1 **Title page: Pneumococcal Colonisation Rates in Patients Admitted to a UK Hospital**

2 **with Lower Respiratory Tract Infection – a prospective case-control study.**

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Abstract

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

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

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

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

Introduction

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

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

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

Materials and Methods

Screening and Recruitment

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

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

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

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

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

Sampling

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

DNA extraction and qPCR

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

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

Binax

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

Results

Screening and recruitment

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

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

Sampling: Rate of colonisation

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

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

Sampling: Density of colonisation by *q*PCR

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

Clinical data

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

List of abbreviations:

Lower respiratory tract infection (LRTI)
Nasal wash (NW)
Quantitative real time *lytA* Polymerase Chain Reaction (*q*PCR)
Community acquired pneumonia (CAP)
Accident and emergency (A&E)
Acute medical admissions unit (AMAU)

Pulmonary embolus (PE)

Congestive cardiac failure (CCF)

Adult acute respiratory distress syndrome (ARDS)

Competing interests:

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

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Figure legends:

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

Tables:

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

	LRTI (n=19)	Control (n=19)	p value
Gender: Male n (%)	9 (47.4)	9 (47.4)	1.000 *
Age Years ± SD	64.47 ± 15.78	64.58 ±14.50	0.954 β
Smoker/ ex-smoker n (%)	15 (78.9)	10 (52.6)	0.170 α
23 PPV *Pneumovax* 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) ± SD	10.14 ± 3.14	10.36 ± 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

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

454 Table 2: Pneumococcus identification (by culture, *q*PCR) and density (by *q*PCR) 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 *q*PCR 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
*q*PCR positive n (%) at detection limit	10 (52.6)	8 (42.1)	0.516 *
Density (by *q*PCR) copies/ml (geometric mean) [95% CI]	3066 [1225 – 7675]	2208 [244 – 19972]	0.408 β
Clinically relevant density (by *q*PCR) >8000 copies/ml	3	4	0.999 α

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