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Association between pneumococcal load and disease severity in adults with pneumonia

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Determination of pneumococcal load by quantitative PCR may be useful for diagnostic and prognostic purposes. We hypothesized that higher pneumococcal load would be associated with increased pneumonia severity. Therefore, we tested serum, sputum and urine specimens from 304 adults with community-acquired pneumonia by using a quantitative *lytA* pneumococcal real-time PCR assay. The association between pneumococcal load and disease severity was assessed using several markers of severity: CURBage score, PSI risk class, intensive care unit admission, in-hospital death and admission duration. For PCR-positive specimens, the bacterial loads were higher in sputum specimens [median 8.55×10^5 copies ml^{-1}; interquartile range (IQR) 4.70×10^4–4.69×10^6 copies ml^{-1}] than either serum (median 180 copies ml^{-1}; IQR 165–8970 copies ml^{-1}) or urine (median 623 copies ml^{-1}; IQR 510–650 copies ml^{-1}). Detection of pneumococcal DNA in serum was associated with severe disease, and there was evidence of a dose–response effect with increased bacterial load being associated with increased severity. The same observations were not observed for other specimen types. This study adds to an increasing body of evidence suggesting that determination of pneumococcal load has a clinical utility. Further work is needed to determine whether measuring pneumococcal load in respiratory specimens from adults will differentiate colonization from coincidental carriage.

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INTRODUCTION

Streptococcus pneumoniae is one of the most important human pathogens, causing a spectrum of diseases, including pneumonia, bacteraemia, meningitis, sinusitis and otitis media. Effective management and surveillance of pneumococcal disease is hampered by the difficulty in accurately diagnosing infections caused by *S. pneumoniae*. Currently available laboratory tests have either too low sensitivity to diagnose pneumococcal disease and/or fail to differentiate between the presence of *S. pneumoniae* as a colonizer or as an invasive pathogen (Werno & Murdoch, 2008). PCR assays have improved the ability to detect many pneumonia-causing pathogens, although the application of PCR to diagnose pneumococcal disease has been limited. However, there is emerging evidence that a high pneumococcal load detected in some clinical specimens may be associated with worse patient outcomes, including septic shock and mortality (Waterer & Rello, 2011).

In the present study, we tested a variety of clinical specimens from adults with community-acquired pneumonia (CAP) using a quantitative pneumococcal real-time PCR assay. We hypothesized that a higher pneumococcal load would be: (i) detected in specimens from patients with positive blood cultures, sputum cultures and urinary antigen test results for pneumococcus; and (ii) associated with increased disease severity.

Abbreviations: CAP, community-acquired pneumonia; CI, confidence interval; IQR, interquartile range; OR, odds ratio; PSI, pneumonia severity index.

METHODS

Subjects. From July 1999 to July 2000, 304 adult patients aged ⩾18 years admitted to Christchurch Hospital, Christchurch, New Zealand, were enrolled in a study of CAP. Inclusion and exclusion criteria were based on those used in previous studies (British Thoracic Society, 1987; Neill *et al.*, 1996). Detailed descriptions of the study, including patient demographics and clinical presentations, have been previously published. Important characteristics of the study population included that 29 % of the patients had chronic obstructive pulmonary disease (COPD), 13 % suffered from asthma and 19 % were current smokers (Laing *et al.*, 2001; Murdoch *et al.*, 2001, 2003; Jennings *et al.*, 2008).

All patients presented with an acute illness with clinical features of pneumonia and radiographic shadowing in one lobe or at least a lung segment, which was neither pre-existing, nor attributable to a known cause. Patients were only included if pneumonia was the main reason for admission and not a terminal event, and when the affected lung tissue was not due to pneumonia distal to bronchial obstruction, bronchiectasis or previous tuberculosis.

The study was approved by the Canterbury Ethics Committee.

Specimens. Serum, sputum and urine specimens were collected from the patients on admission to hospital and were stored at −80 °C

until tested. The quality of sputum for culture was assessed in accordance with accepted criteria (Murray & Washington, 1975) and only sputa of acceptable quality were cultured.

Sample DNA extraction and PCR amplification. Between 0.5 and 1 ml of thawed serum, sputum and urine underwent DNA extraction with the Promega DNA extraction kit (Abbott Laboratories) according to the manufacturer's instructions. The extraction was performed on an automated M2000sp platform (Abbott Laboratories).

Qualitative real-time PCR amplification. The real-time duplex PCR included the *lytA* gene target as well as an in-house inhibitor control (unpublished). The pneumococcal *lytA* gene sequence was detected using the lytA-CDC forward primer (5′-ACGCAATCTAGCAG-ATGAAGCA-3′) and reverse primer (5′-TCGTGCGTTTTAATTC-CAGCT-3′) as previously described and a FAM-labelled lytA-CDC reversed probe (5′-6FAM–CTCCCTGTATCAAGCGTTTTCGGCA–BBQ) with a reverse strand modification of the CDC probe used in previous publications (Carvalho *et al.*, 2007).

Real-time duplex PCR assays were performed in a final volume of 30 µl, containing 10 µl DNA, on the ABI 7500 RT-PCR system (Applied Biosystems) with Abbott optical 96-well reaction plates and adhesive covers. The PCR mixture contained 2× Universal TaqMan Gene Expression Master Mix (Applied Biosystems) with each primer at a concentration of 0.5 µM, and probes were used at a concentration of 0.2 µM. A positive *S. pneumoniae* extraction and a 'no template' control (molecular grade water) were included in each amplification run. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Analysis of qualitative results was performed with ABI 7500 software version 2.0.1.

Quantitative real-time-PCR amplification. All positive samples underwent a quantitative single-plex RT-PCR assay with the same PCR conditions and *lytA*-targeting primers and probes. An internal control was not included at this stage. However, 50× Rox dye fluorochrome was included in each run to verify PCR efficiency across the optical 96-well plate. The standard curve was set up using 10-fold dilutions of a known concentration of *S. pneumoniae*, established by colony plate count. The standard included six concentrations from 1.8×10^6 to 1.8×10^1 c.f.u. ml^{-1}. Analysis of quantitative data was performed with ABI 7500 software v 2.0.1.

Statistical analysis. Data were analysed with Stata/IC 11.0 software (StataCorp). Dichotomous variables were analysed with McNemar's test and continuous variables with the Wilcoxon rank sum test. Logistic regression analysis was used to assess independent associations with severe disease. Five different measures of severity were assessed: (i) CURBage score (Lim *et al.*, 2003) >2, (ii) pneumonia severity index (PSI) risk class (Fine *et al.*, 1997) IV or V, (iii) intensive care unit admission, (iv) in-hospital death and (5) hospital stay >5 days. The other variables included in each model were: *lytA* PCR results (binary variable or grouped as 0, 1–1000, 1001–10^5 and >10^5 c.f.u. ml^{-1}), age (years), gender (binary variable), duration of symptoms prior to admission (days), status as a previous or current smoker (binary variable) and antibiotic use prior to admission (binary variable). For all logistic regression analyses, likelihood ratio tests were used to compare models with and without interaction terms.

RESULTS

Of the 304 enrolled patients, 48 % were female and 52 % were male. At the time of enrolment, blood was drawn for various routine investigations, including microbiological culture. Of the 304 patients, 303 provided serum, 196 provided sputum and 256 provided urine specimens, which were used for PCR testing, and 274 patients had blood cultures taken on admission, which were available for comparison to PCR results from serum. Overall, 173 patients had a complete set of serum, sputum and urine samples available for PCR testing.

Analytical sensitivity of quantification

The lower limit of detection in the PCR was <10 copies of the *lytA* gene, which is in agreement with previously published data (Carvalho *et al.*, 2007). The lowest quantifiable dilution of *lytA* in clinical specimens was reproducible to the level of 10^2 c.f.u. ml^{-1}.

Comparison of qualitative PCR targeting the *lytA* gene with other diagnostic tests

Table 1 shows the comparison of qualitative PCR results targeting the *lytA* gene with other diagnostic tests on the same patients. Statistically significant differences were noted between the performance of the *lytA* gene-targeting PCR and all other tests under comparison. The PCR detected *S. pneumoniae* in all but two sputum specimens that were culture-positive, but also detected *S. pneumoniae* in an additional 51 culture-negative specimens. PCR detected *S. pneumoniae* in serum from 6 out of the 11 patients with positive blood cultures and in urine from only 4 out of the 69 patients with positive urinary antigen tests.

Quantitative PCR results

For PCR-positive specimens, the *S. pneumoniae* bacterial loads were higher in sputum specimens [median 8.55×10^5 copies ml^{-1}; interquartile range (IQR) 4.70×10^4–4.69×10^6 copies ml^{-1}] than either serum (median 180 copies ml^{-1}; IQR 165–8970 copies ml^{-1}) or urine (median 623 copies ml^{-1}; IQR 510–650 copies ml^{-1}) specimens.

Figs 1 and 2 show the bacterial loads in all PCR-positive specimens for two specimen types and for culture-positive and -negative results. It is of note that bacterial loads were statistically significantly higher in culture-positive compared to culture-negative sputum specimens and in serum from patients with positive blood cultures compared to those with negative blood cultures. Positive and negative urine antigen detection tests were compared to bacterial loads using the PCR assay to detect the *lytA* gene but they did not show any correlation between higher bacterial loads and positive antigen tests in the urine. Only six data points were analysed (data not shown).

PCR results and antibiotic use

Eighty-eight patients had received antibiotics prior to admission, including amoxicillin (12), coamoxyclav (31), macrolides (32) and other antibiotics (13). Detection of *S. pneumoniae* by PCR in sputum was statistically significantly

Table 1. Comparison of qualitative *lytA* PCR results with those of other diagnostic tests on the same sample types.

Values represent number of samples that tested positive or negative by diagnostic tests. Statistically significant differences were noted between all three comparisons (sputum culture, $P<0.0001$; blood culture, $P=0.02$; urine antigen, $P<0.0001$).

		***lytA* PCR on sputum**	
		Negative	Positive
Sputum culture	Negative	97	51
	Positive	2	46
		***lytA* PCR on serum**	
		Negative	Positive
Blood culture	Negative	246	17
	Positive	5	6
		***lytA* PCR on urine**	
		Negative	**Positive**
Urine antigen	Negative	185	2
	Positive	65	4

less common in patients who had been administered antibiotics prior to hospitalization and specimen collection [odds ratio (OR) 0.26; 95 % confidence interval (CI) 0.13 to 0.52, $P<0.0001$]. There was no association between prior antibiotic use and PCR positivity in either serum (OR 0.43; 95 %CI 0.15 to 1.33, $P=0.13$) or urine (OR 2.55; 95 % CI 0.50 to 13.00, $P=0.24$) specimens. There were also no statistically significant differences between bacterial loads with or without antibiotic use for each of the three specimen types (data not shown).

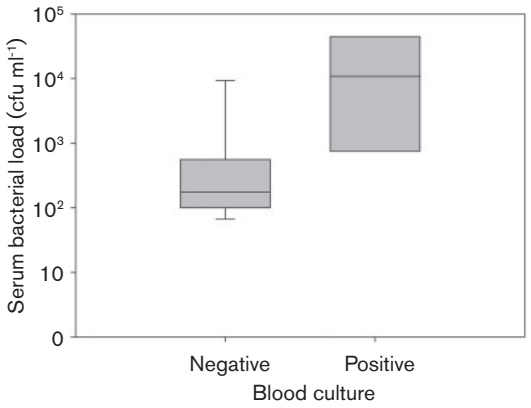

Fig. 1. *S. pneumoniae* bacterial load as determined by PCR targeting the *lytA* gene in serum from blood-culture-positive and -negative patients. The box and whisker plots show the median, 25th and 75th percentiles, and 10th and 90th percentiles (negative results only). <10 data points were obtained for positive patients; therefore, 10th and 90th percentiles are not shown. $P=0.02$ for the difference between medians.

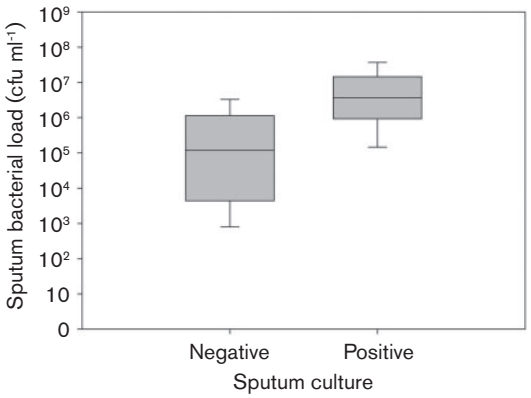

Fig. 2. *S. pneumoniae* bacterial load as determined by PCR targeting the *lytA* gene in sputum from sputum-culture-positive and -negative patients. The box and whisker plots show the median, 25th and 75th percentiles, and 10th and 90th percentiles. $P=0.02$ for the difference between medians.

Association of PCR results with disease severity

Overall, 71 patients had a CURBage score >2, 146 were PSI risk class IV or V, eight were admitted to the intensive care unit, 11 died in hospital and 160 had a hospital stay greater than 5 days. Table 2 shows the associations between qualitative PCR results and other pneumococcal diagnostic tests (blood culture, sputum culture, urinary antigen) with disease severity after adjusting for age, gender, duration of symptoms, smoking history and prior antibiotic use. The inclusion of an interaction term between sputum pneumococcal PCR and smoking significantly improved the model for severe disease as assessed by PSI risk class assignment. No other interaction terms significantly improved any of the models. Table 3 shows the results for the same models run with quantitative PCR results, showing the association between pneumococcal load and disease severity.

Detection of pneumococcus in serum by PCR was associated with four markers of severe disease; with three markers (CURBage score, PSI risk class and ICU admission) there were increased odds of severe disease with increasing bacterial load. Detection of pneumococcus in urine associated with severe disease was established with the CURBage score only, while detection in sputum was only associated with severe disease when assessed by using the PSI risk class in patients who had never smoked. The inclusion of additional variables for the detection of other viral or bacterial pathogens did not significantly change any of these findings (data not shown).

Table 2. Association between qualitative PCR results and other pneumococcal diagnostic tests

Outcome	Specimen and test	OR (95 % CI)	*P*-value
Severe disease (CURBage score)	Serum PCR	2.91 (1.07–7.96)	0.04
	Sputum PCR	1.59 (0.73–3.43)	0.24
	Urine PCR	14.00 (1.72–114.02)	0.01
	Blood culture	3.71 (0.96–14.37)	0.06
	Sputum culture	1.21 (0.52–2.81)	0.66
	Urinary antigen	2.78 (1.35–5.73)	0.006
Severe disease (PSI risk class)	Serum PCR	6.85 (1.99–23.52)	0.002
	Sputum PCR (previous/current smoker)	2.54 (1.05–6.17)	0.04
	Sputum PCR (never smoked)	0.14 (0.01–1.38)	0.09
	Urine PCR	2.12 (0.22–20.52)	0.52
	Blood culture	2.52 (0.42–15.18)	0.31
	Sputum culture	1.65 (0.69–3.96)	0.26
	Urinary antigen	1.88 (0.88–4.01)	0.10
ICU admission	Serum PCR	10.48 (2.23–49.34)	0.003
	Sputum PCR	5.32 (0.59–47.56)	0.14
	Urine PCR	*	
	Blood culture	19.72 (3.23–120.21)	0.001
	Sputum culture	1.12 (0.20–6.40)	0.90
	Urinary antigen	2.41 (0.57–10.20)	0.23
Death	Serum PCR	1.75 (0.20–15.73)	0.62
	Sputum PCR	0.93 (0.12–6.87)	0.94
	Urine PCR	*	
	Blood culture	3.46 (0.33–36.53)	0.30
	Sputum culture	*	
	Urinary antigen	3.31 (0.69–15.84)	0.13
Length of admission >5 days	Serum PCR	9.92 (2.96–33.28)	<0.001
	Sputum PCR	0.84 (0.42–1.68)	0.63
	Urine PCR	2.66 (0.32–22.00)	0.36
	Blood culture	11.02 (1.22–99.63)	0.03
	Sputum culture	0.76 (0.36–1.60)	0.48
	Urinary antigen	1.37 (0.72–2.63)	0.33

*Convergence not achieved.

DISCUSSION

A main finding of this study was that detection of pneumococcal DNA in serum was associated with severe disease in adults with CAP and, for some variables, increased bacterial load was associated with increased disease severity. We did not observe an association between bacterial load and disease severity for sputum specimens, except for one group of patients; previous and current smokers, who were more likely to be in one of the higher PSI risk classes if pneumococcal DNA was detected in sputum. There was an association between pneumococcal DNA in urine and disease severity as assessed by the CURBage score, but this was not evident with other markers. The significance of the latter two findings is unclear and may represent chance findings; therefore, these tests need to be replicated in other patient populations.

The findings from serum PCR in this study are consistent with other recent reports. Among Spanish adults with CAP, high pneumococcal loads in blood were associated with increased risk of both septic shock and mortality (Rello *et al.*, 2009). This study also found that there was a marked increase in mortality above a bacterial load of 10^5 c.f.u. ml^{-1}. The relatively small number of deaths in our study will have limited our ability to demonstrate any associations with mortality. Pneumococcal load in blood has also been associated with the development of systemic inflammatory response syndrome in adults with CAP (Peters *et al.*, 2009). In children with meningitis and pneumonia, pneumococcal load was also associated with mortality as well as inflammatory markers (Carrol *et al.*, 2007).

The use of PCR as a diagnostic tool for pneumococcal disease has had mixed results. Qualitative PCR has been useful for detection of pneumococcal DNA in blood specimens, especially from children (Azzari *et al.*, 2011; Marchese *et al.*, 2011), but detection in respiratory specimens has been difficult to interpret because of the inability to differentiate colonization from infection (Dagan *et al.*, 1998; Murdoch *et al.*, 2003). The choice of PCR assay can have a noticeable

Table 3. Association between pneumococcal load and disease severity

Pneumococcal loads in urine specimens were not included in this analysis as all were <1000 c.f.u. ml^{-1}.

Outcome	Specimen	Pneumococcal load (c.f.u. ml^{-1})	OR (95 % CI)	*P*-value
Severe disease (CURBage score)	Serum	0	1.00	
		1–1000	1.53 (0.42–5.57)	0.52
		1001–10^5	9.80 (1.76–54.50)	0.009
Severe disease (PSI risk class)	Serum	0	1.00	
		1–1000	5.22 (1.30–21.01)	0.02
		1001–10^5	13.58 (1.55–118.86)	0.02
	Sputum (previous/current smoker)	0	1.00	
		1–1000	0.35 (0.03–4.47)	0.42
		1001–10^5	4.68 (0.97–22.49)	0.05
		>10^5	2.73 (1.01–7.36)	0.05
	Sputum (never smoked)	0	1.00	
		1–1000	0.02 (0.0002–1.35)	0.07
		1001–10^5	0.40 (0.02–9.36)	0.57
		>10^5	0.10 (0.007–1.40)	0.09
ICU admission	Serum	0	1.00	
		1–1000	8.44 (1.26–56.58)	0.03
		1001–10^5	13.55 (1.95–94.16)	0.008
Length of admission >5 days	Serum	0	1.00	
		1–1000	12.90 (2.87–58.01)	0.001
		1001–10^5	6.15 (0.96–39.50)	0.06

effect on results. In a previous study, we used a different assay that targeted the pneumolysin (*ply*) gene (Murdoch *et al.*, 2003). This assay has since been shown to have relatively low specificity (Whatmore *et al.*, 2000; Messmer *et al.*, 2004; Suzuki *et al.*, 2005; Carvalho *et al.*, 2007), especially compared with pneumococcal *lytA*-based PCR assays (Sheppard *et al.*, 2004; Abdeldaim *et al.*, 2010), and, thus, the use of *ply*-based quantitative PCR assays has been questioned with regard to its clinical usefulness (Abdeldaim *et al.*, 2009). Consequently, the *lytA* gene was chosen as the target for the quantitative PCR assay in the present study. This gene is thought to be present in most, if not all, pneumococcal serotypes; although, it is acknowledged that occasional non-pneumococcal strains may also harbour the *lytA* gene (Carvalho *et al.*, 2007; Abdeldaim *et al.*, 2008).

In the present study, using PCR we detected *S. pneumoniae* in almost all culture-positive samples and in many culture-negative sputum specimens, presumably reflecting the increased sensitivity of PCR as a method of detection. In contrast, serum specimens from five of the 11 patients with positive blood cultures were PCR-negative and an additional 17 serum specimens from blood culture-negative patients were PCR positive. These results may reflect a combination of increased sensitivity of PCR and the low-level intermittent nature of pneumococcal bacteraemia with occasional sampling errors causing false-negative results. We also detected pneumococcal DNA in relatively few urine specimens. The low rate of *lytA* gene detection in blood cultures and urine may also indicate a sensitivity issue with the PCR assay, which is inherently difficult to investigate due to the lack of a robust 'gold standard'. Although PCR is generally thought to be less affected than culture by prior antibiotic treatment, we noted that detection of *S. pneumoniae* by PCR in sputum samples, but not in other specimens, was less common in patients who had been administered antibiotics prior to specimen collection.

Others have reported that plasma and pleural fluid bacterial loads are lower after prior antibiotic use (Muñoz-Almagro *et al.*, 2011). The application of quantitative PCR enabled the estimation of pneumococcal burden in clinical specimens. In general, our findings were not surprising with pneumococcal loads being considerably higher in sputum specimens than in positive serum or urine specimens. In addition, bacterial loads based on PCR targeting the *lytA* gene were higher in culture-positive compared to culture-negative sputum specimens and in serum from patients with positive blood cultures compared to those with negative blood cultures.

The limitations of this study are the incompleteness of sputum, serum and urine sets for all patients, and the relatively small number of PCR-positive serum and urine samples. The relatively small number of patients who were

admitted to the intensive care unit and the small number of mortalities limited our ability to analyse these variables as markers of disease severity. Another limiting factor of the study was the use of serum instead of whole blood. The latter specimen type has the theoretical advantage of also capturing intracellular organisms but such samples were unavailable to test in our study. In addition, sample deterioration may have occurred through storage resulting in false-negative results. Finally, the lack of nasopharyngeal swabs from the patients prevents us from evaluating the utility of measuring pneumococcal load in this specimen type. Recent data have shown some interesting associations between nasopharyngeal pneumococcal load and the presence of radiologically confirmed pneumonia and viral co-infection in children (Vu *et al.*, 2011) and it would be interesting to assess the association between this and disease severity in adults as well. Reports have demonstrated an association between invasiveness and severity of disease with certain pneumococcal serotypes (Rodríguez *et al.*, 2011). Serotype or molecular data of the pneumococcal isolates in this study could have provided further information about the association of strains with severity of disease; however, these data were not available. The strength of the present study is the use of specimens from a very well-characterized group of adults with CAP.

This study adds to an increasing body of evidence, suggesting that determination of pneumococcal load has a clinical utility. Higher pneumococcal load in serum and, possibly, urine was associated with increased disease severity in adults with pneumonia. Further work is needed to determine whether measuring pneumococcal load in respiratory specimens from adults will differentiate colonization from coincidental carriage.

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