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Pneumococcal Colonisation is an Asymptomatic Event in Healthy Adults using an Experimental Human Colonisation Model.

--Manuscript Draft--

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Full Title:	Pneumococcal Colonisation is an Asymptomatic Event in Healthy Adults using an Experimental Human Colonisation Model.
Short Title:	
Corresponding Author:	Ryan Edwin Robinson, MBChB Liverpool School of Tropical Medicine Liverpool, Liverpool UNITED KINGDOM
Keywords:	pneumococcal pneumonia; asymptomatic colonisation
Abstract:	Introduction Pneumococcal colonisation is regarded as a pre-requisite for developing pneumococcal disease. In children previous studies have reported pneumococcal colonisation to be a symptomatic event and described a relationship between symptom severity/frequency and colonisation density. The evidence for this in adults is lacking in the literature. This study uses the experimental human pneumococcal challenge (EHPC) model to explore whether pneumococcal colonisation is a symptomatic event in healthy adults. Methods Healthy participants aged 18-50 were recruited and inoculated intra-nasally with either Streptococcus pneumoniae (serotypes 6B, 23F) or saline as a control. Respiratory viral swabs were obtained prior to inoculation. Nasal and non-nasal symptoms were then assessed using a modified Likert score between 1 (no symptoms) to 7 (cannot function). The rate of symptoms reported between the two groups was compared and a correlation analysis performed. Results Data from 54 participants were analysed. 46 were inoculated with S. pneumoniae (29 with serotype 6B, 17 with serotype 23F) and 8 received saline (control). In total, 14 became experimentally colonised (30.4%), all of which were inoculated with serotype 6B. There was no statistically significant difference in nasal (p= 0.45) or non-nasal symptoms (p=0.28) between the inoculation group and the control group. In those who were colonised there was no direct correlation between colonisation density and symptom severity. In the 22% (12/52) who were co-colonised, with pneumococcus and respiratory viruses, there was no statistical difference in either nasal or non-nasal symptoms (virus positive p=0.74 and virus negative p=1.0). Conclusion Pneumococcal colonisation using the EHPC model is asymptomatic in healthy adults, regardless of pneumococcal density or viral co-colonisation.
Order of Authors:	Ashleigh Trimble
	Victoria Connor
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	Duolao Wang
	Stephen Gordon
	Daniela Ferreira
	Angela Hyder-Wright
	Andrea Collins

Opposed Reviewers:	
Response to Reviewers:	Review Comments to the Author Reviewer #1: The manuscript by Trimble and colleagues present additional results from an experimental human challenge model, describing whether experimental pneumococcal colonisation in healthy adults is a symptomatic event. This manuscript is a resubmission from 2016 (but due to time limits is now a new submission) which has addressed some of the queries from the initial review. This study reports some interesting data but is nevertheless limited mainly due to the small sample size, especially for the secondary analyses. Some points for consideration: 1. The pneumococcal colonisation rate was 30.4% (14/46), which was solely due to serotype 6B, and actually 48% if only those who were inoculated with 6B are included. This seems quite high for an otherwise healthy adult population. The authors state that this model mimics natural pneumococcal exposure but the high carriage rate may suggest otherwise. Can the authors provide some comment on the colonisation rate they observed with respect to what has been reported among UK healthy adults or more generally? Some explanation of this would greatly benefit the manuscript. The authors also argued against providing some comment on the lack of 23F colonisation but I tend to think that this would also be worth noting in the discussion, given that both these are commonly carried serotypes. Thank you for your feedback, the EHPC model is able to artificially induce the otherwise naturally occurring phenomenon of pneumococcal carriage by pipetting the live bacteria directly into the participant's nasopharynx. As the EHPC inoculation is more efficient than the 'natural process' the rate of colonisation is higher. This has now been added to the discussion. We have added an explanation for the difference in colonisation rate between serotypes as requested. 2. Figures 2 and 3 could be combined since they are both essentially reporting the same data but with a different comparison. Also, it should be Fisher's exact test, not Fischer's exact test. Thank you, we have now corrected the spelling of Fisher's exact test. We feel that combining the two figures together may affect the clarity of the underlying message, and therefore have respectfully chosen to keep them separate. We have however added a legend to all figures to make them easier to interpret. 3. Can the authors comment on the apparent high rate of nasal and non-nasal symptoms in the control group and whether they believe this could have contributed to the lack of any clinical symptoms being demonstrated for the other groups? An independent assessor may have been useful here. We recognise that there is a high number of symptoms in the control group. We believe this may be due in part to the small study sample size but also reflects the high sensitivity of the Likert scale used to record any symptoms. Since the symptoms were self-reported by participants and they were blinded to their intervention group, we feel that an independent assessor would have been unlikely to improve this. 4. There are a couple of instances where what is reported in the results is inconsistent with the data or discussion. For example, on lines 192-193 it states that "This study does not however report the effect of co-colonisation (SPN and virus) on symptoms" when this is clearly shown in Figure 5. Also on lines 207-208, it states that "…experimental SPN colonisation does not increase nasal or non-nasal symptoms" when in the results (lines 156-157) it states that "Experimental SPN colonisation rates were higher in the presence of virus….($p<0.05$)". Please correct. The p-value, if indeed significant, should be included in the Figure. Thank you, the comment regarding symptoms from co-colonisation refers to the Rodrigues at al study. This has been made clearer in the text to avoid confusion.

	In the discussion we note that experimental SPN colonisation does not increase nasal or non-nasal symptoms. In the results section we note that experimental SPN colonisation was higher in the presence of virus. This is referring to two separate points. Participants who are positive for the presence of a virus and SPN ('co-colonised') did not appear to have greater rates of symptoms. The figure relating to this data (Figure 5) has been edited to include the relevant p- values and a legend has been added for clarity. 5. What were the viruses that were detected, and were there any associations between specific viruses? A variety of viruses were detected, however since these were only present in very small numbers, we were unable to ascertain any relationship between symptoms and these specific viruses. In future studies we aim to further investigate this relationship in more detail. For most of the reported p-values there are no = sign. Please include. Thank you, this has now been added. Reviewer #2: General comments This study uses a human challenge model to examine if symptoms are experienced in the context of pneumococcal colonisation among adults. The structure of the paper is acceptable, however there could be improvement made in the finer details, e.g. error bars/confidence intervals and raw numbers I think it is also important to mention the limitations of this study e.g. not an RCT, low sample size, and only one serotype successfully colonised. Consequently I think some of the statements in the discussion should have the language softened, as this study is not the definitive study which demonstrates that colonisation is asymptomatic among adults. Rather it might suggest this, but further work using more serotypes, in larger RCTs is needed. Thank you, we have modified the manuscript to take this into account. Changes have been made to the abstract, discussion and conclusion to recognise this. Specific comments Abstract Methods section line 46 " ... reported between groups was compared ..." to groups were compared Thank you, this has now been updated. Results section line 49 S. pneumoniae (29 with serotype 6B, 17 with serotype 23F ... Thanks, this has been updated Conclusion section line 58 Pneumococcal colonisation using the EHPC was asymptomatic ... Thanks, this has also been updated as requested. Introduction line 69 - correct typo, H. influenzae Thanks, this has now been updated line 70-73 - could be better worded

	This has been re-worded for clarity. lines 83-87 - suggest rearranging this paragraph e.g. The experimental pneumococcal challenge model (EHPC) mimics natural colonisation in healthy ....vaccines in randomised control trials. We aimed to use the EHPC to investigate if the process is symptomatic.... Thank you this has now been updated. Methods Was a sample size calculation conducted? Thank you for this feedback. We have not performed a sample size calculation as this was performed as a pilot study. In a larger follow up study we will ensure that a formal sample size calculation is done. Results line 134-137 please provide actual numbers, proportions, confidence intervals, and p-values. These have been added where required. figures - please add in confidence intervals. Thank you, these have been included where required. A legend has been added to aid the interpretation. Discussion Lines 164 and 212 I would tone down the language here e.g. This study provides evidence supporting the hypothesis that SPN colonisation among adults is asymptomatic Thank you, this has been updated. We have taken this into account and altered the discussion and conclusion to reflect this. line 171 Other than sample size, key limitations also include: the study wasn't randomised, only one serotype was assessed in this study, and pre-existing immunity. The lack of randomisation to the allocated group and the use of a single successful serotype has been added as key limitations. As we used SPN 6B, a serotype that is not otherwise present in the community, it is unlikely that the participants have pre-existing immunity to this serotype. A line has been added to the discussion in order to address this. line 213
Additional Information:	
Question	**Response**
Financial Disclosure Enter a financial disclosure statement that	Bill and Melinda Gates Grand Challenges Exploration Programme II. DF, SG

describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research articles from *PLOS ONE* for specific examples. This statement is required for submission and **will appear in the published article** if the submission is accepted. Please make sure it is accurate. **Unfunded studies** Enter: *The author(s) received no specific funding for this work.* **Funded studies** Enter a statement with the following details: • Initials of the authors who received each award • Grant numbers awarded to each author • The full name of each funder • URL of each funder website • Did the sponsors or funders play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript? • **NO** - Include this sentence at the end of your statement: *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.* • **YES** - Specify the role(s) played. * typeset	Descriptor number: 10.12 The funders did not play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Competing Interests Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any competing interests that could be perceived to bias this work—acknowledging all financial support and any other relevant financial or non-financial competing interests. This statement **will appear in the published article** if the submission is accepted. Please make sure it is accurate. View published research articles from *PLOS ONE* for specific examples.	The authors have declared that no competing interests exist

NO authors have competing interests Enter: *The authors have declared that no competing interests exist.* **Authors with competing interests** Enter competing interest details beginning with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests: [insert competing interests here]* * typeset	
Ethics Statement Enter an ethics statement for this submission. This statement is required if the study involved: • Human participants • Human specimens or tissue • Vertebrate animals or cephalopods • Vertebrate embryos or tissues • Field research Write "N/A" if the submission does not require an ethics statement. General guidance is provided below. Consult the submission guidelines for detailed instructions. **Make sure that all information entered here is included in the Methods section of the manuscript.**	All participants gave written, informed consent. Ethical permission was granted by local NHS Research and Ethics Committee (REC) (11/NW/0592 Liverpool-East).

Format for specific study types **Human Subject Research (involving human participants and/or tissue)** • Give the name of the institutional review board or ethics committee that approved the study • Include the approval number and/or a statement indicating approval of this research • Indicate the form of consent obtained (written/oral) or the reason that consent was not obtained (e.g. the data were analyzed anonymously) **Animal Research (involving vertebrate animals, embryos or tissues)** • Provide the name of the Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board that reviewed the study protocol, and indicate whether they approved this research or granted a formal waiver of ethical approval • Include an approval number if one was obtained • If the study involved *non-human primates*, add *additional details* about animal welfare and steps taken to ameliorate suffering • If anesthesia, euthanasia, or any kind of animal sacrifice is part of the study, include briefly which substances and/or methods were applied **Field Research** Include the following details if this study involves the collection of plant, animal, or other materials from a natural setting: • Field permit number • Name of the institution or relevant body that granted permission	
Data Availability Authors are required to make all data underlying the findings described fully available, without restriction, and from the time of publication. PLOS allows rare exceptions to address legal and ethical concerns. See the PLOS Data Policy and FAQ for detailed information.	Yes - all data are fully available without restriction

A Data Availability Statement describing where the data can be found is required at submission. Your answers to this question constitute the Data Availability Statement and **will be published in the article**, if accepted. **Important:** Stating 'data available on request from the author' is not sufficient. If your data are only available upon request, select 'No' for the first question and explain your exceptional situation in the text box. Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	
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and contact information or URL). • This text is appropriate if the data are owned by a third party and authors do not have permission to share the data. * typeset	
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16/12/19

Dear PLOS ONE.

RE: Pneumococcal Colonisation is an Asymptomatic Event in Healthy Adults using an Experimental Human Colonisation Model

We would like to re-submit the above titled manuscript to PLOS ONE. Thank you for the helpful feedback from the peer reviewers. We feel this has made it a much stronger manuscript. A point-by-point rebuttal has been provided to aid the peer review process. We look forward to further feedback and hope that this is now deemed ready for publication.

I will act as corresponding author pre-publication and act on behalf of all authors. All authors have seen and approved this manuscript and contributed significantly to the work.

Yours sincerely

Dr Andrea Collins and Prof Daniela Ferreira

Title Page

List Title: Pneumococcal Colonisation is an Asymptomatic Event in Healthy Adults using an Experimental Human Colonisation Model.

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Author contributions:

AC, AW, DF, SG - writing the protocol

AC, DF, AW, SG - ethics submission

AC, AW, CH, DF, SG - study co-ordination

AC, AW, SG - clinical cover including on call responsibility

AC, AW, CH - recruiting and consenting participants

AC, AW, CH - sample collection

AC, AW, CH, AT, DW - data collection and management

AC, AW, SG, DF, AT, DW- statistical planning and analysis

DF - bacterial inoculum preparation and sample processing

SG - co-ordination of DMSC communications

AC, AW, SG, DF, AT, DW, VC, RR, DM- manuscript preparation

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At a Glance Commentary: The Experimental Human Pneumococcal Colonisation (EHPC) model has been established to test current and new pneumococcal vaccines. Literature suggests that pneumococcal colonisation in adults is an asymptomatic process but there is limited evidence to support this; therefore, we addressed the question using the EHPC model.

Abstract

254/ 300 words

Introduction

Pneumococcal colonisation is regarded as a pre-requisite for developing pneumococcal disease. In children previous studies have reported pneumococcal colonisation to be a symptomatic event and described a relationship between symptom severity/frequency and colonisation density. The evidence for this in adults is lacking in the literature. This study uses the experimental human pneumococcal challenge (EHPC) model to explore whether pneumococcal colonisation is a symptomatic event in healthy adults.

Methods

Healthy participants aged 18-50 were recruited and inoculated intra-nasally with either *Streptococcus pneumoniae* (serotypes 6B, 23F) or saline as a control. Respiratory viral swabs were obtained prior to inoculation. Nasal and non-nasal symptoms were then assessed using a modified Likert score between 1 (no symptoms) to 7 (cannot function). The rate of symptoms reported between the two groups was compared and a correlation analysis performed.

Results

Data from 54 participants were analysed. 46 were inoculated with *S. pneumoniae* (29 with serotype 6B, 17 with serotype 23F) and 8 received saline (control). In total, 14 became experimentally colonised (30.4%), all of which were inoculated with serotype 6B. There was no statistically significant difference in nasal (p= 0.45) or non-nasal symptoms (p=0.28) between the inoculation group and the control group. In those who were colonised there was no direct correlation between colonisation density and symptom severity. In the 22% (12/52) who were co-colonised, with pneumococcus and respiratory viruses, there was no statistical difference in either nasal or non-nasal symptoms (virus positive p=0.74 and virus negative p=1.0).

Conclusion

Pneumococcal colonisation using the EHPC model is asymptomatic in healthy adults, regardless of pneumococcal density or viral co-colonisation.

Introduction

Streptococcus pneumoniae (pneumococcus, SPN) frequently colonises the human nasopharynx, with 40-95% of infants and 10-25% of adults being colonised at any one time(1). Pneumococcal/SPN colonisation rates also vary with geographical location, genetics and socioeconomic background(2). SPN colonisation is a dynamic process. Although multiple SPN serotypes can both simultaneously and

sequentially colonise, one serotype is usually the predominant current coloniser(3). In addition interspecies competition occurs between resident flora and potential colonisers including *S.pneumoniae*, *H.influenzae* and *S.aureus(4).*

Colonisation of the nasopharynx is considered a pre-requisite for SPN infections including pneumonia, sepsis, meningitis and otitis media. However, most colonisation episodes will not lead to subsequent disease. Pneumococcal colonisation is also thought to be the predominant source of immunological boosting against SPN infection in both children and adults(5, 6).

SPN colonisation appears to be asymptomatic in murine models(7) and in adults, however the current data are limited(8). Previous studies in children have demonstrated mild nasal symptoms following SPN colonisation however when adjusted for age this relationship was weak(9). Other studies have reported a relationship between symptom severity, pneumococcal density and pneumococcal/viral co-colonisation in children(10). Pneumococcal colonisation may cause nasal symptoms in two ways; the bacteria could induce host secretions and inflammatory responses or in co-colonised individuals (pneumococcus and virus) due to viral proliferation inducing rhinitis(9). Some studies have concluded that the presence of respiratory viruses and/or other bacteria within the nasopharynx are the main cause of symptoms; this colonisation in turn increases the rate of pneumococcal colonisation(9).

. The novel experimental pneumococcal challenge model (EHPC) model mimics natural pneumococcal colonisation in healthy human adults and has been used to effectively study mucosal immunity and as a platform to test the efficacy of pneumococcal vaccines in randomised control trials(11, 12). We aimed to use the EHPC model to investigate if the process of nasopharyngeal pneumococcal colonisation leads to symptoms.

Methods

We recruited non-smoking healthy participants aged 18-60 years old (self-selection) as part of a larger EHPC dose ranging study between 24th May 2012 to 23rd October 2012, with follow up until January 2013. A modified CONSORT flow diagram is shown in Figure 1. Specimen collection and sample processing were conducted in Liverpool, UK. All participants gave written, informed consent. Ethical permission was granted by local NHS Research and Ethics Committee (REC) (11/NW/0592 Liverpool-East, date 11/10/2011). This study was retrospectively registered on the ISRCTN database (ISRCTN85403723) as this was not a mandatory requirement at the time of recruitment. All ongoing and related trials for this intervention are now prospectively registered with ISRCTN. Exclusion criteria included natural pneumococcal colonisation at baseline, any chronic medical condition or regular medication (study participation could put the participant at increased risk of pneumococcal disease) and regular contact with an at-risk individual such as young children (study participation could put the at-risk individual at increased risk of pneumococcal disease).

Participants were nasally inoculated with $8x10^4$, $1.6x10^5$, or $3.2x10^5$ mid-log phase colony forming units (CFU) *S. pneumoniae* (prepared as previously described)(6). Bacterial inoculation density was confirmed by serial dilutions of the inoculation stock onto blood agar (Oxoid). Two serotypes were used; 6B and 23F, both were fully sensitive to penicillin. 46 participants were allocated to be inoculated with *S. pneumoniae* (SPN 6B or 23F) as part of a dose-ranging study and 8 participants inoculated with saline as a control group. They were blinded to their groups.

Pre-inoculation oropharyngeal swabs were assayed for respiratory viruses using multiplex Polymerase Chain reaction (PCR) as previously published (13). The PCR assay panel detected Influenza A and B, Respiratory syncytial virus, Human metapneumovirus, Human rhinovirus, Parainfluenza viruses 1-4 and Coronaviruses OC43, NL63, 229E and HKU1. Nasopharyngeal colonisation was assessed in nasal washes (Nacleiro technique, as previously described) collected at day 2, 7 and 14 post inoculation(14). Pneumococcal colonisation status and density in nasal washes was determined by classical culture as previously described(6, 14).

Participants were prompted to complete a daily symptom log on the day of inoculation (baseline) and daily for 7 days post-inoculation. The symptom log consisted of a 7-point visual analogue scale (a type of Likert scale) which assessed five nasal and five non-nasal symptoms(15). The only modification to the validated questionnaire was the removal of 'mental function' as a non-nasal symptom (Figure 2). Scores ≥2 were considered 'symptomatic'. The score awarded at inoculation (day 0) was considered their baseline score, the participant was considered symptomatic if the score went above baseline and ≥ 2.

Graphical and statistical analyses were performed using GraphPad version 5.0 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel, with a p-value of <0.05 considered significant. Rates of symptoms reported between groups were compared using Fisher's exact tests and Chi square where appropriate. Correlation analysis was performed using Spearman's rank text. The daily symptom logs were collected at the next scheduled visit following completion.

Results

Fifty-five participants were recruited with an age range of 19-49 years old over a 6- month period from24/04/2012-23/10/2012. Participants with incomplete symptom severity score logs were excluded, therefore data from 54 participants were analysed. 46 participants were inoculated with SPN (29 with 6B, 17 with 23F) and 8 with saline (control group). Participants inoculated with 6B, 23F and saline were similar in age and gender distribution. In total, 14 participants became experimentally colonised (30.4%), all of which were inoculated with serotype 6B. None of the participants in the control group developed natural SPN colonisation during the study. Overall 72% (39/54) of participants reported either or both nasal or non-nasal symptoms during the 7 days post-inoculation. Of these symptoms, similar rates of nasal and non-nasal symptoms were reported; 59% (32/54) of participants reported nasal symptoms and 56% (30/54) reported non-nasal symptoms.

No statistical difference was seen between number of participants who reported symptoms in the experimental SPN positive or negative group. Similar rates of experimental SPN positive participants reported nasal symptoms (71%, 10/14) and non-nasal symptoms (57%, 8/14) compared to experimental SPN negative participants (50%, 16/32 in nasal (OR 2.50 [95% CI: 0.65- 9.66] *P*= 0.212) and non-nasal (OR 1.33 [95% CI: 0.38- 4.73] *P*= 0.754). See Figure 3.

Nasal SPN inoculation did not lead to greater rates of reported symptoms when compared to the control (saline inoculation) group, as show in Figure 4. Nasal symptoms were reported by 75% (6/8) of participants inoculated with saline compared to 57% (26/46) of those who were inoculated with SPN, no statistical difference was seen (p= 0.45). Similarly, no statistical difference was seen with the reporting of non-nasal symptoms 24/46 (52%) post-SPN inoculation compared to post-saline inoculation 6/8 (75%), (p= 0.28). Participants that reported 'any symptom' were higher in the control group 100% (8/8) compared to 67% (31/46) in the inoculation group, this was not statistically significant (p= 0.09).

Of the 14 participants colonised with SPN, colonisation density was measured at days 2 and 7. No direct correlation was seen between SPN density and the mean symptom severity score at day 2 and day 7 for nasal (p= 0.86 Spearman's correlation) and non-nasal symptoms (p= 0.83 Spearman's correlation), Figure 5.

Viral colonisation data was available for 96% (52/54) participants at baseline. Viral colonisation was detected in 22% (12/52) of participants, 2 were inoculated with saline and 10 with SPN [serotype 23F (n=2) and 6B (n=8)]. There was no increase in nasal or non-nasal symptoms respectively in virus positive 8/12 (67%) and 7/12 (58%) respectively compared to virus negative participants 23/40 (58% for both symptoms), p= 0.74 and p= 1.0. Experimental SPN colonisation rates were higher in the

presence of virus 6/10 (60%) compared to 8/35 (23%) in virus negative participants (p= <0.05). Virus and SPN positive (Co-colonised) participants did not report greater rates of nasal or non-nasal symptoms [4/6, (60%) for both symptoms],when compared to SPN positive only [nasal symptoms 6/8 (75%), OR 0.67 [95% CI: 0.06- 6.88], P= 1.000), non-nasal symptoms 4/8 (50%), OR 2.00 [95% CI: 0.22-17.90] P= 0.627] and virus positive only [nasal symptoms 3/4 (75%) OR 3.23 [0.30- 35.13] *P*= 0.600, non-nasal symptoms 2/4 (50%] OR 0.93 [95% CI: 0.11- 7.59] *P*= 1.000). This is shown in Figure 6 .

Discussion

This study, with a clear methodology, provides evidence supporting the hypothesis that experimental pneumococcal (SPN) colonisation in adults is an asymptomatic event. This novel use of a human challenge model allowed the study of pneumococcal colonisation and symptomology in a controlled environment. The carriage rate of pneumococcus in this study was 30.4% and all with SPN6B. This is higher than the 'natural rate', due to the artificial introduction of the bacteria directly into the nasopharynx. This SPN 6B (BHN418) is known from epidemiological studies to have a very low prevalence in the community, therefore the participants are unlikely to have been exposed to it previously. The carriage rate for those inoculated with 23F was 0 %. The reasons for the variability in carriage rate between serotypes is unclear but thought to be related to the evasion of mucociliary clearance, host nutrient availability and niche competition[8].

The strengths of this study are the robust methodology used to assess symptom severity(15) , the lack of recall bias (due to prospective daily data log completion) and the use of a control group. Using this novel human challenge model, the exact day of pneumococcal inoculation and the onset and termination of each SPN colonisation episode was known allowing association between symptoms and pneumococcal presence and density over time. The main limitations of our study was the total sample size (n=54), the lack of randomisation for group allocation and the use of a single serotype of SPN.

Although a previous study in adults used a small sample size (n=14) and did not include the methods used to support this conclusion(16), it agrees with our data that pneumococcal colonisation in healthy adults is indeed asymptomatic. Higher symptom severity scores were not a predictor for colonisation.

SPN colonisation is more common in children; therefore, a limitation of this work is the lack of generalisability of results to all age groups, however reasonable evidence exists that SPN colonisation in children does cause nasal symptoms(10, 17). Another limitation is that only one serotype was

assessed in this study, SPN 6B. This particular serotype is not thought to be present in the community and therefore it is very unlikely that the participants would have pre-existing immunity from previous exposure. A previous study suggested that the presence of symptoms could be dependent on the serotype of pneumococcus(17). The authors reported that colonisation with serotype 19F was strongly associated with symptoms such as coryza, sneezing, cough and expectoration. However, these children were recruited from a paediatric hospital emergency room, the study did not report on the diagnosis given to these patients therefore upper or lower respiratory infection may have been the cause of these symptoms rather than solely SPN colonisation(17).

Rodrigues et al found that rhinitis symptoms, rates of colonisation with SPN and *Haemophilus Influenzae (Hi)* in pre-school children decreased with age. Symptoms of rhinitis were reported using the Symptoms of Nasal Outflow Tally (SNOT) score. Both SPN and *Hi* colonisation were strongly associated with increased SNOT scores in children <5 years (p= 0.002 and 0.001) whereas colonisation with *S. aureus* was negatively associated with SNOT scores (p=0.04). Interestingly, 40% of asymptomatic children (low SNOT score) were in fact SPN colonised. However, when the data was analysed considering age, the association between SPN colonisation and SNOT scores was weak (p= 0.06) whereas the association between SNOT scores and *Hi* colonisation remained strong (p= 0.003). This suggest that *Hi* may stimulate rhinitis in children to increase transmission(9). The study by Rodrigues et al does not, however, report the effect of co-colonisation (SPN and virus) on symptoms.

Our results suggest that in adults co-colonisation (SPN and virus) is also an asymptomatic process with similar rates of nasal and non-nasal symptoms reported in all groups. Our results did show that asymptomatic viral infection at baseline was associated with the acquisition of SPN colonisation in adults. This is in keeping with results in children which found that asymptomatic viral infections had a large effect on SPN colonisation(18). They reported that the proportion of children with SPN colonisation was higher during prompted visits for review of upper respiratory tract infections (URTI) symptoms rather than for regular planned study follow up visits. Due to the small sample size of SPN

and virus co-colonisers (n=6), it is difficult to make strong assumptions about the symptomology of co-infection from our study. Viral swabs were also only performed at baseline (up to 7 days prior to inoculation) therefore we cannot assess correlation between symptoms and viral status at each point, nor was density measured.

In conclusion we have provided evidence to support the hypothesis that neither nasopharyngeal inoculation nor experimental pneumococcal colonisation cause nasal or non-nasal symptoms in adults. Our results suggest that asymptomatic viral infection prior to nasopharyngeal inoculation or experimental SPN colonisation does not increase nasal or non-nasal symptoms. A better understanding of the process of viral co-infection in adults and the symptoms caused by viral infection prior to or following acquisition of SPN colonisation is needed and would add to this study's preliminary data. A key question, given the difference between adults and children, is the association between colonisation symptoms and transmission; our study indicates that pneumococcal colonisation in adults is asymptomatic, but does not address transmission dynamics.

Acknowledgements

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Figure 1

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Figure 1; Modified CONSORT diagram

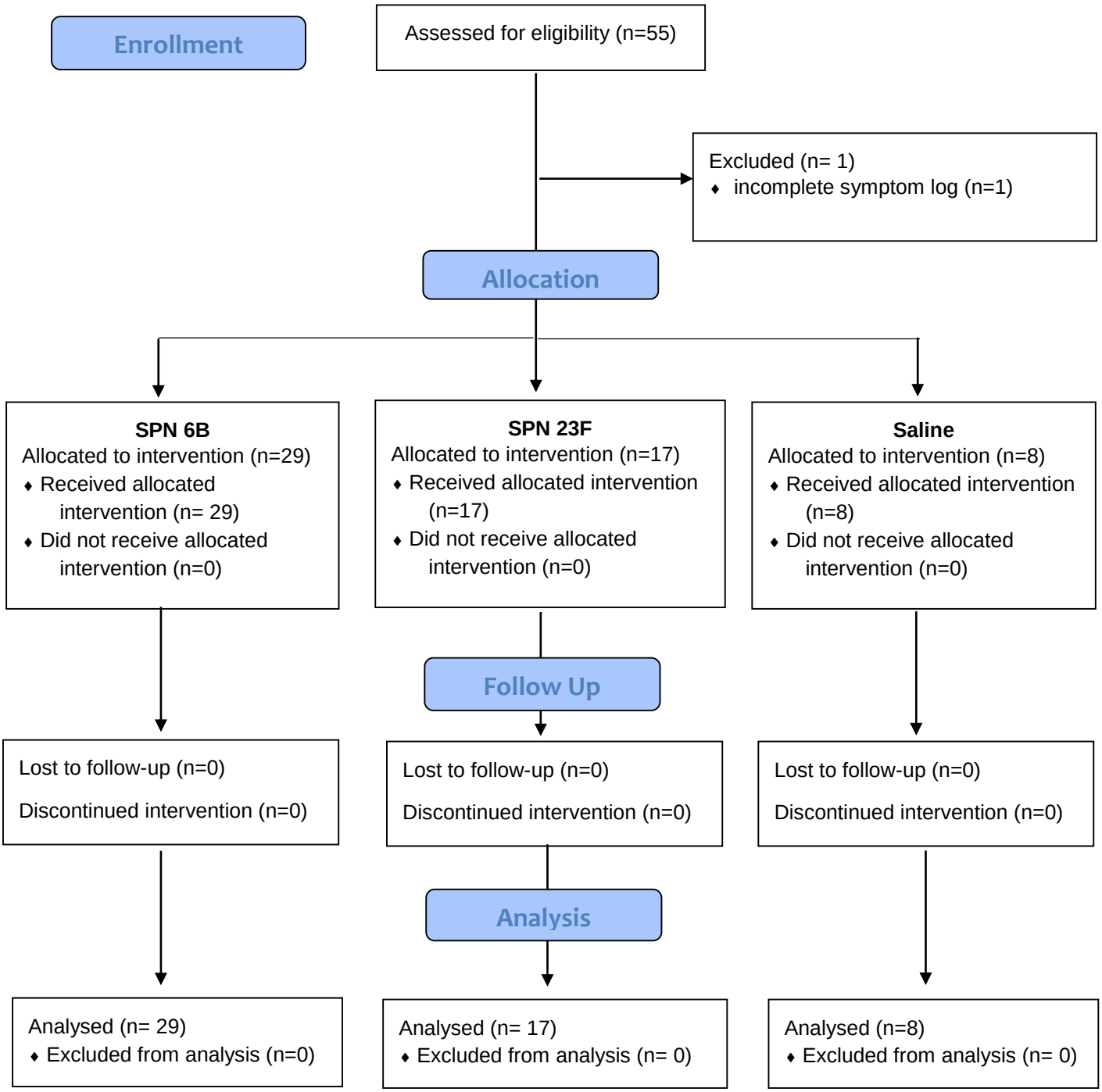

Figure 2: Participant Symptom Log

Nasal Symptoms							
Sneezing	1	2	3	4	5	6	7
Runny nose	1	2	3	4	5	6	7
Congestion	1	2	3	4	5	6	7
Itchy nose	1	2	3	4	5	6	7
Postnasal drip	1	2	3	4	5	6	7
Non-Nasal Symptoms							
Eye symptoms	1	2	3	4	5	6	7
Throat symptoms	1	2	3	4	5	6	7
Cough	1	2	3	4	5	6	7
Ear symptoms	1	2	3	4	5	6	7
Headache	1	2	3	4	5	6	7

Severity score	
1-2	None to occasional limited episode
3-4	Mild to steady symptoms but easily tolerable
5-6	Moderately bothersome or symptoms hard to tolerate/may interfere with daily activities and/or sleep
7	Unbearably severe or symptoms are so bad/cannot function all of the time

A severity score <2 was considered asymptomatic.

Figure 3 Click here to access/download;Figure;Fig 3 symptoms.docx

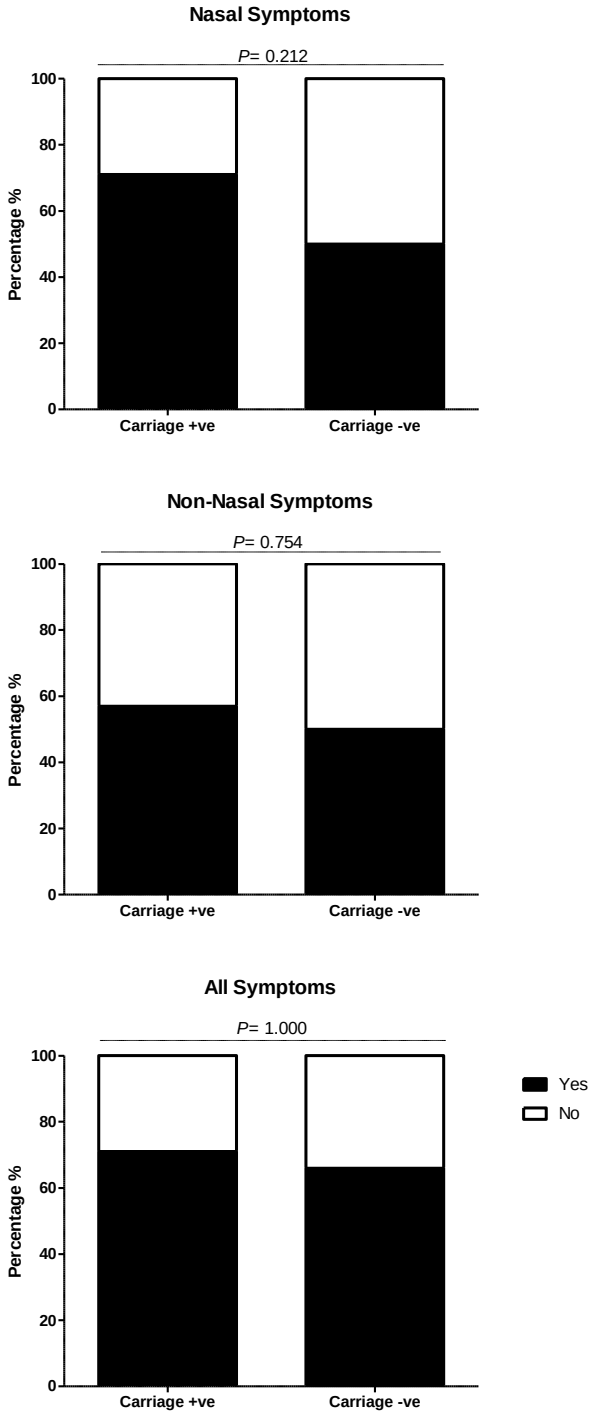

Figure 3: Comparison of nasal, non-nasal and all symptoms between experimental SPN positive and negative participants. Each bar chart shows the percentage of carriage positive (N= 14) and carriage negative (N= 32) participants, who reported symptoms (nasal, non-nasal and all symptoms) after inoculation with *Streptococcus pneumoniae* serotypes 6B or 23F. Participants were deemed symptomatic if they scored >2, or >1 point above baseline for any of the five nasal or non-nasal symptoms in the visual analogue scale. The number of participants reporting symptoms between carriage positive and negative status were statistically compared using Fishers Exact and deemed significant if *P*= ≤0.05. There was no significant difference in the number of participants reporting nasal (OR 2.50 [95% CI: 0.65- 9.66] *P*= 0.212), non-nasal (OR 1.33 [95% CI: 0.38- 4.73] *P*= 0.754) or all

symptoms (OR 1.31 [95% CI: 0.33 to 5.16] *P*= 1.000) between carriage positive and carriage negative participants.

Figure 4

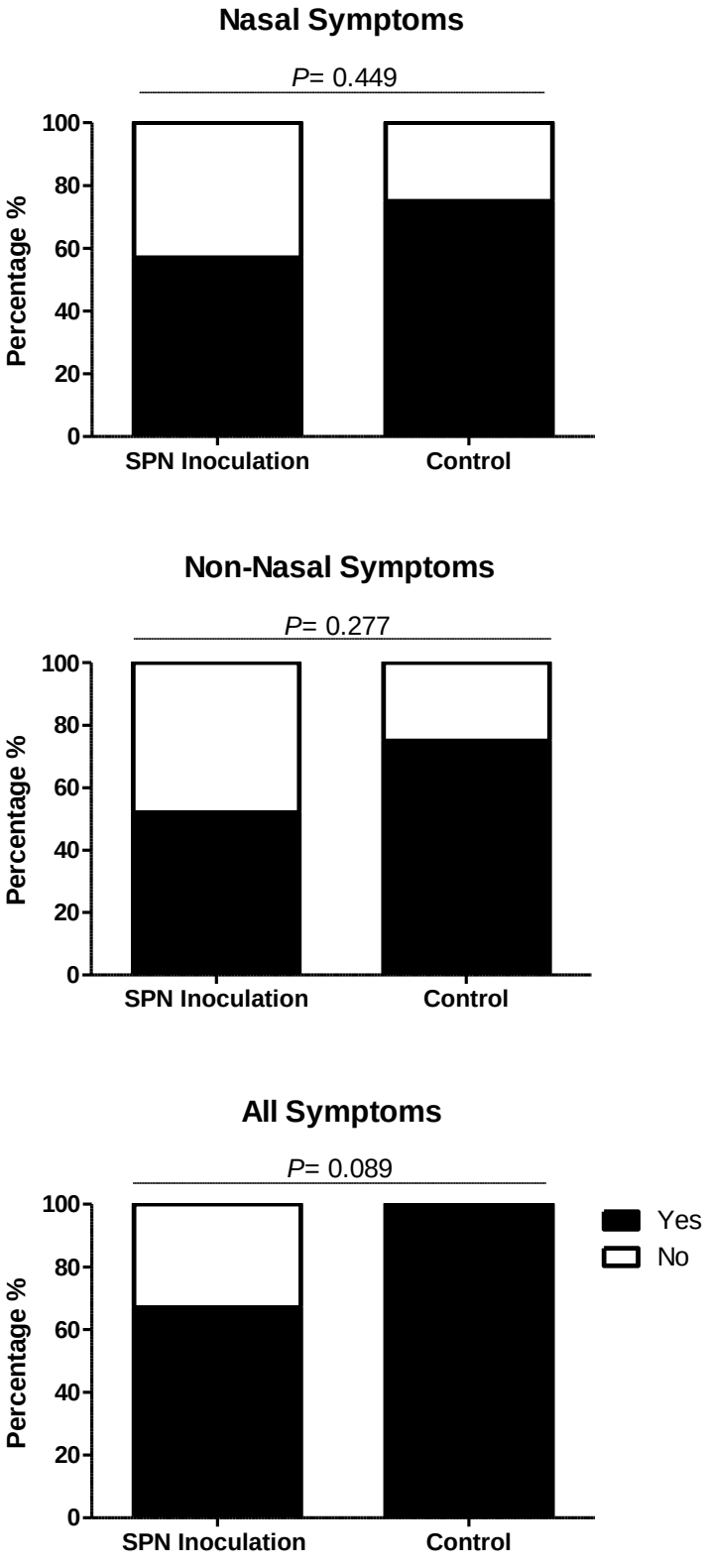

Figure 4: Comparison of nasal, non-nasal and all symptoms between participants inoculated with *S. pneumoniae* compared to those inoculated with normal saline (control). Each bar chart shows the percentage of participants inoculated with *S. pneumoniae* (SPN) serotypes 6B and 23F (N= 46) and normal saline (control) (N= 8) reporting symptoms (nasal, non- nasal and all symptoms) after inoculation. Participants were deemed symptomatic if they scored >2, or >1 point above baseline for any of the five nasal or non-nasal symptoms on the visual analogue scale. The number of participants reporting symptoms between inoculation with SPN and control were statistically compared using Fishers Exact and deemed significant if *P*= ≤0.05. There was no significant difference in the number of participants reporting nasal (OR 0.43 [95% CI: 0.08- 2.38] *P*= 0.449), non-nasal (OR 0.36 [95% CI: 0.07- 2.00] *P*= 0.277) and all symptoms (OR 0.12 [95% CI: 0.01- 2.21] *P*= 0.089)

Figure 5 Click here to access/download;Figure;Fig 5 symptoms.docx

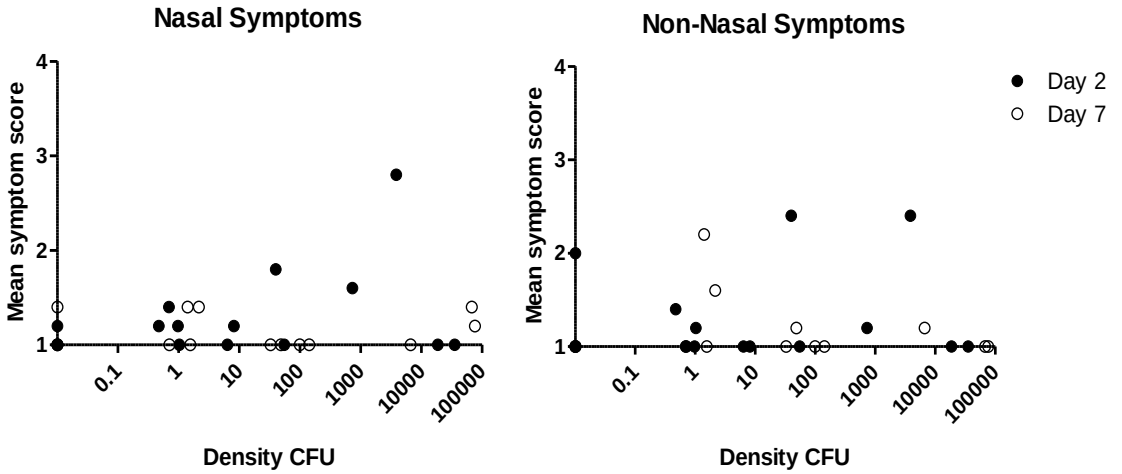

Figure 5: Correlation between pneumococcal colonisation density (SPN positive participants) and mean nasal symptom severity scores at days 2 and 7. Spearman's correlation was used to statistically analyse the correlation between bacterial colonisation density and the participants symptoms score on the visual analogue scale. Participants were deemed symptomatic if they scored >2, or >1 point above baseline for any of the five nasal or non-nasal symptoms in the visual analogue scale. No direct correlation was seen between SPN density and the mean symptom severity score at day 2 and day 7 for nasal (p= 0.86) or non-nasal symptoms (p= 0.83).

Figure 6

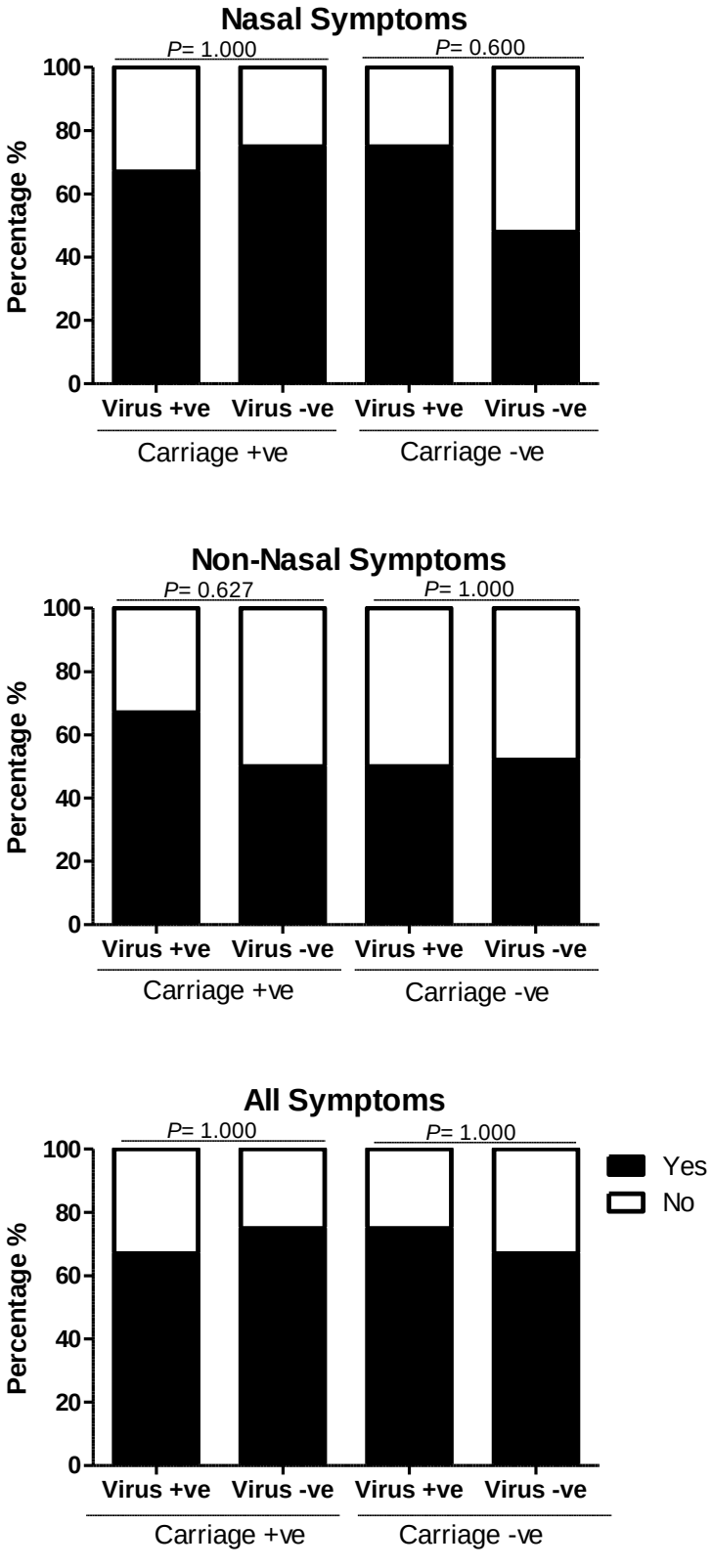

Figure 6. Comparison of nasal, non-nasal and all symptoms in carriage positive and negative participants with and without viral infection. Each bar chart shows a comparison of the percentage of carriage positive and negative participants, after inoculation with *S. pneumoniae* serotype 6B or 23F, who reported symptoms (nasal, non-nasal and all symptoms), between those infected with a virus and those without viral infection. Participants were deemed symptomatic if they scored >2, or >1 point above baseline for any of the five nasal or non-nasal symptoms on the visual analogue scale. The number of participants reporting symptoms between those infected with a virus and those without viral infection were statistically compared using Fishers Exact and deemed significant if $P = \leq 0.05$. There was no significant difference in the number of participants reporting nasal, non-nasal and all symptoms between those with viral infection and those without viral infection in both the carriage positive (nasal symptoms OR 0.67 [95% CI: 0.06- 6.88], $P = 1.000$, non-nasal symptoms OR 2.00 [95% CI: 0.22- 17.90] $P = 0.627$, all symptoms OR 0.67 [95% CI: 0.06- 6.88] $P = 1.000$) and carriage negative groups (nasal symptoms OR 3.23 [0.30- 35.13] $P = 0.600$, non-nasal symptoms OR 0.93 [95% CI: 0.11- 7.59] $P = 1.000$, all symptoms OR 1.50 [95% CI: 0.14- 16.55] $P = 1.000$).

Supporting Information (TREND checklist)

Title Page

List Title: Pneumococcal Colonisation is an Asymptomatic Event in Healthy Adults using an Experimental Human Colonisation Model.

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AC, DF, AW, SG - ethics submission

AC, AW, CH, DF, SG - study co-ordination

AC, AW, SG - clinical cover including on call responsibility

AC, AW, CH - recruiting and consenting participants

AC, AW, CH - sample collection

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AC, AW, CH, AT, DW - data collection and management

AC, AW, SG, DF, AT, DW- statistical planning and analysis

DF - bacterial inoculum preparation and sample processing

SG - co-ordination of DMSC communications

AC, AW, SG, DF, AT, DW, VC, RR, DM - manuscript preparation

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At a Glance Commentary: The Experimental Human Pneumococcal Colonisation (EHPC) model has been established to test current and new pneumococcal vaccines. Literature suggests that pneumococcal colonisation in adults is an asymptomatic process but there is limited evidence to support this; therefore, we addressed the question using the EHPC model.

Abstract

254/ 300 words

Introduction

Pneumococcal colonisation is regarded as a pre-requisite for developing pneumococcal disease. In children previous studies have reported pneumococcal colonisation to be a symptomatic event and described a relationship between symptom severity/frequency and colonisation density. The evidence for this in adults is lacking in the literature. This study uses the experimental human pneumococcal challenge (EHPC) model to explore whether pneumococcal colonisation is a symptomatic event in healthy adults.

Methods

Healthy participants aged 18-50 were recruited and inoculated intra-nasally with either *Streptococcus pneumoniae* (serotypes 6B, 23F) or saline as a control. Respiratory viral swabs were obtained prior to inoculation. Nasal and non-nasal symptoms were then assessed using a modified Likert score between 1 (no symptoms) to 7 (cannot function). The rate of symptoms reported between the two groups was compared and a correlation analysis performed.

Results

Data from 54 participants were analysed. 46 were inoculated with *S. pneumoniae* (29 with serotype 6B, 17 with serotype 23F) and 8 received saline (control). In total, 14 became experimentally colonised (30.4%), all of which were inoculated with serotype 6B. There was no statistically significant difference in nasal (p= 0.45) or non-nasal symptoms (p=0.28) between the inoculation group and the control group. In those who were colonised there was no direct correlation between colonisation density and symptom severity. In the 22% (12/52) who were co-colonised, with pneumococcus and respiratory viruses, there was no statistical difference in either nasal or non-nasal symptoms (virus positive p=0.74 and virus negative p=1.0).

Conclusion

Pneumococcal colonisation using the EHPC model is asymptomatic in healthy adults, regardless of pneumococcal density or viral co-colonisation.

Introduction

Streptococcus pneumoniae (pneumococcus, SPN) frequently colonises the human nasopharynx, with 40-95% of infants and 10-25% of adults being colonised at any one time(1). Pneumococcal/SPN colonisation rates also vary with geographical location, genetics and socioeconomic background(2). SPN colonisation is a dynamic process. Although multiple SPN serotypes can both simultaneously and

sequentially colonise, one serotype is usually the predominant current coloniser(3). In addition interspecies competition occurs between resident flora and potential colonisers including *S.pneumoniae*, *H.influenzae* and *S.aureus(4).*

Colonisation of the nasopharynx is considered a ~~important as~~ ~~the~~ pre-requisite for SPN infections including pneumonia, sepsis, meningitis and otitis media. However, m~~M~~ost colonisation episodes will not lead to subsequent disease. Pneumococcal c~~C~~olonisation is also thought to be the predominant source of immunological boosting against SPN infection in both children and adults(5, 6).

SPN colonisation appears to be asymptomatic in murine models(7) and in adults, however the current data are limited(8). Previous studies~~-~~ in children have demonstrated mild nasal symptoms following SPN colonisation however when adjusted for age this relationship was weak(9). Other studies have reported a relationship between symptom severity, pneumococcal density and pneumococcal/viral co-colonisation in children(10). Pneumococcal colonisation may cause nasal symptoms in two ways; the bacteria could induce host secretions and inflammatory responses or in co-colonised individuals (pneumococcus and virus) due to viral proliferation inducing rhinitis(9). Some studies have concluded that the presence of respiratory viruses and/or other bacteria within the nasopharynx are the main cause of symptoms; this colonisation in turn increases the rate of pneumococcal colonisation(9).

~~We have used the novel experimental pneumococcal challenge model (EHPC) to investigate if the process of nasopharyngeal pneumococcal colonisation is symptomatic, causing either nasal or non-nasal symptoms~~. The novel experimental pneumococcal challenge model (EHPC)~~is~~ model mimics natural pneumococcal colonisation in healthy human adults and has been used to effectively study mucosal immunity and as a platform to test the efficacy of pneumococcal vaccines in randomised control trials(11, 12). We aimed to use the EHPC model to investigate if the process of nasopharyngeal pneumococcal colonisation leads to symptoms.

Methods

We recruited non-smoking healthy participants aged 18-60 years old (self-selection) as part of a larger EHPC dose ranging study between 24th May 2012 to 23rd October 2012, with follow up until January 2013. -A modified CONSORT flow diagram is shown in Figure 1. Specimen collection and sample processing were conducted in Liverpool, UK. All participants gave written, informed consent. Ethical permission was granted by local NHS Research and Ethics Committee (REC) (11/NW/0592 Liverpool-East, date 11/10/2011). This study was retrospectively registered on the ISRCTN database (ISRCTN85403723) as this was not a mandatory requirement at the time of recruitment. All -ongoing and related trials for this intervention are now prospectively registered with ISRCTN. Exclusion criteria included natural pneumococcal colonisation at baseline, any chronic medical condition or regular medication (study participation could put the participant at increased risk of pneumococcal disease) and regular contact with an at-risk individual such as young children (study participation could put the at-risk individual at increased risk of pneumococcal disease).

Participants were nasally inoculated with $8x10^4$, $1.6x10^5$, or $3.2x10^5$ mid-log phase colony forming units (CFU) *S. pneumoniae* (prepared as previously described)(6). Bacterial inoculation density was confirmed by serial dilutions of the inoculation stock onto blood agar (Oxoid). Two serotypes were used; 6B and 23F, both were fully sensitive to penicillin. 46 participants were allocated to be inoculated with *S. pneumoniae* (SPN 6B or 23F) as part of a dose-ranging study and 8 participants inoculated with saline as a control group. They were blinded to their groups.

Pre-inoculation oropharyngeal swabs were assayed for respiratory viruses using multiplex Polymerase Chain reaction (PCR) as previously published (13). The PCR assay panel detected Influenza A and B, Respiratory syncytial virus, Human metapneumovirus, Human rhinovirus, Parainfluenza viruses 1-4 and Coronaviruses OC43, NL63, 229E and HKU1. Nasopharyngeal colonisation was assessed in nasal washes (Nacleiro technique, as previously described) collected at day 2, 7 and 14 post inoculation(14). Pneumococcal colonisation status and density in nasal washes was determined by classical culture as previously described(6, 14).

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Participants were prompted to complete a daily symptom log on the day of inoculation (baseline) and daily for 7 days post-inoculation. The symptom log consisted of a 7-point visual analogue scale (a type of Likert scale) which assessed five nasal and five non-nasal symptoms(15). The only modification to the validated questionnaire was the removal of 'mental function' as a non-nasal symptom (Figure 2~~1~~). Scores ≥2 were considered 'symptomatic'. The score awarded at inoculation (day 0) was considered their baseline score, the participant was considered symptomatic if the score went above baseline and ≥ 2.

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Graphical and statistical analyses were performed using GraphPad version 5.0 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel, with a p-value of <0.05 considered significant. Rates of symptoms reported between groups were compared using Fisher's exact tests and Chi square where appropriate. Correlation analysis was performed using Spearman's rank text. The daily symptom logs were collected at the next scheduled visit following completion.

Results

Fifty-five participants were recruited with an age range of 19-49 years old over a 6- month period from 24/04/2012-23/10/2012~~May-October 2014~~. Participants with incomplete symptom severity score logs were excluded, therefore data from 54 participants were analysed. 46 participants were inoculated with SPN (29 with 6B, 17 with 23F) and 8 with saline (control group). Participants inoculated with 6B, 23F and saline were similar in age and gender distribution. In total, 14 participants became experimentally colonised (30.4%), all of which were inoculated with serotype 6B. None of the participants in the control group developed natural SPN colonisation during the study.

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Overall 72% (39/54) of participants reported either or both nasal or non-nasal symptoms during the 7 days post-inoculation. Of these symptoms, similar rates of nasal and non-nasal symptoms were

reported; ~~.~~ 59% (32/54) of participants reported nasal symptoms and 56% (30/54) reported non-nasal symptoms.

No statistical difference was seen between number of participants who reported symptoms in the experimental SPN positive or negative group~~s~~. Similar rates of experimental SPN positive participants reported nasal symptoms (71%, 10/14) and non-nasal symptoms (57%, 8/14) compared to experimental SPN negative participants (50%, 16/32 in nasal (OR 2.50 [95% CI: 0.65- 9.66] *P*= 0.212) and non-nasal (OR 1.33 [95% CI: 0.38- 4.73] *P*= 0.754). See Figure 3~~2~~. ~~-~~

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Nasal SPN inoculation did not lead to greater rates of reported symptoms when compared to the control (saline inoculation) group, as show in Figure 4~~3~~. Nasal symptoms were reported by 75% (6/8) of participants inoculated with saline compared to 57% (26/46) of those who were inoculated with SPN, no statistical difference was seen (p= 0.45). Similarly, no statistical difference was seen with the reporting of non-nasal symptoms 24/46 (52%) post-SPN inoculation compared to post-saline inoculation 6/8 (75%), (p= 0.28). Participants that reported 'any symptom' were higher in the control group 100% (8/8) compared to 67% (31/46) in the inoculation group, this was not statistically significant (p= 0.09).

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Of the 14 participants colonised with SPN, colonisation density was measured at days 2 and 7. No direct correlation was seen between SPN density and the mean symptom severity score at day 2 and day 7 for nasal (p= 0.86 Spearman's correlation) and non-nasal symptoms (p= 0.83 Spearman's correlation), Figure 5~~4~~.

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Viral colonisation data was available for 96% (52/54) participants at baseline. Viral colonisation was detected in 22% (12/52) of participants, 2 were inoculated with saline and 10 with SPN [serotype 23F

(n=2) and 6B (n=8)]. There was no increase in nasal or non-nasal symptoms respectively in virus positive 8/12 (67%) and 7/12 (58%) respectively compared to virus negative participants 23/40 (58% for both symptoms), p= 0.74 and p= 1.0. Experimental SPN colonisation rates were higher in the presence of virus 6/10 (60%) compared to 8/35 (23%) in virus negative participants (p= <0.05). Virus and SPN positive (Co-colonised) participants did not report greater rates of nasal or non-nasal symptoms [4/6, (60%) for both symptoms],when- compared to SPN positive only [nasal symptoms 6/8 (75%), OR 0.67 [95% CI: 0.06- 6.88], P= 1.000), non-nasal symptoms 4/8 (50%), OR 2.00 [95% CI: 0.22- 17.90] P= 0.627] and virus positive only [nasal symptoms 3/4 (75%) OR 3.23 [0.30- 35.13] *P*= 0.600, non-nasal symptoms 2/4 (50%] OR 0.93 [95% CI: 0.11- 7.59] *P*= 1.000). This is shown in Figure 6 .

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Discussion

This study, with a clear methodology, provides evidence supporting the hypothesis ~~confirms~~ that experimental pneumococcal (SPN) colonisation in adults is an asymptomatic event. This novel use of a human challenge model allowed the study of pneumococcal colonisation and symptomology in a controlled environment. The carriage rate of pneumococcus in this study was 30.4% and all with SPN6B. This is higher than the 'natural rate', due to the artificial introduction of the bacteria directly into the nasopharynx. This SPN 6B (BHN418) is known from epidemiological studies to have a very low prevalence in the community, therefore the participants are unlikely to have been exposed to it previously. The carriage rate for those inoculated with 23F was 0 %. The reasons for the variability in carriage rate between serotypes is unclear but thought to be related to the evasion of mucociliary clearance, host nutrient availability and niche competition[8].

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The strengths of this study are the robust methodology used to assess symptom severity(15) , the lack of recall bias (due to prospective daily data log completion) and the use of a control group. Using this novel human challenge model, the exact day of pneumococcal inoculation and the onset and termination of each SPN colonisation episode was known allowing association between symptoms and pneumococcal presence and density over time. The main limitations of our study was the total sample size (n=54), the lack of randomisation for group allocation and the use of a single serotype of SPN.~~-~~

Although a previous study in adults used a small sample size (n=14) and did not include the methods used to support this conclusion(16), it agrees with our data that pneumococcal colonisation in healthy adults is indeed asymptomatic. Higher symptom severity scores were not a predictor for colonisation.

SPN colonisation is more common in children; therefore, a limitation of this work is the lack of generalisability of results to all age groups, however reasonable evidence exists that SPN colonisation in children does cause nasal symptoms(10, 17). Another limitation is that only one serotype was

assessed in this study, SPN 6B. This particular serotype is not thought to be present in the community and therefore it is very unlikely that the participants would have pre-existing immunity from previous exposure. A previous~~One~~ study suggested that the presence of symptoms could be dependent on the serotype of pneumococcus(17). The authors reported that colonisation with serotype 19F was strongly associated with symptoms such as coryza, sneezing, cough and expectoration. However, these children were recruited from a paediatric hospital emergency room, the study did not report on the diagnosis given to these patients therefore upper or lower respiratory infection may have been the cause of these symptoms rather than solely SPN colonisation(17).

Rodrigues et al found that rhinitis symptoms, rates of colonisation with SPN and *Haemophilus Influenzae (Hi)* in pre-school children decreased with age. Symptoms of rhinitis were reported using the Symptoms of Nasal Outflow Tally (SNOT) score. Both SPN and *Hi* colonisation were strongly associated with increased SNOT scores in children <5 years (p= 0.002 and 0.001) whereas colonisation with *S. aureus* was negatively associated with SNOT scores (p=~~–~~0.04). Interestingly, 40% of asymptomatic children (low SNOT score) were in fact SPN colonised. However, when the data was analysed considering age, the association between SPN colonisation and SNOT scores was weak (p= 0.06) whereas the association between SNOT scores and *Hi* colonisation remained strong (p= 0.003). This suggest that *Hi* may stimulate rhinitis in children to increase transmission(9). The study by Rodrigues et al~~is study~~ does not, however, report the effect of co-colonisation (SPN and virus) on symptoms.

Our results suggest that in adults co-colonisation (SPN and virus) is also an asymptomatic process with similar rates of nasal and non-nasal symptoms reported in all groups. Our results did show that asymptomatic viral infection at baseline was associated with the acquisition of SPN colonisation in adults. This is in keeping with results in children which found that asymptomatic viral infections had a large effect on SPN colonisation(18). They reported that the proportion of children with SPN colonisation was higher during prompted visits for review of upper respiratory tract infections (URTI)

symptoms rather than for regular planned study follow up visits. Due to the small sample size of SPN and virus co-colonisers (n=6), it is difficult to make strong assumptions about the symptomology of co-infection from our study. Viral swabs were also only performed at baseline (up to 7 days prior to inoculation) therefore we cannot assess correlation between symptoms and viral status at each point, nor was density measured.

In conclusion we have provided evidence to support the hypothesis ~~shown~~ that neither nasopharyngeal inoculation nor experimental pneumococcal colonisation cause nasal or non-nasal symptoms in adults. Our results suggest that asymptomatic viral infection prior to nasopharyngeal inoculation or experimental SPN colonisation does not increase nasal or non-nasal symptoms. A better understanding of the process of viral co-infection in adults and the symptoms caused by viral infection prior to or following acquisition of SPN colonisation is needed and would add to this study's preliminary data. A key question, given the difference between adults and children, is the association between colonisation symptoms and transmission; our study indicates~~confirms~~ that pneumococcal colonisation in adults is asymptomatic, but does not address transmission dynamics.

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16/12/19

Dear PLOS ONE

RE: **Pneumococcal Colonisation is an Asymptomatic Event in Healthy Adults using an Experimental Human Colonisation Model**

Thank you for your peer review comments dated the 01/12/19. We are grateful for the helpful feedback and have addressed each detailed point below. The responses are highlighted in bold.

Review Comments to the Author

Reviewer #1:

The manuscript by Trimble and colleagues present additional results from an experimental human challenge model, describing whether experimental pneumococcal colonisation in healthy adults is a symptomatic event. This manuscript is a resubmission from 2016 (but due to time limits is now a new submission) which has addressed some of the queries from the initial review. This study reports some interesting data but is nevertheless limited mainly due to the small sample size, especially for the secondary analyses.

Some points for consideration:

1. The pneumococcal colonisation rate was 30.4% (14/46), which was solely due to serotype 6B, and actually 48% if only those who were inoculated with 6B are included. This seems quite high for an otherwise healthy adult population. The authors state that this model mimics natural pneumococcal exposure but the high carriage rate may suggest otherwise. Can the authors provide some comment on the colonisation rate they observed with respect to what has been reported among UK healthy adults or more generally? Some explanation of this would greatly benefit the manuscript. The authors also argued against providing some comment on the lack of 23F colonisation but I tend to think that this would also be worth noting in the discussion, given that both these are commonly carried serotypes.

Thank you for your feedback, the EHPC model is able to artificially induce the otherwise naturally occurring phenomenon of pneumococcal carriage by pipetting the live bacteria directly into the

participant's nasopharynx. As the EHPC inoculation is more efficient than the 'natural process' the rate of colonisation is higher. This has now been added to the discussion. We have added an explanation for the difference in colonisation rate between serotypes as requested.

2. Figures 2 and 3 could be combined since they are both essentially reporting the same data but with a different comparison. Also, it should be Fisher's exact test, not Fischer's exact test.

Thank you, we have now corrected the spelling of Fisher's exact test. We feel that combining the two figures together may affect the clarity of the underlying message, and therefore have respectfully chosen to keep them separate. We have however added a legend to all figures to make them easier to interpret.

3. Can the authors comment on the apparent high rate of nasal and non-nasal symptoms in the control group and whether they believe this could have contributed to the lack of any clinical symptoms being demonstrated for the other groups? An independent assessor may have been useful here.

We recognise that there is a high number of symptoms in the control group. We believe this may be due in part to the small study sample size but also reflects the high sensitivity of the Likert scale used to record any symptoms. Since the symptoms were self-reported by participants and they were blinded to their intervention group, we feel that an independent assessor would have been unlikely to improve this.

4. There are a couple of instances where what is reported in the results is inconsistent with the data or discussion. For example, on lines 192-193 it states that "This study does not however report the effect of co-colonisation (SPN and virus) on symptoms" when this is clearly shown in Figure 5. Also on lines 207-208, it states that "…experimental SPN colonisation does not increase nasal or non-nasal symptoms" when in the results (lines 156-157) it states that "Experimental SPN colonisation rates were higher in the presence of virus….($p<0.05$)". Please correct. The p-value, if indeed significant, should be included in the Figure.

Thank you, the comment regarding symptoms from co-colonisation refers to the Rodrigues at al study. This has been made clearer in the text to avoid confusion.

In the discussion we note that experimental SPN colonisation does not increase nasal or non-nasal symptoms. In the results section we note that experimental SPN colonisation was higher in the presence of virus. This is referring to two separate points. Participants who are positive for the presence of a virus and SPN ('co-colonised') did not appear to have greater rates of symptoms. The figure relating to this data (Figure 5) has been edited to include the relevant p- values and a legend has been added for clarity.

5. What were the viruses that were detected, and were there any associations between specific viruses?

A variety of viruses were detected, however since these were only present in very small numbers, we were unable to ascertain any relationship between symptoms and these specific viruses. In future studies we aim to further investigate this relationship in more detail.

For most of the reported p-values there are no = sign. Please include.

Thank you, this has now been added.

Reviewer #2: General comments

This study uses a human challenge model to examine if symptoms are experienced in the context of pneumococcal colonisation among adults. The structure of the paper is acceptable, however there could be improvement made in the finer details, e.g. error bars/confidence intervals and raw numbers

I think it is also important to mention the limitations of this study e.g. not an RCT, low sample size, and only one serotype successfully colonised. Consequently I think some of the statements in the discussion should have the language softened, as this study is not the definitive study which demonstrates that colonisation is asymptomatic among adults. Rather it might suggest this, but further work using more serotypes, in larger RCTs is needed.

Thank you, we have modified the manuscript to take this into account. Changes have been made to the abstract, discussion and conclusion to recognise this.

Specific comments

Abstract

Methods section line 46

" ... reported between groups was compared ..." to groups were compared

Thank you, this has now been updated.

Results section line 49

S. pneumoniae (29 with serotype 6B, 17 with serotype 23F ...

Thanks, this has been updated

Conclusion section line 58

Pneumococcal colonisation using the EHPC was asymptomatic ...

Thanks, this has also been updated as requested.

Introduction

line 69 - correct typo, H. influenzae

Thanks, this has now been updated

line 70-73 - could be better worded

This has been re-worded for clarity.

lines 83-87 - suggest rearranging this paragraph e.g.

The experimental pneumococcal challenge model (EHPC) mimics natural colonisation in healthy ....vaccines in randomised control trials. We aimed to use the EHPC to investigate if the process is symptomatic....

Thank you this has now been updated.

Methods

Was a sample size calculation conducted?

Thank you for this feedback. We have not performed a sample size calculation as this was performed as a pilot study. In a larger follow up study we will ensure that a formal sample size calculation is done.

Results

line 134-137

please provide actual numbers, proportions, confidence intervals, and p-values.

These have been added where required.

figures - please add in confidence intervals.

Thank you, these have been included where required. A legend has been added to aid the interpretation.

Discussion

Lines 164 and 212

I would tone down the language here e.g.

This study provides evidence supporting the hypothesis that SPN colonisation among adults is asymptomatic

Thank you, this has been updated. We have taken this into account and altered the discussion and conclusion to reflect this.

line 171

Other than sample size, key limitations also include: the study wasn't randomised, only one serotype was assessed in this study, and pre-existing immunity.

The lack of randomisation to the allocated group and the use of a single successful serotype has been added as key limitations. As we used SPN 6B, a serotype that is not otherwise present in the community, it is unlikely that the participants have pre-existing immunity to this serotype. A line has been added to the discussion in order to address this.

Yours sincerely,

Dr Andrea Collins and Prof Daniela Ferreira