Video Article

Experimental Human Pneumococcal Carriage

Jenna F. Gritzfeld[1], Angie D. Wright[1,2,3], Andrea M. Collins[1,2,4], Shaun H. Pennington[1], Adam K.A. Wright[5], Aras Kadioglu[6], Daniela M. Ferreira[1], Stephen B. Gordon[1]

[1]Respiratory Infection Group, Liverpool School of Tropical Medicine

[2]Royal Liverpool and Broadgreen, University Hospital Trust

[3]Comprehensive Local Research Network

[4]NIHR Biomedical Research Centre in Microbial Diseases, Royal Liverpool and Broadgreen University Hospitals NHS Trust

[5]Institute of Lung Health, Respiratory Biomedical Unit, University Hospitals of Leicester NHS Trust & University of Leicester

[6]Department of Clinical Infection Microbiology & Immunology, Institute of Infection & Global Health, University of Liverpool

Correspondence to: Jenna F. Gritzfeld at j.gritzfeld@liv.ac.uk

URL: http://www.jove.com/video/50115
DOI: doi:10.3791/50115

Keywords: Infection, Issue 72, Medicine, Immmunology, Microbiology, Infectious Diseases, Anatomy, Physiology, Biomedical Engineering, *Streptococcus pneumoniae*, carriage, nasal wash, inoculation, human, vaccine studies, pneumonia, volunteer selection, clinical

Date Published: 2/15/2013

Citation: Gritzfeld, J.F., Wright, A.D., Collins, A.M., Pennington, S.H., Wright, A.K., Kadioglu, A., Ferreira, D.M., Gordon, S.B. Experimental Human Pneumococcal Carriage. *J. Vis. Exp.* (72), e50115, doi:10.3791/50115 (2013).

Abstract

Experimental human pneumococcal carriage (EHPC) is scientifically important because nasopharyngeal carriage of *Streptococcus pneumoniae* is both the major source of transmission and the prerequisite of invasive disease. A model of carriage will allow accurate determination of the immunological correlates of protection, the immunizing effect of carriage and the effect of host pressure on the pathogen in the nasopharyngeal niche. Further, methods of carriage detection useful in epidemiologic studies, including vaccine studies, can be compared.

Aim

We aim to develop an EHPC platform that is a safe and useful reproducible method that could be used to down-select candidate novel pneumococcal vaccines with prevention of carriage as a surrogate of vaccine induced immunity. It will work towards testing of candidate vaccines and descriptions of the mechanisms underlying EHPC and vaccine protection from carriage[1]. Current conjugate vaccines against pneumococcus protect children from invasive disease although new vaccines are urgently needed as the current vaccine does not confer optimal protection against non-bacteraemic pneumonia and there has been evidence of serotype replacement with non-vaccine serotypes[2-4].

Method

We inoculate with *S. pneumoniae* suspended in 100 µl of saline. Safety is a major factor in the development of the EHPC model and is achieved through intensive volunteer screening and monitoring. A safety committee consisting of clinicians and scientists that are independent from the study provides objective feedback on a weekly basis.

The bacterial inoculum is standardized and requires that no animal products are inoculated into volunteers (vegetable-based media and saline). The doses required for colonization (10^4-10^5) are much lower than those used in animal models (10^7)[5]. Detecting pneumococcal carriage is enhanced by a high volume (ideally >10 ml) nasal wash that is relatively mucus free. This protocol will deal with the most important parts of the protocol in turn. These are (a) volunteer selection, (b) pneumococcal inoculum preparation, (c) inoculation, (d) follow-up and (e) carriage detection.

Results

Our current protocol has been safe in over 100 volunteers at a range of doses using two different bacterial serotypes[6]. A dose ranging study using *S. pneumoniae* 6B and 23F is currently being conducted to determine the optimal inoculation dose for 50% carriage. A predicted 50% rate of carriage will allow the EHPC model to have high sensitivity for vaccine efficacy with small study numbers.

Video Link

The video component of this article can be found at http://www.jove.com/video/50115/

Protocol

1. Volunteer Selection and Screening

1. Healthy volunteers are recruited through poster advertisements and on the University website according to the NHS Research and Ethics Committee guidance.
2. The inclusion criteria for participation are:
    1. Adults aged 18-60 years.
    2. Speaks fluent English and is able to communicate easily by both mobile telephone and text messaging.
3. The exclusion criteria for participation are:
    1. Close contact with at risk individuals (children, immunosuppressed adults, elderly, chronic ill health).
    2. Current smoker or significant smoking history (>10 pack years).
    3. Asthma or other respiratory disease.
    4. Pregnancy.
    5. Allergy to penicillin or amoxicillin.
    6. Current involvement in another clinical trial unless observational or in non-interventional phase.
    7. Unable to give fully informed consent.
4. An initial screening visit, approximately one week before inoculation, includes a focused clinical history and targeted clinical examination.
5. If a previously unrecognized abnormality is found, the volunteer will be excluded from the study and appropriate investigation will be arranged through primary care.
6. During the initial screening visit a full blood count is obtained to ensure that the white cell count is within normal range before the inoculation visit. A nasal wash is performed to exclude natural pneumococcal carriers (see 6.1).
7. Prior to inoculation, volunteers are educated on the risks involved with participation in the study and are provided with an emergency patient leaflet, digital thermometer, emergency telephone numbers and 3 day course of amoxicillin.

2. Preparation of Inoculation Stocks

1. Preparation of the inoculation stocks must be done in a clean environment. The pipettes used should only be used for inoculum preparation. All glass and plastic ware should be sterile. We recommend that inoculation stocks be prepared in a dedicated room, using a dedicated fumehood and incubator.
2. Pneumococcal strains are inoculated onto a blood agar plate. Plates are incubated at 37 °C in 5% CO_2 overnight. The following day all the bacteria is scraped from the blood agar plate and inoculated into a bead stock preservation system. These bead stocks are used for inoculation stock preparation. Test the bead stocks for contamination and colony uniformity before preparing an inoculation stock.
3. To prepare the inoculation stock, inoculate a blood agar plate with two beads from the desired pneumococcal serotype bead stock, streaking to cover the entire plate. Incubate plates at 37 °C in 5% CO_2 overnight. All further incubations will use these same conditions. Also incubate the Vegitone media overnight to ensure sterility.
4. The next morning swab half the plate, taking care not to remove any blood agar, and add to 12 ml of pre-warmed Vegitone broth. Repeat using the other half of the plate. Leave the two 12 ml vials at 37 °C for 2 hr or until a change in turbidity becomes apparent, whichever comes first.
5. Add the 12 ml to 40 ml of pre-warmed Vegitone broth and mix thoroughly. Repeat for the other 12 ml vial. This is time point = 0. Remove 100 µl to read the optical density (OD) at 600 nm. Remove 20 µl for quantification of bacteria by colony forming unit (CFU) count using a variation of the method by Miles and Misra (M&M)[7]. Incubate both vials.
    1. For the M&M, divide a blood agar plate into six sections. Add 180 µl of sterile PBS to six wells of a 96 well plate (U-bottom). Add 20 µl of the broth to the top well and mix. Serially dilute the sample 1:10 six times and discard 20 µl from the sixth well. Place three 10 µl drops from the top well into the first section of the blood agar plate. Repeat for the five other wells. Ensure the plate is dry before it is inverted and incubated.
    2. The next day count the number of visible colonies in each dilution section and record. Using the dilution section with a count between 30 and 300, divide the number of visible colonies by three to get an average.
    3. Multiply the average number of colonies by the dilution factor and then divide by 10 (the amount of the 10 µl drop). This gives CFU/µl.
    4. Multiply this number by 1,000 to get CFU/ml.
6. Perform OD 600 readings and quantification of bacteria by M&M method every hour until early mid-log phase is reached, an OD of 0.25.
7. Once this OD is reached, add sterilized 10% glycerol to one vial and prepare 1 ml aliquots. Store aliquots at -80 °C.
8. For a more concentrated stock, centrifuge the other vial at 3,345 x g for 15 min and remove the supernatant. Resuspend the pellet in 22.5 ml of Vegitone medium and add 10% glycerol. Prepare 1 ml aliquots and store at -80 °C.
9. Leave stocks at -80 °C for at least 48 hr before thawing and quantifying.
10. Quantify the frozen bacterial stock by the M&M method. Test three stock tubes individually to ensure reproducibility. Use the value obtained to dilute the stock to the desired inoculum concentration in saline, as described below.
11. For an inoculation, the quantity of pneumococci in the inoculum is calculated before and after the inoculation. This quantification of bacteria by the M&M method is used to determine the average number of pneumococci in the inoculum which accounts for the time it takes to complete the entire procedure.
    1. Prepare an inoculum, diluting to the desired concentration, and perform quantification by the M&M method for the "pre-inoculation" count.

2. In our Clinical Research Facility it takes the team 30 min to complete the entire inoculation procedure. Pneumococcal suspensions in saline will usually show a decreased count in this interval. To account for this, we let the diluted inoculum sit at room temperature for 30 min and then perform a "post-inoculation" quantification of the bacteria by the M&M method.
3. Take the average of the before and after to determine the final inoculated concentration. Adjust the "sitting" time according to your clinical protocol but keep the interval as brief as possible.

12. All new stocks should be sent to a reference laboratory for confirmation of stock purity and identity.

3. Inoculum Preparation

1. Preparation of the inoculum should begin 30 min before the scheduled volunteer inoculation appointment.
2. Take one of the previously made stock tubes out of the -80 °C freezer, thaw it and spin the tube in the microcentrifuge at 17,000 x g for 3 min.
3. Warm blood agar plates in the 37 °C incubator. Prepare a 96 well plate for bacterial quantification by M&M method and prepare dilution tubes according to the desired dose.
4. Remove the broth supernatant from the centrifuged tube and resuspend the pellet in 1 ml of saline. This step removes the broth from the inoculum.
5. Repeat the centrifugation step.
6. Remove the saline supernatant and resuspend the pellet in 1 ml of saline.
7. Perform dilution set for desired inoculum concentration and quantify by M&M method.
8. Take the inoculum to the volunteer appointment and after inoculating the volunteers, repeat the quantification at the lab. Label the tube with the date and inoculum dose and store at -80 °C.
9. To ensure consistency from inoculation to inoculation, it is important to use the same pipettes and dedicated laboratory equipment every time.

4. Inoculation

1. Volunteers should be seated comfortably in a semi-recumbent position.
2. Using a P200 pipette and sterile filter tips draw up 100 µl of the inoculum and insert the tip just inside the nasal cavity.
3. Slowly expel the inoculum onto the nasal mucosa using a circular motion.
4. It is crucial that the pipette tip does not come in contact with the nasal mucosa as a disruption in the integrity of the epithelium could result in the bacteria entering the bloodstream.
5. It is also vital that the inoculum is not placed too far back or it will run down the throat; it should reside inside the nasal cavity.
6. Repeat for the other naris.
7. Have the volunteer remain in the semi-recumbent position for 10 min without sniffing or blowing the nose. This allows time for the bacteria to disperse across the mucosa.

5. Monitoring

1. Volunteers are monitored daily following inoculation. This includes a daily text message sent by the volunteer to investigators before 2 pm.
2. If the text is not received by 2 pm the volunteer will be contacted to ensure his/her well-being. If he/she does not respond, the allocated next of kin will be contacted to ensure the volunteer's well-being.
3. Volunteers are asked to report on any upper respiratory tract symptoms at every appointment. Clinical staff will ask the volunteer to describe his/her symptoms and will follow up any complaints.
4. The antibiotics given to the volunteers are only to be taken under three circumstances; in the event that they are unwell and are instructed to take them by the research team, if they are carrying pneumococcus at the end of the study, or if they are unwell and unable to contact the research team. The volunteer is encouraged to keep this emergency pack with them at all times during the study and is requested to contact the research team on a daily basis for the next 7 days.
5. Our team is available 24/7 with nurse contact during working hours and two doctors available out of hours. All queries are dealt with by telephone call and/or immediate review. Provisions are available for immediate, direct admission to an Infectious Disease ward, if needed.

6. Nasal Wash (NW) Procedure

1. Nasal washes (NW) are performed at an initial screening visit, as well as 48 hr, 7, and 14 days post- inoculation. The NW method used is taken from Naclerio *et al.*[8] Volunteers naturally colonized by *S. pneumoniae* are excluded from inoculation and follow up.
2. The volunteer is seated comfortably and the head is tilted back 30° from the vertical.
3. Ask the volunteer to take a deep breath in and hold their breath whilst pushing their tongue up and backwards against the roof of the mouth. Whilst in this position, the volunteer is asked to signal that they are ready.
4. A syringe filled with 20 ml saline is inserted into the anterior nasal space and 5 ml of saline is expelled. The volunteer then leans forward immediately and expels the fluid by exhaling rapidly through their nose into a foil bowl.
5. Repeat this procedure 3 more times so that each naris has been washed twice and the full 20 ml has been used.
6. Pool all samples together in a centrifuge tube and send to the laboratory at room temperature for processing.

7. Nasal Wash Processing

1. Centrifuge the samples for 10 min at 3,345 x g.
2. Remove the supernatant and store as 1 ml aliquots in labelled Eppendorf tubes at -80 °C.
3. Add 100 µl of STGG medium[9] to the pellet and mix thoroughly. Ensure that the total volume in the tube at this point is determined. This dilution will be used in calculating the CFU of a carriage positive NW.

4. Plate a 20 µl drop of the STGG containing the resuspended pellet onto a blood agar plate containing gentamicin and streak the entire plate.
    1. If the NW is post-inoculation, remove 10 µl from the STGG containing the resuspended pellet and use for bacterial quantification by M&M method.
5. Add another 800 µl of STGG to the NW tube and mix thoroughly.
6. Plate 25 µl onto a blood agar plate and 25 µl onto a chocolate agar plate, streaking the entire plate.
7. Divide the remaining amount bacteria suspended in STGG medium into 2 cryovials and store at -80 °C.
8. Incubate the plates at 37 °C overnight in 5% CO_2.
9. Examine the plates the next day for pneumococcus and other potential respiratory pathogens.
10. Pneumococci are identified on plates by colony morphology. Alpha haemolytic colonies with draughtsman morphology are sub-cultured onto blood agar with an optochin disc and incubated overnight.
11. Presumptive pneumococcal colonies that are optochin sensitive are Gram stained to confirm Gram positive diplococci and serotyped using the Statens Serum Institut Pneumotest-Latex kit.

Representative Results

We have experience inoculating 159 people, of which 35 have carried pneumococcus. The lowest dose we achieved carriage with using serotype 6B was 11,100 CFU/100 µl per naris, the highest dose was 313,000 CFU/100 µl per naris. The lowest dose we achieved carriage with using serotype 23F was 9,000 CFU/100 µl per naris, the highest dose was 84,500 CFU/100 µl per naris.

Our method of carriage assessment is a nasal wash, chosen because a recent study[10] showed that 91% of volunteers found nasal wash to be more comfortable than nasopharyngeal swab; the nasal wash was also more likely to detect pathogens using microbiological culture. Microbial flora recovered on blood agar plates inoculated with NW can be variable and difficult to read. Gentamicin is used on the first blood agar plate to select for pneumococcus. The chocolate agar plate and the second blood agar plate are used to detect other potential respiratory pathogens which may help or hinder pneumococcal colonization. An example of a blood agar plate inoculated with NW that is easy to read is shown in **Figure 1a**; a difficult plate containing numerous kinds of flora is shown in **Figure 1b**.

At every appointment volunteers were asked to describe any upper respiratory tract symptoms, if present, and comparisons between carriers and non-carriers were highlighted. Of the 145 people recently inoculated with pneumococcus, 28 complained of symptoms (**Table 1**). Eight of these were carriers, twenty were not. The most common symptoms were non-specific nasal symptoms (**Table 1**). Of the cases in which there were reported systemic symptoms, including fever and/or malaise, none of the investigations undertaken showed evidence of pneumococcal disease and symptoms were consistent with concurrent viral infection except in one case of tonsillitis. Two pneumococcal positive volunteers were treated with antibiotics. One complained of sore throat and ear ache and, after meeting with the study doctor, was advised to take antibiotics as a precaution. The other volunteer was diagnosed clinically with tonsillitis. All volunteers had rapid resolution of symptoms.

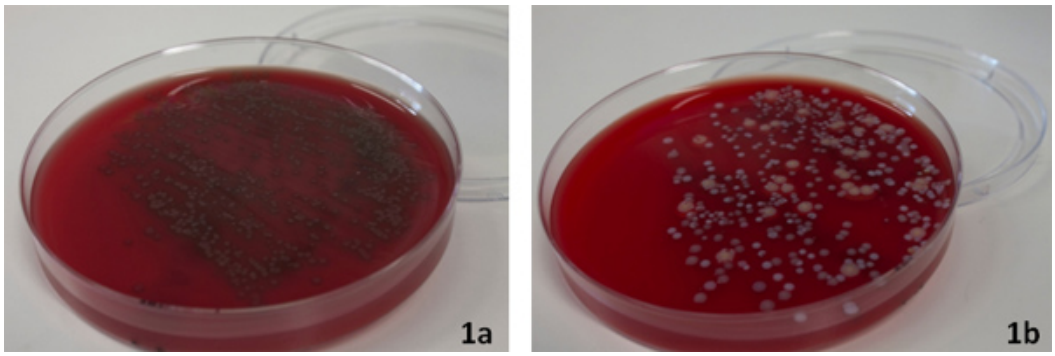

Figure 1. Blood agar plate inoculated with NW. Blood agar plates inoculated with NW can be difficult to read. **1a** is an example of a plate that is easy to read with pneumococci clearly visible. **1b** is a plate with lots of co-colonizing flora making it more difficult to detect if pneumococci is present.

Symptoms		Carriage (n=32)	No Carriage (n=112)
All symptoms		24%	18%
Systemic	Fever	3%	4%
	Malaise*	9%	4%
Local	Ears	9%	3%
	Nose	0	10%
	Throat	12%	3%

Table 1. Characterization of volunteer reported symptoms following *S. pneumoniae* 6B inoculation. All complaints were reviewed by the external safety committee and, following full investigations, no symptoms were attributed to pneumococcal disease. The most common symptoms were sore throat, stuffed nose and flu-like illness.

Discussion

The EHPC platform has a number of potential uses including use as a mucosal vaccine model and as a surrogate of protection for testing novel protein vaccines. The method is dependent upon a consistent inoculation technique and a high quality NW.

Reproducibility can be an issue with the inoculum. Owing to the variable nature of the frozen bacterial aliquots, it can be difficult to replicate the desired dose. A halving or doubling of the desired dose is considered within range. It is crucial that the bacterial quantification be done immediately prior to- and following inoculation, in order to accurately determine quantification of bacteria by CFU count. For this reason it may be helpful if volunteer appointments occur simultaneously so that only one inoculum need be prepared and the quantification plating done prior to and after inoculation happens no more than 30 min apart.

The NW method requires the cooperation of the volunteer and would not be an ideal method in children. If the yield is less than 5 ml, the procedure can be repeated using up to an extra 20 ml. Wearing dentures may reduce the NW yield. If the volunteer has a blocked/congested nose and the saline is running out anteriorly after insertion, blow the nose, insert the syringe a little further back, and tilt the head back more if needed.

The NW is a technique that gets better with practice. Some volume will usually be lost so the aim is for a return of at least 10 ml. If the volunteer can taste saline during the NW then the connection between the posterior nasopharynx and oropharynx has not been adequately closed by the volunteer's tongue. If the volunteer swallows most of the saline, explain the procedure again, emphasizing the pushing of the tongue against the roof of the mouth. Then repeat the procedure to increase the volume returned.

If the NW contains a substantial amount of mucus, it is best to remove it before centrifugation. Vortex the sample so as to loosen anything that might be bound to the mucus and then remove as much of the large pieces as possible while removing as little saline as possible.

A previous EHPC study in the U.S.A. was completed safely and had no adverse events[11]. To ensure the continued safety of the EHPC platform, we have set up an emergency protocol which is based around 24 hr access to the research team. Volunteers are given contact details, allowing them access to the research team 24 hr a day. Any medical care needed is provided in the Royal Liverpool University Hospital. As a further measure of protection, a safety committee consisting of clinicians and scientists from outside the research group receives a weekly safety report. The report contains the bacterial dose each volunteer received and whether any symptoms or illness were reported. This report allows the safety of the study to be externally critiqued without bias. The safety committee are also available 24 hr a day in the unlikely event of an emergency.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was funded by the Bill and Melinda Gates Foundation (Grand Challenge Exploration award to SBG), the National Institutes for Health Research (NIHR), Biomedical Research Centre in Microbial Diseases, and the NIHR Comprehensive Local Research Network. We acknowledge the NWDA for infrastructural support. We thank Prof. J. N Weiser, University of Pennsylvania and Prof. P Hermans, University of Nijmegen for the pneumococcal isolates. Thanks to the EHPC Safety Committee for their support and advice, to all our volunteers for their participation and to Mathieu Bangert for taking the photos.

References

1. Ferreira, D.M., Jambo, K.C., & Gordon, S.B. Experimental human pneumococcal carriage models for vaccine research. *Trends in Microbiology.* **19**, 464-470, doi:10.1016/j.tim.2011.06.003 (2011).
2. Mera, R., Miller, L.A., Fritsche, T.R., & Jones, R.N. Serotype replacement and multiple resistance in *Streptococcus pneumoniae* after the introduction of the conjugate pneumococcal vaccine. *Microbial Drug Resistance.* **14**, 101-107, doi:10.1089/mdr.2008.0782 (2008).
3. Tocheva, A.S., *et al.* Declining serotype coverage of new pneumococcal conjugate vaccines relating to the carriage of *Streptococcus pneumoniae* in young children. *Vaccine.* **29**, 4400-4404, doi:10.1016/j.vaccine.2011.04.004 (2011).
4. Weinberger, D.M., Malley, R., & Lipsitch, M. Serotype replacement in disease after pneumococcal vaccination. *Lancet.* **378**, 1962-1973, doi:10.1016/S0140-6736(10)62225-8 (2011).
5. Wu, H.Y., *et al.* Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb. Pathog.* **23**, 127-137, doi:10.1006/mpat.1997.0142 (1997).
6. Wright, A.K.A., Ferreira, D.M., Gritzfeld, J.F., Wright, A.D., Armitage, K., Jambo, K.C., Bate, E., El Batrawy S., Collins, A., & Gordon, S.B. Human Nasal Challenge with *Streptococcus pneumoniae* is Immunising in the Absence of Carriage. *PloS Pathogens.* **8** (4), e1002622 (2012).
7. Miles, A.A., Misra, S.S., & Irwin, J.O. The estimation of the bactericidal power of the blood. *The Journal of Hygiene.* **38**, 732-749 (1938).
8. Naclerio, R.M., *et al.* Mediator release after nasal airway challenge with allergen. *Am. Rev. Respir. Dis.* **128**, 597-602 (1983).
9. O'Brien, K.L. & Nohynek, H. Report from a WHO working group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*. *Pediatr. Infect. Dis J.* **22**, 133-140, doi:10.1097/01.inf.0000048676.93549.d1 (2003).
10. Gritzfeld, J.F., Roberts, P., Roche, L., El Batrawy, S., & Gordon, S.B. Comparison between nasopharyngeal swab and nasal wash, using culture and PCR, in the detection of potential respiratory pathogens. *BMC Research Notes.* **4**, 122, doi:10.1186/1756-0500-4-122 (2011).
11. McCool, T.L., Cate, T.R., Moy, G., & Weiser, J.N. The immune response to pneumococcal proteins during experimental human carriage. *J. Exp. Med.* **195**, 359-365 (2002).