

The use of multiplex real-time PCR improves the detection of the bacterial etiology of community acquired pneumonia

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Abstract. Community-acquired pneumonia (CAP) is still a major cause of morbidity and mortality especially to children and compromised hosts, such as the old and those with underlying chronic diseases. Knowledge of pathogens causing CAP constitutes the basis for selection of antimicrobial treatment. Previous data have shown that etiological agents can be identified in only up to 50% of patients, but this figure can be improved by using polymerase chain reaction (PCR). This study was designed to evaluate multiplex real-time PCR as a method for rapid differential detection of five bacterial causes of CAP (*Streptococcus pneumoniae*, *Burkholderia pseudomallei* and atypical bacterial pathogens namely *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae* and *Legionella pneumophila*) in CAP patients attending Hospital Tengku Ampuan Afzan (HTAA)/ Kuantan, Pahang, Malaysia. Two previously developed multiplex real-time PCR assays, duplex for the differential detection of *S. pneumoniae* and *B. pseudomallei* and triplex for the atypical bacterial pathogens, were used to detect a bacterial cause of CAP in blood and respiratory samples. Thus, 46 blood and 45 respiratory samples collected from 46 adult CAP patients admitted to HTAA were analysed by multiplex real-time PCR assays and conventional methods. The microbial etiology of CAP could be established for 39.1% (18/46) of CAP patients by conventional methods and this was increased to 65.2% (30/46) with the additional use of real-time PCR. The most frequently detected pathogens were *S. pneumoniae* (21.7% - all by PCR alone), *Klebsiella pneumoniae* (17.3%), *B. pseudomallei* (13% - 83% of them positive by PCR alone and 17% by both culture and PCR), *Pseudomonas aeruginosa* (6.5%), *M. pneumoniae* (6.5% - all by serology), *C. pneumoniae* (4.3% - all positive by both PCR and serology), *L. pneumophila* (2.1% - all by PCR alone), *Escherichia coli* (4.3%). *Haemophilus influenzae*, *Acinetobacter lwoffii* and *Acinetobacter baumannii* were detected by conventional methods (2.1% for each).

INTRODUCTION

Community acquired pneumonia (CAP) remains a leading cause of morbidity and a significant cause of mortality worldwide, with up to 30% of patients requiring hospitalization (Wattanathum *et al.*, 2003; Aydogdu *et al.*, 2010). The mortality from pneumonia was estimated to be 8–14% for hospitalized patients in the United States and 7.3% in Asia (Song *et al.*, 2008). The differential detection and identification of these infections is thus considered a public health priority.

Although many pathogens have been associated with CAP, it is a small range of key pathogens that cause most cases. *Streptococcus pneumoniae* was proven to be the predominant pathogen causing CAP responsible for about two-thirds of all cases of bacteraemic pneumonia (Wattanathum *et al.*, 2003). *Burkholderia pseudomallei* is also a common cause of pneumonia especially in endemic areas including Southeast Asia and Northern Australia (Cheng *et al.*, 2005; Raja, 2008). More recently; *Mycoplasma pneumoniae*,

Chlamydophila pneumoniae (*Chlamydia pneumoniae*), and *Legionella pneumophila* are also found to be common relative to other pathogens causing CAP. These organisms are often called “atypical pathogens” (Lui *et al.*, 2009; Trubnikov *et al.*, 2009; Cunha, 2010).

Management of CAP requires prompt diagnosis in order to initiate early proper antibiotic therapy based on the knowledge of the likely etiologic agent. On the other hand, the increase of resistant bacteria, including CAP pathogens, is a worldwide health problem (Ho *et al.*, 2009). One of the effective measures to prevent new emergence of resistant bacteria is to use only sufficient quantity of appropriate antibiotics to eliminate the target pathogens. Therefore, rapid and precise determination of causative pathogens is critical.

The standard laboratory methods for CAP diagnosis are culture and serology. Although many etiologic studies have been performed, the etiology remains unknown in approximately one-half of the cases (Jokinen *et al.*, 2001; File, 2003). This is mainly due to limitations of conventional diagnostic methods. Results of blood cultures are only positive for 2%–10% of CAP patients, the value of sputum culture findings are questionable in most cases and serologic testing requires convalescent-phase samples, therefore, allows a retrospective diagnosis only. Moreover, for patients who were treated by antibiotics prior to hospitalization, the culture method may give false-negative results, and thus, the causative agent cannot be determined from clinical specimens (Lidman *et al.*, 2002).

PCR is an attractive tool for diagnosing the cause of pneumonia, because it can detect minute amounts of nucleic acid from potentially all pneumonia pathogens, does not depend on the viability of the targeted microbe, is probably less affected by previous antimicrobial therapy than are culture-based methods, and provides results quickly (Chan *et al.*, 2007; Nolte, 2008). These advantages may make PCR the front-runner for the ideal diagnostic test for pneumonia and for most other infections as well (Marois *et al.*, 2010). Recently, multiplex real-time PCR methods have been developed providing an attractive

alternative to conventional PCR in clinical diagnostic laboratories (Brittain-Long *et al.*, 2008; Westh *et al.*, 2009).

This study was designed to evaluate two multiplex real-time PCR assays for rapid differential detection of five bacterial pathogens commonly causing community acquired pneumonia (*S. pneumoniae*, *B. pseudomallei* and atypical bacterial pathogens namely *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila*).

MATERIALS AND METHODS

Study protocol has been approved by Ethics Review Committee of Faculty of Medicine/ International Islamic University Malaysia (IIUM), Ministry of Health and the Medical Director of Hospital Tengku Ampuan Afzan (HTAA)/ Kuantan, Pahang, Malaysia. In addition; written informed consent was obtained from each patient before enrolment in the study.

Clinical samples (blood and respiratory) were collected from patients with CAP during the first 6 months of the study (October 2009 until March 2010). Those samples were then analysed in the following 4 months by the two multiplex real-time PCR assays previously developed in our laboratory (accompanying article in the same issue of this journal) for the differential detection of *S. pneumoniae* and *B. pseudomallei* in a duplex assay and the three atypical bacterial pathogens in a triplex assay. Besides, these samples were analysed by conventional microbiological tests and the results were compared with PCR results.

Origin of the clinical samples

Hospitalized adult patients admitted to the medical wards of Hospital Tengku Ampuan Afzan (HTAA)/ Kuantan, Pahang, Malaysia with a primary diagnosis of CAP were recruited.

Patients included were those aged 15 years or older with the presence of at least two signs or symptoms of pneumonia which include: new or increased cough, new or increased production of purulent sputum, body temperature higher than 37.8°C,

pleuritic chest pain, shortness of breath or crackles on auscultation in addition to chest radiograph taken at the time of admission showing new infiltrates or consolidation that could not be attributed to some other etiology.

Excluded patients were those younger than 15 years old, those who had been hospitalised for more than 48 hours prior to recruitment, patients transferred from another hospital or discharged from an acute care facility during the previous 2 weeks, cases of post-obstructive pneumonia due to lung cancer, pneumonia in immunosuppressed patients including: patients with human immunodeficiency virus (HIV) infection or acquired immunodeficiency syndrome, severe neutropenia of less than 1.0×10^9 cells/L, patients on immunosuppressive therapy including corticosteroid therapy with a dosage of more than 20 mg prednisolone-equivalent per day for 2 weeks or more, and solid-organ or bone marrow transplantation patients, patients with clinical and/or radiological evidence indisputably suggestive of TB and patients who did not agree to participate in the study.

Both respiratory (sputum or throat swab) and blood samples were collected from each patient during the 1st 48 hours of admission. Besides, acute serum sample was collected and each patient was asked to return within 3-8 weeks to give convalescent serum sample.

Conventional microbiological tests

Standard methods were used for isolation of aerobic and anaerobic bacteria from sputum, throat swab and blood samples. All sputum specimens were Gram stained and examined microscopically for the presence of WBCs, epithelial cells and bacteria. Purulence was measured by microscopy and was acceptable if there was >25 WBCs and <10 squamous epithelial cells. Results from sputum cultures were only considered significant if the above Gram-stain criteria were satisfied (Song *et al.*, 2008).

Antibody titers for *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* in patients' acute- and convalescent phase sera were determined. SERODIA[®]-MYCO II particle-agglutination test (Fujirebio, Japan)

was used for the detection of total antibodies against *M. pneumoniae*. An antibody titer ≥ 160 was considered as positive (Ngeow *et al.*, 2005). For *C. pneumoniae*, qualitative IgG and IgM ELISA kits (Vircell, Spain) were used to detect the presence of antibodies against the organism and semiquantitative protocol was used to determine IgG antibody titer in positive serum samples. Qualitative ELISA kits for IgG and IgM (Vircell, Spain) were also used to detect the presence of antibodies against *L. pneumophila* serogroup 1.

DNA extraction

Sputasol (Oxoid, UK) was used for sputum homogenization prior to DNA extraction. About 1ml of sputum was washed by normal saline which was removed later and an equal volume of Sputasol solution was added to the washed sputum with brief shaking followed by incubation in a 37°C shaking water bath for 30 minutes until liquefaction is complete. Homogenized sputum was transferred into microcentrifuge tube and centrifuged at 8,000 rpm for 2 minute and 200 μ l of homogenized sputum including the pellet was used for DNA extraction. DNA extraction was done using QIAmpDNA mini kit (Qiagen, Germany) according to the manufacturer recommended protocol (Blood and Body Fluids Spin Protocol). Final elution volume used was 200 μ l according to the manufacturer recommended protocol.

Throat swabs were collected and stored in Stuart transport medium (Nuova Aptaca, Italy). Each sample was soaked in 1.5 ml of Phosphate Buffered saline and incubated for 10 minutes at room temperature on a shaker. The resultant solution was transferred into a 2.0 ml microcentrifuge tube and centrifuged for 30 min at full speed. Supernatant was discarded carefully not to dislodge the pellet which was used for DNA extraction. DNA extraction was done using QIAmpDNA mini kit (Qiagen, Germany) according to the manufacturer recommended protocol (Tissue Spin Protocol). Final elution volume used was 50 μ l instead of the 200 μ l mentioned in the recommended protocol in order to increase the final DNA concentration in the eluate.

DNA extraction from whole blood samples was done using QIAmpDNA mini kit

(Qiagen, Germany) according to the manufacturer recommended protocol (Blood and Body Fluids Spin Protocol). Final elution volume used was 100 µl instead of the 200 µl mentioned in the recommended protocol in order to increase the final DNA concentration in the eluate.

Molecular testing of the clinical samples

Optimized duplex and triplex real-time PCR reaction mixture and cycling protocols were followed. Each clinical sample was tested in duplicate by both duplex and triplex real-time PCR using CFX96 Real-time PCR detection system (BioRad, USA) with subsequent data analysis using CFX manager software.

Criteria for determination of CAP microbial etiology

Depending on conventional microbiological methods; the etiologic diagnosis of agents causing CAP were considered as being definite, presumptive, and unknown (Wattanathum *et al.*, 2003). A definite etiology was established if one of the following conditions was present:

- a) Blood or pleural fluid culture yielding the presence of a bacterial or fungal pathogen.
- b) Seroconversion (i.e. a four-fold increase in IgG titres) for *L. pneumophila* or *C. pneumoniae*.
- c) A single increased IgM titer for *L. pneumophila* or *C. pneumoniae*.

A presumptive etiology was considered if any of the following conditions was present:

- a) Growth of a predominant bacterial pathogen from culture of sputum or other respiratory tract specimen in combination with similar findings on Gram staining.
- b) An antibody titer ≥ 160 for *M. pneumoniae* in either the acute-phase or convalescent-phase serum specimens.

Patients, in whom more than one microorganism was detected, according to the above-mentioned criteria, were defined as having mixed infection. When no causative agent was found according to the criteria, the

pneumonia was classified as having unknown etiology.

Recently, with the spread of modern molecular testing methods and their application in diagnostic microbiology; detection of microbial DNA in patient's sample was considered to be a support for probable microbial diagnosis (Johansson *et al.*, 2010).

RESULTS

Patient characteristics

During the 6 months period of sample collection, a total number of 97 patients with a primary diagnosis of CAP were screened; however, only 46 patients fulfilled the inclusion criteria and thus were recruited in this study. The mean age (\pm SD) was 54.7 (± 15.3) years (range 17–80 years old).

For the purpose of serological testing, acute phase serum was collected from each of the 46 patient. However, convalescent phase sera were collected from only 29 patients (63%) because 12 patients (26.1%) were unable or unwilling to come for follow up and the remaining 5 patients (10.9%) died either during hospitalization (4 patients) or at home (1 patient).

For real-time PCR testing, one blood and one respiratory sample were collected from each patient (with the exception of one patient who died before giving a respiratory sample). Thus 46 blood and 45 respiratory samples (29 sputa and 17 throat swabs) were collected and tested by both multiplex real time PCR assays.

Fourteen (30.4%) of patients received antibiotics prior to hospital admission and all of the 46 patients (100%) received antibiotic therapy during the first 48 hours of hospitalization. Unfortunately, the exact timing of sample collection relative to antibiotic administration during hospitalization was not documented.

Microbial etiology established by conventional methods

Blood culture revealed relevant microbial etiology of CAP for only 4 (8.7%). Isolated organisms were two *Escherichia coli*

(4.3%), one *B. pseudomallei* (2.2%) and one *Pseudomonas aeruginosa* (2.2%). Positive culture results were obtained from 12 (26%) of patients' sputa while none could be obtained from throat swabs. The organisms isolated from sputum samples were *Klebsiella pneumoniae* in 8/46 (17.3%) sputa, *P. aeruginosa* in 2/46 (4.3%), *Haemophilus influenzae*, *Acinetobacter lwoffii* and *Acinetobacter baumannii* were detected in one (2.2% for each).

Particle-agglutination test was used for the detection of total antibodies against *M. pneumoniae*. Antibody titer ≥ 160 in acute phase serum was used in previous Asian studies as a cutoff point to define the presence of acute infection (Chan *et al.*, 2001; Ngeow *et al.*, 2005). According to this definition only 3/46 (6.5%) of our patients were seropositive.

ELISA test was used to detect the presence of antibodies against *L. pneumophila* serogroup 1 (IgG and IgM). Both IgG and IgM antibodies were absent in the tested serum samples indicating the absence of acute infection with *L. pneumophila* serogroup 1. No serologic test

was attempted to test the presence of antibodies against other *L. pneumophila* serogroups (2-15) as no kit was available to cover those serogroups (Javed *et al.*, 2010).

ELISA test was also used to detect the presence of IgG and IgM antibodies against *C. pneumoniae*. Interpretation of serological data may be problematic due to the high prevalence of *Chlamydoiphila* antibodies in adult population (Menendez *et al.*, 1999). Positive serological results were obtained for only 2/46 (4.3%) of our patients. Both of them were IgM positive in their acute phase sera in addition to having ≥ 4 folds rise in the IgG titers between acute and convalescent phase sera.

Microbial etiology established by molecular methods

46 blood and 45 respiratory samples were tested in duplicate by duplex and triplex real-time PCR (figure 1 and 2).

Duplex real-time PCR results showed that 6 (13%) patients were positive for *B. pseudomallei* (Two patients had positive PCR for both blood and respiratory samples, two

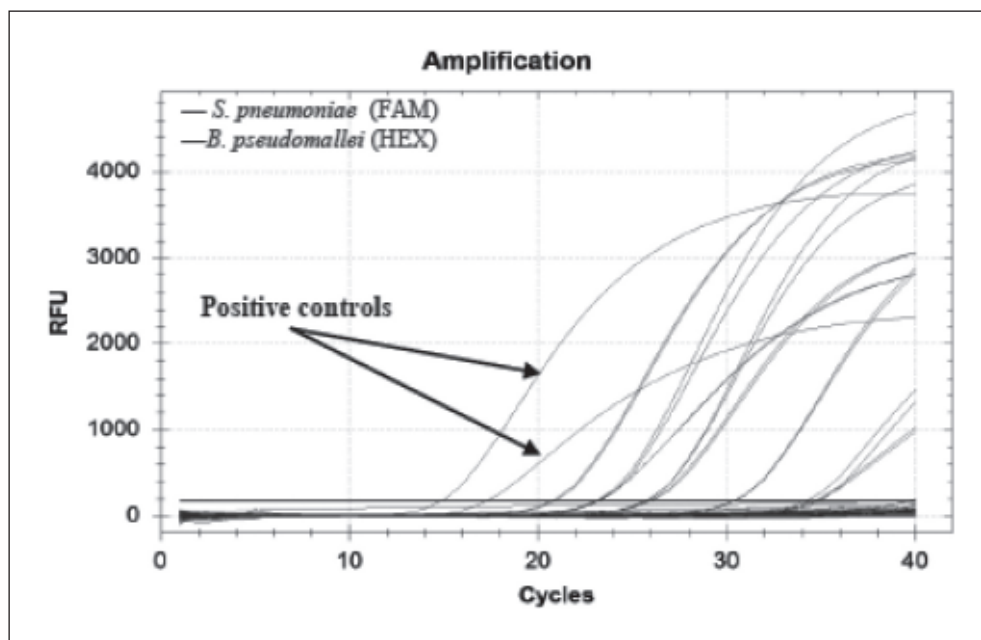


Figure 1. Duplex real-time PCR for *S. pneumoniae* and *B. pseudomallei* showing amplification profile of some positive clinical samples (blood and respiratory samples). The X and Y axes represent amplification cycles and relative fluorescence units (RFU) respectively

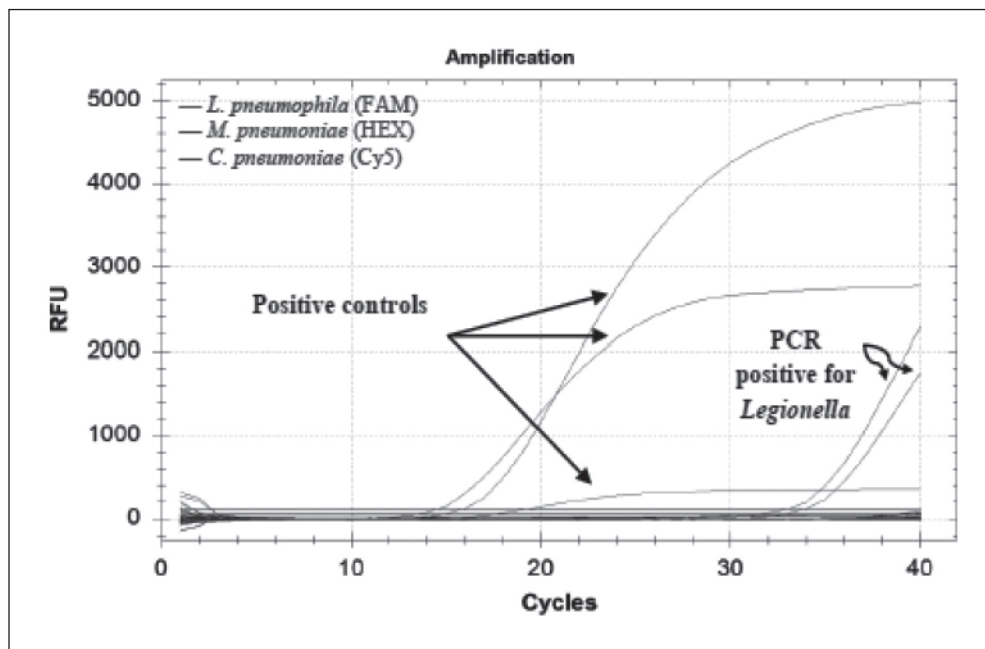


Figure 2. Triplex real-time PCR for *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae*. The X and Y axes represent amplification cycles and relative fluorescence units (RFU) respectively. The relatively low RFU of *C. pneumoniae* amplification curves is related to fluorescence properties of the reporter dye (Cy5)

had positive blood PCR only, one had positive sputum PCR only and one positive PCR in throat swab only). 4 of these 6 patients died during the 1st 48 hours of hospitalization. One of them only has positive blood culture providing definitive diagnosis of his illness and the other 3 died without known microbial etiology.

For *S. pneumoniae*, 10 (21.7%) respiratory samples were PCR positive with negative blood samples. There was one patient having both *B. pseudomallei* and *S. pneumoniae* detectable in his throat swab by duplex real-time PCR. This patient died rapidly during hospitalization as mentioned above. Patients' blood and/or respiratory samples with positive duplex real-time PCR results are described in Table 1.

According to the triplex real-time PCR results; only one (2.2%) patient was with positive sputum PCR for *L. pneumophila*. Despite having negative serology results, he had clinical features suggestive of legionella infection (Edelstein, 2008).

Two patients were confirmed to have *C. pneumoniae* as both serology and respiratory PCR were positive. Three other patients had serological evidence of *M. pneumoniae* infection; however, PCR was negative for their blood and respiratory samples. Patients' samples with positive triplex real-time PCR results are described in Table 2.

Microbial etiology established by both conventional and molecular methods

Microbial etiology of CAP could be established for 18/46 (39.1%) patients by conventional methods but the figure was increased to 30/46 (65.2%) with the additional use of real-time PCR. The most frequently detected CAP pathogens with their detection methods are described in Table 3. Mixed infection was identified in 8/46 (17.6%) of cases as in Table 4.

Table 1. Patients' samples with positive results in duplex real-time PCR

Study ID	Duplex Real-time PCR			
	Mean Ct value			
	<i>Burkholderia pseudomallei</i>		<i>Streptococcus pneumoniae</i>	
	Blood	Respiratory samples	Blood	Respiratory samples
4	-ve*	-ve	-ve	30.41(Throat swab)
10	-ve	-ve	-ve	34.57 (Sputum)
16	34.52	No sample	-ve	No sample
19	-ve	-ve	-ve	25.98 (Sputum)
23	-ve	25.38 (Throat swab)	-ve	-ve
29	-ve	-ve	-ve	25.69 (Sputum)
31	-ve	23.04 (Sputum)	-ve	-ve
32	-ve	-ve	-ve	23.17 (Sputum)
33	35.54	35.75 (Throat swab)	-ve	28.21 (Throat swab)
36	-ve	-ve	-ve	30.38 (Sputum)
39	-ve	-ve	-ve	20.81 (Sputum)
40	34.43	35.03 (Sputum)	-ve	-ve
41	33.06	-ve	-ve	-ve
42	-ve	-ve	-ve	24.94 (Sputum)
45	-ve	-ve	-ve	22.27 (Sputum)

* -ve = negative.

Table 2. Patients' samples with positive results in triplex real-time PCR

Study ID	Triplex Real-time PCR					
	Mean Ct value					
	<i>Legionella pneumophila</i>		<i>Mycoplasma pneumoniae</i>		<i>Chlamydophila pneumoniae</i>	
	Blood	Respiratory samples	Blood	Respiratory samples	Blood	Respiratory samples
1	-ve*	-ve	-ve	-ve	-ve	36.13 (Sputum)
20	-ve	33.4 (Sputum)	-ve	-ve	-ve	-ve
43	-ve	-ve	-ve	-ve	-ve	35.73 (Sputum)

* -ve = negative

Table 3. Detection rates of single CAP pathogens as determined by multiplex real-time PCR, culture and serology

Microorganisms	% Microbial detection	Detection method
<i>Streptococcus pneumoniae</i>	21.7%	All by PCR alone
<i>Klebsiella pneumoniae</i>	17.3%	Culture
<i>Burkholderia pseudomallei</i>	13%	83% by PCR alone and 17% by both culture and PCR
<i>Pseudomonas aeruginosa</i>	6.5%	Culture
<i>Mycoplasma pneumoniae</i>	6.5%	All by serology
<i>Chlamydophila pneumoniae</i>	4.3%	All by both PCR and serology
<i>Escherichia coli</i>	4.3%	Culture
<i>Legionella pneumophila</i>	2.2%	All by PCR alone
<i>Haemophilus influenzae</i>	2.2%	Culture
<i>Acinetobacter baumannii</i>	2.2%	Culture
<i>Acinetobacter lwoffii</i>	2.2%	Culture

Table 4. Detection rates of mixed CAP pathogens as determined by multiplex real-time PCR, culture and serology

Microorganisms	Frequency	Percent
<i>Klebsiella pneumoniae</i> + <i>Legionella pneumophila</i>	1	2.2
<i>Klebsiella pneumoniae</i> + <i>Chlamydomphila pneumoniae</i>	1	2.2
<i>Klebsiella pneumoniae</i> + <i>Haemophilus influenzae</i>	1	2.2
<i>Klebsiella pneumoniae</i> + <i>Burkholderia pseudomallei</i>	1	2.2
<i>Streptococcus pneumoniae</i> + <i>Burkholderia pseudomallei</i>	1	2.2
<i>Streptococcus pneumoniae</i> + <i>Pseudomonas aeruginosa</i>	1	2.2
<i>Mycoplasma pneumoniae</i> + <i>Pseudomonas aeruginosa</i>	1	2.2
<i>Escherichia coli</i> + <i>Acinetobacter baumannii</i>	1	2.2
Total	8	17.6

DISCUSSION

Molecular diagnostic techniques are promising tools for the rapid etiologic diagnosis of many infections including CAP. Multiplex real-time PCR technology enables simultaneous amplification of multiple gene targets in the same sample, saving both time and cost. Thus, simultaneous detection of multiple CAP pathogens is both possible and desirable for rapid diagnosis of pneumonia (Templeton *et al.*, 2005; Johansson *et al.*, 2010).

In this study, clinical samples (blood and respiratory samples) collected from 46 CAP patients were tested by two multiplex real-time PCR assays, duplex for the simultaneous detection and differentiation of *S. pneumoniae* and *B. pseudomallei* and triplex for the atypical bacterial pathogens (*L. pneumophila*, *M. pneumoniae* and *C. pneumoniae*).

A microbial etiology could be established for 39.1% (18/46) of CAP patients depending on conventional methods alone (culture for typical bacterial pathogens and serology for atypical ones). This figure was increased to 65.2% (30/46) with the additional use of multiplex real-time PCR. Similar findings were reported previously with a range of microbial detection from 43% to 89% when real-time PCR results were added to those of conventional methods

(Templeton *et al.*, 2005; Johansson *et al.*, 2010). This wide range is due to the difference in inclusion criteria, sampling and methodology followed in each study.

Consistent with most previous studies in Asia and worldwide (Song *et al.*, 2008); *S. pneumoniae* was the most common etiologic agent of CAP in our study. It was detected in 10/46 (21.7%) cases by PCR alone in patients with negative blood and respiratory cultures. Failure to isolate *S. pneumoniae* was reported previously and was attributed to prior antibiotic therapy that may impair the diagnostic validity of both sputum and blood cultures in addition to the delay of sample processing that can reduce the isolation rates and increase indigenous flora (Bartlett *et al.*, 1998; Lim *et al.*, 2009). Several studies found that sputum cultures became quickly negative for *S. pneumoniae* during antibiotic treatment in contrast to PCR which remained positive in spite of ongoing treatment (Stralin *et al.*, 2005; Kee *et al.*, 2010). Another reason for the PCR positivity in culture-negative samples could be its higher sensitivity compared to culture. Thus, our findings support earlier suggestions that pneumococci cause the majority of conventional tests – negative CAP cases (Ruiz-Gonzalez *et al.*, 1999; Johansson *et al.*, 2010). We were able to obtain positive PCR results from respiratory samples but not from blood. This can be due to low level or absence

of pneumococcal bacteriemia especially that all blood cultures were negative. Another possibility is that these patients developed a low-level transient bacteraemia that was rapidly cleared by the innate immune system (Menendez *et al.*, 1999; Hohenthal *et al.*, 2008).

Burkholderia pseudomallei was identified in 6/46 (13%) patients, however, a successful isolation by culture was achieved for only one patient. This may be due to several factors including sample type, timing of collection and antecedent use of antibiotics. Moreover, the presence of pathogenic bacteria in nonsterile specimens such as sputum and throat swabs may be overlooked due to the speedier growth of other commensal organisms. Many investigators have reported low levels of *B. pseudomallei* in blood samples from patients with septicemic melioidosis (Tiangpitayakorn *et al.*, 1997; Simpson *et al.*, 1999). In a study conducted by Walsh *et al.* (1995), 45% of septicemic melioidosis patients have less than 1 CFU/ml in their blood samples. This low level of bacteria in the blood presents a challenge, even to the sensitive PCR assay and may explain why five out of six PCR positive patients had negative blood and respiratory cultures for *B. pseudomallei*. The PCR-based diagnosis was supported when it was realized that all of the 6 cases were diabetic with positive clinical and radiological findings. This was even further supported by the high mortality rate reported as 4/6 (67%) died within the 1st 48 hours of hospital admission due to fulminant pneumonia with septicemia. Similar high mortality rates were previously reported in Pahang and other endemic regions in Malaysia and Southeast Asia (How *et al.*, 2006, 2009; Supaprom *et al.*, 2007; How *et al.*, 2009). It was suggested that early accurate detection of *B. pseudomallei* may be accomplished by assaying concentrated samples such as respiratory samples (Novak *et al.*, 2006). Our findings agree with this suggestion as concentrated respiratory samples from 4 patients were positive by PCR. But, PCR was also positive for blood samples of 4 cases as well (two of them had positive respiratory and blood PCR).

Unfortunately, the number of positive cases was too small for a valid statistical analysis to decide the superiority of one sample type over the other.

Legionella pneumophila has been reported as one of the most common atypical pathogens causing CAP around the world; however, the frequency of CAP due to *L. pneumophila* was found to be relatively low in Asian communities (Liam *et al.*, 2007; Song *et al.*, 2008). In the present study, sputum PCR was positive for one patient only (2.2%) with supportive clinical and laboratory findings but tested negative for *L. pneumophila* serogroup 1 antibodies. Commercially available ELISA kit used to test serum samples of patients recruited in the study was specific for detection of antibodies against serogroup 1 only and thus cannot detect antibodies against other *L. pneumophila* serogroups. However, the set of primers and probe used in this study was designed to allow the detection of all serogroups (1–15) as they targeted the *mip* gene which is similarly present in all serogroups (Joly *et al.*, 2006; Stolhaug *et al.*, 2006). Thus there is a possibility that the PCR positive patient had *L. pneumophila* belonging to a serogroup other than 1. A similar finding was reported by Morozumi *et al.* in 2006 who reported one positive real-time PCR adult patient suspected of having the infection, later confirmed to have *L. pneumophila* serogroup 5 by real-time PCR of a urine sample. Animal studies have indicated that PCR testing of whole blood samples may be a useful non-invasive method for the diagnosis of legionella pneumonia (Aoki *et al.*, 2003). However, no previous study, known to us, was done using PCR to test human whole blood samples. In this study, the patient with positive sputum PCR had negative blood PCR. Collection of the blood sample on the second day of his admission to hospital may explain his blood PCR negativity as it has been proved that legionella DNA only peaks early in the acute stage of infection (Diederer *et al.*, 2007). Besides, antibiotics administration was also shown to be a factor affecting the amount of *Legionella* DNA available in patients' clinical samples.

Previous Asian studies reported low incidence of *C. pneumoniae* among hospitalized CAP patients (Liam *et al.*, 2007; Song *et al.*, 2008). This was explained by the fact that *C. pneumoniae* often causes mild clinical disease that is more likely to be treated in outpatients clinics. Our results agreed with these studies as only 2/46 (4.3%) of our CAP patients tested positive by triplex real-time PCR and ELISA.

In most previous Asian studies, an antibody titre ≥ 160 in acute phase serum was arbitrarily chosen as presumptive evidence of a recent for *M. pneumoniae* infection (Rastawicki *et al.*, 2002; Kung *et al.*, 2007;). However, a cut off titer ≥ 640 was used to define acute *Mycoplasma* infection in a recent Japanese study using the same PA kit (Otomo *et al.*, 2008). This was based on previous reports from Japan stating that the specificity of the PA test is equal to 99.3% when a cut off titer of 640 is used for testing a single acute serum sample (Yamazaki *et al.*, 2006; Narita, 2007). Due to the lack of information on the population background antibody levels in Malaysia, we depended on the cut off titer of 160 recommended by most previous Asian studies to define an acute infection. According to this definition, only 3/46 (6.5%) of our patients were considered seropositive. However, PCR testing of their blood and throat swabs (other respiratory samples could not be obtained) revealed negative results. It was reported that throat swabs are less informative samples for *M. pneumoniae* DNA detection as compared to other types of respiratory specimens (Dorigo-Zetsma *et al.*, 1999). The early disappearance of *M. pneumoniae* due to antimicrobial therapy or the host immune response may also explain negative PCR results in those patients with otherwise serologically confirmed mycoplasma respiratory tract disease (Daxboeck *et al.*, 2003). Another possible explanation is that the cut off titer (160) used to serologically define an acute infection may be low leading to false-positive results. In addition, the low

number of patients infected with *M. pneumoniae* in the current study could be the relatively older age (mean 54.7 ± 15.3 years) of our patients, as this pathogen is often considered to be more common in children and young adults (Marrie, 2000; Stralin *et al.*, 2005)

There are some limitations to our study. The number of patients and thus the number of positive specimens was unfortunately too small to perform elaborate statistical analysis. Serum sample collection was incomplete because not all patients came back to give the convalescent phase sera. The exact timing of sample collection relative to antibiotic administration was not accurately documented. Finally, there was difficulty in obtaining representative respiratory samples from some patients. These issues are probably inherent in all clinical studies enrolling patients with CAP.

In conclusion, we demonstrated that multiplex real-time PCR is useful in identifying CAP causative agents. By supplementing traditional diagnostic methods with real-time PCR, a higher microbial detection rate was achieved for both typical and atypical pneumonias. Further larger scale prospective studies are needed to establish standardized real-time PCR methods that are robust and simple enough to be used outside the setting of research laboratories i.e. in diagnostic reference and hospital-based laboratories.

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