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1 **Title page: Pneumococcal Colonisation Rates in Patients Admitted to a UK Hospital**
2 **with Lower Respiratory Tract Infection – a prospective case-control study.**

3 **Authors: Andrea M Collins^{1,2#}, Catherine M K Johnstone², Jenna F Gritzfeld², Antonia**
4 **Banyard², Carole A Hancock¹, Angela D Wright^{2,3}, Laura Macfarlane¹, Daniela M**
5 **Ferreira², Stephen B Gordon²**

6 **Affiliations:** ¹Respiratory Infection Group, Royal Liverpool and Broadgreen University
7 Hospital Trust, Prescott Street, Liverpool, L7 8XP, UK

8 ²Respiratory Infection Group, Liverpool School of Tropical Medicine, Pembroke Place,
9 Liverpool, L3 5QA, UK

10 ³Local Comprehensive Research Network, Liverpool, UK

11 **Corresponding author# :** Dr Andrea Collins, Liverpool School of Tropical Medicine,
12 Pembroke Place, Liverpool, L3 5QA. 0151 705 3712, andrea.collins@liverpool.ac.uk

13 **Alternative corresponding author:** Professor Stephen Gordon, Liverpool School of
14 Tropical Medicine, Pembroke Place, Liverpool, L3 5QA. 0151 705 3169,
15 Stephen.gordon@lstmed.ac.uk

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18 andrea.collins@liverpool.ac.uk, cathjohnstone@hotmail.com, jenna.gritzfeld@lstmed.ac.uk,
19 antonia.banyard@cruk.manchester.ac.uk, Chancock@liverpool.ac.uk,
20 adwright@liverpool.ac.uk, lauraclairemcfarlane@hotmail.co.uk,
21 daniela.ferreira@lstmed.ac.uk, stephen.gordon@lstmed.ac.uk.

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23

24 **Abstract**

25 **Background:** Current diagnostic tests are ineffective at identifying the aetiological pathogen
26 in hospitalised adults with lower respiratory tract infection (LRTI). The association of
27 pneumococcal colonisation with disease has been suggested as a means to increase
28 diagnostic precision. We compared pneumococcal colonisation rate and density of nasal
29 pneumococcal colonisation by a) classical culture and b) quantitative real time *lytA*
30 Polymerase Chain Reaction (*qPCR*) in patients admitted to hospital in the UK with LRTI
31 compared to control patients.

32 **Methods:** 826 patients were screened for inclusion in this prospective case-control study. 38
33 patients were recruited, 19 with confirmed LRTI and 19 controls with another diagnosis.
34 Nasal wash (NW) was collected at the time of recruitment.

35 **Results:** Pneumococcal colonisation was detected in 1 LRTI patient and 3 controls ($p=0.6$)
36 by classical culture. Using *qPCR* pneumococcal colonisation was detected in 10 LRTI
37 patients and 8 controls ($p=0.5$). Antibiotic usage prior to sampling was significantly higher in
38 the LRTI than control group 19 v. 3 ($p<0.001$). Using a clinically relevant cut-off of >8000
39 copies/ml on *qPCR* pneumococcal colonisation was found in 3 LRTI patients and 4 controls
40 ($p > 0.05$).

41 **Conclusions:** We conclude that neither prevalence nor density of nasal pneumococcal
42 colonisation (by culture and *qPCR*) can be used as a method of microbiological diagnosis in
43 hospitalised adults with LRTI in the UK. A community based study recruiting patients prior to
44 antibiotic therapy may be a useful future step.

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48 **Introduction**

49 Recent studies suggest that detection and quantification of nasal pneumococcus by
50 quantitative real time *lytA* Polymerase Chain Reaction (qPCR) could be used to identify
51 pneumococcus as the aetiological pathogen in adults with pneumonia [1] and could be
52 useful as a disease severity marker [2]. In that study, South African patients with community
53 acquired pneumonia (CAP) were more frequently colonised than controls using classical
54 culture (44.9 v. 11.7%) and qPCR (62.8 v. 19.8%) and, in addition, patients with
55 pneumococcal CAP were also noted to have higher colonisation density than asymptomatic
56 controls [1]. By applying a cut off of 8000 copies/ml to the qPCR data Albrich et al [1] found
57 that 52.5% of patients were considered to have pneumococcal CAP, compared with 27.1%
58 diagnosed using standard tests.

59

60 The association of pneumonia and pneumococcal colonisation has been previously noted in
61 children, in whom those with radiological pneumonia were more frequently colonised with
62 pneumococci than those without [3] and had higher density colonisation than those with
63 bronchitis or without disease [4]. In contrast, in the elderly very low colonisation rates have
64 been shown; 0.3% in pneumococcal vaccine naive hospitalised Australians (by classical
65 culture) [5] (of which 10 had respiratory infection) and 2.3% in a Portuguese community
66 cohort [6]. In developed countries, pneumococcal colonisation rates in healthy adults are
67 between 1 - 18%, and are affected by age, immune status, antibiotic use, household
68 composition and contact with children [7, 8]. There are no published data on pneumococcal
69 colonisation in hospitalised patients with respiratory infection in the UK.

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71 We therefore aimed to determine the rate and density of pneumococcal colonisation by a)
72 classical culture and b) qPCR in hospitalised adult patients with LRTI when compared with
73 age and gender-matched controls in a developed country setting.

74

75 **Materials and Methods**

76 **Screening and Recruitment**

77 We recruited hospitalised adults with LRTI at the Royal Liverpool and Broadgreen University
78 Hospital from January - July 2013 within 72 hours (hrs) of admission. The syndrome of LRTI
79 was defined as; symptoms of respiratory infection with clinical signs +/- radiological
80 consolidation; therefore meeting a British Thoracic Society (BTS) definition of pneumonia as
81 used in community (GP) practice. Clinical signs of LRTI included ≥ 2 of: cough,
82 breathlessness, pleuritic chest pain, fever, increased or new sputum production. Exclusion
83 criteria were: patients with infective or non-infective exacerbations of chronic obstructive
84 pulmonary disease (IECOPD), asthma or bronchiectasis (without radiological consolidation),
85 aspiration pneumonia, oxygen saturations $< 86\%$ on air, suspected tuberculosis (TB) and
86 neutropenia. Patients with IECOPD were excluded as exacerbations are known to commonly
87 be due to viruses (up to 60%) [9, 10], *Haemophilus influenzae* (up to 33%) [11] and
88 *Moraxella catarrhalis* (around 10%) [12] rather than pneumococcus. Patients who had been a
89 hospital inpatient for ≥ 72 hrs or had recently been discharged from hospital ≤ 14 days before
90 were excluded since it is likely their nasal flora would have altered due to hospital
91 exposures. Patients with oxygen saturations $< 86\%$ on air were excluded since it was felt
92 unsafe to remove their oxygen in order to perform a nasal wash (NW).

93 A carefully selected control group of hospitalised patients with no signs of respiratory
94 infection were recruited within 7 days (where possible or as soon after as possible) of the
95 LRTI patient. The control group were matched for age (within 10 years of the LRTI patient)
96 and gender. Exclusion criteria were: oxygen saturations $< 86\%$ on air, neutropenia, ≥ 7 days
97 after admission and recent hospital discharge ≤ 14 days.

98 The study team were in regular communication with the hospital capacity team, the ward
99 based case managers, nursing and medical co-ordinators in accident and emergency (A&E),
100 the acute medical admissions unit (AMAU) and the respiratory wards from Monday to

101 Thursday. Through regular education, information dissemination events, and daily interaction
102 with the study team, key staff were made aware of the study and its aims, objectives, and
103 potential participants. A list of potential participants was generated on a daily basis in
104 combination with these personnel. To recruit LRTI patients we targeted screening to AMAU,
105 respiratory and infectious disease wards, for control participants we targeted surgical wards.

106 Patient eligibility was confirmed by review of the medical records; with permission of the
107 attending team, patient consent was sought prior to recruitment. Baseline clinical data of
108 age, gender, history of presenting complaint, past medical history, vaccination history,
109 antibiotic prescription, and contact with children (defined as at least alternate day contact
110 with children aged ≤ 10 yrs) were recorded. NW and urine samples were collected within 12
111 hours of recruitment. The study was approved by Liverpool East, North-west NHS Research
112 Ethics Committee (12/NW/0713) and registered with ClinicalTrials.gov (NCT01861184).

113 **Sampling**

114 NW was collected on the day of recruitment with a maximum of 20 mls of normal saline
115 instilled into the nasopharynx as previously described[13, 14]. A minimum of 5 mls of normal
116 saline was recovered and processed in all cases. Briefly, samples were immediately
117 centrifuged at high speed (3345 x g) to obtain a bacterial pellet [14]. Pellets were
118 resuspended in 100 μ l of skimmed-milk tryptone glucose glycerol (STGG) medium and the
119 total volume of the suspension was determined. Samples were then serially diluted on blood
120 agar and CFUs/mL of NW were determined the next day. In a subset of samples that
121 exceeded 7 mls a proportion of the sample (3 – 5 mls) was removed and centrifuged at 836
122 x g to obtain cellular material after which the supernatant was re-added to the rest of the
123 sample for the high speed spin.

124 **DNA extraction and qPCR**

125 DNA was extracted from 200 µl of the NW bacterial pellet stored in STGG using the QIAamp
126 DNA mini kit and the Centers for Disease Control protocol [15]. Briefly, the pellet was
127 resuspended in 200 µl TE buffer containing 0.04 g/ml lysozyme and 75 U/ml mutanolysin
128 (Sigma) and incubated at 37°C for 1 hr. Following incubation, 20 µl of proteinase K and 200
129 µl of Buffer AL were added to the sample which was vortexed and then incubated at 56°C for
130 30 min. The sample was then centrifuged briefly and 260 µl of ethanol was added. All
131 subsequent steps followed the manufacturer's instructions. DNA was eluted in 100 µl of
132 QIAGEN elution buffer and stored at -20°C.

133 Colonisation density was determined by targeting the pneumococcal autolysin *lytA* gene
134 [16]. A no-template control, a negative extraction control (parallel extraction of TE buffer) and
135 a *S. pneumoniae* (BHN418) positive control were included in each run. DNA was amplified
136 with the Mx3005P system (Stratagene) and data was analysed using the instrument
137 software. A sample was considered positive if both duplicates had a cycle threshold (C_T)
138 value below a mean 35. Values of >8000 copies/ml were considered clinically relevant as
139 per Albrich and colleagues [1].

140 **Binax**

141 An immunochromatographic membrane test (ICT) (BinaxNOW *Streptococcus pneumoniae*;
142 Binax) was performed on all patient's unconcentrated urine specimens, according to the
143 manufacturer's recommendations.

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149 **Results**

150 **Screening and recruitment**

151 We screened 826 patients and recruited 19 LRTI and 19 control (age, gender and season
152 matched) patients. 217 were 'potential' LRTI patients, of which 198 were not eligible (Figure
153 1). 58 patients did not have a syndrome of LRTI (acute exacerbation [AE] COPD n=22, AE
154 bronchiectasis n = 5, AE asthma n=3, AE pulmonary fibrosis n= 1 and alternative diagnoses
155 including pulmonary embolus (PE), congestive cardiac failure (CCF), sepsis of unknown
156 cause and adult acute respiratory distress syndrome (ARDS) n = 30), 36 patients did not
157 have capacity to consent (predominately due to dementia or acute delirium), 48 patients
158 were identified >72 hours after admission and 17 after a recent hospital discharge ≤14 days
159 before, 20 patients declined to participate and 2 'next of kin' refused permission for their
160 relative to participate, 10 oxygen saturations <86% on air, 12 aspiration pneumonia, 14 were
161 excluded for other reasons.

162 We planned to recruit 100 patients to each arm of the study, but stopped recruiting on the
163 grounds of futility after interim analysis noted 100% antibiotic usage prior to recruitment in
164 the LRTI group and low rates of colonisation (on culture) and due to high screen failure rate
165 (778/816, 95.4%). Recruiting age matched controls was difficult, especially for the younger
166 LRTI patients (aged 36 - 46 yrs old). In 9 cases, the time between the recruitment of the
167 LRTI patient and control was >7 days (range 9 - 43). We know from our Experimental
168 Human Pneumococcal Colonisation (EHPC) studies that antibiotic usage terminates
169 pneumococcal colonisation, meaning that continued recruitment in this population was
170 unethical.

171 **Sampling: Rate of colonisation**

172 All patients were successful at providing a nasal sample. One patient was unable to replicate
173 the NW technique (as per protocol) and therefore had a nasopharyngeal swab instead. NW
174 volumes were not significantly different between LRTI patients and controls (Table 1).

175 Pneumococcal colonisation was detected using classical microbiology in 1 LRTI patient and
176 3 controls ($p=0.6$). Using *qPCR* 10 LRTI patients and 8 controls were positive ($p=0.516$)
177 [Table 2]]. One of the controls was positive for colonisation by culture but considered
178 negative by *qPCR* as the C_T value was >35 .

179 **Sampling: Density of colonisation by *qPCR***

180 For *qPCR* a cut off value of >8000 copies/ml was used to define clinical relevance [1]. In our
181 study, 3 LRTI patients and 4 controls had values >8000 copies/ml. Of the 3 LRTI patients,
182 only 1 was culture positive; of the 4 controls, 2 were culture positive (Table 2). Of the 4
183 patients overall who were culture positive, 3 had >8000 copies/ml, 1 in the LRTI and 2 in the
184 control group.

185 **Clinical data**

186 Antibiotic usage prior to sampling was significantly higher in LRTI patients than controls 19 v.
187 3 ($p<0.001$). Radiological consolidation was present in 7 out of 19 LRTI patients; only 2 out
188 of 38 urine samples were positive using BinaxNOW. None of the LRTI patients recruited
189 were pneumococcal sputum or blood culture positive. There were no statistical differences
190 between the groups with regards to smoking, contact with children age <10 yrs old or 23 PPV
191 *pneumovax* vaccination (Table 1).

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198 **Discussion**

199 The anticipated high rate of pneumococcal colonisation (by culture +/- qPCR) in the LRTI
200 group was not found, given that antibiotic usage (pre-admission/pre-recruitment) was
201 significantly different between LRTI and control groups, with all LRTI patients having
202 received at least 2 doses prior to NW, this is likely to have resulted in culture negativity.
203 However we also found no significant difference in colonisation rates using qPCR and
204 colonisation density between the LRTI and control groups. There was also no significant
205 difference in colonisation rates in polysaccharide vaccinated (23 PPV *Pneumovax*) and
206 unvaccinated patients, consistent with previous literature reporting that the vaccine does not
207 protect against colonisation [17, 18].

208 Large numbers of patients were referred as 'potential LRTI'. Alternative diagnoses such as
209 PE, CCF, non-infective exacerbation of pulmonary fibrosis, sepsis of unknown cause and
210 aspiration pneumonia were common. This diagnostic imprecision has important implications
211 for the use of NW as a diagnostic technique since it would lead to many inappropriate
212 samples being collected. We have previously demonstrated that confusion is common in
213 LRTI patients (>20%) [19]. LRTI rates increase with age (63% CAP admissions were aged
214 >65 and 25% ≥85yrs old) [20] as do rates of comorbidities (including dementia), therefore
215 recent hospital admission is also common.

216 The main strength of this study is the large number of screened patients; the LRTI patients
217 are well phenotyped and the controls are matched in age, gender and time with similar
218 smoking habits, 23 PPV *pneumovax* vaccination rates and child contact. Our cohort was not
219 'CAP' by strict definition of radiological consolidation, instead a broad study group of LRTI
220 was chosen due to its clinical relevance in UK hospital practice and admissions, making
221 these results very generalisable. Nationally, GP antibiotic prescribing for LRTI is very high,
222 but lower for clinically diagnosed CAP (due to usual immediate hospitalisation) [21].

223 Accurately diagnosing pneumonia is challenging; inter-doctor variability in reporting of
224 radiological pneumonia is common [22]. Studies of patients that have radiological
225 pneumonia as an inclusion criterion may be less applicable to everyday hospital medicine.
226 LRTI may be a more useful term for this clinical syndrome, particularly in instances where
227 guidelines suggest clinical rather than radiological diagnosis [20]. Liverpool is in the North-
228 west of England, and has the second highest LRTI rate (age standardised episodes/1000
229 person years) and the third highest CAP rate nationally. [21] It is therefore an ideal area for
230 recruiting to respiratory infection studies, although community antibiotic prescription rates
231 are high. The Royal Liverpool hospital has ~1400 admissions per year that are coded as
232 'pneumonia', approximately 20% of these cases are not community acquired or have no
233 radiological features of pneumonia.

234 Limitations of the study include that this is a single centre study which may reduce the
235 generalisability of the results specifically in areas where community antibiotic prescription
236 rates are lower, that we were unable to fully recruit to the study despite high numbers
237 screened and that the NW technique, rather than nasopharyngeal swab, for pneumococcal
238 isolation may not have been ideal in this elderly population, since the research nurses noted
239 poor technique and lower yields than in the cohort of healthy volunteers in which we
240 commonly use this technique (data not shown). Nevertheless, patient comfort is higher [23]
241 and sensitivity for colonisation density is very high [24]. We know from our Experimental
242 Human Pneumococcal Colonisation (EHPC) studies that antibiotic usage terminates
243 pneumococcal colonisation; after interim analysis noted 100% antibiotic usage in the LRTI
244 group prior to recruitment and low rates of colonisation (on culture), the study was stopped
245 as continued recruitment in this population was unethical.

246 Previous studies have shown colonisation rates of 44.9% and 62.8% in patients with
247 radiologically confirmed CAP compared to 11.7% and 19.8% in controls, by culture and
248 qPCR respectively [1]; in comparison we detected colonisation of 5% and 15.8% (>8000
249 copies/ml) in patients with LRTI and 15.8% and 21.0% (>8000 copies/ml) in controls. We

250 therefore noted high rates of PCR positivity in both groups and low rates of culture positivity
251 in our LRTI patients compared with the CAP patients in this previous study. The differences
252 between the two studies may be due to the fact that our patient cohort was considerably
253 older (64.5 v. 38.4 yrs old) [1], had low rates of radiologically confirmed pneumonia (36.8%),
254 high rates of prior antibiotic treatment, high rates of contact with children and are presumed
255 HIV uninfected (overall HIV incidence is low in Liverpool - 15 per 100000, with a prevalence
256 of 0.17% in 2011 [unpublished local data]). Previously in Liverpool we found natural
257 colonisation rates in healthy non-smoking volunteers of 10% by classical culture (25/249,
258 age 23 yrs old [SD \pm 5.7]) [unpublished data]. The higher rate (15.8%) in this cohort may be
259 related to their high rates of contact with children and smoking history.

260 *q*PCR can deliver results within a few hours (usually 3-6hrs) and could impact the critical
261 phase of early clinical care [25], however it does not distinguish between viable (live) and
262 non-viable (dead) bacteria or determine whether the bacteria is a pathogen or a coloniser
263 [26, 27]. Specificity can also be an issue with *q*PCR and there have been concerns that *lytA*
264 may not discriminate between *S. pneumoniae* and *S. viridans*, however *lytA* is currently the
265 most widely used target gene for pneumococcus and we have previously shown that our
266 assay specificity [24] is in line with that reported by others [16].

267 Within this cohort all LRTI patients had taken antibiotics prior to sampling, which likely
268 accounts for the higher positivity rate of *q*PCR over culture. Prior antibiotic treatment can
269 lower plasma and pleural fluid PCR loads [28] as well as sputum and blood culture positivity.
270 It is not known how rapidly pneumococcus will be undetectable by *q*PCR in the NW of those
271 previously colonised with pneumococcus after antibiotic therapy.

272 Albrich and colleagues suggest that a density of 10^3 - 10^4 may be the critical density at which
273 colonisation leads to infection [1]; however we have found densities as high or higher in our
274 cohort of healthy volunteers after experimental colonisation without infection [24, 29].
275 Colonisation density was not different in LRTI and controls, we also found high mean

276 densities $\geq 10^3$ in those without infection (n = 4 controls). It is possible therefore that if
277 colonisation is dense and in the setting of the correct clinical syndrome then the
278 pneumococcus is a likely pathogen. Again an important difference between the two study
279 groups may be HIV infection status. Only 10.5% (2/19) of our LRTI group were Binax
280 positive compared to 72.7% in patients with non-bacteraemic CAP in another study [1].
281 Binax results remain positive for at least 7 days after the initiation of antibiotic treatment [30];
282 notably our urine samples were taken up to 72hrs after admission but often several days
283 after antibiotics had been started. Previous antibiotic therapy has been noted to decrease
284 culture and *qPCR* positivity by up to 50% [1].

285

286 **Conclusion**

287 We have shown that pneumococcal colonisation (assessed by culture and *qPCR*) cannot be
288 used as a method of diagnosis in pneumococcal blood culture negative hospitalised adults
289 with LRTI in the UK, since such patients have already received community antibiotics and
290 the laboratory test is non-discriminatory. Further, the number of adults tested for 'potential
291 LRTI' on screening would be impracticable in terms of staff resource. A community based
292 study recruiting patients prior to antibiotic therapy may however be a useful future step.

293

294 **List of abbreviations:**

295 Lower respiratory tract infection (LRTI)
296 Nasal wash (NW)
297 Quantitative real time *lytA* Polymerase Chain Reaction (*qPCR*)
298 Community acquired pneumonia (CAP)
299 Accident and emergency (A&E)
300 Acute medical admissions unit (AMAU)

301 Pulmonary embolus (PE)

302 Congestive cardiac failure (CCF)

303 Adult acute respiratory distress syndrome (ARDS)

304

305 **Competing interests:**

306 No authors have any competing interests to declare. The authors have had no support from
307 any organisation for the submitted work, no financial relationships with any organisations
308 that might have an interest in the submitted work in the previous three years and no other
309 relationships or activities that could appear to have influenced the submitted work.

310

311 **Author contributors:**

312 A M Collins was involved in writing and submitting the protocol and ethics, study co-
313 ordination, data collection, statistical planning and analysis and manuscript preparation.

314 A Banyard was involved in sample processing and manuscript editing.

315 C M K Johnstone was involved in screening and recruiting participants, sample collection
316 and processing and manuscript editing.

317 A D Wright was involved in study co-ordination, screening and recruiting participants, sample
318 collection, data collection, statistical analysis and manuscript editing.

319 J F Gritzfeld was involved in protocol writing, sample processing, data collation and
320 interpretation, and manuscript preparation.

321 L Macfarlane was involved in study co-ordination, screening and recruiting participants,
322 sample collection and manuscript editing.

323 C A Hancock was involved in study co-ordination, screening and recruiting participants,
324 sample collection and manuscript editing.

325 D M Ferreira was involved in writing the protocol and ethics submission, laboratory co-
326 ordination, sample processing and storage and manuscript editing.

327 S B Gordon was chief investigator and was involved in editing the protocol, ethics
328 submission and manuscript preparation.

329 D Shaw was involved in screening and recruiting participants and sample collection.

330 S H Pennington was involved in sample processing.

331 A M Collins is the guarantor of the above.

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443 **Figure legends:**

444 Figure 1: Screening and recruitment flowchart. Reasons for non-recruitment for lower
445 respiratory tract infection (LRTI) patients are detailed. Total no. screened n = 826. Note
446 multiple reasons for non-recruitment per patient were possible.

447 **Tables:**

448 Table 1: Baseline demographics, antibiotic Status, nasal wash volume returned and
 449 evidence of pneumococcal disease investigation results of patients with lower respiratory
 450 tract infection (LRTI) and age and gender matched hospitalised controls.

451

	LRTI (n=19)	Control (n=19)	p value
Gender: Male n (%)	9 (47.4)	9 (47.4)	1.000 *
Age Years \pm SD	64.47 \pm 15.78	64.58 \pm 14.50	0.954 β
Smoker/ ex-smoker n (%)	15 (78.9)	10 (52.6)	0.170 α
23 PPV <i>Pneumovax</i> n (%)	7 (36.8)	8 (42.1)	0.740 *
Contact with children n (%)	10 (52.6)	12 (63.2)	0.511 *
Antibiotics at time of recruitment n (%)	19 (100)	3 (15.8)	0.0001 α
Nasal wash volume returned (ml) \pm SD	10.14 \pm 3.14	10.36 \pm 4.83	0.855 β
Evidence of pneumococcal disease: Binax urine test positive n (%)	2 (10.5)	0 (0)	0.486 α
Evidence of pneumococcal disease: Blood or sputum culture positive n %	0 (0)	N/A	N/A

452 *Chi Square, β Mann Whitney U test, α Fisher's Exact, SD standard deviation, PPV

453 polysaccharide vaccine

454 Table 2: Pneumococcus identification (by culture, qPCR) and density (by qPCR) in patients
 455 with lower respiratory tract infection (LRTI) and age and gender matched hospitalised
 456 controls.
 457 Note low rates of culture positivity and high rates of qPCR positivity in both LRTI and control
 458 groups.
 459

	LRTI (n=19)	Control (n=19)	p value
Culture positive n (%)	1 (5)	3 (15.8)	0.604 α
qPCR positive n (%) at detection limit	10 (52.6)	8 (42.1)	0.516 *
Density (by qPCR) copies/ml (geometric mean) [95% CI]	3066 [1225 – 7675]	2208 [244 – 19972]	0.408 β
Clinically relevant density (by qPCR) >8000 copies/ml	3	4	0.999 α

460 α Fisher's Exact, *Chi squared, β Mann Whitney U test, qPCR quantitative polymerase chain
 461 reaction

