ABSTRACT

*Streptococcus pneumoniae* is a major cause of morbidity and mortality, presenting with invasive infections such as lobar pneumonia, bacteremia and meningitis. The emergence of penicillin resistant strains since the 1970s has been life threatening and the number of cases that have been reported since then are mushrooming. The evolution of this species of bacteria has enabled it to develop resistance to many other antibiotics such as the macrolides and the fluoroquinolones. The increase in incidence of multi-drug resistance among *S. pneumoniae* necessitates a better understanding of the mechanisms of antibiotic resistance and a need for a tool to identify the presence of these genes within the strains in order to institute appropriate antimicrobial therapy. Penicillin and fluoroquinolone resistance was the main focus of this study. Penicillin resistance in *S. pneumoniae* is due to production of altered penicillin-binding proteins (PBPs), which are essential in cell wall synthesis. In order to study the mechanisms of penicillin resistance, the distribution of penicillin binding proteins (*pbp1a, pbp2b, pbp2x*) were first studied and associated to antibiotic profiles. The investigations showed that alteration in either one or more of these genes causes penicillin resistance with *pbp1a* being essential for the development of high level penicillin resistance. It has been reported that the cell wall of the resistant strains have an abnormal chemical composition, indicating a structural difference of the muropeptides. Branching of the muropeptides has been suggested as a cause of reduced binding of penicillin to the organism in resistant strains. This involves an operon encoded by the *murM* and *murN* genes. In this, it was shown that the penicillin resistant strains had an additional allele when compared to published sequences suggestive of a branched muropeptide structure. Further analysis of the cell wall using the Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Spectroscopy (NMR), showed that there was lack of
branching of the muropeptides in the sensitive strains with an absence of an aromatic structure. The higher transmittance value in the sensitive strain also postulates presence of molecular vibration of a larger mass as compared to the resistant strain, which had smaller vibrational energy. Pneumococci also require choline residues for the structure of the cell wall which can also be replaced by ethanolamine, which is a component of the pneumococci teichoic acid (muropeptides). In this study, an additional peak at 3.89 ppm, representing amino acids, betaine, glycerol phosphorylcholine, glycerol phosphoethanolamine, ethanolamine, glycerol, and glycerol-3-phosphate was only observed in the resistant strain, suggesting that the presence of the ethanolamine component has reduced the autolysis activity of the strain, causing it to be more tolerant to penicillin. The amino acid composition in the resistant strains indicates the presence of disaccharide tetrapeptide units covalently linked to teichoic acid chains. The variation in the nucleotide sequence of the murMN operon, together with the cell wall analysis using FTIR and NMR suggest that the branching structure of the cell wall may be the cause of reduced affinity of the cell wall to β-lactam drugs. In order to study the mechanisms of antibiotic resistance in S. pneumoniae, microarray was employed to study a wider range of genes that may play a role in the development of antibiotic resistance. Preliminary findings show that genes postulated to play a role in antibiotic resistance are mainly transport and growth factors such as the choline binding proteins which are expressed in the resistant strains but not expressed in the sensitive or intermediate strains. These proteins assist in the extrusion of the antibiotic from the bacteria, hence resulting in resistance. In this study, choline binding genes were expressed in the sensitive strain but not in the resistant strain. This could be a factor triggering autolysis and cell death as compared to the resistant strain that failed to lyse,
and hence remained tolerant. The expression of these genes could be further linked to triggering of the autolytic pathway, which may lead to the development of antibiotic resistance. In order to elucidate further the mechanisms of penicillin resistance, the similar microarray experiments was carried out using strains exposed to penicillin. Functional genes with significant expression levels were noted in genes encoding transport, transcription regulation, two component signal transduction, ribosomal proteins and cell surface proteins. Genes which are involved in the biosynthesis of the cell wall envelope such as penicillin binding proteins, choline binding proteins and D-alanylation of cell wall were noted to have significant expression levels upon penicillin stress. Hence, antibiotic stress has an effect on the bacterial physiology and gene regulation.

*S. pneumoniae* have been noted to be resistant to fluoroquinolone in countries in South East Asia, though in Malaysia to date there has been no resistance to fluoroquinolones. In order to study the mechanisms of fluoroquinolone resistance, two different mechanisms (point mutation in the QRDRs and the efflux pump) involved in fluoroquinolone were investigated to better understand the mechanisms as well as monitor the development of fluoroquinolones in Malaysia. *S. pneumoniae* isolates in Malaysia were characterized for mutations in the Quinolone Resistance Determining Regions (QRDRs) by PCR and sequenced, following which a real-time PCR method was developed to detect point mutations in the *gyrA* gene which was found to be common (gyrASer81-Phe) amongst the Malaysian isolates. 56 isolates of the 100 *S. pneumoniae* isolates were categorised to have reduced susceptibility to ciprofloxacin ($\geq 2\mu g/ml$). PCR amplification for presence of the *gyrA, parC, gyrB* and *parE* genes was carried out, of which the PCR product of 8 representative strains with various susceptibility to fluoroquinolones were sequenced. 2 out of the 8 isolates that were
sequenced were shown to have a point mutation in the \textit{gyrA} gene at position Ser81. There were no mutations detected within the \textit{gyrB} and \textit{parE}. The efflux pump is another mechanism by which \textit{S. pneumoniae} resistance to fluoroquinolones develops. Using Real-time PCR, 38 of the 56 strains had expression of the \textit{pmrA} gene. The \textit{pmrA} gene was expressed in only one of the 2 strains which had the mutation but the antibiotic susceptibility profiles of the two strains were similar. The other strain had low level expression. Therefore, this example suggests that there was no correlation seen between overexpression/low level expression of the \textit{pmrA} gene and mutation in the \textit{gyrA} gene. This would suggest that there would be an interplay of other multi-drug efflux pumps simultaneously in the development of antibiotic resistance in \textit{S. pneumoniae}. This also suggests that newer fluoroquinolones such as the ciprofloxacin, levofloxacin, gatifloxacin and moxifloxacin might not be substrates for the \textit{pmrA} efflux.

With the understanding of the mechanisms of antibiotic resistance, rapid methods were then developed so as to identify and detect antibiotic resistance strains using conventional and real-time PCR. Two sets of conventional multiplex PCRs (quintuplex and triplex PCR) to identify and characterize the antibitoic resistance genes, \textit{ermB}, \textit{pbp1A}, \textit{gyrA}, \textit{mefE} simultaneously with the \textit{S. pneumoniae} species specific pneumolysin gene and the common eubacteria gene were developed. The conventional multiplex PCR was then converted into a real-time format which enables detection of three genes simultaneously comprising the antibiotic resistance genes; \textit{ermB} and \textit{pbp2B} and pneumolysin gene (\textit{ply}), a \textit{S. pneumoniae} species specific gene. Both the assays were evaluated using 120 bacterial cultures and 20 direct blood cultures isolates. The results obtained by using these assays correlated to the antibiotic profiles that were reported using the standard laboratory methods. The assays also showed to be sensitive and specific when tested against a wide range of other bacteria. The
development of such a rapid, sensitive and specific technique has an advantage over the conventional method, which has a longer turnaround time. Detection of *S. pneumoniae* and its antibiotic resistance genes from a bacterial culture is possible within 3-4 hours using the conventional multiplex PCR assay but takes only an hour using the real-time multiplex PCR assay. This allows rapid identification of the antibiotic resistance genes, thus allows better prediction of the appropriate drug therapy.
ABSTRAK


*S. pneumoniae* yang resistan terhadap ‘fluroquinolone’ pula telah dilaporkan di negara-negara Asia Tenggara melainkan Malaysia. Ini menjadi perangang untuk lebih memahami mekanisma yang terlibat dalam resistensi kepada antibiotik ini. Dua mekanisma yang dikaji termasuk mutasi pada siri jujukan yang terdapat dalam ‘Quinolone Resistance Determining Region’ (QRDR) dan pem efflux. Isolat-isolat *S. pneumoniae* yang diperolehi di Malaysia dikaji untuk kewujudan mutasi. Untuk tujuan ini, PCR dan ‘sequencing’ digunakan. Kemudian teknik Real-time PCR dibentuk untuk mengesan mutasi tersebut. Hasil kajian ini mendapati mutasi pada gen *gyrA* pada kedudukan Ser81-Phe dijumpai dalam isolat-isolat Malaysia. 56 daripada 100 isolat yang dikaji menunjukkan nilai MIC yang lebih tinggi (> 2µg/ml) terhadap ‘ciprofloxacin’ (reduced susceptibility). Teknik PCR telah digunakan untuk mengesan gen *gyrA*, *gyrB*, *parC* dan *parE*. Hasil PCR daripada...

Dengan pengetahuan tentang mekanisma-mekanisma antibiotik resistan, teknik yang lebih cepat dibentuk untuk mengesan organisma ini dan juga gen-gen yang terlibat dalam resistan terhadap antibiotik. Teknik yang dibentuk menggunakan PCR dan Real-Time PCR. Dua set PCR (triplex dan quintuplux) dibentuk untuk mengesan gen pnumolysin, ply yang spesifik terhadap S. pneumoniae dan gen ermB, pbp1A, gyrA dan mefE. Real-Time PCR pula dibentuk untuk mengesan gen-gen ermB, ply dan pbp2b. Kedua-dua teknik ini diuji menggunakan 120 kultur bakteria dan 20 kultur darah (blood cultures). Hasil ujian tersebut menunjukan bahawa gen yang dikesan dalam isolat tersebut bersesuaian dengan laporan makmal dan profil antibiotik yang diperolehi menggunakan teknik makmal yang konvensional. Teknik-teknik ini juga adalah didapati sensitif dan
spesifik setelah diuji terhadap bakteria-baktaria lain. Dengan pembentukan teknik yang sebegini, masa untuk mengesan organisma dalam konteks diagnostik dapat disingkatkan. Teknik PCR dapat memberi keputusan yang dikehendaki dalam masa 3-4 jam manakala ia hanya mengambil masa 1 jam menggunakan teknik Real-Time PCR. Kesimpulannya, penyingkatan masa ini membolehkan ramalan yang lebih tepat bagi memilih antibiotik dalam mengubati pesakit.
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<td>Asian Network for Surveillance of Resistant Pathogens</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BaCl₂</td>
<td>Barium Chloride</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
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<tr>
<td>bp</td>
<td>Basepair</td>
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<td>C</td>
<td>Cytosine</td>
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<tr>
<td>CAP</td>
<td>Community – acquired pneumonia</td>
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<tr>
<td>CDC</td>
<td>Centre for Disease Control and Prevention</td>
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<tr>
<td>cfu</td>
<td>Colony forming unit</td>
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<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>CRO</td>
<td>Ceftriaxone</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>Cefuroxime</td>
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<td>deoxynucleotide</td>
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<td>Hydrogen chloride</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Dihydrogen sulphate</td>
</tr>
<tr>
<td>I</td>
<td>Intermediate</td>
</tr>
<tr>
<td>KCL</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LEV</td>
<td>Levofloxacin</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHA</td>
<td>Mueller Hinton Agar</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory concentration</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50/90&lt;/sub&gt;</td>
<td>MIC at which 50% or 90% of growth are inhibited</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOX</td>
<td>Moxifloxacin</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NPS</td>
<td>Nasopharyngeal secretion</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Pbp or pbp</td>
<td>Penicillin binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
</tbody>
</table>