Department for Environment, Food and Rural Affairs

Application for consent to release a GMO – organisms other than higher plants

Part A1: Information required under schedule 2 of the Genetically Modified (Deliberate Release) Regulations 2002

Part I

General information

1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release.

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Experimental Human Pneumococcal Challenge (EHPC) Model: ***S****treptococcus pneumoniae* **N**asopharyngeal **E**xperimental challenge study of **A**ttenuated **S**trains – proof of concept in healthy adults V2.0 25th July 2018

Research Assistant
Liverpool School of Tropical Medicine (as above)

Post Doctoral Research Assistant
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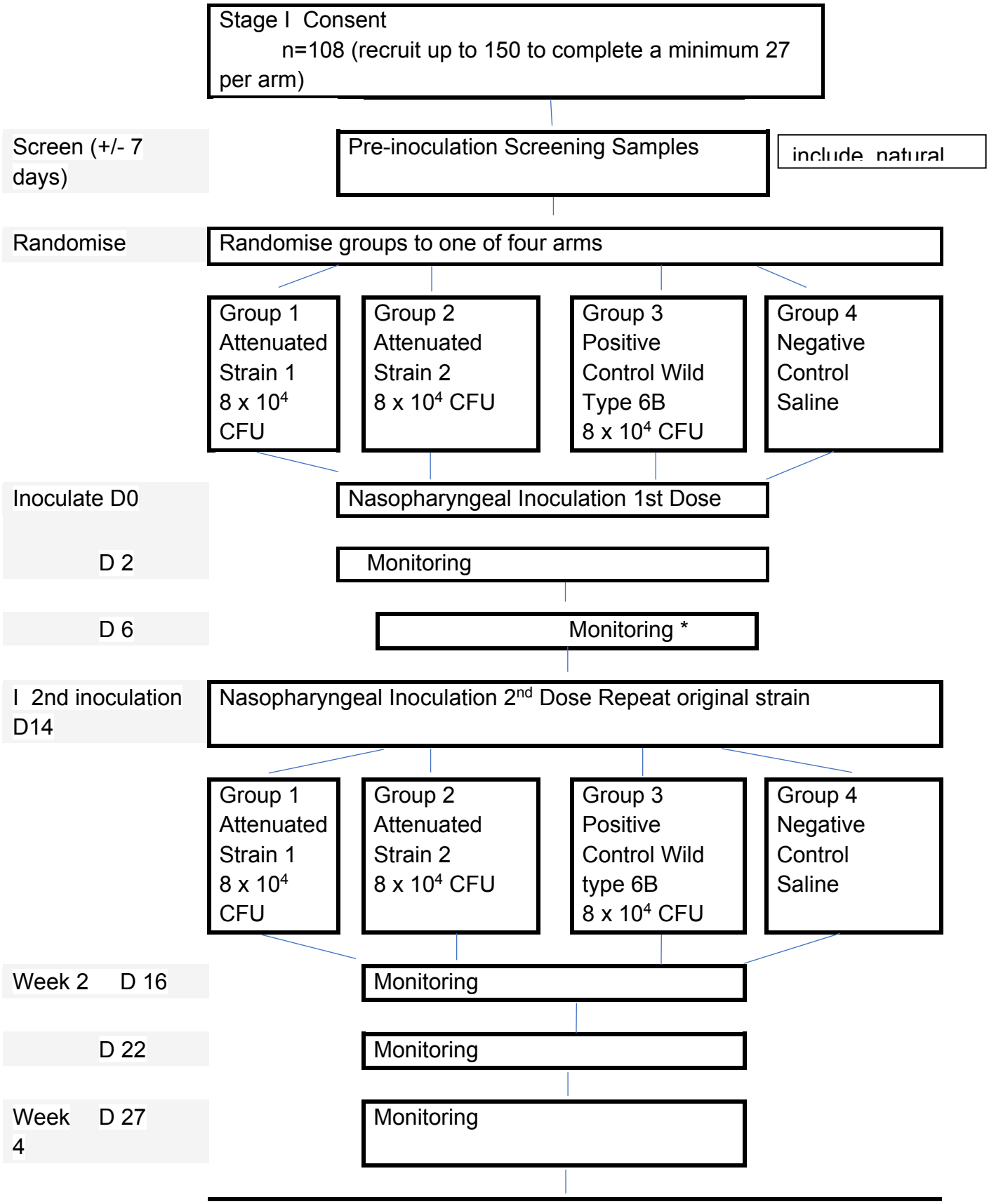
2. The title of the project.

SNEAS Clinical Trial: Experimental Human Pneumococcal Challenge Model (EHPC): ***S****treptococcus Pneumoniae* **N**asopharyngeal **E**xperimental carriage clinical trial of **A**ttenuated **S**trains – proof of concept in healthy volunteers

Funder: This research is part of a Programme Grant funded by the Medical Research Council title: 'Enhancing mucosal immunity to *Streptococcus pneumoniae* by nasal administration of live strains attenuated in virulence.'

Trial Flow Chart for Participants



Week 7 D 36	Monitoring All participants: antibiotics (Amoxicillin)
Challenge after +- 6mnth	Stage II Consent and re-screen for challenge all groups
D0	**Challenge** Nasopharyngeal inoculation wild type *S. pneumoniae 6B* 8 x 10^4 CFU
D 2	Monitoring
D 6	Monitoring
D 14	Monitoring* Antibiotic (Amoxicillin) if one sample is carriage positive

* Shedding will be monitored in a proportion of participants at a minimum of Day 6 (see section 77).

This schedule may be revised after Interim analysis to include nasal cells (table 7) and /or a sub-group of home samples

Part II

Information relating to the organisms

Characteristics of the donor, parental and recipient organisms

3. Scientific name and taxonomy.
Streptococcus pneumoniae (S. pneumoniae)
In this application, the Genetically Modified Organism (GMO) is an attenuated strain of a *S. pneumoniae* serotype 6B wild type strain (parent) that is almost genetically identical, with the only difference being deletions of two genes and their replacement with antibiotic resistance cassettes (encode resistance to spectinomycin and kanamycin). The mutations result in strains that have reduced virulence in animal models of systemic disease but are still able to colonise the nasopharynx, although this is likely to be for a shorter period of time than the wild-type parental strain.
Taxonomy
Domain: Bacteria:
Class: Cocci
Order: Lactobacillales
Genus: Streptococcus
Species: *S. pneumoniae*
Strain: 6B BHN418

4. Usual strain, cultivar or other name.
Capsular serotype 6B strain BHN418
5. Phenotypic and genetic markers.
Serotype 6B capsule – Quellung reaction with type specific antisera
6. The degree of relatedness between the donor and recipient or between parental organisms.
We will use a genetically modified strain of the above 6B strain containing deletions of two genes; hence the GM modified strain will be 99.9% genetically identical to the parental 6B *S. pneumoniae* strain. This study will evaluate whether the selected attenuate strains are able to colonise the nasopharynx and are safer for those who are vulnerable as they have less virulence in humans than the wild-type. As the duration of colonisation is anticipated to be shorter than the wild-type we plan an adaptive design for this trial. Four strains will be prepared with two mutations in each strain. Initially participants will be randomised to a control or one of two attenuated strains. In the event that a strain does not generate any immune response the Independent Data Safety Monitoring Committee following interim analysis will advise whether to replace either or both strain with another. To support this four strains each with a double mutation have been prepared. Individual gene deletions removed

all DNA for the target genes from their start to their stop codon, replacing the deleted sequence with the antibiotic resistance cassette in the same location.
The mutant strains for this study, gene maps and the primers used to make the overlap extension gene deletion constructs are listed in confidential Appendix I.

7. The description of identification and detection techniques.
Following the intranasal inoculation of volunteers with either the attenuated S. pneumoniae strains or the wild type 6B BHN 418 strain (extensively used in previous clinical trials of the EHPC model) nasal washes will be collected. The samples will be processed using our published protocol (Gritzfeld, Jove: https://www.jove.com/video/50115/experimental-human-pneumococcal-carriage) and will be plated on blood agar plate supplemented with gentamicin or selective antibiotic (spectinomycin / kanamycin). The latter will allow growth of the GM attenuated strains but not the wild type parent strain, as resistance requires the strain to contain the antibiotic resistance cassette used for gene replacement. Following overnight incubation at 37°C in 5% CO2, alpha-haemolytic, draughtsman shaped colonies will be sub-cultured for optochin sensitivity and the serotype will be tested using a latex agglutination kit (Staten Serum Institute) for the encapsulated strains (wild type and one of the mutants). A multiplex PCR targeting lytA and the antibiotic resistant gene (or knocked-out genes) will be used as a verification step of the detection of the mutant strain.

Detection Clinical (see Fig 1): Participants are screened for a natural carriage using nasal wash prior to the initial inoculation. Following the initial nasopharyngeal inoculation on Day 0 (D0) they have five follow up visits including a nasal wash on days 2, 6, 16, 22, 27 and 36. All volunteers are challenged after 6 months with nasal wash to identify bacteria on days 2, 7, 14 following re-inoculation.

8. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques.
Classical microbiology culturing for the detection of wild type pneumococci is a reliable technique and approaches the levels of qPCR sensitivity for *S. pneumoniae* detection [12]. However, the colonisation of the nasopharynx with very low numbers of *S. pneumoniae* (‘*S. pneumoniae* density’) limits their detection by culture methods (low limits of detection). Therefore, lytA and/or capsular polysaccharide specific gene qPCR have been designed and broadly utilised, allowing for very high accuracy on *S. pneumoniae* detection and identification (Appendix II & III). Molecular techniques-qPCR enable the detection of 0.1 copies of *S. pneumoniae* DNA/ ml, whereas the classical microbiology techniques have a higher threshold of detection, usually equal with 1 CFU/ml.
GM attenuated strains containing mutations will be identified and their density quantified by qPCR, specifically designed for each mutant strain using the primers

information listed in Appendix I. The combination of the microbiological and molecular methods described in item 7 enables the identification of both parent and the GM attenuated strains with 100% accuracy.

9. The description of the geographic distribution and of the natural habitat of the organisms including information on natural predators, prey, parasites and competitors, symbionts and hosts.

S. pneumoniae is only found as a human oropharynx and/or nasopharyngeal commensal; there is no environmental reservoir and *S. pneumoniae* is not a commensal of non-human mammalian species, with the exception of horses which are colonised by a subset of *S. pneumoniae* strains that do not include the 6B strain BHN418. Natural competitors include all other human nasopharyngeal commensals including other naturally occurring *S. pneumoniae* strains.

10. The organisms with which transfer of genetic material is known to occur under natural conditions.

This is largely restricted to other *S. pneumoniae* strains. Transfer of genetic material could occur to other nasopharyngeal commensals but has to our knowledge never been described, and recent data suggest that genetic exchange from closely related streptococci such as *Streptococcus mitis* is unidirectional into *S. pneumoniae* but not vice versa (Kilian MBio. 2014 Jul 22;5(4):e01490-14. doi: 10.1128/mBio.01490-14). These data suggest there is a substantial barrier that inhibits the transfer of genetic material from *S. pneumoniae* to even closely related nasopharyngeal commensal species.

11. Verification of the genetic stability of the organisms and factors affecting that stability.

Stability is assessed by multiple rounds of culture without antibiotic selection. For all four of the GM attenuated strains after 3 rounds of broth culture without selective pressure (increase in CFU by 250000% over 8 hours) 100 of 100 colonies retained resistance to both streptomycin and kanamycin, showing a high degree of mutant stability. We have shown genetic stability of the wild type BHN418 *S. pneumoniae* during experimental colonization [1].

12. The following pathological, ecological and physiological traits:

a. the classification of hazard according to existing Community rules concerning the protection of human health and the environment

S. pneumoniae is a category 2 pathogen (http://www.hse.gov.uk/pubns/misc208.pdf)

b. the generation time in natural ecosystems, and the sexual and asexual reproductive cycle

Generation time during nasopharyngeal colonisation is unclear – probably about 1 hour.

c. information on survivability, including seasonability and the ability to form survival structures, including seeds, spores and sclerotia

There is no environmental source of *S. pneumoniae*, which is unable to produce spores and only survives for a short period in the environment. A systematic review

in 2006 found limited data on how long *S. pneumoniae* persists on inanimate surfaces [2].
An EHPC pilot study exposed the hands of healthy adult participant's (n=63) to a well-characterised, fully sequenced penicillin-sensitive *S. pneumoniae* serotype 6B and then observed for the development of *S. pneumoniae* carriage. Drying of the bacteria tended to lead to lower density of colonisation compared to transmission while the bacteria was still wet. Microbiological culture suggested that transmission of *S. pneumoniae* was more likely when the bacteria was transmitted while still wet (1/20 transmissions with dry bacteria vs 7/20 with wet bacteria). Using molecular methods (qPCR) both the wet and dry groups having approximately 50% colonisation rates but considerably lower density in the dry group. However, these data were not statistically significant as this is limited as a pilot study (unpublished data, Connor LSTM appendix II and German appendix III).
d. pathogenicity, including infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organisms and possible activation of latent viruses (proviruses) and ability to colonise other organisms
S. pneumoniae commonly colonises the human nasopharynx and is not known to be toxic or allergenic at this site. When colonised with wild-type 6B *S. pneumoniae* there is a low risk of pathogenicity, we have inoculated over 1000 human volunteers over a 9-year period (including 30 participants over 65 years of age, this age is known to be at higher risk of *S. pneumoniae* infections) with no cases of S. pneumoniae disease. The GM modifications are designed to markedly reduce the virulence of the parental 6B strain even further and therefore are markedly less likely to lead to infection than the wild type strain. The attenuated virulence of the GM strains has been confirmed in mice (Appendix I). *S. pneumoniae* is unable to colonise non-humans (except for horses) or activate proviruses.
e. antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy
The GM *S. pneumoniae* will be resistant to spectinomycin (very rarely used in humans, restricted to patients with multi-drug resistant mycobacterial infection) and kanamycin (rarely used in humans, generally restricted to patients with multi-drug resistant mycobacterial infections or on rare occasions Gram-negative infections that are largely hospital acquired).
f. involvement in environmental processes including primary production, nutrient turnover, decomposition of organic matter and respiration
Not applicable – no environmental reservoir.
13. The sequence, frequency of mobilisation and specificity of indigenous vectors and the presence in those vectors of genes which confer resistance to environmental stresses.
Antibiotic resistance genes are integrated into the chromosome and are not expressed in genetically mobile elements or inserted into prophages. As discussed

above genetic exchange of chromosomal genes with other *S. pneumoniae* is possible, but the data indicate that this is very rare with other even closely related nasopharyngeal commensals.
14. The history of previous genetic modifications.
There have been no previous genetic modifications to the wild type 6B BHN418 *S. pneumoniae* strains prior to the gene deletions in the GM attenuated strains.

Characteristics of the vector

15. The nature and source of the vector.
No vector has been used.
16. The sequence of transposons, vectors and other non-coding genetic segments used to construct the genetically modified organisms and to make the introduced vector and insert function in those organisms.
None – the mutant strains were made by recombination of DNA constructs made using overlap extension PCR and isolated antibiotic resistance gene cassettes.
17. The frequency of mobilisation, genetic transfer capabilities and/or methods of determination of the inserted vector.
Not applicable – no inserted vector.
18. The degree to which the vector is limited to the DNA required to perform the intended function.
Not applicable – no inserted vector.

Characteristics of the modified organisms

19. The methods used for the modification.
Target genes were deleted by recombination of DNA constructs using the natural *S. pneumoniae* DNA uptake and recombination competence mechanisms and established laboratory protocols involving administering exogenous competence stimulating peptide [3-5]. The DNA constructs were made using overlap extension PCR[6] to combine chromosomal DNA fragments that flank the target gene with the spectinomycin or kanamycin resistance genes using established protocols [7-9]. The antibiotic resistant genes were integrated into the chromosome replacing the gene deletion and are not expressed in genetically mobile elements or inserted into prophages. The antibiotic cassettes used were chosen to create resistance to antibiotics that are rarely used in humans and to which the bacteria are very unlikely to be exposed to during the clinical trial.
Mutants were selected using growth on agar plates containing kanamycin and / or spectinomycin and confirmed by PCR and DNA sequencing. As discussed above mutant stability has been checked by multiple rounds (>3) of growth to stationary phase without selection.
20. The methods used:

a. to construct inserts and introduce them into the recipient organism
The DNA constructs were made using overlap extension PCR to combine chromosomal DNA fragments that flank the target gene with the spectinomycin or kanamycin resistance genes.
b. to delete a sequence
Transformation used competence stimulating peptide to stimulate *S. pneumoniae* uptake of gene deletion DNA constructs and allow them to undergo recombination at the target gene site. Deletions were introduced into the wild type 6B strain sequentially in two separate transformation reactions.

21. The description of any insert and/or vector construction.
The deleted genes are all required for the full virulence of S. pneumoniae 6B strain in mouse models of infection (see confidential Appendix I for details on each gene deletion and the effects of the mutations in animal models of infection).
22. The purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function.
The inserted antibiotic cassettes were made by PCR and have been sequenced after transformation into *S. pneumoniae*; they show 100% identity to the source DNA and published DNA sequences for the antibiotic resistance cassettes
23. The methods and criteria used for selection
Transformants were made by sequential transformation with the deletion cassettes and selected using antibiotic selection with kanamycin or spectinomycin depending on which resistance gene was included in the deletion construct. The identity of the transformants was confirmed by PCR and sequencing, and where possible laboratory tests of phenotype related to loss of the targeted gene.
24. The sequence, functional identity and location of the altered, inserted or deleted nucleic acid segments in question and, in particular, any known harmful sequence.
The only inserted sequences will be the spectinomycin or kanamycin resistance genes.

Characteristics of the genetically modified organisms in their final form

25. The description of genetic traits or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed.
The exact constructs were defined by laboratory work in Professor Brown's Laboratory at UCL as part of an ongoing research project. The mutations result in *S. pneumoniae* strains that have reduced virulence in animal models of systemic disease but are still able to colonise the nasopharynx, although generally for a shorter period of time than the wild-type parental strain (Appendix 1).
26. The structure and amount of any vector or donor nucleic acid remaining in the final construction of the modified organisms.

The GM attenuated strains' chromosome each contain two antibiotic resistance genes for spectinomycin and kanamycin, totalling 847 and 1037 bp respectively.
27. The stability of the organism in terms of genetic traits.
For all four of the GM attenuated strains after 3 rounds of broth culture without selective pressure (increase in CFU by 250000% over 8 hours) 100 of 100 colonies retained resistance to both streptomycin and kanamycin, showing a high degree of mutant stability.
28. The rate and level of expression of the new genetic material in the organism and the method and sensitivity of measurements of that rate and level.
There is constitutive expression of antibiotic resistance proteins; this is shown by the mutant strain being resistant to the relevant antibiotics when grown on blood agar plates containing antibiotics.
29. The activity of the gene product.
Not applicable.
30. The description of identification and detection techniques, including techniques for the identification and detection of the inserted sequence and vector.
Detection
The initial screening visit will assess participants for natural carriage using nasal wash and throat swabs. Following the initial nasopharyngeal inoculation on Day 0 participants are followed up and have a nasal wash on days 2, 6, 14, 16, 22, 27 and 36. Participants are challenged with *6B S. pneumoniae* (wild type) after 6 months then attend follow up visits that include nasal wash to identify bacteria on days 2, 7,14.

Identification
The samples will be processed using our published protocol (Gritzfeld, JOVE: https://www.jove.com/video/50115/experimental-human-pneumococcal-carriage) and will be plated on blood agar plate supplemented with gentamycin or selective antibiotic (spectinomycin/kanamycin). The latter will allow growth of the attenuated strain but not the wild type parent strain, as it is only included in the antibiotic resistance cassette used for gene replacement. Following overnight incubation at 37°C in 5% CO2, alpha-haemolytic, draughtsman shaped colonies will be sub-cultured for optochin sensitivity and the serotype will be tested using a latex agglutination kit (Staten Serum Institute). A multiplex PCR targeting on *lytA* and the antibiotic resistant gene (or knocked-out genes) will be used as a verification step of the detection of the mutant strain.

31. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques.
See section 8
32. The history of previous releases or uses of the organisms.

As far as we are aware this clinical trial is the first release in humans of GM *S. pneumoniae*. The wild type 6B parental strain has been used in over eleven clinical trials including 1000 healthy volunteers in the EHPC programme at Liverpool School of Tropical Medicine over the past 9 years.

33. In relation to human health, animal health and plant health:

S. pneumoniae is a predominately a human restricted pathogen, and there are no implications for animal or plant health from this application.

a. the toxic or allergenic effects of the non-viable organisms and/or their metabolic products

The parent organism commonly colonises the oropharynx and or nasopharynx; it is not known to be toxic or allergenic. Previous EHPC studies have not identified any related reactions.

b. the comparison of the organisms to the donor, recipient or (where appropriate) parental organism regarding pathogenicity

The parental wild type 6B *S. pneumoniae* strain only has low pathogenic potential in humans, and there have been no infection related side effects in over 1000 human subjects inoculated with this strain using the EHPC model. The GM strains have significantly lower pathogenicity than the parental wild type strain when assessed using mouse models of infection (as described above).

c. the capacity of the organisms for colonization

Colonisation of the nasopharynx with wild type *S. pneumoniae* is common with 40-95% of infants and 10-25% of adults colonised at any one time [10, 11] [11-13]. Adaptive immune response (especially to protein antigens) are thought to contribute to the large fall in *S. pneumoniae* carriage rate at the end of infancy [14]. In previous EHPC studies of healthy adults, nasal inoculation of $8x10^4$ CFU of a *6B S. pneumoniae* strain in 0.1mls saline resulted in nasopharyngeal carriage at a density typical of natural carriage in approximately 50% of subjects commonly for a period of seven days but generally no longer than 4 weeks.

Attenuated strains in mice often have a reduced duration of colonisation compared to wild type bacteria, this may affect the efficacy of using attenuated mutants for preventing *S. pneumoniae* infections. The mouse data on colonisation for the GM *S. pneumoniae* strains are shown in Appendix I. The data suggests that two of the GM strains (strains 1 and 2) will be unlikely to colonise as high a proportion of human subjects or reach the same density of colonisation as the wild type 6B strain, whereas the other two GM strains may do so. They are very unlikely to colonise to a higher level.

d. if the organisms are pathogenic to humans who are immunocompetent:

i. diseases caused and mechanisms of pathogenicity including invasiveness and virulence

Disease caused: *S. pneumoniae* is associated with several diseases included in hospital admission statistics for the UK in 2013-2014 [15-19] [20]:

- Pneumonia (189 000 admissions, approximately 40% due to *S. pneumoniae*, overall mortality 18%)
- Septicaemia
- Meningitis
- Exacerbation of COPD (117 000 admissions, mortality 14%, 25% of cases are thought to be associated with *S. pneumoniae*)
- Otitis media

Mechanisms for pathogenicity and virulence: Nasopharyngeal carriage is a pre-requisite of infection and the primary reservoir for transmission [21-23]. Colonisation by *S. pneumoniae* requires the pathogen to adhere to the epithelial lining of host's respiratory tract and avoid multiple host defence systems. For successful colonisation or transmission the pathogen must either penetrate the mucosal barrier, avoid mucociliary clearance, bind to epithelial carbohydrates and proteins, obtain nutrients from host while evading cellular and humoral immunity and finally exploit the hosts responses to allow replication and invasion across host barriers [24]. Although the data are incomplete, previous publications and our present understanding of how the mutations reduce the virulence of the GM attenuated strains is in confidential Appendix I:

ii. communicability

S. pneumoniae is unable to colonise non-humans (except horses) or activate proviruses.

In the developed world adult to adult transmission of *S. pneumoniae* is rare. Due to the high carriage rates in children there is a higher risk of child to adult transmission [25, 26]. All participants are aged 18 – 50 years and are excluded if they have contact with children under the age of 5 years or other at risk groups. Outbreaks of *S. pneumoniae* infection are uncommon and restricted to high risk populations in crowded group accommodation settings such as barracks, nursing home residents [27], day care centres [28, 29], prisons [30] and residents in homeless shelters [31]. Importantly, with the wild type 6B strain we found that there was **no hand to nose transmission once *S. pneumoniae* has dried on skin** (Connor, unpublished see Appendix II). The EHPC model has previously included partners and volunteers who house share yet the post inoculation nasal washes have not shown communicability in nasal washes performed on these cohabitees during the six week follow up period.

iii. infective dose

In the previous clinical trials using the EHPC model, participants were inoculated with a dose of $8x10^4$ CFU 6B *S. pneumoniae* strain in 0.1mls saline pipetted 0.5 µL into each nostril. This was based on the EHPC dose finding study [32]. This dose will be replicated for this study, given on two occasions separated by 14 days (wild type 6B or GM attenuated strains), followed by a further dose after 6 months (wild type 6B to all subjects). There have been no cases of active *S. pneumoniae* infection or

related serious adverse events reported. Participants are followed up for a period of at least 4 weeks post inoculation. This dose leads to successful colonisation in approximately 50% of participants. It is anticipated that the immune response to the first dose will reduce the carriage for the second dose.

iv. host range and possibility of alteration

Host Range: *S. pneumoniae* is predominately only found as a human nasopharyngeal commensal. Nasopharyngeal carriage is the predominant source of immunising exposure and immunological boosting against *S. pneumoniae* infection in both children and adults. Infection of horses occurs with a subset of *S. pneumoniae* strains that do not include the 6B strain used for this study.

Alteration: as *S. pneumoniae* are naturally transformable, we have mutated two virulence genes to minimise the chance of revertants developing.

v. possibility of survival outside of human host

There is no environmental reservoir and *S. pneumoniae* not a commensal of non-human mammalian species, with the exception of horses which are colonised by a subset of *S. pneumoniae* strains that do not include the 6B strain BHN418.

There is limited data on how long *S. pneumoniae* persists on inanimate surfaces [2]. However, in an EHPC pilot study where healthy adult participant's hands were exposed to a well-characterised, fully sequenced penicillin-sensitive pneumococci and then observed for the development of *S. pneumoniae* carriage; on dry hands there was no further transmission despite contact with the nose (unpublished data, Connor). This is consistent with a laboratory based study that found a one log decrease in bacterial counts when the inoculum was left to air dry compared to a wet swab (unpublished, Germain).

vi. presence of vectors or means of dissemination

Transmission of *S. pneumoniae* from person-to-person is thought to occur due to airborne respiratory droplets. Epidemiological data provides evidence that transmission is influenced by overcrowding and concurrent viral respiratory tract infections [33, 34]. The EHPC team found that when *S. pneumoniae* was put onto dry hands there was less nasal transmission following hand to nose contact (see Q12 Connor, unpublished). There are no arthropod or animal vectors involved in *S. pneumoniae* transmission.

vii. biological stability

The GMO bacteria will be a double mutant in order to maintain biological stability. Stability testing has been performed by culture through multiple generations without antibiotic selection and has shown the mutations are very stable with no evidence of reversion (see above).

viii. antibiotic-resistance patterns

To minimise the risk of transfer of novel genetic material to other bacteria within the host or the host's contacts we will use spectinomycin or kanamycin as the antibiotic

marker for this proposal; both antibiotics are very rarely used in clinical practice in the UK and only for very complex infections treated in a hospital setting. It is unlikely that even if spectinomycin or kanamycin resistance is transferred to other bacteria that it will be maintained in the absence of selective pressure. All EHPC participants that are experimental *S. pneumoniae* carriers will be given penicillin to eradicate *S. pneumoniae* 36 days after colonisation; hence the potential maximum duration of GM carriage will be time limited. In practice most subjects will clear the GM strains well within this 36 day period.

ix. allergenicity

This organism commonly colonises the nasopharynx and is not known to be toxic or allergenic.

x. availability of appropriate therapies

EHPC has safety guidelines are based on established processes developed during eleven clinical trials for monitoring of participants and provision of additional clinical care when required (Box 4 p38). In the event the participant is concerned about their healthy they are advised to follow their normal route of health care to avoid any delay in treatment. At the end of follow up the participants who have carried the bacteria during the trial will be advised to take antibiotics by day 36 aiming to clear carriage. Participants are screened when challenged after 6 months. If the attenuated strain is present at this stage then the clinical team will advise participants regarding antibiotic treatment to clear carriage at the end of follow up. This may include either a five day course of amoxicillin or an alternative antibiotic such as Clarithromycin.

e. Other product hazards

Not applicable

Part III

Information relating to the conditions of release

The release

34. The description of the proposed deliberate release, including the initial purpose or purposes of the release and any intention to use the genetically modified organisms as or in a product in the future.

Initial purpose of the release:

The purpose is to conduct a clinical trial in healthy volunteers to determine whether nasopharyngeal colonisation of adults with an attenuated *S. pneumoniae* strain increases serotype-independent protective adaptive immunity. Ultimately, this could potentially be used to for future development of vaccines to prevent *S. pneumoniae* lung infections.

The reason for attenuating the strain is that administering wild-type *S. pneumoniae* in subjects who are more susceptible to *S. pneumoniae* could on occasion cause disease. A safer alternative would be to use attenuated strains that are unable to cause serious infections by mutating the virulence factors whilst maintaining the ability to boost adaptive immunity. Two or more virulence genes are mutated to minimise the chance of revertants developing. To stimulate cross-strain protection immunity will need to target protein rather than capsular antigens.

The first stage of the research is construction and testing of the mutant phenotypes using laboratory assays and mouse models of infection to confirm they are safe to use yet retain the ability to stimulate a significant immune response after colonising the nasopharynx. These experiments have shown that the deleted genes targeted in the GM strains results in strains that are attenuated in virulence in a mouse model of pneumonia with sepsis, but still stimulate a protective immune response when inoculated into the mouse nasopharynx. Four double mutant strains have been constructed, two of which will be chosen for use in the clinical trial. The other two are reserve strains in case interim trial data analysis suggest one of the original two strains used are inappropriate due to lack of immunogenicity.

We propose a clinical trial utilising the established Experimental Human Challenge Model (EHPC). This involves nasal administration of two double GM mutant attenuated *S. pneumoniae* strains in healthy human volunteers n=150 (fig 1). Four groups of up to 35 participants per group will be randomised for comparison to:

- Attenuated strain 1
- Attenuated strain 2
- Positive control: unmutated bacteria (wild type 6B *S. pneumoniae*)
- Negative control: mock inoculation (saline).

As mentioned above, an interim analysis after the first 40 participants (10 per arm) will determine whether there is an immune response to each attenuated strain. If no immune response is present in one or both of the attenuated GM strain arms then the IDSMC may advise to replace the attenuated strain with one or both of the reserve attenuated strain(s). Then continue to complete a definitive study with the attenuated strain (s).

Table 1 Primary Objectives and Endpoints

	Objectives	Endpoints (SAP section 10)
Primary	To test the efficacy of nasal administration of attenuated *S. pneumoniae* strains to induce mucosal immunity thereby prevent future colonisation with wild type *S. pneumoniae* using the EHPC model.	Superiority design comparing attenuated strains with the saline control: the presence of wild type 6B *S. pneumoniae* in nasal wash at any time point post challenge detected using classical microbiology.
Secondary	To compare bacteriological and immunological parameters associated with prevention of mucosal infection To assess attenuated strain colonisation characteristics including density and duration Bacteriological safety of attenuated strains Evaluate shedding by the transfer of bacteria from the nose for a sub-group.	Antibodies and cellular adaptive immune responses local and systemic to *S. pneumoniae* pre and post colonization. The presence, density and duration of *S. pneumoniae* following inoculation in nasal wash collected on days, 2, 6 , 16, 22, 27 or 36 and after challenge on day 2, 6, 14 using classical microbiological and qPCR techniques Sequencing and *in vitro* testing of bacteria recovered to ensure genome and phenotypic stability Shedding of *S. pneumonia*e following cultures taken as a minimum for on Day 6 during Stage I.

Clinical Trial in Healthy Volunteers as proof of concept

Inoculation: young healthy volunteers 18 to 50 years of age (minimum of 108 completed participants up to a maximum of 150 to allow for natural carriage and loss to follow up) will be inoculated in the Accelerator Research Clinic (ARC) in Liverpool School of Tropical Medicine then return to the wider community following each appointment. They will have the bacteria ($8x10^4$ *S. pneumoniae* CFU in 0.1ml saline) inoculated into their nose. The inoculation is repeated 14 days later (see question 54). This booster is required as a reduced proportion of subjects are likely to be successfully colonised with the GM attenuated strains compared to the wild type 6B strain, and this is supported by the pre-clinical data for at least two of the GM strains. Hence the GM strains may be less immunogenic than the wild type 6B strain, and this can be partially overcome by using two rather than one inoculations.

Follow up appointments: Nasal wash fluid and blood samples are collected at follow up visits over the next few weeks (fig 1). Nasal cells may be added following the interim analysis. The nasal wash includes the insertion of saline into the nose then this and secretions drip into a sterile bowl. Nasal cells are collected using a rhinoprobe to make a light scratch on the inferior turbinate (if required following interim analysis). For a sub-group nasosorption are collected. This is a type of filter paper that collects secretions from the lining of the nose.

Challenge (after 6 months): all volunteers will then be challenged by intranasal inoculation of wild type 6B *S. pneumoniae* to see whether the immune response to previous colonisation with the attenuated strain prevents subsequent colonisation with 'normal' wild type 6B *S. pneumoniae*. The immune response to *S. pneumoniae* colonisation in the volunteers will be assessed using conventional tests of antibody and white cell responses and the nasal wash fluid and blood samples. The laboratory team will be blinded at this stage by allocating new study numbers to participants.

Results: The primary endpoint is reduction of colonisation after challenge at 28 weeks with wild-type *S. pneumoniae* strain to 15% for EHPC subjects given the attenuated strains compared to 50% for the negative control group, assessed by recovery of bacteria from nasal washes.

Safety: A safety protocol is established for the conduct of the EHPC model (Box 4 section 86) Over a period of nine years over 1000 EHPC healthy adult participants have been inoculated with wild type *S. pneumoniae* without any related serious adverse events. The attenuated strains are anticipated to be less virulent therefore less likely to cause serious side effects; they will probably also have lower carriage

Experimental Human Pneumococcal Challenge (EHPC) Model: ***S****treptococcus pneumoniae* **N**asopharyngeal **E**xperimental challenge study of **A**ttenuated **S**trains – proof of concept in healthy adults V2.0 25th July 2018

rates. Safety reports are distributed weekly to the Trial Management Group and when required to the independent DSMC and National Research Ethics Committee.

In future: Information from this clinical trial could contribute towards developing vaccines for pneumonia, specifically for prevention of pneumonia in high risk adults. At present we are not expecting to develop the live attenuated strain approach for use as a vaccine for infants.

The Investigator will ensure that this study is conducted in accordance with relevant regulations and with Good Clinical Practice. The MHRA have confirmed that this proposal is not a Clinical Trial of an Investigational Medicinal Product (IMP) as defined by the EU Directive 2001/20/EC and no submission to the Clinical Trials Unit at the MHRA is required (email dated 11th January 2018).

35. The intended dates of the release and time planning of the experiment including frequency and duration of releases.
The intended start date of the release is estimated as September 2018. We aim to complete the trial by June 2020 but may continue until June 2022 for example to allow replacement of participants due who do not complete follow up or due to recruitment delays. This is subject to regulatory and governance approvals as per Gantt chart:

Table 2 Gantt Chart

Planned Activities	Date								
	06/18	09/18	12/18	03/19	06/19	09/19	12/19	03/20	06/20
Clinical Trial Approvals finalise DEFRA / ACRE /Ethics / R&D / HRA									
Stage I Pre-interim analysis Inoculate n= 40									
Interim Analysis									
Stage I Post- interim analysis Inoculate n= 68 (up to max 140)									
Stage II Challenge 6 months post initial inoculation									
Statistical Analysis /Publication									

The first groups inoculated will be limited to 5 – 10 participants to review the carriage and safety data. Symptoms are monitored daily for the first week then at each clinic visit. Based on previous EHPC studies with the wild type 6B we aim increase this to approximately 10 – 15 participants per week. The target is 108 completed participants but up to 150 participants may be inoculated due to drop out and natural carriage. Approximately half will have a GM attenuated strain with all participants being recruited over a period of up to 4 months. Natural carriers are estimated as 10% they will be included but identified by nasal wash on screening. The number of experimental carriage positive participants are estimated as 40 – 50% (n= 70) who will be challenged after 6 months with the wild type 6 B strain commencing March 2019. However, if there is a high dropout rate for the challenge we will continue for a further period of up to 12 months.

36. The preparation of the site before the release.

The inoculation preparation and inoculation of participants and their follow up visits will be conducted within the Accelerator Building at Liverpool School of Tropical Medicine (LSTM). Participant visits are undertaken in the Accelerator Research Clinic (ARC) a dedicated patient clinical research facility in the Accelerator Building. Participants are inoculated with the wild type 6B *S. pneumoniae* after a period of six months then the follow up visits in the Accelerator Research Clinic (LSTM).

The following approvals in addition to ACRE/Defra will be confirmed prior to commencing the research:

- Ethical approval from the National Research Ethics Service.
- Approval by the Research and Development Governance Team (LSTM)
- Authorisation by the Sponsor (LSTM)
- Independent Data Safety Monitoring Committee

The Medicines and Healthcare Products Regulatory Agency (MHRA) have already confirmed that this trial does not require a Clinical Trial Authorisation.

The EHPC team include registered health professionals in addition to laboratory scientists who have conducted eleven clinical trials over nine years mainly in healthy adult volunteers. The Chief Investigator will delegate roles to the research team on completion of study specific training in the protocol, standard operating procedures and Good Clinical Practice. A laboratory handbook will be prepared to standardise the inoculation and sampling procedures specific to this trial. The EHPC Safety Protocol for the wild type is well established and monitored the clinical and laboratory teams including weekly reports (Box 4 p38). The research team are trained in the EHPC model and the requirements for preparing and disposal of the inoculum and other clinical waste consistent with EHPC and Liverpool School of Tropical Medicine Standard operating procedures (appendix IV and V). Clinical SOP's are consistent with NHS standards for the handling and disposal of clinical waste. This team are experienced in conducting clinical trials within the Research Governance Framework

including approvals required by the Health Research Authority and the National Research Ethics Service.

37. The size of the site.

The preparation of and inoculation of participants will be undertaken within the Accelerator Research Clinic and laboratory in the Liverpool Life Sciences Accelerator Building, a dedicated clinical research facility for research appointments. Immediately following inoculation on the same day participants will be discharged to the community returning to the clinic for study visits (Fig. 1).

38. The method or methods to be used for the release.

Preparation of inoculum: The inoculum will be prepared by the EHPC laboratory and transferred immediately after preparation to the adjacent Accelerator Research Clinic (both on the 3rd Floor Accelerator Building). Discarding Inoculum: excess of inoculum will be autoclaved as per LSTM clinical waste policy (SOP 004 appendix IV and V), then incinerated.

Administration to participants: Health professionals trained in the inoculation procedure and safety monitoring will administer the inoculum. Nasal inoculation of $8x10^4$ CFU of a 6B *S. pneumoniae* strain in 0.1mls saline are pipetted or sprayed into each nostril. Staff administering inoculum will wear protective gloves and aprons. A safe inoculation system is in place as the laboratory scientist checks the quantity of inoculum with the clinical team in the cubicle with the patient. To minimise the risk of spillage a nurse/doctor guards the outside of the bed space to prevent unplanned interruptions.

Clinical waste: The research clinic is a dedicated facility with sharps bins for pipette ends, aprons, gloves and access to handwashing plus sanitising gel for researchers and including participants.

Consumable items and the inoculum are handled and disposed of consistent with SOPs on clinical waste in line with NHS standards and overseen by LSTM Health and Safety Officer (appendix IV).

39. The quantity of organisms to be released.

In total a minimum of 108 participants who complete follow up are required and up to 150 participants may be inoculated including controls as shown in fig 1. Of these 70 will be administered the attenuated strains on two occasions 8×10^4 CFU with a fourteen day interval between the first and second inoculation (Fig 1). Based on previous EHPC data we anticipate that approximately 50% of those initially inoculated may continue to carry this bacteria post inoculation.

In most cases the participant's natural immunity will clear the bacteria prior to day 35 however all participants who have carried the study bacteria as shown on nasal wash will be advised to take a course of antibiotics provided. The sensitivity of these bacteria to the antibiotic will be confirmed prior to commencing the trial by Public Health England.

Participants will be challenged after a period of six months with the wild type 6B *S. pneumoniae.*

40. The disturbance of the site, including the type and method of cultivation, mining, irrigation, or other activities.
Not applicable
41. The worker protection measures taken during the release.
Clinical and laboratory researchers are provided with training in all clinical and laboratory procedures including inoculation, sampling and safety aspects and attend mandatory training. Prior to any research activity they are formally delegated by the Chief Investigator to assess their competence.
Laboratory disposal of the inoculum. After the completion of inoculation session, the remaining volume of inoculum is safely transferred to the laboratory facilities. A fraction is used on dilution plate to determine the dose received and the excess of inoculum is discarded in 1% aqueous solution of Virkon. Similarly, tips and dilution tubes are decontaminated in Virkon and all the solid waste is autoclaved as per LSTM clinical waste policy (SOP 004) (appendix IV).
Clinical Team: administration of the inoculum and follow up procedures (including nasal washes) are undertaken in a dedicated clinical area with facilities for hand washing, hand gel, aprons, gloves and masks (if required). Clinical waste bins and sharps bins will be available for safe disposal of pipette ends or consumable items. Researchers at increased risk of infection will be exempt from administration or follow up (including those who are pregnant).
During the inoculation of the participant's the laboratory staff member escorts the clinical team to check the correct dose of inoculum. A third person remains outside the cubicle to ensure there are no interruptions during the procedure to minimise the risk for example of spillage. A standard operating procedure consistent with spillage and clinical waste is in place (LSTM SOP 004 appendix IV).
Liverpool School of Tropical Medicine has a dedicated Biological Safety Manager.
42. The post-release treatment of the site.
Participant in the ARC Clinic: participants are assessed for carriage (nasal colonisation of the bacteria) based on results of nasal washes on days 0, 2, 7, 9,14,21, 28 and 36 then again following challenge on days 2, 7 and 14. Carriage is anticipated in approximately 50% of participants.

Accelerator Research Facility and Laboratory:
The inoculum and samples are transferred between the clinical area and the laboratory that are adjacent rooms and will be packaged consistent with Category B standards (HSE). Waste disposal and cleaning will be consistent with the site SOPs for biological and clinical waste (LSTM SOP 004).
43. The techniques foreseen for elimination or inactivation of the organisms at the end of the experiment or other purposes of the release.
Laboratory: After the completion of inoculation session, the remaining volume of inoculum is safely transferred to the laboratory facilities. A faction is used on dilution

plate to determine the dose received and the excess of inoculum is discarded in 1% aqueous solution of Virkon. Similarly, tips and dilution tubes are decontaminated in Virkon and all the solid waste is autoclaved as per LSTM clinical waste policy
44. Information on, and the results of, previous releases of the organisms and in particular, releases on a different scale or into different ecosystems.
This is the first use of *S. pneumoniae* GMO in human studies.
The EHPC model has completed eleven clinical trials (1000+ participants) with the parent strain *S. pneumoniae* by this team over the last nine years without any related adverse event reports. By attenuating this strain the aim is to make it less virulent than the wild type and is anticipated to have less carriage based on pre-clinical data.

The environment (both on the site and in the wider environment)

45. The geographical location and national grid reference of the site or sites onto which the release will be made, or the foreseen areas of use of the product.
Geographical Location:
The release will take place in a dedicated Accelerator Research Clinic, 3rd Floor Accelerator Building shared between Liverpool School of Tropical Medicine and the Royal Liverpool Hospital 1 Daulby Street Liverpool L7 8XZ
Grid Reference: SJ 35782 90814
Participants will be inoculated in the Accelerator Research Clinic a shared facility between Liverpool School of Tropical Medicine and the Royal Liverpool University Teaching Hospital then return after teach appointment to the wider community. The attenuated strain will not be released by other sites. In the period between inoculation follow up participants may travel anywhere they choose in the UK. Antibiotics are prescribed for carriage positive participants at the end of Stage I. Participants may then travel overseas until stage.
46. The physical or biological proximity of the site to humans and other significant biota.
The site is Liverpool city centre and urban area in the North West of England.
47. The proximity to significant biotopes, protected areas or drinking water supplies.
Not applicable
48. The climatic characteristics of the region or regions likely to be affected.
Not applicable
49. The geographical, geological and pedological characteristics.
Not applicable
50. The flora and fauna, including crops, livestock and migratory species.
Not applicable
51. The description of target and non-target ecosystems likely to be affected.
S. pneumoniae is only found as a human nasopharyngeal commensal; there is no known environmental reservoir and *S. pneumoniae* not a commensal of non-human mammalian species, with the exception of horses which are colonised by a subset of

S. pneumoniae strains that do not include the 6B BHN418 strain. Natural competitors including other human nasopharyngeal commensals.

52. The comparison of the natural habitat of the recipient organisms with the proposed site or sites of release.

The natural habitat of wild type *S. pneumoniae* is the human nasopharynx. Study participants who consent to the clinical trial will be the recipient of a GM attenuated strain or the wild type 6B strains.

53. Any known planned developments or changes in land use in the region which could influence the environmental impact of the release.

Not applicable

Part IV

Information relating to the interactions between the organisms and the environment

Characteristics affecting survival, multiplication and dissemination

54. The biological features which affect survival, multiplication and dispersal.
Survival: *S. pneumoniae* can only survive for prolonged periods as a commensal of the nasopharynx is its natural habitat. Although it spreads between humans by indirect and direct contact it is thought that it can only survive in the environment and on skin surfaces for hours, and this is supported by our unpublished data as discussed above (appendix II & III).
Multiplication:
S. pneumoniae replication will only occur during colonisation of the nasopharynx and not within the environment. Colonisation of the nasopharynx can be prolonged in infants lasting several months for some *S. pneumoniae* strains (including serotype 6B strains) but this period is shorter in adults, down to days or weeks. In the EHPC model we have found that approximately 20% of experimental carriers (corresponding to 10% of all research subjects) remained carriage positive on nasal wash cultures after 28 days [32]. For the GM attenuated strains carriage proportion, density and duration will either be reduced compared to subjects given wild type 6B strain or the same; it is very unlikely that the mutations will increase carriage, especially given the mouse colonisation data that shows markedly reduced colonisation for two of the four strains (see above / appendix I).
Dispersal: There are limited data on how long *S. pneumoniae* persists on inanimate surfaces [2]. One report found that dessicated bacteria can live for up to 28 days in the environment [35]. However, these data seem exceptional and our own work suggests *S. pneumoniae* is usually unable to survive in the environment for a prolonged period. In an EHPC pilot study healthy adult participant's hands were exposed to a well-characterised, fully sequenced penicillin-sensitive *S. pneumoniae* to asses whether this can lead to nasopharyngeal carriage. The results showed that after the bacteria were allowed dry on hands there was no further transmission despite direct contact with the nose (unpublished V Connor LSTM, Appendix II & E German III). This is consistent with a laboratory based study that found a one log drop in bacterial counts when *S. pneumoniae* were left to air dry compared to a wet swab (unpublished Esther Germain).

55. The known or predicted environmental conditions which may affect survival, multiplication and dissemination, including wind, water, soil, temperature and pH.
The incidence of *S. pneumoniae* has a pronounced peak during winter periods [36], probably related to co-existent viral upper respiratory tract infections. As discussed above *S. pneumoniae* survival in the environment is limited.

56. The sensitivity to specific agents.
The high sensitivity of the wild type strain to clinically relevant antibiotics has been confirmed; amoxicillin is currently recommended in the protocol. The attenuated GM strains will be tested for sensitivity to amoxicillin by the Public Health England.

Interactions with the environment

57. The predicted habitat of the organism.
The natural habitat of *S. pneumoniae* is the human nasopharynx. Healthy volunteers will be the recipients of the organism when inoculated nasally.
58. The studies of the behaviour and characteristics of the organisms and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms and greenhouses.
Not applicable
59. The capability of post-release transfer of genetic material:
a. from the genetically modified organisms into organisms in affected ecosystems
Transfer of genetic material could occur to other nasopharyngeal commensals but has to our knowledge never been described, and recent data suggest that genetic exchange from closely related streptococci such as *Streptococcus mitis* is unidirectional into *S. pneumoniae* but does not occur in the reverse direction (Kilian MBio. 2014 Jul 22;5(4):e01490-14. doi: 10.1128/mBio.01490-14). These data suggest there is a substantial barrier that inhibits the transfer of genetic material from *S. pneumoniae* to even closely related nasopharyngeal commensal species.
b. from indigenous organisms to the genetically modified organisms
Transfer of genetic material could occur from other nasopharyngeal commensals to the *S. pneumoniae* strains used for this study is theoretically possible, but seems to be a very rare event. For example *S. pneumoniae* penicillin resistance due to genetic transfer of penicillin binding proteins from other streptococci took several years to develop after penicillin was introduced and is only maintained when there is very high levels of penicillin use.
60. The likelihood of post-release selection leading to the expression of unexpected or undesirable traits in the genetically modified organisms.
Despite over 1000 subjects, there has been not one incident when *S. pneumoniae* recovered from a colonised subject has developed an unexpected phenotype or undesirable genetic trait. This would be even less likely for the GM strains as their duration and density of colonisation is expected to be reduced compared to the wild type 6B strain.
61. The measures employed to ensure and to verify genetic stability, the description of genetic traits which may prevent or minimise dispersal of genetic material and methods to verify genetic stability.
As described above mutant stability testing has been demonstrated using repeated culture without selection. The genetic identity of bacteria recovered from colonised individuals will be assessed using PCR on DNA extracted directly from nasal

washes; using primers that anneal to the antibiotic cassette genes or the deleted genes combined with upstream or downstream sequences will detect whether revertants may have occurred with a high degree of sensitivity. Positive PCRs using deleted gene primers will demonstrate the presence of this gene in the nasopharynx; whether this is due to a revertant or co-colonisation with another *S. pneumoniae* strain will require cross referencing with culture and serotyping results and capsular gene PCR.

62. The routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact and burrowing.

Transmission: *S. pneumoniae* is a frequent colonizer of the nasopharynx transmitted person to person via respiratory droplets. The strain administered in this trial will be genetically modified to attenuate virulence. An EHPC pilot study comparing hand to nose transmission found that there was no transmission once the bacteria have dried on the skin.

Surfaces: The data on survival on surfaces in ambient temperatures and humidity is limited [37]. Dessication tolerance has been shown to be essential for long-term survival on dry surfaces of up to 28 days [38]. Our pilot study exposed healthy adult participant's hands with well-characterised, fully sequenced penicillin-sensitive pneumococci and observe them for the development of *S. pneumoniae* carriage. They found that when the bacteria were dry on hands there was less transmission despite contact with the nose (see appendices unpublished V Connor). This is consistent with an EHPC laboratory based study that observed a one log drop in bacterial counts when the inoculum was left to air dry compared to a wet swab (unpublished Esther Germain).

63. The description of ecosystems to which the organisms could be disseminated.

Transmission is limited to humans.

64. The potential for excessive population increase of the organisms in the environment.

S. pneumoniae nasopharyngeal colonisation events are time limited, and in adults usually have a duration of less than a few weeks. Data with the 6B strain from the EHPC model confirms this, with only 10% of subjects having any evidence of colonisation 36 days after nasopharyngeal inoculation. Furthermore, subjects are given amoxicillin to clear any persisting colonising *S. pneumoniae*. Hence expansion of the population of administered *S. pneumoniae* does not occur, and this will also be true for the GM strains especially as they often have a reduced duration of colonisation in mice compared to wild-type bacteria [39-41]. Therefore, we do not anticipate an the genetically modified *S. pneumoniae* strains will establish themselves as independent populations in the environment or as a human commensal.

65. The competitive advantage of the organisms in relation to the unmodified recipient or parental organism or organisms.
The GM attenuated strain does not have a survival advantage over the wild type 6B strain, and is in fact at a strong competitive disadvantage.
66. The identification and description of the target organisms if applicable.
As discussed above, the GM organisms are identified by culture (double resistance to streptomycin and kanamycin) and by PCR using primers to the antibiotic resistance genes.
67. The anticipated mechanism and result of interaction between the released organisms and the target organisms if applicable.
There is no planned interaction between the GM strains and the wild type 6B strain; each strain will be inoculated into separate groups of subjects. Although all groups will be rechallenged after 6 months with the wild type 6B strain this is after all subjects have been treated with amoxicillin to clear colonising bacteria and is a considerable length of time longer than colonisation is likely to last. In addition, before the administration of the wild type 6B strains after 6 months all subjects will be checked for persisting colonisation with *S. pneumoniae*.
68. The identification and description of non-target organisms which may be adversely affected by the release of the genetically modified organisms, and the anticipated mechanisms of any identified adverse reaction.
PCR to amplify the resistance gene cassettes from nasal washes will be used as a sensitive screen for transfer of antibiotic resistance to other nasopharyngeal bacteria. There is no other adverse effect on nasopharyngeal commensal population that will be specific for the GM strains, and no data has shown that the wild type 6B strain causes adverse effects on nasopharygeal commensals.
69. The likelihood of post-release shifts in biological interactions or in the host range.
This is negligible; the likely interactions with nasopharygeal fauna is as described above and it is difficult to think of any major events that might alter these.
70. The known or predicted interactions with non-target organisms in the environment, including competitors, prey, hosts, symbionts, predators, parasites and pathogens.
Not applicable
71. The known or predicted involvement in biogeochemical processes.
Not applicable
72. Any other potentially significant interactions with the environment.
Not applicable

Part V

Information on monitoring, control, waste treatment and emergency response plans

Monitoring techniques

73. Methods for tracing the organisms and for monitoring their effects.
Location: The inoculum stock of the different strains (attenuated strains and wild type 6B) will be prepared under aseptic conditions in a dedicated laboratory room (Inoculum preparation room), located in the main laboratory area of the Accelerator Building (LSTM). The room contains a designated safety cabinet, incubator and set of pipettes for this procedure.
Preparation of Inoculum bacterial stock: The preparation and transfer of the inoculum is undertaken by the laboratory team who prepare inoculum as per https://www.jove.com/video/50115/experimental-human-S. pneumoniae -carriage (appendix V). Briefly, each of the bacterial strain (attenuated or wild type 6B) will grow in liquid culture up to mid-log phase, using growth media without the addition of animal products. Bacterial inoculum stock will be frozen at -80°C in aliquots of glycerol-enriched media. Post freezing bacterial stocks concentration will be measured by thawing several frozen aliquots and quantifying the bacterial numbers (colony forming units [CFU] per ml) by Miles and Misra (M&M). Stocks **purity** will be assessed in-house by culturing and results will be confirmed by DNA sequencing (Sanger Institute). Also, serotyping and penicillin sensitivity will firstly be carried out in our laboratory and then will be confirmed in a reference laboratory (Public Health England). Both DNA sequencing, serotyping and antibiotic profile are key elements of the *S. pneumoniae* strains **characterisation**.
Furthermore, bacterial stock **stability** in regards to concentration will be assessed at regular interval (bi-monthly) by M&Ms. On experimental days, aliquots of inoculum stock will be thawed, washed twice, and re-suspended in saline at an appropriate density for each inoculation dose.
Monitoring nasopharyngeal colonisation:
Nasal washes are included on the screening visit to assess presence of natural carriage and on each study visit (Fig 1). Nasal washes will be plated on to culture media containing Gentamycin or selective antibiotics (streptomycin/kanamycin) and incubated overnight at 37°C in 5% CO_2. Colonies will be confirmed as *S. pneumoniae* using classical techniques including (i) typical draughtsman-like colony morphology (ii) the presence of α-haemolysis (iii) optochin sensitivity and (iv) solubility in bile salts. Serotyping is completed by SSI agglutination anti-sera kit. Isolates will be frozen at -
80ºC for storage and reference laboratory confirmation. Results from the cultured nasal wash will also be confirmed using PCR based methods of bacterial detection.

Laboratory monitoring: the samples will be processed using our published protocol (Gritzfeld, Jove: https://www.jove.com/video/50115/experimental-human-S.

Quality Control Box 1	
Quality Measure	Methods
Characterisation and Purity	**Preparation** https://www.jove.com/video/50115/experimental-human-pneumococcal-carriage**):** **Inoculum bacterial stock preparation:** The inoculum stock of the different strains (attenuated strains and wild type 6B) will be prepared under aseptic conditions in a dedicated laboratory room (Inoculum preparation room with a designated safety cabinet, incubator and pipettes for this procedure.) Quality control DNA sequencing, serotyping and antibiotic profile are key elements of the *S. pneumoniae* strains characterisation: Bacterial stocks concentration will be measured by thawing several frozen aliquots and quantifying the bacterial numbers (colony forming units [CFU] per ml) by Miles and Misra (M&M). Bacterial stock **stability** in regards to concentration will be assessed at regular interval (bi-monthly) by M&Ms. P**urity** will be assessed in-house by culturing to confirm the serotype and penicillin sensitivity of inoculum. External Quality control: Results will be confirmed by DNA sequencing including the assessment of known viruses (Sanger Institute). Serotyping and penicillin sensitivity will firstly be confirmed in a reference laboratory (Public Health England). Administration of inoculum: At each inoculation session one *S. pneumoniae* strain will be prepared to minimise the risk of contamination.
Stability	**In vitro:** Post inoculation the purity is reconfirmed as the remaining inoculum serotype is retested following administration to each cohort. **In vivo:** Attenuated strains: revertants are minimised by attenuation of double mutants sequencing of bacteria from carriage positive participants administered the attenuated strain
Potency	The EHPC portfolio have inoculated over 1000 healthy volunteers. The established dose for the 6B wild type bacteria is 8 x 10^4 CFU based on a dose finding study [42].Culturing and molecular techniques will monitor reversion during in vivo colonisation. Carriage in approximately 80% of positive participants cleared by day 28 [32]. Antibiotics are provided for participants who have not cleared carriage at this stage.

pneumoniae -carriage) and will be plated on blood agar plate supplemented with gentamycin or selective antibiotic (spectinomycin/kanamycin). The latter will allow growth of the attenuated strain but not the wild type parent strain, as it is only included in the antibiotic resistance cassette used for gene replacement. Following overnight incubation at 37°C in 5% CO2, alpha-haemolytic, draughtsman shaped colonies will be sub-cultured for optochin sensitivity and the serotype will be tested using a latex agglutination kit (Staten Serum Institute). A multiplex PCR targeting on lytA and the antibiotic resistant gene (or knocked-out genes) will be used as a verification step of the detection of the mutant strain.

74. Specificity (to identify the organisms and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques.

The GMOs that are cultured from nasal wash samples from volunteers can be easily confirmed to be the test and control strains through standard diagnostic microbiology techniques. The combination of the microbiological and molecular methods described above enables the detection and identification of both parent and attenuated strain with 100% accuracy.

75. Techniques for detecting transfer of the donated genetic material to other organisms.

PCR to amplify the resistance gene cassettes from nasal washes will be used as a sensitive screen for transfer of antibiotic resistance to other nasopharyngeal bacteria.

76. Duration and frequency of the monitoring.

The study is planned to commence September 2018 pending approvals and complete within 12 months (Box 1 p31). Duration and frequency of monitoring of carriage includes 7 visits over 36 days post inoculation (Fig 1). Carriage status is reported weekly for review by both the clinical and laboratory team. Carriage will be monitored and compared to anticipated carriage rates found in previous EHPC studies of wild type *S. pneumoniae.* The Trial Management Group include the clinical and laboratory team who meet weekly to review the carriage and safety data and report to the Trial Steering Groups and Independent DSMB.

Control of the release

77. Methods and procedures to avoid and/or minimise the spread of the organisms beyond the site of release or the designated area for use.

Minimise Shedding in the community (Box 2 p33):

Adult to adult transmission is rare and in the last 1000 participants of EHPC model there have been partners and volunteers who house share yet the post inoculation nasal washes have not identified communicability. Participants are screened to exclude those or their close contacts who are at a higher risk of infection (Box 3 section 86). Participants are discharged on the day of inoculation to the wider

community and may still be colonised during the follow up period. An EHPC pilot study comparing hand to nose transmission found that there was no transmission once the bacteria have dried on the skin. There may be a higher risk of younger child to adult transmission as over 50% of children carry *S. pneumoniae [11],* but participants will not be in routine contact with children under 5 years of age. Safety monitoring during this period is described in the EHPC safety protocol (Box 4 p38). Shedding will be monitored by either cough sample or swabbing the hand for a sub-group on Day 6. The Trial Management Group will determine whether further samples are required at visits during Stage I. This may be informed for example by carriage data from the nasal wash.

Methods to Minimise Shedding Box 2

Minimising Shedding (see EHPC Safety Protocol Box 1) Box 2
Selection of Strains to minimise shedding of bacteria: Shedding seems to be related to density of colonisation. Attenuated strains are anticipated to be less virulent, colonize for a shorter period and with lower density. Therefore, they are less likely to be shed from the colonised subjects [43]. **Revert to wild type:** There is a risk that as *S. pneumoniae* are naturally transformable; therefore two or more virulence genes are mutated to minimise the chance of attenuated strains reverting to wild type. [10, 39, 40, 44]. **Transfer of novel genetic material** to other bacteria within the host or the host's contacts is minimised by using spectinomycin and kanamycin as the antibiotic markers – these antibiotics are very rarely used in clinical practice in the UK and only in readily identifiable patients that are not eligible for inclusion in this trial. It is unlikely that even if spectinomycin or kanamycin resistance was transferred to other bacteria that they will be maintained in the absence of antibiotic selective pressure. **Inoculation:** the inoculation schedule will start slowly to allow evaluation of both safety data and carriage rates from the first 10 participants administered each attenuated strain for a period of 7 days will be completed prior to further inoculation of other participants with that attenuated strain. **Evaluating shedding:** transmission from the nose will be monitored to inform future studies. This will include minimally invasive methods for example swabbing the hand or coughing on a culture plate to be determined during the study. **Laboratory Preparation:** The laboratory is adjacent to the Accelerator Research Clinic reducing the risk of transporting inoculum. The preparation and transfer of the inoculum is undertaken by the laboratory team who prepare inoculum as per https://www.jove.com/video/50115/experimental-human-S. pneumoniae -carriage **Discarding inoculum:** excess of inoculum will be autoclaved as per LSTM clinical waste policy (SOP 004), then incinerated. Standard EHPC Inoculation Procedures: **Research team:** wear protective aprons, gloves and are excluded from if at higher risk of infection. **Clinical waste:** The research clinic is a dedicated facility with sharps bins for pipette ends, aprons, gloves and access to handwashing plus sanitising gel for researchers and including participants. Consumable items and the inoculum are handled and disposed of consistent with SOPs on clinical waste in line with NHS standards and overseen by LSTM Health and Safety Officer.

78. Methods and procedures to protect the site from intrusion by unauthorised individuals.
The entrance to the Accelerator Building is staffed by a reception and security team during office hours and in the evening. Swipe access is required for staff to the separate floors. Participants will escorted to the Accelerator Research Clinic.
79. Methods and procedures to prevent other organisms from entering the site.
The inoculation of participants will be planned in groups of up to 5 participants at a time to be administered the same inoculum. This will reduce the risk of error associated with the preparation and administration and avoid contamination.

Laboratory Preparation: The laboratory is adjacent to the Accelerator Research Clinic reducing the risk of transporting inoculum. The preparation and transfer of the inoculum is undertaken by the laboratory team who prepare inoculum as per https://www.jove.com/video/50115/experimental-human-S. pneumoniae -carriage

Discarding inoculum: excess of inoculum will be autoclaved as per LSTM clinical waste policy (SOP 004), then incinerated.

Waste treatment

80. Type of waste generated.
Clinical and laboratory waste may include blood/ nasal wash samples, sharps, syringes, pipette ends, disposable aprons, gloves, microbiological waste (agar plates etc) and masks. Clinical waste bags and bins are used for any consumable items associated with sampling and inoculation. This will be consistent with the LSTM Standard Operating Procedures for clinical waste disposal and handling.

81. Expected amount of waste.
The amount of waste will depend on the stage of the trial. We plan to have 70 participants inoculated with attenuated strains. Each participant has approximately 35 samples during the follow up period. We may require 2 - 3 clinical waste bags and or sharps bins per day during the follow up visits managed by standard operating procedures currently in place at the LSTM (see question 82).
82. Description of treatment envisaged.
Any waste generated in the laboratories that is classified as clinical waste is placed within specifically labelled yellow clinical waste sacks which are housed in yellow bins clearly marked “clinical waste”. The yellow sacks are then taken to the clinical waste skips. The Laboratory Manager or deputy fills in the required paperwork and affixes the correct waste type label to the skip. This procedure involves placing the yellow sack/s into a wheelie bin which is then used to transport the sacks to the clinical waste skips. The service lift should be used when transporting waste between floors. The wheelie bin is required to be cleaned thoroughly with disinfectant at least once a month, and after any spillage within. Once a week a

commercial waste disposal company comes on site to collect the waste and take it for incineration.
After the completion of inoculation session, the remaining volume of inoculum is safely transferred to the laboratory facilities. A faction is used on dilution plate to determine the dose received and the excess of inoculum is discarded in 1% aqueous solution of Virkon. Similarly, tips and dilution tubes are decontaminated in Virkon and all the solid waste is autoclaved as per LSTM clinical waste policy (SOP 004).

Emergency response plans

83. Methods and procedures for controlling the organisms in case of unexpected spread.
Unexpected spread during the procedure.
This risk of spillage is minimised during transit and the inoculation procedure.

- the inoculum is prepared in the dedicated research laboratory adjacent to the Accelerator Research Clinic
- a buddy system the laboratory scientist and the clinician check the inoculum and administer at the patient's side
- A safety person is allocated to ensure there are no interruptions during the procedure and will wear a red apron in the area of inoculation to alert others.
- In the event of spillage the area will be cleaned using 70% ethanol. The staff will wear protective gloves and aprons then dispose of the waste in a clinical waste bin.

Unexpected Spread in the Community:
S. pneumoniae is commonly identified in healthy volunteers estimated as 10% based on healthy volunteers screened for EHPC clinical trials. This attenuated strain is anticipated to have lower carriage rates (see question 54), therefore, the risk of unexpected spread is anticipated to be lower than the parent organism that commonly colonises health adults.
In the event that a participant is unwell or has symptoms they are advised to notify the study team. A research doctor is on call 24 hour 7 days for advice. Participants may be advised to attend a triggered clinician referral to assess them. If required further cultures will be available via the adjacent NHS Hospital Microbiology department. The Chief Investigator/Sponsor will determine whether a serious adverse event possibly related to the intervention related adverse event then inform the Independent Data Safety Monitoring Committee and National Research Ethics Service.
84. Methods, such as eradication of the organisms, for decontamination of the areas affected.
Virkon may be used for decontamination.
85. Methods for disposal or sanitation of plants, animals, soils and any other thing exposed during or after the spread.

Any waste generated in the laboratories that is classified as clinical waste is placed within specifically labelled yellow clinical waste sacks which are housed in yellow bins clearly marked “clinical waste” as per LSTM SOP 004.

86. Methods for the isolation of the areas affected by the spread.

Clinical Area: Community: The wild type bacteria are commonly found in healthy adults and more common in young children and we anticipate the attenuates strain will be less virulent. Participants may travel in the wider community following visits. In the event of any symptoms they are advised to contact the clinical team or if concerned for their health to access the usual route of health care. However, the participants are provided with a course of antibiotics and if indicated they may commence the course immediately without delay. At the end of the follow up period all participants will take antibiotics to reduce or clear carriage.

All participants are required to provide contact details including a close friend or family member during the study and will be contactable if for any reason we require them to either take antibiotics earlier or attend further assessments.

87. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect.

The EHPC Model with the wild type bacteria is well established and will be implemented in this clinical trial including screening, exclusion criteria and implementation of an established safety protocol. Additional measures for this study include evaluating safety after the first 5 - 10 participants. Shedding will be monitored by collecting samples from a sub-group of participants (for example this may include swabbing their hands or coughing on a culture plate). The result will be used to inform future studies. **Screening:** to minimise risk to participants who consent they will initially have a health screen including medical history, full blood count, vital signs, pregnancy test (females), cardiac and respiratory assessment. If their circumstances change for example if a family member if unwell they may withdrawn from the study and take the antibiotics earlier to reduce or clear carriage. The aim is to exclude participants or their close contacts who are at a higher risk of infection with established inclusion and exclusion (Box 3 p37 p). vulnerable to infection:

Participant Inclusion and Exclusion: Box 3
Inclusion * • Healthy volunteers • Age 18 – 50 years • Capacity to give informed consent • Ability to speak fluent English Exclusion: • Research participant: o currently involved in another study unless observational or non- interventional o participant in a previous EHPC trial at any time • **Vaccination:** *S. pneumoniae* vaccination (routine in UK babies born since 2005 or US 2001) • **Allergic:** known allergy to penicillin or amoxicillin or gentamicin • **Health history:** o Chronic ill health including, immunosuppressive history, diabetes, asthma (on regular medication), frequent otitis media or other respiratory disease o Medication that may affect the immune system or clotting e.g. steroids, inflammation altering (eg nasal steroids, roacutane or aspirin) o Long term antibiotics (eg. known active chronic infection) o Splenectomy o Current acute severe febrile illness o Major *S. pneumoniae* illness requiring hospitalization • **Direct caring role or close contact** with individuals at higher risk of infection o Children under 5 years o Chronic ill health or immunosuppressed adults o Adults over the age of 75 years • Smoker: o Current or ex-smoker in the last 6 months o Significant smoking history - more than 20 cigarettes per day for 10 years or the equivalent (>10 pack years) • Women of child-bearing potential (WOCBP) who are: o not deemed to have sufficient /effective birth control/ confirmed abstinence o pregnant • **History of drug or alcohol abuse** * **Natural carriers** are included following screening prior to inoculation based on nasal wash results. It is anticipated that 10-15% participants of screened participants will have natural *S. pneumoniae* colonisation at the time of recruitment as demonstrated by the initial nasal wash. If a participant is a natural carrier after the initial inoculation they may continue in the study even if this occurs prior to the booster inoculation. Experimental carriers at the time of re-screening will continue as planned.

EHPC Safety Protocol	
Screening	Exclude adults or their close contacts with potential risk factors for invasive infection based on history, vital signs, cardiac/respiratory assessment and full blood count.
Participant: Safety Information Leaflet	A safety information leaflet includes: • contact details for the research team available 24/7 • advised to report early signs of infection or to seek urgent health care if concerned • if/when to take the antibiotics provided • to report adverse events including unrelated hospital admissions for the duration the follow up period until their last visit. • Maintain regular hand washing. Safety guidance is presented during consent then verbally on the day of inoculation. **Close friend or family:** participants are encouraged to inform a close contact that they are taking part in a trial and a copy of the safety leaflet is provided for them including contact details for the research team. We advise participants that if they are unwell to contact their family or friend so they are not alone and to inform the clinical research team.
Symptoms and Access to Health care	**Urgent Care:** to avoid any delay in diagnosis, participants are advised to attend their usual health care facility if concerned about their health, as their condition may not necessarily be related to the inoculation. Also, to inform the research team. **Daily checks:** following each inoculation participants text the clinical team to confirm they are well and have no symptoms for 3 days. Thermometers are provided. **Symptoms** will be monitored and recorded systematically at each visit by the clinical research team. **Triggered clinician assessment:** Participants with minor respiratory /ear symptoms may attend the clinic for a triggered assessment by the research nurse/doctor available weekdays 0900 to 1700 and may advise them to seek their usual route of health care. A research doctor is available for advice 24/7 for participants. **General Practitioner:** will be routinely notified of participants participation in the trial.
Antibiotics	**Sensitivity:** the inoculum will be tested to confirm sensitivity to the protocol antibiotics. **A supply of antibiotics (**Amoxicillin 500mg TDS for 3 days) is provided to each participant. To avoid delay in treatment if the participant has symptoms preferably, after discussion with the clinical team, or if the

	participant is unable to contact the research team. **Termination of colonisation:** participants will be advised to take antibiotics in an attempt to clear or reduce carriage post inoculation following **Stage I** all participants at the end of initial follow up on day 36. **Stage II** only those with at least one positive nasal wash on day 14
Monitoring Carriage	**Nasal Wash** results report carriage at each follow up visit. Carriage and safety data are communicated weekly for discussion by the TMG.
Withdrawn	If a participant or their close contacts develop potential risk factors for invasive infection then they may be withdrawn from the study at any time and commence antibiotics to clear/reduce carriage when required.
Monitoring safety	**Trial Oversight:** An established IDSMC and TSC review carriage rates and adverse events. They will be contacted by the CI in the event of any change to anticipated carriage rates or serious adverse events. SAE are reported to the Sponsor and NRES. **TMG:** triggered clinician referrals and non-serious adverse events of interest to the research are recorded on the safety reports and discussed by the clinical and laboratory team weekly.

The EHPC Programme has an independent Data Safety Monitoring Board (DSMB) who provide trial oversight and will make recommendations to the study investigators relating to any ethical or safety reasons. The Chief Investigator may contact the DSMB in any situation where independent advice or review is required. The DSMB will advise the trial team in the event that the trial protocol should be amended or the trial should be stopped. In a serious adverse events occurs that the Chief Investigator classes as possibly related as defined by National Research Ethics Service http://www.hra.nhs.uk/documents/2015/06/safety-progress-reports-procedural-table-non-ctimps.pdf this will be reported to the Sponsor and the Data Safety Monitoring Committee.

Part VI

A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this schedule, and the name of the body or bodies responsible for carrying out the studies.

The Clinical Trial and laboratory will be conducted within Accelerator Research Clinic and Laboratory within the Accelerator Building, **Liverpool School of Tropical Medicine (LSTM).** This is a university higher education institute with standard operating procedures for clinical and laboratory work. The LSTM Health and Safety Officer has provided guidance with this protocol.

The **Experimental Human Pneumococcal Carriage Model** is established over nine years as a method for studying colonisation of *S. pneumoniae* in adults. [10, 32, 45]

The research team are all trained in **Good Clinical Practice** (GCP). Health professionals include registered NHS doctors and nurses. The laboratory team are trained in laboratory safety.

Pre-clinical preparation of the attenuated strain has been undertaken at **University College London**.

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Dated: June 2018

Appendix I

As this section contains highly confidential information it is provided as separate confidential document.

Appendix II

Hands shown to be vehicles for transmission of *Streptococcus pneumoniae* in novel controlled human infection study

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Abstract: (100 words max)

Streptococcus pneumoniae (pneumococcus) is a leading cause of morbidity and mortality worldwide. Colonisation of the nasopharynx by this pathogen is important as a pre-requisite for infection and primary reservoir for transmission. The importance of non-aerosolised modes of spread is unknown, although the bacteria can persist on hands for short periods.
Using a novel controlled human infection model, we have demonstrated the viability of transmission of pneumococcus from hand to nasopharynx, especially in suspension. Good hand hygiene practices, already known to reduce enteric disease, may also prevent the spread of bacteria thought to be primarily spread through aerosolisation.

Background:

Streptococcus pneumoniae (pneumococcus) is the most common cause of acute otitis media, sinusitis and pneumonia worldwide and significantly contributes to meningitis burden [46]. 1.6 million deaths are caused by annually, mostly due to pneumonia in children under the age of 5 in developing countries [46, 47]. Colonisation of the human nasopharynx with pneumococcus is common, with prevalence in cross-sectional surveys of 40-95% in infants and 10-25% in adults [48]. Colonisation is important as the pre-requisite of infection, the primary reservoir for transmission, and immunological boosting against pneumococcal infection in both children and adults[21, 22].
Transmission of *S. pneumoniae* has been postulated by aerosol, droplet, or indirect contact, and outbreaks are well documented in day care centres, prisons and nursing homes [33]. Primary spread through aerosol is suggested by the association

with overcrowding and concurrent viral respiratory tract infections [33, 34], but the relative contribution of transmission modes to colonisation and disease are unknown. We investigated potential for manual transmission of pneumococcus to induce nasopharyngeal colonisation.

Methods:

We enrolled 63 healthy adult volunteers, between April-May 2017, to our well-characterised controlled human infection model (EHPC), modified slightly from previous published protocols [49] to assess "hand-to-nose" transmission.
Briefly, volunteers were administered pneumococcus (mid-log phase dose of $3.2x10^6$colony-forming units (CFU) of *S. pneumoniae* serotype 6B prepared as previously described) onto their fingertip or dorsum of their hand. Bacterial density was confirmed by serial dilutions of the inoculum on blood agar (Oxoid). Volunteers were directed to either sniff the bacterial residue or to make direct contact with the nasal mucosal surface (pick/poke their nose), and to do so either while the bacteria were wet or shortly after drying. Pre-inoculation throat swabs were assayed for respiratory viruses (adenovirus, influenza A and B, coronavirus, respiratory syncytial virus/human metapneumovirus, rhinovirus and parainfluenza 1-4), using multiplex Polymerase Chain Reaction (PCR) as previously published [50].
Nasopharyngeal colonisation was assessed in nasal washes collected at 2, 6 and 9 days post inoculation. Pneumococcal density in nasal washes was determined by culture and confirmed using quantitative PCR with primers for lytA, and for *S. pneumoniae* serotype 6A/B.
All participants gave written, informed consent. Ethical permission was sought and obtained from the Liverpool East NHS Research Ethics Committee (17/NW/0054).

Results

Forty volunteers were randomly allocated to the four different transmission group; 1) sniffing wet bacterial residue ['wet sniff'] 2) sniffing bacterial residue after air-drying of the hands ['dry sniff'] 3) pick/poke nose with finger exposed to wet bacteria residue ['wet poke'] 4) pick/poke nose with finger exposed to dried bacteia residue [dry poke].
Eight individuals (20%) were found to be colonised with 6B serotype pneumococcus at follow up visits by culture, with highest rates in the 'wet poke' (4/10, 40%), and 'wet sniff' (3/10, 30%) groups. Drying of the bacteria on the skin before "sniff" or "poke" led to 1/10 and 0/10 participants becoming colonised respectively (See Figure 1). Groups were too small to compare however, acquisition following transmission with wet bacteria was significantly higher than dry (P 0.04).
Median post-exposure colonisation densities (CFU/ml of nasal wash) of the 'wet sniff', 'wet poke' and 'dry sniff' groups were 5.6E+01 (range 4.33E-01-3.71E+06), 4.72E+00 (range 4.51E-01-1.25E+02) and 2.42E+00 (range 1.16E+00-9.55E+00)

respectively. The ‘wet sniff’ group was expanded to improve precision-estimates of rates, resulting in total of 6/33 participants becoming colonised (18%). No viruses were detected in any throat swabs taken before pneumococcal inoculation.

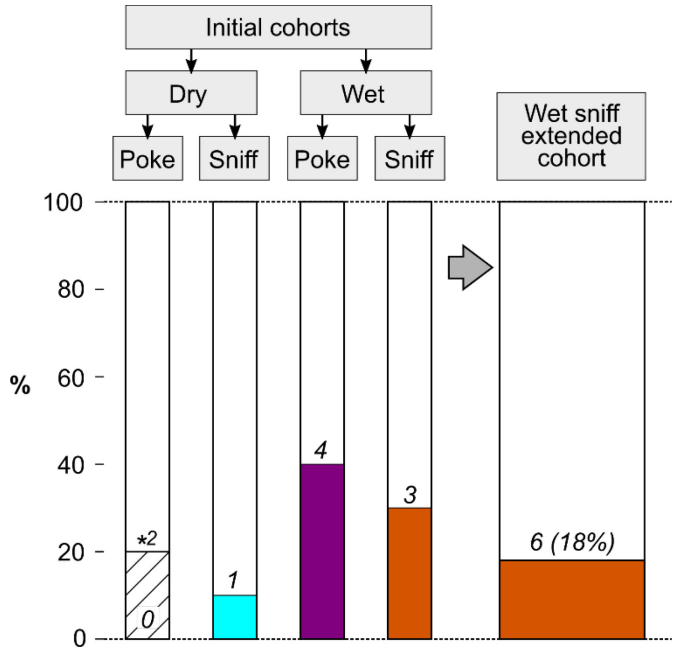


Figure 1: Colonisation rates following classical culture of nasal wash samples at any time point after exposure in each strain 6b transmission group. *No but 2 experimental carriers of natural found in dry poke group (serotype participants became type group D) carriers of pneumococci after exposure to 6B. 19 and non-vaccine

Overall, 252 nasal wash samples were available for analysis. qPCR detection (lytA) resulted in higher colonisation detection rates compared with culture (35/63 [56%] vs 13/63 [23%] respectively, p=0.0001). This was most apparent in ‘dry poke’ group, in which *S. pneumoniae* 6B was detected by PCR (lytA qPCR confirmed with 6a/b specific qPCR) in 7/10 (70%) at any time point, with detectable pneumococcal DNA in nasal wash at 9 days after inoculation. See Figure 2 for full results.

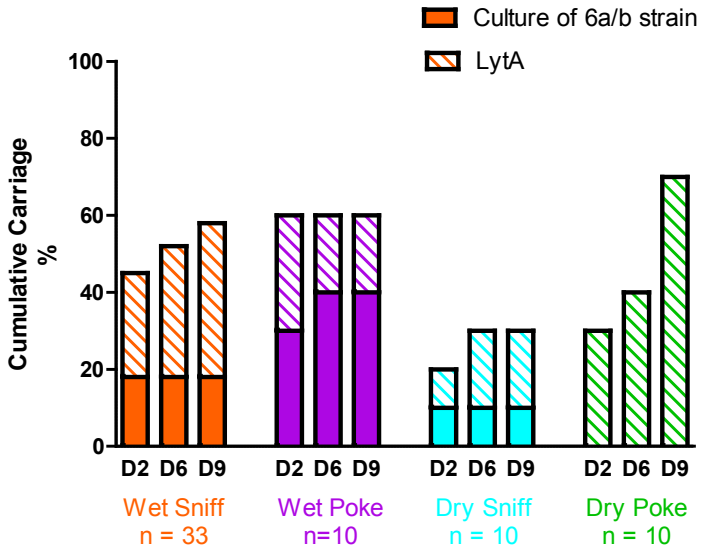


Figure 2: Comparison of culture (6a/b strain only) and lytA qPCR results for different transmission methods

Discussion 364 words

In this first study to examine transmission of pneumococcus using a controlled human infection model, we show that hands can be vehicles for transmission of pneumococcus into the nose and that this transmission can lead to nasopharyngeal colonisation. Wet particles increase transmission and sniffing transmission method led to increased density of colonisation.

We were unable to investigate the relationship between colonisation acquisition and concurrent viral infection due to the absence of viral detection in our participants. In a previous study asymptomatic co-infection increased odds of pneumococcal colonisation (75% virus positive became colonised vs. 46% virus-negative participants, p=0.02),[50] and other experiments have shown that viruses (for example rhinovirus) facilitate acquisition and transmission between individuals [51].

This novel use of a human challenge model allowed for the study of pneumococcal colonisation in a controlled environment following transmission of the pathogen from hands into the nose. Children are the primary reservoirs for community pneumococcal transmission and although an adult population was used, it probably represents potential for hand-nose transmission in paediatric populations.

We determined pneumococcal colonisation and density by both culture and qPCR, results suggest these two methods complement each other and reduces that chance of missing pneumococcal carriage. Our data supports previous studies suggesting that qPCR may be superior at detecting subclinical or low-density colonisation. However, qPCR cannot inform on the viability of pneumococci in the nasopharynx, qPCR detection when culture results are negative could represent live bacteria causing low-density colonisation or dead pneumococcal debris.

This study reinforces the imperative for good hand hygiene practices. The hands have been implicated as vehicles for transmission of many pathogens and viruses, and this study suggests that pneumococci can be transmitted by a similar process. Better understanding the duration of survival of pneumococci on the hands in nasal secretions and the frequency of hand contamination would support hand hygiene advice. Improving knowledge about the process of pneumococcal shedding from the nose in humans during colonisation and factors which promote shedding are also important areas for further work. This modification of the experimental human pneumococcal challenge model has several potential uses including testing of current or new hand cleaning interventions to ensure that it reduces transmission of this important bacterial pathogen.

Acknowledgements:

This work received financial support from Unilever and The Medical Research Council (MRC). Funders did not have any input into the study design. The authors thank the respiratory research team; Trial Steering Committee members: Professor Stephen Gordon, Malawi–Liverpool–Wellcome Trust Clinical Research Programme and Professor Neil French and the Liverpool School of Tropical Medicine (LSTM) Respiratory group.

Appendix IV

STANDARD OPERATING PROCEDURE:

CLINICAL WASTE DISPOSAL

SOP Number	SOP-LSTM-004	Effective Date:	22.03.10
Version number:	1.2	Approved by:	LUG
Superseded Version Number:	1.1	Date:	
Originator:	Tadge Szestak	Next Review Date:	29.03.14
Reviewer:	David Simpkin	Review Date:	07.09.12

1. PURPOSE

1.1 This document outlines the procedures involved in the preparation of clinical waste generated within the LSTM in readiness for its disposal off site.
1.2 It is the responsibility of the research staff employing this procedure to be sufficiently familiar with this SOP prior to carrying out any disposal of clinical waste.

2. RESPONSIBLE PERSONNEL

2.1 Any member of laboratory staff and research students who are working with material, which when being disposed of is classified as clinical waste.

3. PROCEDURE

3.1 Clinical waste is defined as any waste which consists wholly or partly of:

- Human or animal tissue
- Blood or other body fluids
- Excretions
- Drugs or other pharmaceutical products other than controlled or cytotoxic drugs
- Swabs or dressings
- Syringes, needles or other sharp instruments which, unless rendered safe, may prove hazardous to any person coming into contact with it

- Any other waste arising from the research & diagnostic laboratories, which may cause infection to any person coming into contact with it

3.2 Any waste generated in the laboratories that is classified as clinical waste is placed within specifically labelled yellow clinical waste sacks which are housed in yellow bins clearly marked “clinical waste”.
3.3 The sacks must have LSTM and research group details written on them in indelible ink.
3.4 Any waste that has been autoclaved is treated as clinical waste.
3.5 The bags must not be allowed to get more than ¾ full.
3.6 Once this level is reached the neck must be secured using a tag. Each tag is uniquely numbered and a set is assigned to each research group. In this way waste streams can be tracked if need be.
3.7 Following the correct procedure, the yellow sacks are then taken to the clinical waste skips. This procedure involves placing the yellow sack/s into a wheelie bin which is then used to transport the sacks to the clinical waste skips. The service lift should be used when transporting waste between floors.
3.8 The wheelie bin is required to be cleaned thoroughly with disinfectant at least once a month, and after any spillage within.
3.9 The clinical waste skips are in two locations currently in the School.

- One is in the clinical waste & recycling room on the ground floor of the CTID building (CT031) and
- the second is outside the back of the 66-wing in a padlocked stockade. The key for this stockade is held with the Snake Venom group on the top floor of the Old School.

3.10 Once the bags are deposited, the paperwork requires to be filled in stating the number of clinical waste bags deposited from each particular research group.
3.11 Once a week a commercial waste disposal company comes on site to collect the waste and take it for incineration.
3.12 In readiness for this, the Laboratory Manager or deputy fills in the required paperwork and affixes the correct waste type label to the skip.

Appendix V

Appendix A - Inoculum Stock preparation protocol

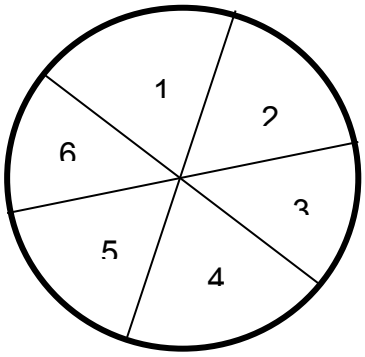
Objective
The purpose of this protocol is to provide guidance to the investigational site involved in EHPC clinical trials in the preparation of a bacterial inoculum stock containing S. pneumoniae. The required precautions and procedures outlines in this document should be followed than handling S. pneumoniae.

Reagents and Materials Required
Blood plates – Oxoid PB0122A
Vegitone broth - Add 9.25g of powder to 250ml distilled H_2O. Autoclave, leave to cool before use.
Sterile glycerol – VWR 1.04093.1000
Sterile loops
Sterile PBS
Sterile Pipettes
Eppendorf tubes
96 well plate (for dilutions) (Corning CLS3367 – Sigma)
Spectrophotometer: FLUOstar Omega plate reader
Incubator dedicated to inoculum strains of S.pneumoniae
Heraeus Megafuge 1.0

Protocol
Day 1
Using bead stock from -80°C freezer, plate on blood agar.
Incubate for 9 to 16 hours (overnight) at 37°C, 5% CO_2.
Prepare Vegitone broth. Leave some in an incubator overnight to ensure sterility

Day 2
Add bacterial colonies to Vegitone, mix thoroughly and aim for 0.15 OD at 620nm. Prepare at least 4 tubes like this
Incubate all tubes at 37°C, 5% CO_2.
Monitor the samples every hour (including at time = 0). Measure the OD value at 620nm.
While waiting for the bacteria to grow, prepare labels in ProCuro that include the serotype and date prepared
When OD reaches 0.30-0.35, top up with Vegitone, bringing the OD back down to 0.15-0.2.
Repeat the above process until you have at least 100 mL TOTAL of liquid bacterial stock at 0.3-0.35
Mix all tubes in a bacterial flask (conical) and check OD again
Pour equal amounts back into falcon tubes
Centrifuge culture at 3345 g or 4000 rpm for 15mins

Pour off the supernatant and resuspend the pellet in the same volume of Vegitone broth that was removed
Transfer to a conical bacterial flask and add 10% glycerol (ensure you use the glycerol for stock prep)
Aliquot 1ml into Eppendorf tubes and store at -80°C. Label tubes
Determination of CFU/ml in the final aliquots (M&M plates)
Divide an agar plate into six sections and label them 1 to 6 as below



Add 180ul of sterile saline to 6 wells (A to F) on a 96 well non-treated plate.
Add 20ul of inoculum into well A and mix by pipetting up and down.
Dilute 1:10 from A to F by mixing contents of well A 3-5x and transferring 20ul to well B.
Change the tip and repeat to reach well F. Discard 20ul from well F.
Place a 10ul drop from well A into the corresponding section on the plate: Well A = section 1, Well B = section 2, well C = section 3, well D = section 4, well E = section 5, well F = section6.
*Repeat this twice more for a **TOTAL of 3 DROPS PER SECTION**.*
Leave the sample to dry on the plate.
Incubate for 9 to 16 hours at 37°C, 5% CO_2
Day 3
Read M&M plates to determine CFU/ml at each time point.
Count the visible colonies in each section and record. If you cannot count them, record as TMTC (too many to count)

Section	*Dilution Factor*	*# of visible colonies*	*CFU/ml*
1	*10*		
2	*100*		
3	*1000*		
4	*10000*		
5	*100000*		
6	*1000000*		

Divide the number of visible colonies by three
Take the average number of colonies in a droplet, multiply by the dilution factor and then divide by the amount plated (10ul) = CFU/ul
Multiply this number by 1000 to get CFU/ml

Risks/Dangers

S. pneumoniae is an opportunistic pathogen, care should be taken to prevent aerosols and subsequent inhalation of bacteria.