HIV and Influenza Infection Are Associated with Increased Blood Pneumococcal Load: a Prospective, Hospital-Based Observational Study in South Africa, 2009-2011

Running Title: HIV/influenza increase pneumococcal load

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ABSTRACT

Background

Increased pneumococcal loads are associated with severe outcomes. We determined the prevalence of pneumococcal DNA in blood of patients hospitalized with acute lower respiratory tract infection and identified factors associated with invasive pneumococcal pneumonia, bacterial loads and death.

Methods

8523 patients were enrolled as part of prospective hospital-based surveillance. Blood was collected for quantitative pneumococcal (*lyt*A) detection, and nasopharyngeal specimens for detection of influenza and other respiratory viruses by real-time PCR.

Results

Of 6396 (75%) cases with *lyt*A results, 422 (7%) were positive for pneumoccal DNA. HIV and influenza prevalence were 51% (2965/5855) and 8% (485/6358), respectively. On multivariable analysis, HIV infection [adjusted odds ratio (aOR): 2.4, 95% confidence interval (CI): 1.6-3.6], influenza co-infection [aOR: 1.4, CI: 1.2-2.1], oxygen therapy during admission [aOR: 1.6, CI: 1.1-2.3] and in-hospital death [aOR: 2.1, CI: 1.1-4.0] were significantly associated with increased pneumococcal load. Amongst *lytA*-positive patients, adjusting for length of hospitalization, duration of symptoms and oxygen therapy during admission, pneumococcal loads ≥10,000 DNA copies/ml [aOR: 3.6, CI: 1.8-7.2] were associated with increased risk of death.

Conclusions

HIV and influenza virus infection were associated with elevated pneumococcal loads which, in turn, were associated with increased risk of death.

Keywords: *Streptococcus pneumoniae*; pneumococcal pneumonia; bacterial load; HIV; influenza

INTRODUCTION

In 2004, lower respiratory tract infections were the third most common cause of death globally, accounting for approximately 4.2 million deaths [1]. In South Africa, in 2008, influenza and pneumonia ranked the second most common natural cause of death in all ages, accounting for 8% of deaths [2]. *Streptococcus pneumoniae* is a common, and often the predominant, cause of bacterial pneumonia [3,4,5].

The etiological diagnosis of community-acquired pneumonia is difficult to elucidate due to inadequate diagnostic tests. As a result the incidence of pneumococcal pneumonia is underestimated [6]. Real-time PCR offers a fast and sensitive alternative to the gold standard, blood culture, and is not dependent on the viability of the organism. The detection of pneumococcal DNA in blood has been shown to be a potentially useful diagnostic tool [7,8,9,10,11], and PCR compared favourably with culture and serological tests when used to diagnose the pneumococcus in children with lower respiratory tract infections [12,10]. In addition, real-time PCR has been used to quantify bacterial loads in blood, which may serve as useful markers of community-acquired pneumonia [13,14,8,9].

HIV infection is a known risk factor for invasive pneumococcal disease [15,16]. Infection with influenza virus is commonly complicated by secondary pneumococcal infection, resulting in severe disease and increased mortality [17,18]. Respiratory viral infections are specifically associated with increased rates of invasive pneumococcal disease [19,20] and are considered a risk factor for pneumococcal pneumonia [21,22]. Pneumococcal loads in clinical specimens have been shown to be directly related to severity of illness and mortality [13,23,9]. However, there are few data on the effect of HIV infection and respiratory virus co-infections on pneumococcal loads.

We used quantitative real-time PCR to determine the prevalence of pneumococcal DNA in patients hospitalized with acute lower respiratory tract infection and to identify factors associated with invasive pneumococcal pneumonia, pneumococcal loads and death.

MATERIALS AND METHODS

Study design and setting

In South Africa, a low to middle income country of about 50 million people [24], influenza virus circulation occurs mainly during winter (May to August) [25]. In 2006, approximately 11% of the South African population (4% of children <5 years) were infected with HIV [26].

We conducted a prospective hospital-based observational study as part of the Severe Acute Respiratory Illness surveillance programme that started in February 2009 and aims to describe the etiology of and risk factors for acute lower respiratory tract infection in South Africa. Blood specimen collection for the detection of *S. pneumoniae* started in May 2009 through April 2011. The study was implemented at six public referral hospitals at four surveillance sites in four provinces of South Africa.

Patients were enrolled if they met the WHO's acute lower respiratory tract infection case definition and had symptom onset within 7 days from admission [27]. A case in children aged 2 days to <3 months included any hospitalized patient with physician-diagnosed sepsis or physician-diagnosed acute lower respiratory tract infection, irrespective of signs and symptoms. A case in children aged 3 months to <5 years included any hospitalized patient with physician-diagnosed acute lower respiratory tract infection, including bronchitis, bronchiolitis, pneumonia and pleural effusion. A case in individuals aged ≥5 years included any hospitalized patient presenting with manifestation of acute lower respiratory tract infection with recent (≤7 days) onset of fever (>38°C) and cough or sore throat and shortness of breath or difficult breathing with or without clinical or radiographic findings of pneumonia.

A standardized questionnaire was used to collect demographic and clinical information. Vaccination history was verified from the patient's Road to Health card, which was available for review on admission in 69% (1860/2691) of children <5 years of age. Blood cultures were

performed at the discretion of the clinician and results were retrieved from the patient's hospital records.

Sample collection and processing

Whole blood samples were collected in EDTA-containing vacutainer tubes within 24 hours of hospital admission. Respiratory specimens, including oropharyngeal and nasopharyngeal swabs (Dryswab^{TM}, Medical Wire and Equipment, Corsham, England) for patients ≥5 years or nasopharyngeal aspirates for children <5 years, were placed in virus transport medium (Highveld Biological, Johannesburg, South Africa) upon collection. Swabs collected from the same patient were placed in the same virus transport medium. Specimens were stored at 4°C and transported to the National Institute for Communicable Diseases (NICD) for testing.

Detection and quantification of *S. pneumoniae*

DNA was extracted from 200 µl of whole blood using the Roche MagNA Pure LC 2.0 instrument and DNA Isolation kit III for bacteria (Roche, Mannheim, Germany) according to the manufacturer's instructions. Extracted DNA was eluted into 100 µl of elution buffer and stored at -20°C.

A case of invasive pneumococcal pneumonia was defined as the identification of *S. pneumoniae* in the blood specimen using a single-target (*lytA*) quantitative real-time PCR assay adapted from Carvalho et al. [28]. The 25 µl PCR reaction contained 1x TaqMan gene expression mastermix (Applied Biosystems, Foster City, CA, USA), 200 nM each of forward, reverse primer and probe (5'FAM) and 2.5 µl of extracted DNA. Universal cycling conditions were used: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Samples with a cycle threshold (C_t) value of >40 were recorded as negative. A standard curve was prepared using serially diluted DNA extracts from a known quantity (confirmed spectrophotometrically) of *S. pneumoniae* ATCC49619, and used to calculate pneumococcal

loads (DNA copies/ml). For blood-culture positive, *lytA*-negative cases, blood specimens were re-extracted and PCR testing repeated.

Detection of respiratory viruses

Respiratory samples were tested by multiplex reverse-transciption real-time PCR as previously described [29] for the following viruses: influenza type A and B, adenovirus, enterovirus, rhinovirus, human metapneumovirus, respiratory syncytial virus (RSV) and parainfluenza virus types 1-3. The influenza A positive specimens were subtyped using methods from the WHO Collaborating Center for Influenza, Centers for Disease Control and Prevention, US.

Determination of HIV status

HIV results were obtained from a combination of two sources (i) patient clinical records where available and (ii) for consenting patients, an anonymized linked dried blood spot was tested at NICD. For a large number of patients where both results were available, the NICD result was used. Testing included HIV enzyme-linked immunosorbent assay (ELISA) testing for patients ≥18 months and PCR testing for children <18 months if the ELISA was reactive. A comparison of HIV prevalence among patients with anonymized linked testing results (2926/5636; 52%) and patients with clinical records (1348/2674; 50%) revealed no difference (p=0.201).

Statistical analysis

To identify factors associated with *lyt*A positivity, bacterial load and death, we included both potential determinants for, as well as outcomes or characteristics of the primary endpoints of the analysis. Univariate comparisons were performed using logistic regression. In addition, we implemented three multivariable models to identify factors associated with: (i) *lytA* positivity among cases with available *lyt*A results; (ii) pneumococcal load among *lytA*-positive patients; and (iii) mortality among *lytA*-positive patients. The *lytA* positivity and

mortality models were implemented using stepwise backward selection logistic regression. For the mortality analysis, four binary variables were generated for the pneumococcal load (cut-offs: 100; 1,000; 10,000; 100,000 DNA copies/ml) and included in the model as a predictor to investigate the effect of different pneumococcal loads on mortality. For the analysis of factors associated with pneumococcal load, we used a proportional-odds (ordinal) model, which assumes that the ordinal outcome variable represents categories of an underlying continuous variable. The outcome variable (pneumococal load) was categorized into 5 levels as follows: <100; 100 to <1,000; 1,000 to <10,000; 10,000 to <100,000; ≥100,000 DNA copies/ml. The coefficients of the proportional-odds model (and associated odds ratio) measure the effect of a predictor on the log odds of being above a specified level compared with the log odds of being at or below the specified level. In addition, we compared the prevalence (and associated odds ratio) of covariates in each bacterial load level to the lowest bacterial load category (<100 DNA copies/ml).

Covariates with a p-value <0.2 in the univariate analysis were assessed for significance in the multivariable analysis and statistical significance was assessed at p<0.05. Pairwise interactions were assessed by the inclusion of product terms for all variables remaining in the final multivariable additive models. Pneumococcal cases positive on blood culture only were excluded from the analysis since no bacterial load data were available. The statistical analysis was implemented using STATA® version 11 (StataCorp, Texas, USA).

Ethical approval

The protocol was approved by the University of the Witwatersrand Human Research Ethics Committee (M081042) and the University of KwaZulu Natal Biomedical Research Ethics Committee (BF157/08).

RESULTS

Demographic and epidemiologic characteristics of the study population and factors associated with *lytA* positivity

From May 2009 through April 2011, we enrolled 8523 cases and *S. pneumoniae* results were obtained from 6396 (75%) patients. Of these, 2969 (46%) were males and 2753 (43%) were children ≤5 years (Table 1). Patients' age ranged between 2 days and 92 years (median: 24 years). 5855 of the 6396 (92%) patients had known HIV status and 2965 (51%) were HIV-positive (Table 1). HIV prevalence was 13% (243/1943), 23% (97/431), 54% (95/175), 86% (1884/2181) and 57% (646/1125) in the <2, 2-5, 6-18, 19-44 and ≥45 years age groups, respectively. 6358 of the 6396 (99%) cases had available influenza results and 485 (8%) were positive for influenza. An influenza subtype was obtained from 474 (98%) positive samples. Of these, 165 (34%) were influenza A(H1N1)pdm09, 154 (33%) were influenza A(H3N2) and 155 (33%) were influenza B. Five percent (329/6348) of patients reported having received antibiotics in the 24 hours prior to hospital admission.

The overall pneumococcal detection rate was 7% (422/6396). The highest detection rate was observed in the 19-44 years age group (9%; 203/2280) (Table 1). Cases of invasive pneumococcal pneumonia were detected throughout the year (Figure 1). However, higher numbers of cases were detected concomitantly with the circulation of influenza virus (Figure 1) during winter (June to August) (153/1725; 9%) and spring (September to November) (145/1732; 8%) compared to summer (December to February) (52/1351; 4%) and autumn (March to May) (72/1588; 5%) (Table 1 and Figure 1). In 2009, following the introduction of influenza A(H1N1)pdm09 in the country, South Africa experienced two distinct waves of influenza virus circulation. The first wave was dominated by influenza A(H3N2) followed by influenza A(H1N1)pdm09. In the same year a biphasic detection of invasive pneumococcal pneumonia was observed concomitantly with the circulation of the two influenza A subtypes (Figure 1).

In the univariate analysis, amongst other factors (Table 1), HIV (odds ratio (OR): 2.6; 95% confidence interval (CI): 2.1-3.3) and influenza virus (OR: 1.5; CI: 1.1-2.1) infections were significantly associated with *lyt*A positivity. Conversely, RSV and rhinovirus were significantly less associated with *lytA* positivity in the univariate analysis (Table 1). Amongst eligible children, receipt of one (6/183; OR: 0.7; CI: 0.3-1.8) or two (11/229; OR:1.1; CI: 0.5-2.2) doses of the 7-valent pneumococcal conjugate vaccine (PCV7) was not significantly associated with reduced *lytA* positivity compared to the unvaccinated group (34/793).

On multivariable analysis (Table 1), HIV (adjusted odds ratio (aOR): 1.9; CI: 1.5-2.4) and influenza virus (aOR: 1.6; CI: 1.1-2.2) infections remained significantly associated with *lyt*A positivity. In addition, patients with invasive pneumococcal pneumonia were more likely to present late (>2 days from symptom onset) (aOR: 2.0; CI: 1.5-2.7), have longer (≥5 days) hospitalization time (aOR: 1.3; CI: 1.1-1.6), receive antibiotic treatment during admission (aOR: 6.1; CI: 1.5-24.9), require oxygen therapy (aOR: 1.8; CI: 1.4-2.1) and were at greater risk of death (aOR: 1.5; CI: 1.1-2.2).

Factors associated with pneumococcal load among *lytA*-positive cases

Among the 422 *lytA*-positive cases, the overall pneumococcal loads ranged from 1.2 to 6.3 log DNA copies/ml, with a median of 3.1 log DNA copies/ml. The median pneumococcal loads by age group were: 2.8, 3.0, 3.4, 3.3 and 3.1 log DNA copies/ml in the <2, 2-5, 6-18, 19-44 and ≥45 years age groups, respectively. On multivariable analysis (Table 2), HIV and influenza infections were significantly associated with elevated pneumococcal load. The prevalence of HIV infection increased from 50% (16/32) in patients with a pneumococcal load <100 DNA copies/ml to 96% (25/26) in patients with a pneumococcal load ≥100,000 copies/ml (aOR/proportional-odds model: 2.4; CI: 1.6-3.6). Similarly, the prevalence of influenza infection was 6% (2/32) in patients with a pneumococcal load <100 DNA copies/ml compared to 31% (8/26) in patients with a pneumococcal load ≥100,000 DNA copies/ml (aOR/proportional-odds model: 1.4; CI: 1.2-2.1). In addition, individuals with higher

pneumococcal loads were more likely to require oxygen therapy during hospitalization and were at greater risk of death.

A sub-analysis restricted to cases positive for both pneumococcus and influenza revealed no difference in the association of influenza subtypes with pneumococcal load; however the sample size was small. The mean pneumococcal load was 82732 (n=14), 34906 (n=17) and 64487 (n=15) for influenza A(H3N2), A(H1N1)pdm09 and B infected patients, respectively (p=0.127).

Factors associated with mortality among *lytA*-positive patients

The outcome of hospitalization was available for 99% (6374/6396) of patients with *lytA* results. The case-fatality ratio was higher among *lytA*-positive (43/421; 10%) compared to *lytA*-negative patients (300/5953; 5%) ($p<0.001$). On univariate analysis, factors associated with increased mortality among the *lytA*-positive individuals were HIV infection and pneumococcal load ≥10,000 or ≥100,000 DNA copies/ml, as well as different markers of disease severity (Table 3).

On multivariable analysis (Table 3), *lytA*-positive patients with loads ≥10,000 DNA copies/ml (aOR: 3.6; CI: 1.8-7.2) and that presented to hospital late (≥5 days from onset of symptoms) (aOR: 3.4; CI: 1.6-7.0) were at increased risk of death. In addition, patients that died were more likely to require oxygen therapy during admission (aOR: 4.7; CI: 2.1-10.4) and had shorter (<5 days) length of hospitalization (OR: 0.3; CI: 0.2-0.7) indicating a fulminant progression of illness.

Association between *lytA* and blood culture positivity

In total, 18% (1120/6396) of cases had blood culture results available. Of these, 2% (23/1120) were recorded as culture-positive for the pneumococcus. From this same set of

cases, 10% (116/1120) were PCR-positive for the pneumococcus. Of the 23 culture-positive cases, 9 (39%) were negative by real-time PCR. One of these nine patients died.

DISCUSSION

In this study we detected pneumococcal DNA in blood specimens of patients hospitalized with acute lower respiratory tract infection as a marker for pneumococcal pneumonia. HIV infection and influenza co-infection were independently associated with invasive pneumococcal pneumonia and elevated blood pneumococcal loads. In addition, bacterial loads in excess of 10,000 DNA copies/ml were associated with an increased risk of death. Real-time PCR detected four-fold more cases of invasive pneumococcal pneumonia than blood culture alone.

The association between high blood bacterial load and severe disease, including mortality, is well described [13,30,9]. In this study, HIV infection was found to be associated with an increased risk of invasive pneumococcal pneumonia and elevated pneumococcal load. This is consistent with previous findings [13] in which HIV-infected children with pneumonia had higher pneumococcal loads than HIV-uninfected children. Pneumococcal conjugate vaccines (PCV) have been shown to be safe and effective in reducing pneumococcal disease in HIV-infected children [31,32,33] and adults [34]. Currently in South Africa, PCV is advocated for use in children. Based on the results of this study, vaccination of HIV-infected individuals of all ages may reduce the risk of invasive pneumococcal pneumonia and associated mortality in this group.

Similarly, in this study influenza infection was associated with an increased risk of invasive pneumococcal pneumonia and an elevated pneumococcal load. A number of mechanisms have been proposed for the synergistic interaction between influenza and the pneumococcus, including increased bacterial colonization as a result of epithelial cell damage by influenza virus, virus-induced conditions that promote bacterial growth and increased inflammatory responses [35]. Experimentally-infected mice demonstrated a significant reduction in survival rates when co-infected with both pathogens compared to infection with influenza or pneumococcus alone [36]. In addition, increased pneumococcal

load has been observed in squirrel monkeys coinfected with influenza virus [37]. The increased risk of invasive pneumococcal pneumonia and elevated pneumococcal load in influenza-infected patients observed in this study may, in part, explain the lethality of the dual infection [18]. Recent studies of the 1918 influenza epidemic have indicated that the majority of deaths occurred between 7 and 14 days post-infection [38]. The exclusion of cases with duration of symptoms >7 days in this study may therefore have resulted in an underestimation of the number of secondary pneumococcal infections. Studies have reported a similar synergistic interaction in children between the pneumococcus and other respiratory viruses such as RSV [19,39], however this was not observed in our study including all age groups. On multivariable analysis the protective effect of RSV and rhinovirus that was observed in the univariate analysis was not significant, most likely due to the strong age association of these respiratory viruses.

The blood pneumococcal detection rate observed in patients aged ≤18 years during the early introduction of PCV7 (5%) was lower than the 10% described in a similar study performed in Italian children ≤16 years of age [10]. Based on vaccine-probe studies, pneumococcal pneumonia was estimated to account for 8% of clinical pneumonia cases and 36% of chest X-ray positive pneumonia cases in children <5 years [33]. Although still lower than the true burden of pneumococcal pneumonia is thought to be, real-time PCR detected five times as many cases than were detected by blood culture alone. In this study blood cultures were performed only on a subset of cases, however results were similar to findings by Resti et al. [10] in which real-time PCR was four-fold more sensitive than blood cultures when used to diagnose pneumococcal community-acquired pneumonia. Real-time PCR enables diagnosis and serotyping from culture-negative specimens [7,10,11], which may be of particular use in developing countries. In addition, the ability to quantify the pneumococcus enables individuals at risk of severe outcome to be rapidly identified and treated.

PCV7 vaccination was not associated with a reduced risk of pneumococcal disease. Our study design was not appropriate for assessment of PCV7 protection as *lytA*-positive results could not be stratified by serotype due to the lack of serotyping data. In addition, the number of patients, eligible to receive PCV7 and positive for the pneumococcus were too few for meaningful analysis. The pneumococcal detection rate differed significantly amongst the sites on univariate analysis, most likely due to differences in the HIV prevalence and age distribution of the patients presenting to the respective sites. In the multivariable model, accounting for HIV and age, there was no significant difference observed between the sites.

This study has a number of limitations. Firstly, the assay is not likely to detect all cases of pneumococcal pneumonia, as it is dependent on the pneumococcus crossing into the bloodstream. In addition small volumes of blood were tested in the PCR assay, in comparison with blood culture, that may have resulted in false-negative *lytA* results. As a result the true disease prevalence and the magnitude of the identified risk factors for invasive pneumococcal pneumonia may have been underestimated. In addition, the detection of *lyt*A in the blood may reflect transient bacteremias. Limited studies, targeting the *ply* gene, have shown that the pneumococcus can be detected in the blood of healthy individuals [40,41]. However studies targeting the *lyt*A gene, as in our study, did not identify pneumococci in the blood of healthy children colonized with the pneumococcus [42,43]. Secondly, detailed information on clinical characteristics such as oxygen saturation on admission and chest X-ray findings were not available. Thirdly, surveillance programmes such as ours tend to underestimate mortality. This may be due to a number of reasons including the fact that severly ill patients may be less likely to consent to inclusion in the surveillance or a proportion of patients may die before hospital admission or shortly after admission, but prior to being approached by surveillance staff. Therefore our estimates of mortality likely represent a minimum estimate. In addition, we had limited data on CD4+ counts and HIV treatment, therefore the effect of these covariates could not be evaluated.

In conclusion, this study highlights the usefulness of real-time PCR on blood specimens as a marker of pneumococcal pneumonia. In addition, the determination of pneumococcal loads at the time of diagnosis may have a role as a prognostic marker for invasive pneumococcal pneumonia-associated mortality. HIV and influenza virus infection were associated with an increase in pneumococcal loads which, in turn, were associated with an increased risk of death. This may be a pathway by which the immune dysregulation caused by HIV and/or influenza infection results in severe outcome amongst pneumococcal pneumonia patients.

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CONFLICT OF INTEREST

All authors declare that they have no commercial or other association that may pose a conflict of interest.

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Table 1: Univariate and multivariable analysis of factors associated with pneumococcal infection (*lytA* positive) among patients hospitalized with acute lower respiratory tract infection, South Africa, 2009-2011

Variable	Enrolled cases n (%)	*lyt*A positive cases n (% of total) [% *lyt*A positive]	Univariate analysis[a]		Multivariable analysis	
			Odds ratio (95% CI)	p-value	Adjusted odds ratio[b] (95% CI)	p-value
Year	N=6396	N=422				
2009 (May-Dec)	2003 (31)	150 (35) [7]	1			
2010 (Jan – Dec)	3143 (49)	216 (51) [7]	0.9 (0.7-1.1)	0.402		
2011 (Jan – Apr)[c]	1250 (20)	56 (13) [4]	0.6 (0.4-0.8)	0.001		
Gender	N=6369	N=422				
Female	3427 (54)	247 (58) [7]	1			
Male	2969 (46)	175 (42) [6]	0.8 (0.7-0.9)	0.035		
Age group (years)	N=6396	N=422				

<2	2254 (35)	93 (22) [4]	1	
2-5	499 (8)	31 (7) [6]	1.5 (1.1-2.3)	0.043
6-18	194 (3)	4 (1) [2]	0.5 (0.2-1.3)	0.166
19-44	2280 (36)	203 (48) [9]	2.3 (1.8-2.9)	<0.001
≥45	1169 (18)	91 (22) [8]	2.0 (1.5-2.6)	<0.001
Hospital	N=6396	N=422		
Agincourt	1036 (16)	42 (10) [4]	1	
Chris Hani Baragwanath	4681 (73)	325 (77) [7]	1.8 (1.3-2.5)	0.001
Edendale	442 (7)	25 (6) [6]	1.4 (0.9-2.4)	0.177
Klerksdorp	237 (4)	30 (7) [13]	3.4 (2.1-5.6)	<0.001
Season[d]	N=6396	N=422		
Autumn	1588 (25)	72 (17) [5]	1	
Winter	1725 (27)	153 (36) [9]	2.0 (1.5-2.7)	<0.001
Spring	1732 (27)	145 (34) [8]	1.9 (1.4-2.6)	<0.001
Summer	1351 (21)	52 (12) [4]	0.8 (0.6-1.2)	0.358
Influenza virus infection	N=6358	N=420		

No	5873 (92)	374 (89) [6]	1		1	
Yes	485 (8)	46 (11) [10]	1.5 (1.1-2.1)	0.008	1.6 (1.1-2.2)	0.010
Respiratory syncytial virus infection	N=6343	N=420				
No	5559 (88)	387 (92) [7]	1			
Yes	784 (12)	33 (8) [4]	0.6 (0.4-0.8)	0.004		
Rhinovirus infection	N=6343	N=420				
No	4824 (76)	339 (81) [7]	1			
Yes	1519 (24)	81 (19) [5]	0.7 (0.6-0.9)	0.021		
HIV infection	N=5855	N=392				
No	2890 (49)	111 (28) [4]	1		1	
Yes	2965 (51)	281 (72) [9]	2.6 (2.1-3.3)	<0.001	1.9 (1.5-2.4)	<0.001
Antibiotic use (during admission)	N=6392	N=422				
No	273 (4)	4 (1) [1]	1		1	
Yes	6119 (96)	418 (99) [7]	4.9 (1.8-13.3)	0.002	6.1 (1.5-24.9)	0.011
Duration of symptoms	N=6396	N=422				
0-2 days	1909 (30)	65 (15) [4]	1		1	

3-7 days	4487 (70)	357 (85) [8]	2.5 (1.9-3.2)	<0.001	2.0 (1.5-2.7)	<0.001
Length of hospitalization	N=6375	N=421				
0-4 days	3601 (56)	180 (43) [5]	1		1	
≥5 days	2774 (44)	241 (57) [9]	1.8 (1.5-2.2)	<0.001	1.3 (1.1-1.6)	0.022
Received oxygen	N=6380	N=421				
No	4391 (69)	224 (53) [5]	1		1	
Yes	1989 (31)	197 (47) [10]	2.0 (1.7-2.5)	<0.001	1.8 (1.4-2.1)	<0.001
Admitted to ICU[e]	N=6381	N=421				
No	6362 (99)	417 (99) [7]	1			
Yes	19 (1)	4 (1) [21]	3.8 (1.3-11.5)	0.018		
Outcome	N=6374	N=421				
Survived	6031 (95)	378 (90) [6]	1		1	
Died	343 (5)	43 (10) [13]	2.1 (1.5-3.0)	<0.001	1.5 (1.1-2.2)	0.023

[a] Only covariates significant in the univariate analysis are reported. The following covariates were additionally assessed for significance in the univariate analysis: alcohol use, smoking, underlying illness (including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns,

kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer), influenza subtypes, adenovirus infection, enterovirus infection, human metapneumovirus infection, parainfluenza (1-3) virus infection, PCV7 vaccination, antibiotic use 24 hrs prior to admission.

[b] Adjusted odds ratio calculated from observations with available data for all covariates significant at the multivariable analysis (N=5798). Only covariates significant at the multivariable analysis are reported.

[c] Only cases enrolled from January through April 2011 were included in the study and therefore the winter season of 2011 was excluded.

[d] Southern hemisphere seasons were classified as follows: autumn (March through May), winter (June through August), spring (September through November), summer (December through February).

[e] Intensive care unit.

Table 2: Multivariable analysis of factors associated with pneumococcal load (DNA copies/ml) among patients with pneumococcal infection (*lyt*A positive), South Africa, 2009-2011

Variable	***lytA* positive cases with pneumococcal load (DNA copies/ml) of:**					**Proportional-odds model**	
	$<10^2$ n (%)	**10^2 to $<10^3$** n (%) OR[a] (95% CI)	**10^3 to $<10^4$** n (%) OR[a] (95% CI)	**10^4 to $<10^5$** n (%) OR[a] (95% CI)	**$\geq 10^5$** n (%) OR[a] (95% CI)	**Adjusted odds ratio[b,c] (95% CI)**	**p-value**
	N=32	N=141	N=125	N=66	N=26		
HIV infection	16 (50) -	93 (66) 1.9 (0.9-4.2)	88 (70) 2.4 (1.1-5.3)	57 (86) 6.3 (2.2-18.6)	25 (96) 25.0 (2.1-295.9)	2.4 (1.6-3.6)	<0.001
Influenza infection	2 (6) -	9 (6) 1.0 (0.2-10.2)	11 (9) 1.4 (0.3-14.1)	11 (17) 3.0 (0.6-29.3)	8 (31) 6.6 (1.1-68.9)	1.4 (1.2-2.1)	0.002
Oxygen therapy	12 (37) -	62 (44) 1.3 (0.6-2.9)	56 (45) 1.4 (0.6-3.0)	40 (61) 2.6 (1.1-6.3)	17 (65) 3.1 (1.1-9.8)	1.6 (1.1-2.3)	0.018
Patient died	3 (9) -	11 (8) 0.8 (0.2-3.1)	7 (6) 0.6 (0.1-2.4)	11 (17) 1.9 (0.5-7.6)	8 (31) 4.3 (0.9-19.7)	2.1 (1.1-4.0)	0.032

[a] Odds ratio for each category compared with the reference group: <10^2 DNA copies/ml.

[b] Adjusted odds ratio calculated from observations with available data for all covariates significant at the multivariable analysis (N=390).

[c] The following covariates were assessed for significance in the multivariable analysis: age, gender, hospital, season of the year, underlying illness, influenza virus infection, infection with respiratory viruses other than influenza, HIV infection, alcohol consumption, smoking, duration of symptoms, length of hospitalization, antibiotic use 24 hours before admission, antibiotic use during admission, oxygen therapy, admission to the intensive care unit and outcome.

Table 3: Univariate and multivariable analysis of factors associated with death among patients with pneumococcal infection (*lyt*A positive), South Africa, 2009-2011

Variable	Case-fatality ratio n/N (%)	Univariate analysis[a]		Multivariable analysis	
		Odds ratio (95% CI)	**p-value**	**Adjusted odds ratio[b] (95% CI)**	**p-value**
Influenza virus infection					
No	34/373 (9)	1			
Yes	9/46 (20)	2.1 (0.8-4.9)	0.055		
HIV infection					
No	5/111 (4)	1			
Yes	35/280 (12)	2.8 (1.1-9.3)	0.031		
Pneumococcal load (cut off: 10^4 DNA copies/ml)					

$<10^4$	21/322 (7)	1		1	
$\geq10^4$	22/99 (22)	4.1 (2.1-7.8)	<0.001	3.6 (1.8-7.2)	<0.001
Pneumococcal load (cut off: 10^5 DNA copies/ml)					
$<10^5$	33/392 (8)	1			
$\geq10^5$	10/29 (34)	5.7 (2.5-13.3)	<0.001		
Duration of symptoms					
0-4 days	26/331 (8)	1		1	
5-7 days	17/90 (19)	2.7 (1.4-5.3)	0.003	3.4 (1.6-7.0)	0.001
Length of hospitalization					
0-4 days	26/180 (14)	1		1	
≥5 days	17/240 (7)	0.5 (0.2-0.9)	0.016	0.3 (0.2-0.7)	0.002
Received oxygen					
No	9/223 (4)	1		1	
Yes	34/197 (17)	5.0 (2.3-10.6)	<0.001	4.7 (2.1-10.4)	<0.001
Admitted to ICU[c]					

No	41/416 (10)	1	
Yes	2/4 (50)	9.1 (1.3-66.7)	0.029

[a] Only covariates significant in the univariate analysis are reported. The following covariates were additionally assessed for significance in the univariate analysis: gender, age group, alcohol use, smoking, underlying illness (including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer), infection with respiratory viruses other than influenza (respiratory syncytial virus, adenovirus, enterovirus, rhinovirus, human metapneumovirus, parainfluenza virus (types 1-3)), pneumococcal load cut off of 10^2 DNA copies/ml, pneumococcal load cut off of 10^3 DNA copies/ml, antibiotic use 24 hrs prior to admission and antibiotic use during admission.

[b] Adjusted odds ratio calculated from observations with available data for all covariates significant at the multivariable analysis (N=419). Only covariates significant at the multivariable analysis are reported.

[c] Intensive care unit.

Figure 1: Weekly detection rate of Streptococcus pneumoniae (lytA positive) and influenza virus among patients hospitalized with acute lower respiratory tract infection, South Africa, 2009-2011. *Influenza subtype accounted for at least 50% of all influenza subtypes detected during the period