The Use of Polymerase Chain Reaction Amplification for the Detection of Viruses and Bacteria in Severe Community-Acquired Pneumonia

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Key Words

Intensive care unit · Microbiology · Infection · Mortality · Tropical climate

Abstract

Background: Pathogens are often not identified in severe community-acquired pneumonia (CAP), and the few studies using polymerase chain reaction (PCR) techniques for virus detection are from temperate countries. **Objective:** This study assesses if PCR amplification improves virus and bacteria detection, and if viral infection contributes to mortality in severe CAP in a tropical setting, where respiratory pathogens have less well-defined seasonality. **Methods:** In this cohort study of patients with severe CAP in an intensive care unit, endotracheal aspirates for intubated patients and nasopharyngeal swabs for non-intubated patients were sent for PCR amplification for respiratory viruses. Blood, endotracheal aspirates for intubated patients, and sputum for non-intubated patients were analysed using a multiplex PCR system for bacteria. **Results:** Out of 100 patients, using predominantly cultures, bacteria were identified in 42 patients; PCR amplification increased this number to 55 patients. PCR am-

plification identified viruses in 32 patients. In total, only bacteria, only viruses, and both bacteria and viruses were found in 37, 14, and 18 patients, respectively. The commonest viruses were influenza A H1N1/2009 and rhinovirus; the commonest bacterium was *Streptococcus pneumoniae*. Hospital mortality rates for patients with no pathogens, bacterial infection, viral infection, and bacterial-viral co-infection were 16.1, 24.3, 0, and 5.6%, respectively ($p = 0.10$). On multivariable analysis, virus detection was associated with lower mortality (adjusted odds ratio 0.12, 95% confidence interval 0.2–0.99; $p = 0.049$). **Conclusions:** Viruses and bacteria were detected in 7 of 10 patients with severe CAP with the aid of PCR amplification. Viral infection appears to be independently associated with lower mortality.

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Introduction

Community-acquired pneumonia (CAP) is a common disease associated with significant mortality and morbidity [1, 2]. As a microbiological aetiological agent is not found in most patients with CAP [3–6], antimicro-

bial treatment is often empiric. Several factors account for this. Traditional diagnostic methods which primarily employ cultures may not be sufficiently sensitive to detect even the more common bacterial pathogens, and calls have been made for greater development and use of rapid molecular tests [7–9]. Viruses, which have previously often been considered clinically inconsequential and were not evaluated for in routine care, are now recognised as contributing to up to half of all cases of CAP [10].

Pathogens are often not found, even in severe CAP in the intensive care unit (ICU) [3, 11, 12]. Most studies using polymerase chain reaction (PCR) techniques for pathogen detection have involved severe sepsis and bloodstream infections in general rather than CAP per se [13]. The few that have used PCR for viruses in severe CAP were performed in temperate countries and yielded disparate results: viral pathogens were detected in 9, 23, 41, and 49% of patients in studies from Spain [11], the United States [14], South Korea [15], and Finland [16], respectively. To our knowledge, no study has used PCR to detect both viruses and bacteria in severe CAP in a tropical context, where respiratory pathogens have a much less well-defined year-round seasonality.

The primary aim of our study was therefore to assess if the use of PCR techniques, in addition to routine microbiological investigations including cultures, may improve the detection of viral and bacterial pathogens in severe CAP. The secondary aim was to investigate if viruses contributed to mortality in severe CAP in the tropics.

Methods

Study Design

This was a prospective observational cohort study conducted in the medical ICU of our Singaporean university hospital. Informed consent was obtained from the patient or the next-of-kin if the patient was medically unfit to provide consent. Ethics approval was obtained from the National Healthcare Group's Domain Specific Review Board (DSRB 2009/00380).

Inclusion Criteria

All patients who were admitted to the ICU with severe CAP from November 2009 to September 2011 were included. The definition of CAP was an acute infection of the pulmonary parenchyma associated with an acute infiltrate on chest radiograph and two or more of the following: fever ($\geq 38^{\circ}\text{C}$), hypothermia ($< 36^{\circ}\text{C}$), rigors, sweats, new cough, or change in colour of respiratory secretions in a patient with chronic cough, chest discomfort, or dyspnoea. To be considered severe, patients must be in shock, mechanically ventilated, and/or have 3 out of 9 minor severity criteria

as defined by the Infectious Diseases Society of America/American Thoracic Society [2]. All patients were enrolled within 24 h of hospital admission.

Exclusion Criteria

Patients hospitalised within 14 days before the onset of symptoms, immunocompromised patients (with human immunodeficiency virus infection or haematological malignancies, undergoing chemotherapy, or on steroids equivalent to ≥ 10 mg per day of prednisolone for a month), and patients with tuberculosis were excluded.

Collection of Respiratory and Blood Samples

Respiratory samples were collected within 12 h of enrollment. In intubated patients, endotracheal aspirates were collected using a sterile suction catheter inserted at least 24 cm through the endotracheal tube and a suction trap; inline suction and saline instillation were not used [17, 18]. For non-intubated patients, nasopharyngeal swabs were used for viral studies, and sputum was collected for non-viral studies [19]. Blood was collected in a sterile manner before commencing antibiotics.

PCR Amplification Technique for Virus Detection

Endotracheal aspirates and nasopharyngeal swabs were sent for target-enriched reverse transcriptase PCR amplification using the Seeplex RV12 detection kit (Seegene Inc., Seoul, Korea) to identify the following respiratory viruses: influenza virus A and B, parainfluenza virus (types 1–4), respiratory syncytial virus A and B, human metapneumovirus, rhinovirus A/B, adenovirus, and coronavirus (229E/NL63 and OC43) [20]. Influenza virus A positive cases were further subjected to H1N1/2009 and seasonal H1N1/H3N2 subtyping assays for identification [21].

PCR Amplification Technique for Bacteria Detection

Endotracheal aspirates, sputum, and blood samples were analysed using the Seeplex Sepsis DNA test (Seegene Inc.), which is a multiplex PCR system that detects 22 species of *Staphylococcus* spp., 24 species of *Streptococcus* spp., 2 species of *Enterococcus* spp., 10 species of Gram-negative bacteria, and 6 species of fungi [22].

Routine Microbiological Investigations

Endotracheal aspirates and sputum were sent for semi-quantitative cultures, which were deemed positive with moderate to heavy growths of bacteria and few epithelial cells seen on Gram stain examination (≤ 10 per high-power field). Blood cultures were incubated aerobically and anaerobically. Skin contaminants like coagulase-negative staphylococci, *Bacillus* species, *Corynebacterium* species, micrococci, and *Propionibacterium* species were disregarded unless they were deemed clinically significant by the managing physicians or cultured from two or more blood cultures. Serum samples were tested with the *Mycoplasma pneumoniae* IgM enzyme immunoassay. Urine was tested for *Streptococcus pneumoniae* and *Legionella pneumophila* serogroup 1 antigens.

Clinical Management

Patient care was left to the discretion of the managing physicians, who were encouraged to follow the Surviving Sepsis Campaign guidelines [23]. Initial empiric antibiotics typically included a combination of ceftriaxone, azithromycin, and ceftazidime (to cover melioidosis which is endemic in Singapore). Oseltamivir was

Fig. 1. Number of patients with bacteria and/or viruses detected. Data are presented as number of patients with bacteria and/or viruses detected using cultures, PCR, and/or urinary *S. pneumoniae* antigen.

prescribed when influenza was suspected clinically. As the PCR analyses were performed for research, results were not shared with the managing physicians, who optimised and de-escalated antibiotics based on the routine microbiological investigations. The exception to this was the detection of influenza viruses, which was made known to the managing physicians given the potential benefit of oseltamivir and for infection control purposes. Other treatments involved early intubation for respiratory failure and fluid resuscitation and vasopressors when indicated.

Statistical Analysis

Categorical variables were expressed as number (percentage), normally distributed quantitative variables as mean ($\pm$ standard deviation), and non-normally distributed quantitative variables as median (interquartile range). Categorical variables were compared using the χ^2 test or Fisher's exact test and quantitative variables with the t test and the Mann-Whitney U test. Cohen's κ coefficient was used to assess agreement between PCR amplification and routine microbiological investigations including cultures. To assess if viral and/or bacterial infection was independently associated with hospital mortality, a multivariable forward logistic regression analysis was performed using a model which included virus and bacteria identification. To account for the severity of pneumonia on presentation and the severity of critical illness during the first 24 h of ICU admission, respectively, the Pneumonia Severity Index (PSI) points and the Acute Physiology and Chronic Health Evaluation (APACHE) II score were incorporated into the model. A p value <0.05 was considered significant, and SPSS version 20.0 (SPSS Inc., Chicago, Ill., USA) was used.

Results

A total of 100 patients were enrolled, excluding 3 for whom consent was not provided. Using blood and respiratory cultures and urinary *S. pneumoniae* antigen, bacterial pathogens were identified in 42 patients (fig. 1a). Use of PCR amplification for bacteria detection increased this

number to 55 patients (fig. 1b). Use of PCR amplification identified viral pathogens in 32 patients. In total, 37 patients had only bacterial infection, 14 had only viral infection, and 18 had bacterial-viral co-infection (fig. 1c).

Patient characteristics are shown in table 1. Eighty-one patients were intubated and mechanically ventilated. There were no significant differences in clinical characteristics between the 32 patients with viruses detected and the 68 patients without (table 1), except that the former group had a lower white blood cell count. Patients with viruses detected had more rhinorrhea, but this difference did not reach statistical significance. Oseltamivir was prescribed for 31.3% of patients with viruses and for 50.0% of those without ($p = 0.08$). All patients received broad-spectrum antibiotics at the emergency department; all isolated bacteria were susceptible to these antibiotics.

Table 2 lists the viruses identified. Influenza A H1N1 virus was the commonest, followed by rhinovirus. Figure 2 shows the monthly distribution of viruses. There was a minor spike in the number of influenza cases in May, June, and July 2010. Agreement between PCR amplification and cultures for bacteria is shown in table 3. Table 4 lists the bacteria detected, the commonest of which was *S. pneumoniae*. Thirteen patients were PCR positive but culture negative for *S. pneumoniae* in endotracheal aspirates and sputum.

Patient outcomes are shown in table 5. Hospital mortality was lower in the group with viruses detected (3.1%) than in the group without (20.6%) ($p = 0.03$). Hospital mortality rates for the subgroups with no pathogens, bacterial infection, viral infection, and bacterial-viral co-infection were 16.1, 24.3, 0 and 5.6%, respectively ($p = 0.10$). The one non-survivor with a virus detected had a co-in-

Table 1. Patient characteristics

Characteristics	All patients (n = 100)	Virus detected (n = 32)	Virus not detected (n = 68)	p value
Demographics				
Age, years	61.7±17.7	59.9±18.0	62.5±17.7	0.50
Female	42 (42.0)	16 (50.0)	26 (38.2)	0.27
Comorbid illness				
Neoplastic disease	5 (5.0)	0 (0)	5 (7.4)	0.17
Liver disease	1 (1.0)	0 (0)	1 (1.5)	1.00
Heart failure	16 (16.0)	5 (15.6)	11 (16.2)	0.94
Cerebrovascular disease	8 (8.0)	4 (12.5)	4 (5.9)	0.26
Renal disease	22 (22.0)	9 (28.1)	13 (19.1)	0.31
Diabetes mellitus	35 (35.0)	15 (46.9)	20 (29.4)	0.09
Symptoms				
Cough	90 (90.0)	31 (96.9)	59 (86.8)	0.16
Shortness of breath	74 (74.0)	22 (68.8)	52 (76.5)	0.41
Fever	68 (68.0)	25 (78.1)	43 (63.2)	0.14
Increased sputum volume	64 (64.0)	21 (65.6)	43 (63.2)	0.82
Increased sputum purulence	37 (37.0)	10 (31.3)	27 (39.7)	0.41
Rigors	31 (31.0)	13 (40.6)	18 (26.5)	0.15
Chest pain	29 (29.0)	7 (21.9)	22 (32.4)	0.28
Vomiting	20 (20.0)	3 (9.4)	17 (25.0)	0.07
Myalgia	17 (17.0)	6 (18.8)	11 (16.2)	0.75
Rhinorrhea	14 (14.0)	8 (25.0)	6 (8.8)	0.06
Sore throat	13 (13.0)	5 (15.6)	8 (11.8)	0.75
Headache	11 (11.0)	3 (9.4)	8 (11.8)	1.00
Diarrhea	11 (11.0)	3 (9.4)	8 (11.8)	1.00
Vital signs at ED				
Altered mental status	34 (34.0)	8 (25.0)	26 (38.2)	0.19
Heart rate, beats/min	112±24	110±16	113±28	0.60
Respiratory rate, breaths/min	24 (20–28)	22 (20–28)	24 (20–30)	0.52
Systolic blood pressure, mm Hg	113 (90–144)	112 (90–140)	113 (90–145)	0.91
Diastolic blood pressure, mm Hg	64±16	63±13	64±17	0.71
Temperature, °C	37.6±1.1	37.8±1.0	37.5±1.1	0.24
PaO ₂ /FIO ₂ ratio ^a , mm Hg	140 (82–239)	121 (81–220)	158 (84–260)	0.30
Laboratory findings at ED				
Blood urea nitrogen, mmol/l	7.4 (4.7–13.2)	7.4 (4.2–12.7)	7.3 (4.7–13.8)	0.43
Sodium, mmol/l	135 (132–139)	136 (11–139)	135 (132–138)	0.86
Glucose, mmol/l	7.5 (5.8–10.8)	7.0 (5.2–11.6)	7.5 (6.3–9.98)	0.38
Haematocrit, %	37.0±6.3	36.7±6.2	37.1±6.4	0.75
White blood cells, per mm ³	11.82 (8.54–17.49)	9.55 (6.07–14.80)	13.16 (9.31–19.06)	0.01
Platelets, per mm ³	229 (180–306)	222 (167–294)	238 (186–321)	0.26
Arterial pH ^b	7.40 (7.34–7.44)	7.39 (7.34–7.43)	7.41 (7.32–7.45)	0.58
Radiological findings at ED				
Pleural effusion	47 (47.0)	13 (40.6)	34 (50.0)	0.38
Multilobar infiltrates	77 (77.0)	25 (78.1)	52 (76.5)	0.85
Life-sustaining treatment				
Direct ICU admission from ED	72 (72.0)	26 (81.3)	46 (67.6)	0.158
Invasive mechanical ventilation	81 (81.0)	25 (78.1)	56 (82.4)	0.62
Vasopressors	51 (51.0)	17 (53.1)	34 (50.0)	0.77
Severity scores				
Pneumonia severity index points	105.3±39.0	100.5±40.9	107.5±38.2	0.41
APACHE II score	22.9±7.4	21.8±6.9	23.5±7.6	0.27

Data are presented as number (%), mean ± standard deviation, and median (interquartile range). APACHE = Acute Physiology and Chronic Health Evaluation; ED = emergency department; FIO₂ = fraction of inspired oxygen; ICU = intensive care unit; PaO₂ = partial pressure of oxygen. ^a Data on the PaO₂/FIO₂ ratio were unavailable for 5 patients. ^b Data on arterial pH were unavailable for 3 patients.

Table 2. Viral pathogens detected in the study cohort

Viruses	Endotracheal aspirates or nasopharyngeal swabs, n
Influenza A H1N1/2009 virus	13
Human rhinovirus A/B	10
Human metapneumovirus	2
Parainfluenza virus type 3	2
Adenovirus	1
Human coronavirus 229E/NL63	1
Human coronavirus OC43	1
Influenza A H3N2 virus	1
Influenza B virus	1
Parainfluenza virus type 1	1

Data are presented as the number of patients with the specific viruses detected. Viruses were detected in the endotracheal aspirates in all patients except one, in whom both rhinovirus A/B and coronavirus 229E/NL63 were detected in the nasopharyngeal swab.

fection with rhinovirus and pneumococcal bacteraemia. On multivariable analysis, after accounting for severity of illness using the PSI and APACHE II scores, detection of viruses was independently associated with lower hospital mortality (adjusted odds ratio 0.12, 95% confidence interval 0.2–0.99; $p = 0.049$), while detection of bacteria was not associated with hospital mortality.

Discussion

To our knowledge, this is the largest prospective study on severe CAP to date, which used PCR analyses for both viral and bacterial pathogens, and the first of its kind in a tropical setting. We found that PCR amplification increased the detection of pathogens from 42 to 69% of patients, of whom 37% had bacterial infection, 14% had viral infection, and 18% had bacterial-viral co-infection. Viral infection was independently associated with lower hospital mortality.

Cultures lack the sensitivity to identify all bacteria for multiple reasons, including previous antibiotic administration, sampling error, and fastidious bacteria [24]. While PCR amplification may supplement cultures when diagnosing bloodstream infections [25–28], the Infectious Diseases Society of America has called for more work on PCR for respiratory samples in pneumonia [7]. Such studies are lacking in severe CAP [8, 9, 29]. Without quantitative PCR methods [30], it is difficult to differen-

Table 3. Agreement between PCR amplification and cultures for bacteria

	PCR positive, n	PCR negative, n
Blood ¹		
Culture positive	6	11
Culture negative	1	82
Endotracheal aspirates or sputum ²		
Culture positive	18 ³	14
Culture negative	17	51

Data are presented as number of patients. PCR = Polymerase chain reaction. ¹ κ coefficient for blood PCR versus cultures = 0.45, with a standard error of 0.13. ² κ coefficient for endotracheal aspirates and sputum PCR versus cultures = 0.30, with a standard error of 0.10. ³ Eleven patients with cultures and PCR detecting the same bacteria, and 7 patients with cultures and PCR detecting different bacteria.

tiate true infection from colonisation and contamination, especially of upper respiratory tract samples [31–33].

Our study differs from previous investigations in that PCR amplification for bacteria was mostly applied on endotracheal aspirates, thus avoiding contamination by oropharyngeal flora. We found moderate agreement between the Seeplex sepsis DNA test and cultures, with the former detecting more bacterial pathogens in respiratory secretions, the bulk of which were *S. pneumoniae*. Cross-reactivity between *S. pneumoniae* and the closely related viridans streptococci found in the upper respiratory tract is a concern [34], but unlikely nasopharyngeal swabs, they were not used for PCR analyses for bacteria. These findings imply that *S. pneumoniae* is an even commoner cause of severe CAP than suggested by cultures and urine *S. pneumoniae* antigen.

While a recent meta-analysis by Wu et al. [35] found an incidence of viral infections in CAP of 8.6–56.2% [30, 36–40], only a few studies have focused on severe CAP in the ICU: Cilloniz et al. [11] found viruses in 31 out of 362 patients (9%) in Spain; lower respiratory tract samples were available in 86 patients. Wiemken et al. [14] found viruses in 92 out of 393 patients (23%) in the United States, using only nasopharyngeal swabs. Choi et al. [15] found viruses in 26 out of 64 patients (41%) in South Korea, while Karhu et al. [16] found viruses in 24 out of 49 patients (49%) in Finland; both studies used a mix of nasopharyngeal swabs and bronchoalveolar lavage or bron-

Table 4. Bacterial pathogens detected in the study cohort

	Blood samples, n			Endotracheal aspirates or sputum, n		
	only culture positive	only PCR positive	both culture and PCR positive	only culture positive	only PCR positive	both culture and PCR positive
Bacteria in the PCR test menu						
<i>Streptococcus pneumoniae</i>	5	0	2	2	13	7
<i>Klebsiella pneumoniae</i>	0	0	2	4	2	3
<i>Staphylococcus aureus</i>	1	0	1	3	2	1
<i>Escherichia coli</i>	3	0	0	1	3	1
<i>Pseudomonas aeruginosa</i>	0	0	0	2	2	2
<i>Acinetobacter baumannii</i>	0	0	1	1	2	1
<i>Streptococcus agalactiae</i>	0	0	0	0	3	0
<i>Stenotrophomonas maltophilia</i>	0	1	0	0	0	0
<i>Enterococcus faecium/faecalis</i>	0	0	0	0	1	0
Bacteria not in the PCR test menu						
<i>Burkholderia pseudomallei</i>	1	–	–	2	–	–
<i>Haemophilus influenzae</i>	1	–	–	1	–	–
<i>Moraxella catarrhalis</i>	0	–	–	1	–	–
<i>Citrobacter koseri</i>	0	–	–	1	–	–

Data are presented as number of patients with the specific bacteria detected. Some patients had more than one bacterial pathogen detected. PCR = Polymerase chain reaction.

Fig. 2. Monthly distribution of viruses. Data are presented as number of patients.

chial aspirates. In comparison, our study detected viruses in 32 out of 100 patients (32%).

These studies were all performed in temperate regions, where the influenza virus thrives in the winter months [11, 14–16]. Our study was conducted in Singapore, an equatorial island with a stable climate year-round. Just as in the

Spanish and American cohorts [11, 14], influenza dominated; specifically, influenza A H1N1/2009 was our commonest isolate. As our study mostly spanned the period after the national health alert level for the 2009 H1N1 pandemic was lowered in February 2010, the incidence of viral CAP captured likely reflects post-pandemic levels. Tang

Table 5. Outcomes

Characteristics	All patients (n = 100)	Virus detected (n = 32)	Virus not detected (n = 68)	p value
Mortality				
Hospital	15 (15.0)	1 (3.1)	14 (20.6)	0.03
ICU	9 (9.0)	0 (0)	9 (13.2)	0.05
Duration				
Hospital length of stay, days	8 (6–16)	9 (6–14)	8 (5.0–17)	0.94
ICU length of stay, days	4 (2–7)	5 (2–7)	4 (2–7)	0.46
Invasive mechanical ventilation ¹ , days	4 (2–7)	4 (3–7)	4 (2–7)	0.70

Data are presented as number (%) and median (interquartile range). ICU = Intensive care unit.

¹ Eighty-one patients on invasive mechanical ventilation.

et al. [41] found that the incidence of influenza A in Singapore peaks in January and June/July when relative humidity is lowest. Our data support these findings, with influenza cases clustered around the start and middle of the year (fig. 2). Second in frequency was rhinovirus, which was the commonest isolate in the Korean and Finnish cohorts [15, 16]. It is difficult to prove cause and effect by the detection of viruses, especially for rhinovirus and coronavirus in the upper airways [10, 42]. In our study, 31 out of the 32 patients with detected viruses were tested positive in their endotracheal secretions rather than with nasopharyngeal swabs, thus increasing the likelihood of causality.

Viruses may act as a predisposing factor for bacterial infection and can present as co-infection with bacteria [10]. Choi et al. [15] and Karhu et al. [16] found no difference in mortality between patients with bacterial infections, viral infections, and bacterial-viral co-infections. However, we found an independent association with lower mortality for patients with viral infection regardless of bacterial co-infection. While our data do not explain this finding, we note that a previous study by our group found lower mortality for culture-negative versus culture-positive sepsis [43]. Meanwhile, the impact of viral co-infection on outcomes remains unclear [44].

Our study has several strengths. We enrolled patients within 24 h of hospital admission. We collected endotracheal aspirates from all intubated patients and nasopharyngeal swabs and sputum from all non-intubated patients. Our study also has limitations. Although we ensured that blood was collected for cultures and PCR analyses before commencement of antibiotics, this was not necessarily the case for respiratory samples. While this reflects real-life practice, it could have decreased the diagnostic yield for respiratory pathogens. Our study was performed in a single centre, thus potentially limiting its

generalisability. As stated earlier, it is difficult to differentiate infection from colonisation and contamination. Finally, as managing physicians were blinded to the results of the PCR analyses aside from influenza viruses, the impact of these tests on clinical management was not evaluated [7–9].

Our study has several implications. It suggests that a large proportion of patients with severe CAP in a tropical setting who would previously have been considered culture negative are in fact infected by various viruses and/or bacteria. Clinicians resort to empiric antibiotics up front and often arbitrary continuation or de-escalation of antibiotics subsequently, resulting in antibiotic abuse and the emergence of multidrug-resistant bacteria [2, 7, 8, 24]. Greater use of PCR amplification for bacteria may allow more precise antibiotic prescription, and routine use of multiplex PCR kits for viruses [8, 20, 45–48] could provide answers regarding aetiology. However, as specific treatments such as antivirals for respiratory viruses other than influenza do not yet exist, the true utility of these PCR kits remains unclear [10].

In conclusion, the use of PCR amplification in addition to routine microbiological investigations including cultures vastly improves the ability to detect both viral and bacterial pathogens in severe CAP. Detection of viruses appears to be independently associated with lower hospital mortality.

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