typical lung infection titres achieved for NTHi by this method are $\sim 10^4$ 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- κ B regulated pro-inflammatory cytokines, such as TNF- α , IL-1 β 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.

1 2 3 **Mouse immunization and protection experiment**

4
5
6 A typical three step immunization procedure covers a seven week period, followed immediately
7
8 by a seven day infection period with the test bacteria, making a total of eight weeks for each
9
10 experiment. At each step, immunization of a cohort of 12 mice takes up to 30 minutes
11
12 depending upon experience. Obtaining blood samples by retro-orbital bleed will add up to 30
13
14 minutes to the time taken for terminal sampling following bacterial infection. The processing of
15
16 blood to obtain serum samples takes a further two and a half hours.
17
18
19

20 **Antibiotic treatment of NTHi infection**

21
22
23 The protocol for NTHi infection of the *Junbo* mouse, followed by a three dose oral administration
24
25 of antibiotic, spans a seven day time period. Oral gavage takes around 30 minutes for a cohort
26
27 of 12 mice.
28
29

30 **NTHi mouse pulmonary infection**

31
32
33 Following the standard NTHi intranasal inoculation procedure, the infection experiment is
34
35 typically run for a 24 or 48 hour time period prior to terminal sampling of bacteria from the
36
37 animals. Terminal sampling of bacteria from mouse lung homogenate takes about three hours.
38
39
40

41 **Acknowledgements**

42
43
44 This work was supported by the Medical Research Council UK (MC_EX_MR/K014986/1 and
45
46 MC_U142684175). MTC is supported by a BBSRC Institute Strategic Programme Grant
47
48 (BB/J004316/1) to the Roslin Institute.
49
50

51 **Conflicts of Interest**

52
53
54 The authors have no conflict of interest to report.
55
56

57 **Literature Cited**

- 1
2
3 Bakaletz, L.O. 2009. Chinchilla as a robust, reproducible and polymicrobial model of otitis media and its
4 prevention. *Expert review of vaccines* 8:1063-1082.
5
6
7
8 Chang, S.H., Mirabolfathinejad, S.G., Katta, H., Cumpian, A.M., Gong, L., Caetano, M.S., Moghaddam,
9 S.J., and Dong, C. 2014. T helper 17 cells play a critical pathogenic role in lung cancer. *Proc Natl
10 Acad Sci U S A* 111:5664-5669.
11
12
13 Cheeseman, M.T., Tyrer, H.E., Williams, D., Hough, T.A., Pathak, P., Romero, M.R., Hilton, H., Bali, S.,
14 Parker, A., Vizer, L., Purnell, T., Vowell, K., Wells, S., Bhutta, M.F., Potter, P.K., and Brown, S.D.
15 2011. HIF-VEGF pathways are critical for chronic otitis media in Junbo and Jeff mouse mutants.
16 *PLoS Genet* 7:e1002336.
17
18
19
20 Clark, J.M., Brinson, G., Newman, M.K., Jewett, B.S., Sartor, B.R., Prazma, J., and Pillsbury, H.C., 3rd.
21 2000. An animal model for the study of genetic predisposition in the pathogenesis of middle ear
22 inflammation. *The Laryngoscope* 110:1511-1515.
23
24
25
26 Cody, A.J., Field, D., Feil, E.J., Stringer, S., Deadman, M.E., Tsolaki, A.G., Gratz, B., Bouchet, V., Goldstein,
27 R., Hood, D.W., and Moxon, E.R. 2003. High rates of recombination in otitis media isolates of
28 non-typeable Haemophilus influenzae. *Infect Genet Evol* 3:57-66.
29
30
31 De Chiara, M., Hood, D., Muzzi, A., Pickard, D.J., Perkins, T., Pizza, M., Dougan, G., Rappuoli, R., Moxon,
32 E.R., Soriani, M., and Donati, C. 2014. Genome sequencing of disease and carriage isolates of
33 nontypeable Haemophilus influenzae identifies discrete population structure. *Proc Natl Acad Sci
34 U S A* 111:5439-5444.
35
36
37
38 Ercoli, G., Baddal, B., Alessandra, G., Marchi, S., Petracca, R., Arico, B., Pizza, M., Soriani, M., and Rossi-
39 Paccani, S. 2015. Development of a serological assay to predict antibody bactericidal activity
40 against non-typeable Haemophilus influenzae. *BMC Microbiol* 15:87.
41
42
43 Euba, B., Molerres, J., Segura, V., Viadas, C., Morey, P., Moranta, D., Leiva, J., de-Torres, J.P., Bengoechea,
44 J.A., and Garmendia, J. 2015a. Genome Expression Profiling-Based Identification and
45 Administration Efficacy of Host-Directed Antimicrobial Drugs against Respiratory Infection by
46 Nontypeable Haemophilus influenzae. *Antimicrob Agents Chemother* 59:7581-7592.
47
48
49
50 Euba, B., Molerres, J., Viadas, C., Ruiz de los Mozos, I., Valle, J., Bengoechea, J.A., and Garmendia, J.
51 2015b. Relative Contribution of P5 and Hap Surface Proteins to Nontypable Haemophilus
52 influenzae Interplay with the Host Upper and Lower Airways. *PLoS ONE* 10:e0123154.
53
54
55
56 Hardisty-Hughes, R.E., Tateossian, H., Morse, S.A., Romero, M.R., Middleton, A., Tymowska-Lalanne, Z.,
57 Hunter, A.J., Cheeseman, M., and Brown, S.D. 2006. A mutation in the F-box gene, Fbxo11,
58 causes otitis media in the Jeff mouse. *Hum Mol Genet* 15:3273-3279.
59
60

- 1
2
3
4
5 Hood, D., Moxon, R., Purnell, T., Richter, C., Williams, D., Azar, A., Crompton, M., Wells, S., Fray, M.,
6 Brown, S.D., and Cheeseman, M.T. 2016. A new model for non-typeable Haemophilus influenzae
7 middle ear infection in the Junbo mutant mouse. *Disease models & mechanisms* 9:69-79.
8
9
10
11 Juhn, S.K., Jung, M.K., Hoffman, M.D., Drew, B.R., Preciado, D.A., Sausen, N.J., Jung, T.T., Kim, B.H., Park,
12 S.Y., Lin, J., Ondrey, F.G., Mains, D.R., and Huang, T. 2008. The role of inflammatory mediators in
13 the pathogenesis of otitis media and sequelae. *Clinical and experimental otorhinolaryngology*
14 1:117-138.
15
16
17
18 Kaur, R., Casey, J., and Pichichero, M. 2015. Cytokine, chemokine, and Toll-like receptor expression in
19 middle ear fluids of children with acute otitis media. *The Laryngoscope* 125:E39-44.
20
21
22 Langereis, J.D., Stol, K., Schweda, E.K., Twelkmeyer, B., Bootsma, H.J., de Vries, S.P., Burghout, P.,
23 Diavatopoulos, D.A., and Hermans, P.W. 2012. Modified lipooligosaccharide structure protects
24 nontypeable Haemophilus influenzae from IgM-mediated complement killing in experimental
25 otitis media. *mBio* 3:e00079-00012.
26
27
28 Lugade, A.A., Bogner, P.N., Thatcher, T.H., Sime, P.J., Phipps, R.P., and Thanavala, Y. 2014. Cigarette
29 smoke exposure exacerbates lung inflammation and compromises immunity to bacterial
30 infection. *J Immunol* 192:5226-5235.
31
32
33
34 Morey, P., Viadas, C., Euba, B., Hood, D.W., Barberan, M., Gil, C., Grillo, M.J., Bengoechea, J.A., and
35 Garmendia, J. 2013. Relative contributions of lipooligosaccharide inner and outer core
36 modifications to nontypeable Haemophilus influenzae pathogenesis. *Infect Immun* 81:4100-
37 4111.
38
39
40
41 Murphy, T.F. 2003. Respiratory infections caused by non-typeable Haemophilus influenzae. *Curr Opin*
42 *Infect Dis* 16:129-134.
43
44
45 Pang, B., Hong, W., West-Barnette, S.L., Kock, N.D., and Swords, W.E. 2008. Diminished ICAM-1
46 expression and impaired pulmonary clearance of nontypeable Haemophilus influenzae in a
47 mouse model of chronic obstructive pulmonary disease/emphysema. *Infect Immun* 76:4959-
48 4967.
49
50
51
52 Parkinson, N., Hardisty-Hughes, R.E., Tateossian, H., Tsai, H.T., Brooker, D., Morse, S., Lalane, Z.,
53 MacKenzie, F., Fray, M., Glenister, P., Woodward, A.M., Polley, S., Barbaric, I., Dear, N., Hough,
54 T.A., Hunter, A.J., Cheeseman, M.T., and Brown, S.D. 2006. Mutation at the Evi1 locus in Junbo
55 mice causes susceptibility to otitis media. *PLoS Genet* 2:e149.
56
57
58
59
60

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Roos, A.B., Sethi, S., Nikota, J., Wrona, C.T., Dorrington, M.G., Sanden, C., Bauer, C.M., Shen, P., Bowdish, D., Stevenson, C.S., Erjefalt, J.S., and Stampfli, M.R. 2015. IL-17A and the Promotion of Neutrophilia in Acute Exacerbation of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 192:428-437.
- Rye, M.S., Bhutta, M.F., Cheeseman, M.T., Burgner, D., Blackwell, J.M., Brown, S.D., and Jamieson, S.E. 2011. Unraveling the genetics of otitis media: from mouse to human and back again. *Mammalian genome : official journal of the International Mammalian Genome Society* 22:66-82.
- Sethi, S. and Murphy, T.F. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. *Clin Microbiol Rev* 14:336-363.
- Shann, F., Hart, K., and Thomas, D. 1984. Acute lower respiratory tract infections in children: possible criteria for selection of patients for antibiotic therapy and hospital admission. *Bull World Health Organ* 62:749-753.
- Su, Y.C., Mukherjee, O., Singh, B., Hallgren, O., Westergren-Thorsson, G., Hood, D., and Riesbeck, K. 2016. Haemophilus influenzae P4 Interacts With Extracellular Matrix Proteins Promoting Adhesion and Serum Resistance. *J Infect Dis* 213:314-323.
- Tateossian, H., Morse, S., Parker, A., Mburu, P., Warr, N., Acevedo-Arozena, A., Cheeseman, M., Wells, S., and Brown, S.D. 2013. Otitis media in the Tgif knockout mouse implicates TGFbeta signalling in chronic middle ear inflammatory disease. *Hum Mol Genet* 22:2553-2565.
- Unger, B.L., Faris, A.N., Ganesan, S., Comstock, A.T., Hershenson, M.B., and Sajjan, U.S. 2012. Rhinovirus attenuates non-typeable Haemophilus influenzae-stimulated IL-8 responses via TLR2-dependent degradation of IRAK-1. *PLoS Pathog* 8:e1002969.
- Woo, J.I., Kil, S.H., Brough, D.E., Lee, Y.J., Lim, D.J., and Moon, S.K. 2015. Therapeutic potential of adenovirus-mediated delivery of beta-defensin 2 for experimental otitis media. *Innate Immun* 21:215-224.
- Woo, J.I., Oh, S., Webster, P., Lee, Y.J., Lim, D.J., and Moon, S.K. 2014. NOD2/RICK-dependent beta-defensin 2 regulation is protective for nontypeable Haemophilus influenzae-induced middle ear infection. *PLoS ONE* 9:e90933.
- Xu, X., Woo, C.H., Steere, R.R., Lee, B.C., Huang, Y., Wu, J., Pang, J., Lim, J.H., Xu, H., Zhang, W., Konduru, A.S., Yan, C., Cheeseman, M.T., Brown, S.D., and Li, J.D. 2012. EVI1 acts as an inducible negative-feedback regulator of NF-kappaB by inhibiting p65 acetylation. *J Immunol* 188:6371-6380.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Yao, W., Frie, M., Pan, J., Pak, K., Webster, N., Wasserman, S.I., and Ryan, A.F. 2014. C-Jun N-terminal kinase (JNK) isoforms play differing roles in otitis media. *BMC immunology* 15:46.

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.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43

Figure 1



