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Full Length Research Paper

Community acquired pneumonia in Malaysia: Is *Streptococcus pneumoniae* an important pathogen?

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Previous data on etiological agents isolated from adult patients with community acquired pneumonia (CAP) in Malaysia has showed very low percentage of *Streptococcus pneumoniae*. Thus, we used immunochromatography test (ICT) and real-time polymerase chain reaction (PCR) in addition to conventional culture methods for *S. pneumoniae* detection. We found that the detection rate was highest by real time PCR reaction (50%) in contrast to 10% by ICT, 2% from blood and 0% from sputum cultures. This molecular method had contributed to a rise in percentage of *S. pneumoniae* detection accounting for 51.1% of all etiological cases in CAP and the second commonest organism after *Chlamydophila pneumoniae* (63.8%), followed by *M. pneumoniae* (27.7%) and *L. pneumophila* (17%). We have also found that 10.6% of the etiological agents of CAP were not known indicating that other specific organisms including viruses have not been identified. Both ICT and PCR demonstrated sensitivities of 100%, with specificities of 91.3 and 55.6%, respectively, using culture techniques as the "gold standard". Thus from this finding, they will become potential tools in the future for the diagnosis of *S. pneumoniae* in CAP, for the epidemiological importance and prevention as well as for early antibiotic management.

Key words: Community acquired pneumonia, immunochromathography, polymerase chain reaction, *Streptococcus pneumonia*.

INTRODUCTION

Community acquired pneumonia (CAP) is a major cause of morbidity and one of the greatest burdens on the health service in many countries. Most CAP patients are treated mainly in primary care, and about 15% of the cases, hospital admission are required (Lagerstrom et al., 2003).

Streptococcus pneumoniae is the commonest organism detected in many countries in CAP cases. Higher detection rate of *S. pneumoniae* was reported in many countries such as United Kingdom (39%), European countries (19.4%), Australia and New Zealand (38.4%), North America (11.3%), Japan (20.5%), Korea (21.7%), Thailand (22.7%) and Argentina (15.4%). However, a study in Malaysia in 2003 showed lower detection rate of *S. pneumoniae* (3.4%) (Liam, 2005). Other than bacterial causes, other pathogens have become significant independent and co-infecting pathogens in CAP such as respiratory viruses, atypical bacteria (*Mycoplasma pneumoniae*, *Chlamydophila pneumoniae and Legionella pneumophila*) fungi, and parasites (Lode, 2007).

This study was performed to determine the causative agents for community acquired pneumonia with additional methods to detect *S. pneumoniae* using molecular and immunochromatography techniques.

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MATERIAL AND METHODS

We collected samples from patients, 18 years and above with CAP which fulfil at least three out of four criteria which are 1) fever, 2)

cough with or without expectoration, 3) neutrophilia (>7.0 ×10^9 /L) or raised total white cell count (>10.0 × 10^9 /L) and 4) presence of infiltrates on chest radiograph which were consistent with consolidation (Bartlett et al., 2000). We excluded patients diagnosed with opportunistic pneumonia, for example, *Pneumocystis carinii* and pulmonary tuberculosis, neutropenic or immunosuppressed patients due to corticosteroids, chemotherapy or other immunosuppressive agents or the presence of malignancy, leukaemia or lymphoma and hospital acquired pneumonia patient.

We cultured blood and sputum for isolation of bacterial pathogens. Serology methods were used to detect antibody for *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila*. Blood in EDTA bottles were centrifuged at 3,800 rpm for 5 min. Plasma samples were kept at -70°C until required for polymerase chain reaction (PCR) test for *S. pneumoniae*. Urine was collected for immunochromatography (ICT) assay for *S. pneumoniae*.

Sputum was processed in Biosafety Carbinet Class II. Smears and Gram stains were prepared from the sputum sample and examined microscopically. Further isolation and identification of organism was only done from sputum sample which showed less than 10 squamous cell epithelial cells, presence of microorganism and more than 25 polymorphonuclear cells per field at 100 x magnification (low power, 10x objective) to exclude colonizers (Mandell et al., 2005).

Streptococcus pneumoniae identification was confirmed by bile salt solubility test and optochin sensitivity. Antibiotic disc panels for sensitivity testing were chosen according to Clinical and Laboratory and Standards Institute (CLSI) that included oxacillin 1 μg, erythromycin 15 μg, chloramphenicol 30 μg, cefuroxime 30 μg, cefotaxime 30 μg, augmentin (amoxycillin-clavulanic acid) 30 μg and optochin 2 μg. Other causative pathogens were cultured and identified by standard procedure.

Serological technique

We used commercial kits Biolink (BLK) Diagnostic ELISA kit from Badalona Spain for *C. pneumoniae* and *M. pneumoniae* IgM and an indirect immunofluorescence (IF) (BLK Diagnostic, Baladona) for detection of *C. pneumoniae* Ig G (Serodia-MycoII, Fujirebio, Japan) for *M. pneumoniae* Ig G antibody. *Legionella pneumophila* Ig M and Ig G were identified using Serion/Virion ELISA Classic kit (Germany). The tests were performed and interpreted according to the manufactures instructions. Urine samples for pneumococcal C-polysaccharide (PnC) were tested using Binax NOW test kit (Portland, USA).

Polymerase chain reaction (PCR)

Nucleic acid was extracted using the Roche High Pure PCR Template Purification Kit (Roche Molecular Biochemical, Mannheim, Germany) according to the manufacturer's instructions.

The primers SP-S with sequence (5' to 3') of GAC AAT ACA GAA GTG AAG GCG G and SP-AS with sequence (5' and 3') of ATA GGC ACC ACT ATG ATC CAG C (Sigma-Proligo, Proligo Singapore Pty. Ltd) were used to target a 266 base pair fragment of the single copy pneumolysin (ply) gene of *S. pneumoniae* (Walker et al., 1987).

PCR was performed according to Robert et al. (2003), on a close system of Roche LightCycler 2.0 instrument (Martín-Galiano et al., 2003). The PCR reaction mixtures occurred in the presence of SYBER Green I fluorescence dye in glass reaction vessels, coupled with real-time fluorescent to detect the amplicon. Assays were carried out in a 20 μL reaction volume. Reaction reagents were purchased in a preformatted kit (LightCycler FastStart DNA Master for SYBR Green I, Roche Molecular Biochemical, Mannheim, Germany Cat. No. 2 239 264) containing FastStart Taq DNA polymerase and DNA double strand specific SYBR Green I fluorescence dye to detect amplified *S. pneumonia* DNA.

The cycling conditions were as follows: initial denaturation/Taq activation at 95°C for 10 min; followed by 45 cycles of 95°C for 5 s, 65°C for 10 s and 72°C for 15 s. Fluorescence acquisition was performed once per cycle at 65°C annealing stage and all temperature transition rates at 20°C /s. A melting curve was performed at the end of each run using a temperature range of 55°C to 85°C and 0.15°C /s temperature transition rate.

Specificity of the test was performed using positive control [Chromosomal DNA from the *S. Pneumonia* (ATCC 49619)] while *Streptococcus pyogenes* (ATCC 19615), *Escherichia coli* (ATCC 25922), *Streptococcus viridans* (in house control strain) and distilled water were used as negative controls.

To perform the sensitivity of the test, DNA was extracted from a dense suspension of *S. pneumoniae* (ATCC 49619) grown on blood agar plates. The concentration of DNA was determined spectrophotometrically with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). A 10-fold serial dilution scheme ranging from 50 and 0.5 ng/μl was prepared. All the dilutions were run at the same time using the Lightcyler PCR. The amplification curves from each dilution were generated by LightCycler software and plotted on a graph to form a standard linear curve.

All patients' samples were run with a negative water control and positive control [Chromosomal DNA from the *S. Pneumonia* (ATCC 49619)]. All sample materials were handled according to CLSI guidelines (CLSI, 2006).

Interpretation for real time PCR

Melting curve analysis was performed on all samples, and melting point temperature was calculated by averaging the values obtained with control strains for the respective runs (mean ± 0.05). All PCR products displaying melting temperatures outside the acceptance range were regarded nonspecific and hence considered negative.

RESULTS

Sputum and blood culture

The highest yield from the sputum culture were normal mouth flora (83%) followed by *Enterobacter* group 1 β lactamase (GIBL) (2%), Group G *Streptococcus* (2%) and *Pseudomonas aeuroginosa* GIBL (2%). No growth of organisms in 12% of samples, all sixty samples were negative for *S. pneumoniae*. Growth was obtained from 6.4% of blood cultures. *S. pneumoniae* was detected in one sample (1.6%). Other organism isolated included *Burkholderia pseudomallei* (1.6%), *Sternotrophomonas maltophilia* (1.6%) and coagulase negative *Staphylococcus* (CONS) [1.6%]

Immunochromatography (ICT) method

Urinary antigen assays to detect *S. pneumoniae* antigen were performed in 50 (80.6%) out of 62 samples. Ten percent (5/50) of urine sample showed strong positive results. Using blood culture in all 50 samples for *S.*

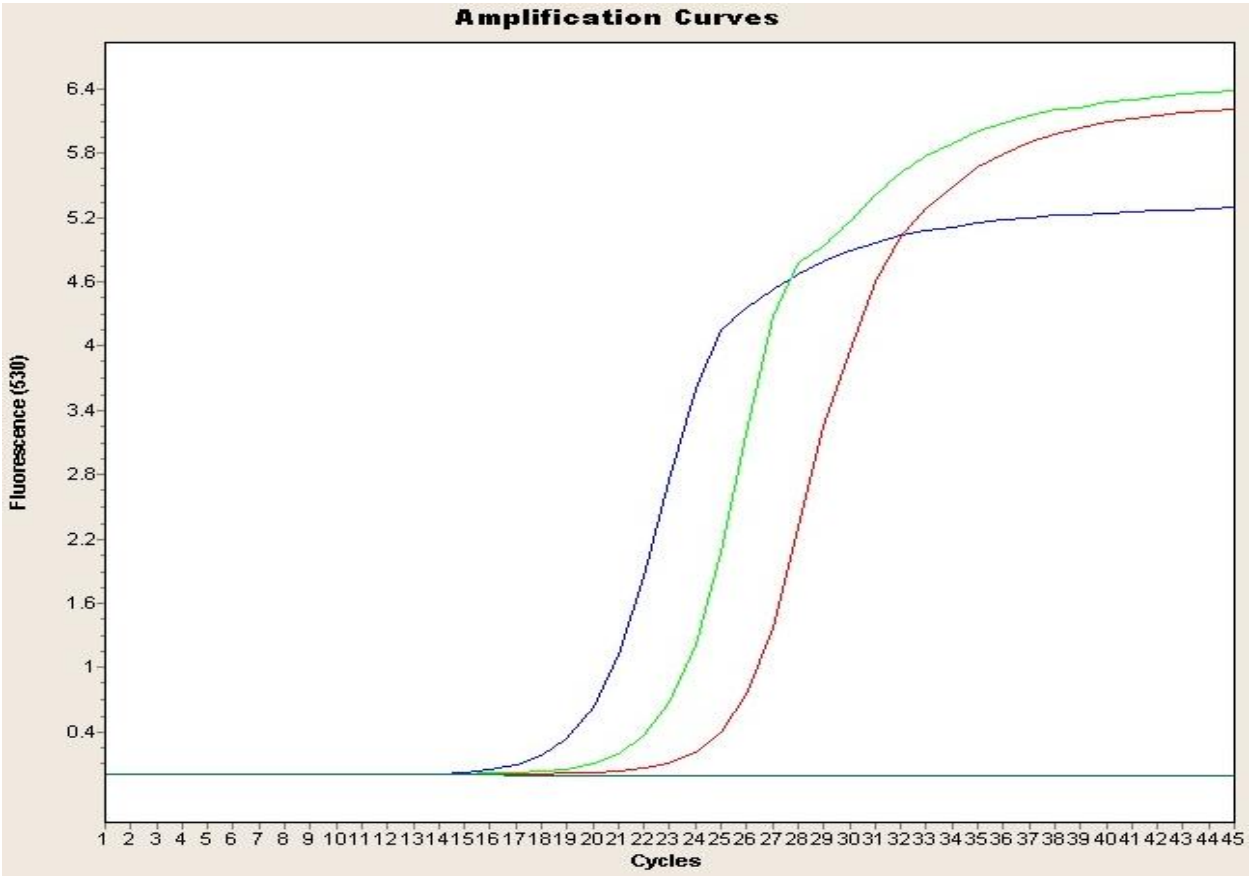

Figure 1. Amplification curve of serial dilutions of *S. pneumoniae* DNA.

pneumoniae detection as gold standard, we found that the sensitivity of ICT was 100% with a specificity of 91.8%.

Molecular (Real-time PCR)

Sensitivity of real-time PCR

Serial dilutions of *S. pneumoniae* DNA were performed from 50 to 0.5 ng/µL and were run at the same time using LightCycler PCR. The amplification curves obtained were shown in Figure 1 displaying the fluorescence values versus the cycle numbers (crossing points). A standard curve was generated from crossing points (Cp) values versus serial dilutions of *S. pneumoniae* DNA (Figure 2). The curve displays a linear trend and intercept at 24.25 at Y-axis. A melting curve analysis showed specific amplifications and melting points (Tm) from each dilution as shown in Table 1. From the results, the mean melting points (Tm) was found to be 85.27 ± 0.12 (SD).

Specificity of the test

Specific melting points (Tm) were obtained from melting curve graphs for each calibrators and positive controls. Over 15 consecutive runs, the mean of Tm values was 84.99 ± 0.41 (SD) and normally distributed (Kolmogrov-Smirnov, p >0.05).

The specificities of the real-time PCR assay were tested using extracted DNA from different bacteria including *S. pneumoniae* (ATCC 49619), *S. pyogenes* (ATCC 19615), *E. coli* (ATCC 25922) and *S. viridans* (in house control strain). *S. pneumoniae* (ATCC 49619) was used as positive control and distilled water was used as negative control in every test. Evaluation of pneumolysin (*ply*) primers at annealing temperatures of 65 and 95°C showed the tested *S. pyogenes* and *S. viridans* had Tm values above 85.2 ± 0.5 (SD) (Figure 3). In other specificity test, melting point of *E. coli* strain was outside the range of melting point for *S. pneumoniae* 84.6 ± 0.5 (SD).

Clinical samples

The result of each sample in the study was analyzed based on Tm point of control positive *S. pneumoniae* and negative control (water) in each run. Any result that was outside the range of Tm of *S. pneumoniae* ± 0.5 (SD)

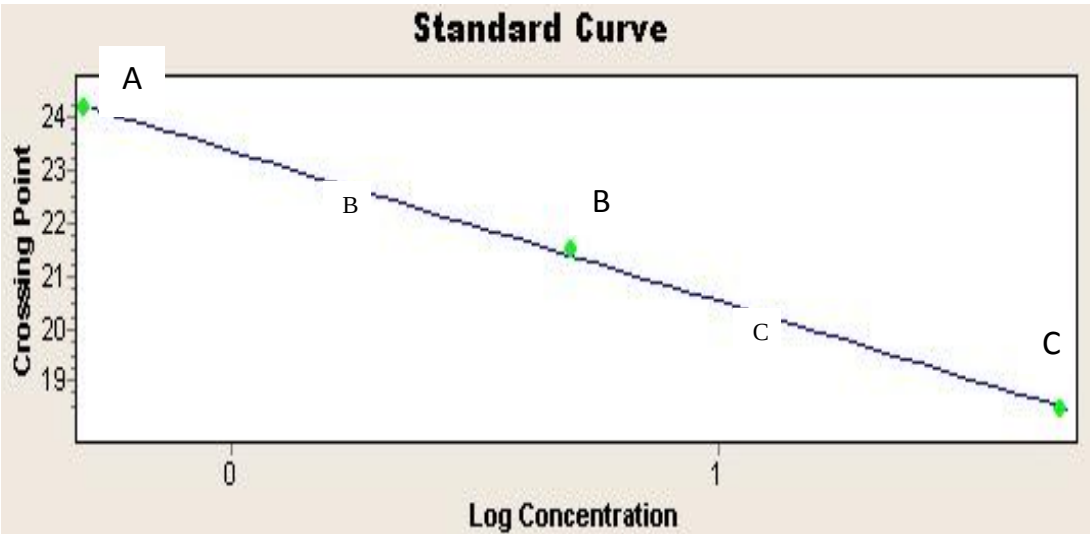

Figure 2. Standard curve showing crossing points (Cp) versus log concentration for *S. pneumoniae* DNA. A: 0.5 ng/µL ; B:5 ng/µL ; C:50 ng/µL.

Table 1. Melting point (Tm) of serial dilution of *S. pneumoniae* DNA.

Concentration (ng/µL)	Melting point (Tm)	Crossing point (C_p, cycles)	Line colour
50.0	85.13	18.45	Blue
5.0	85.34	21.47	Green
0.5	85.36	24.20	Red
Water	Not recorded	Not recorded	Grey

was considered negative. Example of a PCR test result is shown in (Figure 4). From the Figure 4, PCR results were positive in 25 out of 50 samples (50%). Using blood culture results as gold standard, the specificity of our PCR test is 51.0% (95% CI 0.9 to 1.0; p > 0.05). The sensitivity is 100% while positive predictive value (PPV) is 4 % and negative predictive value (NPV) is 100%.

Interpretation

Out of 47 results using all methods including serology for atypical bacteria, single pathogen was detected in 14/47 (29.9%) of patients and two or more pathogens in 28/47 (59.6%) of patients. The most frequent aetiological agents were *C. pneumoniae* 30/47 (63.8%), *S. pneumoniae* 24/47 (51.1%), *M. pneumoniae* 13/47 (27.7%), *L. pneumophilia* 8/47 (17%), Gram negative bacilli (*B. pseudomallei*, *S. maltophila*) 2/47 (4.3%). In 10.6 % (5/47) cases, the microbial aetiology remained unknown.

A few combinations of pathogens were found among adult patients with CAP in the study. The most common mixed infections were *S. pneumoniae* and *C. pneumoniae* (19.1%) followed by *M. pneumoniae* and *C. pneumoniae* (10.6%), and *C. pneumoniae* and *L. pneumophila* (6.4%).

DISCUSSION

This study showed that *C. pneumoniae* 30/47 (63.8%) is the commonest organism followed by *S. pneumoniae* 24/47 (51.1%), *M. pneumonia* 13/47 (27.7%) and *L. pneumophila* (17%) and Gram negative bacilli (*B. pseudomallei*, *Sternothrophomonas maltophilia*) 2/47(4.3%). Higher detection rate for *S. pneumoniae* was obtained using real time PCR when compared with earlier studies in Kuala Lumpur (3.4%) and Pulau Pinang (3.1%) (16,24). Thus, this finding is an agreement with other studies indicating that *S. pneumoniae* is one of the most frequently isolated pathogen in CAP ranging from 6 to 76% (Lorente et al., 2000; Mandell et al., 2007). Although other studies reported higher yield of *S. pneumoniae* detection by blood and sputum 5 - 14% (Bartlett et al., 2000; Campbell et al., 2003; Lentino et al., 1987; Metersky, 2003; Waterer et al., 2001), our finding only showed 1.6% by blood culture and none from the sputum.

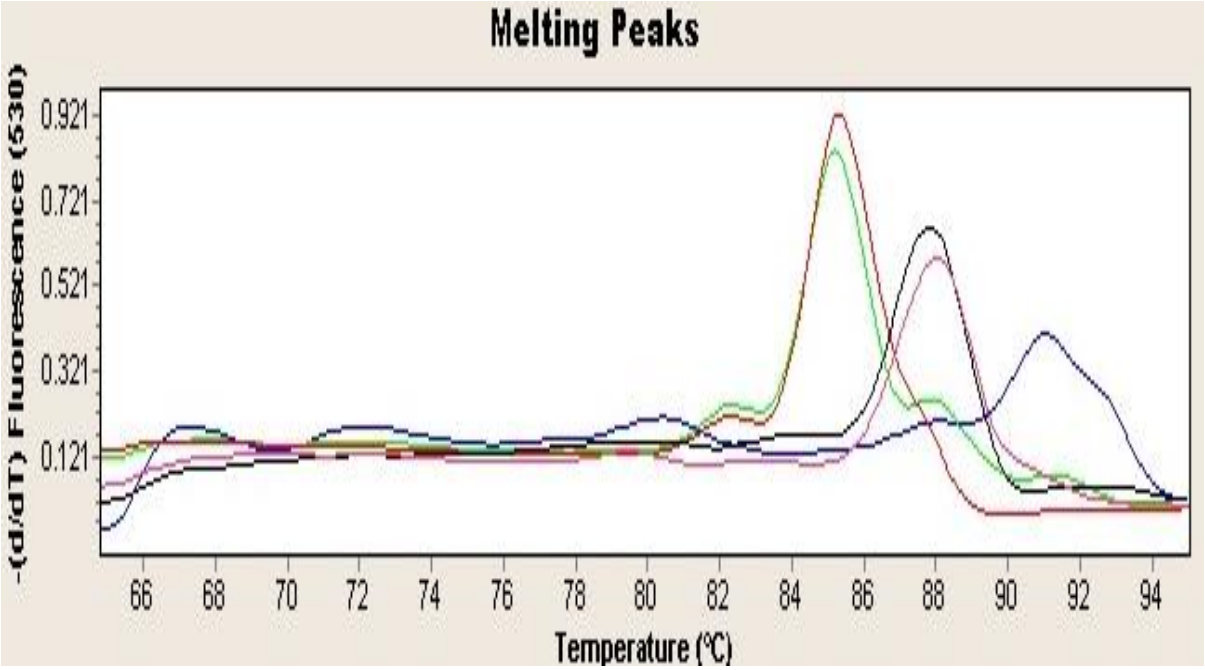

Figure 3. Specificity for *S. pneumoniae* DNA detection by PCR as compared to *S. pyogenes* and *S. viridians*.

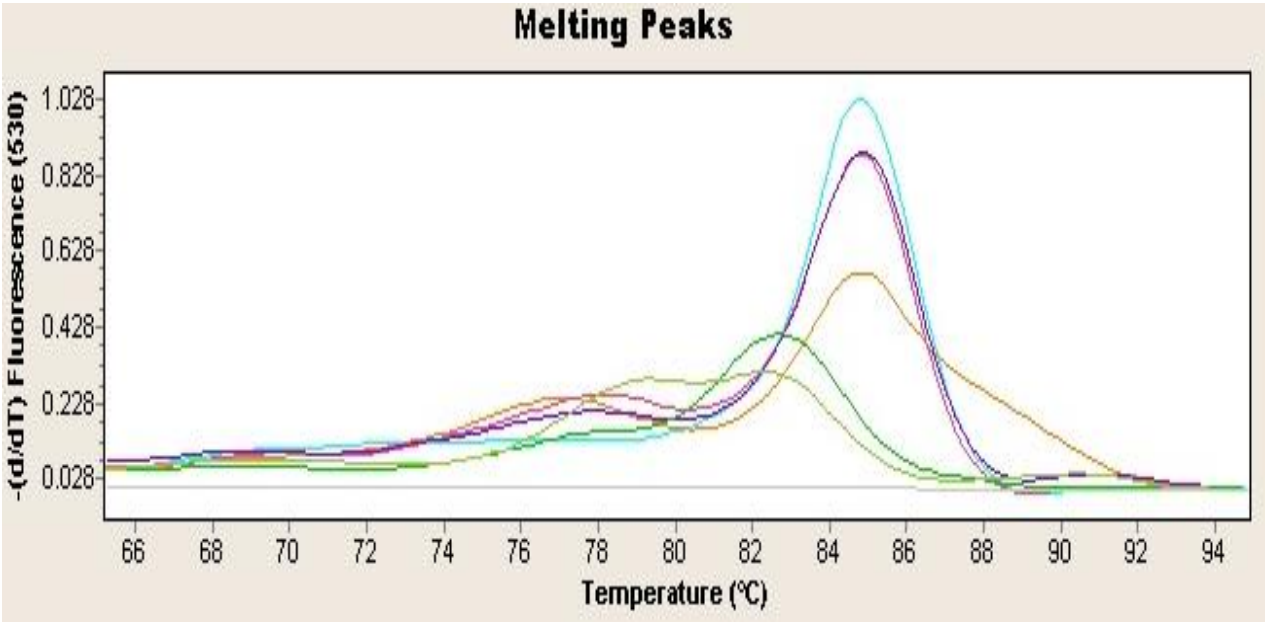

Figure 4. An assay runs for test samples.

The reasons for the low yield of *S. pneumoniae* by this conventional method may be influenced by quality of sputum and blood specimens including collection, transport and processing of samples (Marcos et al., 2003). Antibiotic therapy prior to sampling has shown to reduce the sensitivity of blood cultures in patients with CAP by approximately 50% (Metersky, 2006). False-negative results by culture have been reported not only for CAP, but also for other invasive bacterial diseases (Dominguez, 2001). Although all blood and sputum cultures were obtained before antibiotic administrations in the ward, previous antibiotics treatments provided by

local clinics or self medications were not included in this study. Thus, we cannot analyse the significant relationship between prior antibiotics and culture growth in our results. Another possibility is due to failure of *S. pneumoniae* identification by phenotypical method using optochin susceptibility test. Few studies have discovered optochin resistance in *S. pneumoniae* (Andreas et al., 2001) and optochin-susceptible *Streptococcus mitis* (Martín-Galiano et al., 2003). Therefore, these may lead to the misclassification of the *Streptococcus* species.

The limitations of our culture methods indicate alternative techniques are necessary to increase the sensitivity for *S. pneumoniae* detection. Therefore, ICT and PCR had been chosen as additional methods. We have demonstrated that both of these methods were more sensitivite (100%) using blood culture as gold standard. These results are similar to several publications reporting 50 – 80% sensitivity for ICT (Mandell et al., 2007) and 69 to 100% for PCR methods (Carmen, 2004). In addition, urine for ICT and blood samples for PCR methods were easier to collect in contrast to good quality sputum and invasive respiratory samples.

ICT and PCR are also simple to perform and produce faster results within 15 min by ICT and 1 to 2 h by real-time PCR in contrast to 3 days using conventional culture as proven by previous investigators (Dominguez et al., 2001; James et al., 2001; Marcos et al., 2003.). Fewer operators are required to run the tests therefore the cost of the kits and the reagents can be compensated. The ICT test can also be performed at bed site especially in the emergency room or remote area to provide earlier selection of initial antibiotic therapy (Mandell et al., 2007).

Although the overall sensitivity of the ICT and real-time PCR to diagnose *S. pneumoniae* infection was higher than culture, their specificities were 91.8 and 51.0%, respectively. Several issues should be taken into account when analyzing the meaning of positive ICT and real-time PCR using cultures as the gold standard. The reliance on microbial cultures, either from respiratory samples or from blood, for the diagnosis of *S. pneumoniae* in this situation may be biased by the relatively low sensitivity of cultivation. False positive could be due to low specificity of the new tests but the results could be true positive because they might not properly diagnose with conventional methods.

In contrast to culture, studies have shown that the results of this ICT and PCR methods should not be affected by the administration of antibiotics (Dominguez et al., 2001; Marcoset al., 2003). In addition, studies have also demonstrated positive *S. pneumoniae* antigen in the urine in 83 % of serial specimens from patients after 3 days of therapy (Smith et al., 2003). The results from ICT and PCR may be positive when all other bacteriologic evidence of infection is lacking due to a failure of *S. pneumoniae* isolation, and they do not depend on viable organisms (Dominguez et al., 2001; Lorente et al., 2000). Therefore, the limitation of culture sensitivity as a conventional microbiological method to diagnose *S. pneumoniae* in CAP may not determine if the ICT and PCR results are really true or false.

Murdoch et al. (2003) had demonstrated that *S. pneumoniae* urinary antigens were not detected from 188 control adult patients indicating high specificity of the ICT tests (Murdoch, 2003). However, a study by Dowell et al. (2001) presented positive ICT in healthy children, colonized with *S. pneumoniae*. Nevertheless, we did not include any children in our study. Although *S. pneumoniae* colonizes the oropharynx in 30 to 70% of healthy adults (Haas et al., 1977) it has been shown that the concentration of *S. pneumoniae* in the carrier's oropharynx is below the antigen detection level required for latex agglutination (Dominguez et al., 2001). Therefore, these findings further support the high specificity (91.3%) of the ICT in adults in our present study.

In between the two methods, the detection of *S. pneumoniae* by real-time PCR was 40% greater than by ICT method (50 versus 10%) but ICT technique was more specific than real-time PCR (91.3 versus 55.6%.) However, other studies had demonstrated similar findings with specificity of > 90% for ICT (Mandell et al., 2007) and from 55 to 100% for PCR methods (Carmen et al., 2004; James et al., 2001; Robert van Haeften et al. 2003; Salo et al., 1995). Another benefit of a real time PCR method in comparison to ICT and culture is the closed nature of the system, which reduces the potential for contamination and false positives reported by few studies (Dagan et al., 1998; Isaacman et al., 1998; Lorente et al., 2000; Salo et al., 1995).

Although we are unable to differentiate colonizers and pathogenic *S. penumoniae* infection in CAP, real-time PCR performed in our study may gain further insight into the meaning of CAP patients with negative etiologies. We have found 12.8% positive PCR test in unknown causative organism. Similar outcomes ranging from 16 to 45% have been also demonstrated by other investigators (Lorente et al., 2000). In concordance with previous authors we therefore suggest that a high proportion of *S. pneumoniae* infections are present among patients with pneumonia of unknown etiology.

Our results have shown that our PCR amplification of the pneumolysin (ply) genes was specific for *S. pneumoniae* isolates and negative for *S. viridans*, *S. pyogenes* and *E.coli*. We also run negative control in every PCR test in our procedure to avoid contamination with exogenous material, particularly in the laboratory during the procedure. These indicate that the primers used for detection appeared species specific for *S. pneumoniae*. Our laboratory test using real-time PCR, also produce reproducible results with mean value of melting point (Tm) 84.99 ± 0.41 (SD) over 15 consecutive runs. Two studies on the detection of *S. pneumoniae* in nasopharyngeal secretion by targeting the pneumolysin gene also showed high sensitivity, specificity, and

reproducibility, as well as good correlation with quantitative cultures.

Besides *S. pneumoniae*, other etiological pathogens of CAP have always been difficult to establish. The causative organisms often isolated in as few as 30 - 50% of cases (28, 36). The overall low yield of pathogens by our conventional blood cultures methods (6.4%) suggest the need to improved diagnostic testing in CAP. Atypical organisms (*M. pneumoniae*, *C. pneumoniae* and *L. pneumophilia*) are fastidious to culture and thus, serology methods were chosen for the diagnosis in this study. We did not investigate etiological causes of specific respiratory viruses in our CAP patients as screening for all of them would be costly and laborious. Furthermore, only half (9%) of the pathogenic viruses were identified from the clinical evidence of viral etiologies (18%) (Mandell et al., 2007).

Conclusion

ICT and PCR methods improved the detection of *S. pneumoniae* in CAP patients as compared to conventional culture. *S. pneumoniae* was the second most frequent cause of CAP after *C. pneumonia*. This is similar to results from other Asean countries. ICT and PCR do not differentiate colonizers from actual pathogens. Future technical improvements and control studies in both ICT and PCR methods will probably eliminate and enhance the diagnostic significance of both methods.

Limitations involve smaller sampling and represent a small population in Malaysia. The main difficulties were paired sample for atypical bacteria and the cost for PCR and ICT. Further and continuation from this study is suggested and should include *L. pneumophila* urinary antigen and molecular methods for the atypical bacteria (*M. pneumoniae, C. pneumophila* and *L. pneumoniae*). However, the findings have enhanced the detection rate for *S. pneumoniae* which is still a common pathogen in CAP and should not be neglected especially in the decision of empirical antibiotic treatment.

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