Original Article

Variation of Sequence of Genes Encoding the *MurMN* Operon and Cell Wall Composition in *Streptococcus pneumoniae* Strains of Different Susceptibility Levels to Penicillin

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ABSTRACT

It has been reported that there are structural differences in the muropeptides of the cell wall in penicillin-resistant *Streptococcus pneumoniae*. The cell wall composition and variation of the *murMN* operon sequence of *S. pneumoniae* strains with different penicillin susceptibilities were investigated. PCR amplification and sequencing of the *murM* and *murN* genes were carried out on three selected strains of *S. pneumoniae*. The cell wall was then extracted and elucidated using Fourier Transfer InfraRed (FTIR) Spectroscopy, followed by proton Nuclear Magnetic Resonance (NMR) Spectroscopy. The sequences of the *murM* and *murN* genes were shown to be highly conserved while FTIR and NMR analysis suggested a branching structure of the cell wall and also the presence of ethanolamine in the resistant strain. The variations in *murM* and *murN* genes may have caused modifications in the cell wall structure leading to decreased binding capacity of penicillins and other β-lactam drugs. (*J Infect Dis Antimicrob Agents 2009;26:97-108.*)

INTRODUCTION

Penicillin resistance in *Streptococcus pneumoniae* is due to altered penicillin-binding proteins (PBPs), which are essential in cell wall synthesis.[1-4] This finding was first demonstrated by titration of the penicillin-binding capacity of PBPs in several highly β-

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lactam-resistant isolates from South Africa.[4] Penicillin resistance has been documented in many parts of the world. International Surveillance studies by the Asian Network for Surveillance of Resistant Pathogens (ANSORP) have reported varying levels of penicillin resistance in *S. pneumoniae*: Vietnam (71.4%), Korea (54.0%), Hong Kong (43.2%), Taiwan (38.6%), China (23.4%), Sri Lanka (14.3%), Thailand (26.9%), Singapore (17.1%), Malaysia (29.5%), and Saudi Arabia (10.3%).[5] In the United States, the SENTRY surveillance program documented penicillin-resistant *S. pneumoniae* in 20 percent of clinical isolates causing bloodstream infections in North America.[6] In Europe, the circulated Spanish 23F penicillin-resistant clone was mainly in Spain and Hungary in the 1980s, however dissemination of this clone to other parts of the world was reported in the 1990s with the highest prevalence rate of penicillin resistance being documented in France (57%) and Spain (40%).[7]

PBPs are active-site serine peptidases which catalyze the polymerization as well as cross-linking of peptidoglycan precursors in the assembly of bacterial cell walls.[8,9] The PBPs of resistant strains have been shown to have reduced affinities and or binding capacities to β-lactam drugs. It has also been reported that cell wall peptidoglycans of resistant strains have abnormal chemical compositions, indicating differences in the proportion of branched muropeptides.[10] The presence of branched muropeptides, carrying an alanyl-serine or alanyl-alanine substituent on the lysine epsilon amino group of the stem peptide residues has been shown to be higher in resistant strains than in susceptible strains.[10] This finding was also demonstrated by other researchers from South Africa, Hungary, and the Czech Republic.[10, 11]

Recently, the identification of the *murMN* operon has shed some light into the mechanisms of synthesis of muropeptides and the physiological role of branched peptides. Previous studies have shown that inactivation of the *murMN* operon causes the production of cell wall peptidoglycans composed of linear muropeptides and also causes the complete loss of the resistant phenotype in penicillin-resistant strains.[12] It has also been shown that several penicillin-resistant isolates carry *murM* genes with unique polymorphic regions.[13] The aim of this study was to analyze the *murMN* operon from three Malaysian *S. pneumoniae* strains with varying susceptibilities to penicillin in order to determine any sequence divergence within these strains and its correlation to penicillin resistance. Another aim was to study the variation that may have occurred in the cell wall of these strains using Fourier Transfer Infrared (FTIR) Spectroscopy and Proton Nuclear Magnetic Resonance (NMR) techniques.

MATERIALS AND METHODS

Bacterial strains

Three pneumococcal isolates of different susceptibility levels to penicillin were obtained from clinical specimens processed in the Microbiology Laboratory of University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. The three strains were strain S676 (penicillin-susceptible), strain I81 (penicillin-intermediately susceptible, and strain R98 (penicillin-resistant). Bacterial cultures isolated from these clinical specimens were stored in brain heart infusion broth supplemented with 10 percent glycerol at -80°C without antibiotics until needed. Each stock culture was kept in triplicates to avoid multiple passaging.

Strain identification

Identification of the strains was confirmed by

susceptibility to ethylhydrocupreine disc (optochin), whereby all the strains showed a diameter measurement of ≥ 14 mm.[14] The strains were also shown to be positive by bile solubility testing and to be catalase negative.

Susceptibility testing

Antibiotic susceptibility of the strains was tested on Mueller Hinton Agar (Oxoid) plates containing 5 percent sheep blood (Oxoid), incubated at 37°C with 5 percent CO_2 using the agar dilution method described by the Clinical and Laboratory Standards Institute.[14] The antimicrobial agents used were penicillin, cefotaxime, and ceftriaxone, obtained from Sigma Aldrich (Sigma Chemical Co., St. Louis, Mo., USA). *S. pneumoniae* ATCC 49619 was used as control.

DNA extraction and PCR amplification of *murMN* operon

Genomic DNA was extracted from pure bacterial cultures using a previously described method.[15] Bacterial colonies suspended in 15 µL of H_2O containing 50 mg/mL lysostaphin (Sigma Chemical Co., St. Louis, Mo) were incubated at 37°C for 10 minutes. This was followed by addition of 10 µg/mL proteinase K and 0.1 mM Tris HCL pH 7.5 and incubated at 37°C for another 10 minutes. Subsequently, the suspension was boiled for 5 minutes and finally centrifuged at 13,000 rpm for 2 minutes. The supernatant obtained acts as the template in the PCR reaction.

The primers used in this study were as follows:
murM (Forward): 5'–CTG GAG GAA AGA GAG TAG GA-3'
murM (Reverse) : 5'–CTC TTC TTT CGT GAG TGT AG-3'
murN (Forward) : 5'–GAC TTG CTC TTG ATT T CCGT-3'
murN (Reverse) : 5'–TGT CTC TCC ACC TTT CTA GC-3'

The optimal PCR condition for a 50 µL reaction included 1X PCR buffer, 1.5 mM $MgCl_2$ 0.2 mM dNTP mix, 2 U *Taq* polymerase (Fermentas), and 20 pmol of each primer. The PCR cycling parameters were as follows: an initial denaturation step at 95°C for 5 minutes, 30 cycles of amplification performed as follows: denaturation at 94°C for 5 minutes, annealing temperature at 40°C for 2 minutes and extension temperature at 72°C for 3 minutes and finally completed with an extension at 72°C for 5 minutes. PCR products were purified using the PCR purification kit (Qiagen) and PCR DNA sequencing was carried out using an automated DNA sequencer (an ABI Prism 377 DNA sequencer, Perkin Elmer ABI).

Cell wall preparation

Pneumococcal cell walls were prepared using a previously published procedure with modifications.[16] Briefly, exponentially growing cultures were rapidly chilled by immersing the flasks in an ethanol-ice bath until the temperature dropped from 4°C to 0°C. After the cells were harvested by centrifugation at 4°C, they were suspended in iced phosphate-buffered saline and quickly dropped into boiling sodium dodecyl sulphate (SDS) (4% final concentration) to inactivate any cell wall-modifying enzymes. Then, the cell walls were mechanically broken by shaking with glass beads in a vortex mixer. The cell wall fragments were pelleted at 25,000 g and suspended in buffered saline with 0.05 percent sodium azide, and digested with DNase, RNase, and proteinase K. Peptides from proteases digestion were extracted by boiling in 1 percent SDS and the wall fragments were washed twice with water and incubated for 15 minutes at 37°C, first with 8 M LiCl and then with 100 mM EDTA to remove any material bound by ionic interactions. After the water wash, the fragments were treated with acetone,

suspended in water and lyophilized.

Fourier transform infrared spectroscopy (FTIR spectroscopy)

A small amount of bacterial cell wall was placed on a stainless steel plate and spread evenly using the straight edge of a teflon sheet. The evaporated sample was then placed on a potassium bromide window of spectrometer (Bruker, model IFS 66 v/s). The spectrometer was coupled to an infrared microscope equipped with grazing angle reflectance (GAR) objective of 15X magnification and a mercury-cadmium-tellurite detector and potassium bromide beamsplitter. Bacterial cell wall spectra were collected using the OPUS, version 4.2, Bruker software, over a range of 4,000-400 wavenumber (cm^{-1}) by averaging 50 scans with a resolution of 4 cm^{-1} and absorbance mode. A background spectrum was obtained before each measurement using a clean stainless steel plate at the same instrumental conditions used for the experiment data acquisition.

NMR spectroscopy

One gram of the lyophilized form of the bacterial cell wall was dissolved in deuterium oxide. The same solvent was used to acquire a proton NMR spectrum. Chemical shifts were established by assigning 4.65 ppm to the resonance from the proton of monodeuterated water.

RESULTS

Sequence analysis of the *murM* and *murN* genes

The entire coding region of the *murM* gene was sequenced from three strains of varying susceptibility levels to penicillin. The highest penicillin minimal inhibitory concentration (MIC) value was 4 μg/mL (R98) representing a penicillin-resistant strain, while the penicillin-intermediate (I81) and -susceptible strains (S676) had MIC values of 1.0 μg/mL and 0.032 μg/mL, respectively (Table 1). The sequencing of the *murM* gene yielded nucleotide sequence homology to different *murM* variant alleles that have been characterized previously.[17,18] The *murM* gene of the penicillin-susceptible (S676) and -intermediate (I81) strains was homologous to eight published *murM* variant alleles (*murMB*1, *murMB*3, *murMB*4, *murMB*5, *murMB*6, *murMB*7, *murMB*10, and *murMB*11) (Gene Bank Ascension Numbers: AF281135, AF281136.1, AF281137.1, AF281138.2, AF281139.1, AF281140, DQ100160.1, and DQ100161.1). These alleles were of the strain Pen6 origin (a genetic transformant constructed with the highly penicillin-resistant South African strain 8249 as a DNA donor), which is a resistant phenotype. The penicillin-intermediate strain had a significant level of divergence (29% homology) in the nucleotide sequence to the *murM* variant alleles *murMB*10 and *murMB*11. Interestingly, the resistant strain had an additional *murM* allele, *murMB*9 with a homology of 85 percent (Table 1). This variant allele was not detected in the susceptible and intermediate strains. Table 1 also shows that the intermediate and resistant strains had a higher percentage of homology to the alleles (*murMB*1, *murMB*3, *murMB*4, *murMB*5, *murMB*6, *murMB*7, *murMB*10, *murMB*11, and *murMB*9 (only in the resistant strain), as compared to the penicillin-susceptible strain. The *murM* gene sequence from our isolates had a higher percentage of homology when compared to published sequences of strains R36A (93-99%), while the *murN* gene showed a higher percentage of homology to both R36A (~99%) and Pen6 (~98%).

Variation of cell wall molecular vibration by FTIR spectroscopy

Table 1. Nucleotide sequence homology of the *murM* gene.

Strain	MIC (mg/L)[1]			*murM* variants (% of similarities)								
	PEN	CTX	CRO	*MB*1	*MB*3	*MB*4	*MB*5	*MB*6	*MB*7	*MB*9	*MB*10	*MB*11
S676	0.032	<0.032	0.064	78	79	89	84	89	87	NS	89	91
I81	1.0	2.0	1.0	97	98	98	98	98	98	NS	29	29
R98	4.0	2.0	1.0	84	84	93	90	90	87	85	95	96

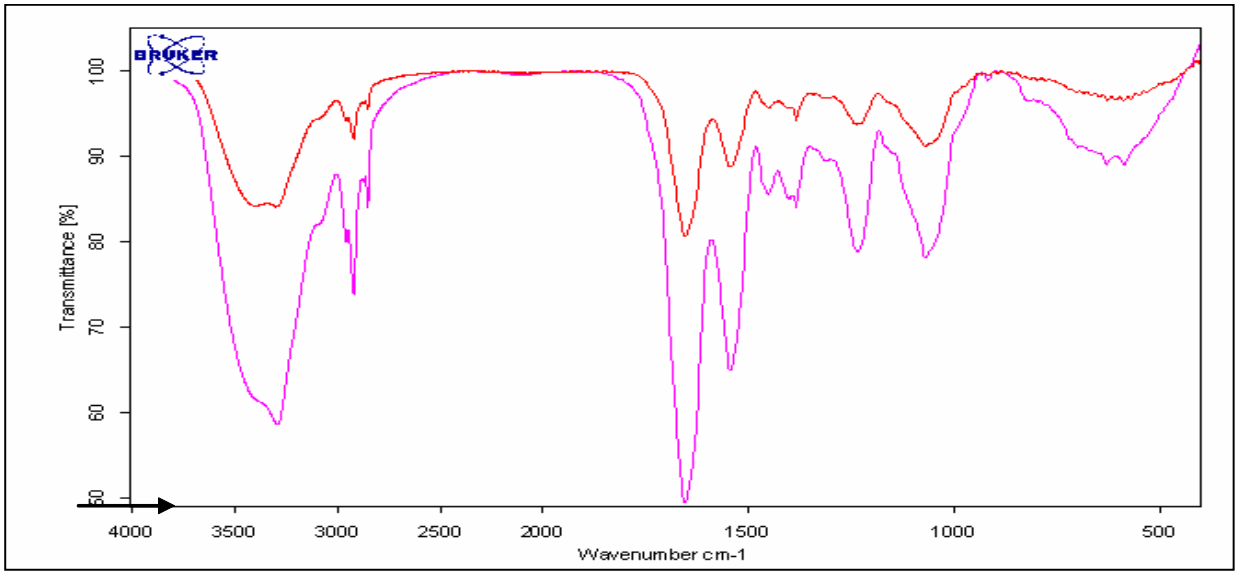
MIC S676: penicillin-susceptible strain, I81: penicillin-intermediate strain, R98: penicillin-resistant strain

[1]Breakpoints recommended by the Clinical and Labolatory Standards Institute guidelines, PEN (S : ≤ 0.06, I : 0.12-1.0,and R: ≥ 2.0 mg/L), CRO (S: ≤ 0.5, I :1.0, and R: ≥ 2.0 mg/L),

CTX (S: ≤ 0.5, I : 1.0, and R: ≥ 2.0 mg/L)

NS: no similarities, PEN: penicillin, CTX: cefotaxime, CRO: ceftriaxone

Using the FTIR technique, visually, the spectra obtained from the molecular vibration of the susceptible (S676) and resistant strain (R98) was similar (Figure 1). This indicates that the composition of the cell wall of both strains is similar in chemical properties. However, the transmittance (%) of the vibration differed in both strains. The susceptible strains were seen to have a higher transmittance of vibration, as compared to the resistant strain (indicated as an arrow in Figure 1). This suggests some variations in the bonding structure of the chemical properties. The higher transmittance value also suggests a stronger molecular vibration, hence postulating a muropeptide of a larger mass. In the resistant strain, a lower transmittance value was noted suggesting the muropeptides of the cell wall to be of a smaller mass. This could be due to the branching of the muropeptide chain. Table 2 shows the assignment of functional groups detected from the FTIR technique. A shoulder peak at 3,293-3,997 wave number/cm indicates the water content of the sample. A functional group detected was the aromatic C=C stretch at 3,299.4 wave number/cm, which was present only in the susceptible strain. The other functional compounds detected were the aliphatic iodo compound (C-I) stretch, CH_2 methylene stretch, C-F stretch, and alkenyl C=C stretch. The ratio of the absorbance/ transmittance of the sensitive strain against the resistant strain was highest for the alkenyl C=C stretch whereas the aliphatic Iodo compound was almost equal in both strains.



Penicillin-susceptible strain (S676)

Penicillin-resistant strain (R98)

Figure 1. Typical spectra of a Fourier Transfer Infrared (FTIR) Spectroscopy.

Table 2. Assignment of the functional groups from the Fourier Transfer Infrared (FTIR) Spectroscopy analysis.

Assignment (functional groups)	S676	Absorbance $A = -\log_{10}T$ (a)	R98	Absorbance $A = -\log_{10}T$ (b)	Ratio (a/b)
H_2O	3997.1528	0.976	3293.6280	0.586	1.666
Aliphatic iodo compound C-I stretch	587.7148	0.965	586.6499	0.890	1.084
CH_2/methylene/C-H asymmetric/symmetric stretch	2921.7156	0.921	2921.1439	0.738	1.248
C-F stretch	1070.6980	0.912	1070.8571	0.781	1.168
Aromatic C=C stretch/C-H acetylenic compound	3299.4204	0.841	-	-	-
Alkenyl C=C stretch	1654.9058	0.807	1655.2540	0.494	1.634

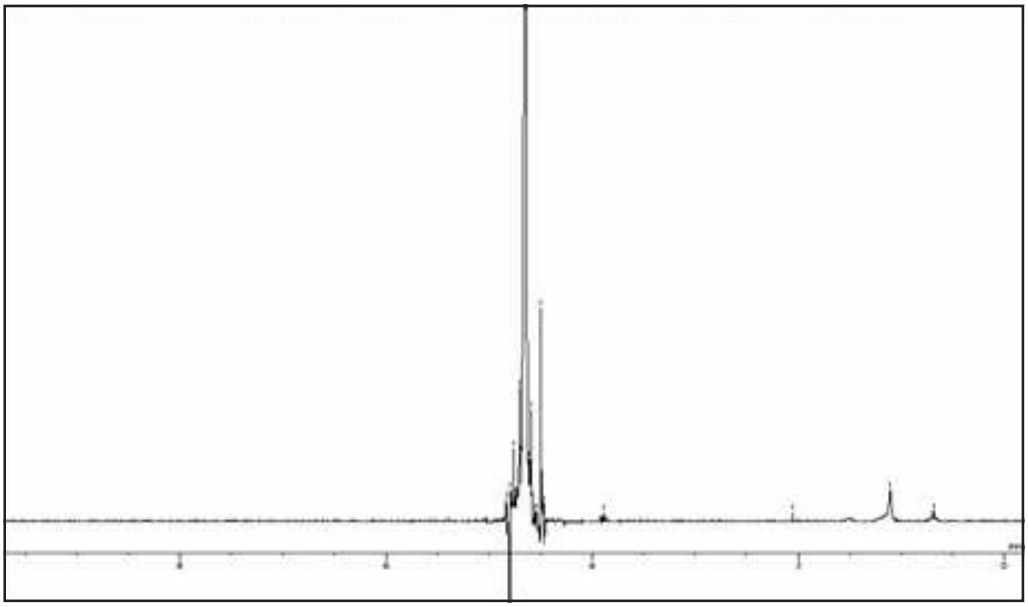
S676: penicillin-susceptible strain, R98: penicillin-resistant strain, T: transmittance, A: absorbance, C: carbon, H: hydrogen, C=C: double bond between 2 carbons, F: fluoride

Variation in the chemical composition of the cell wall structure using NMR

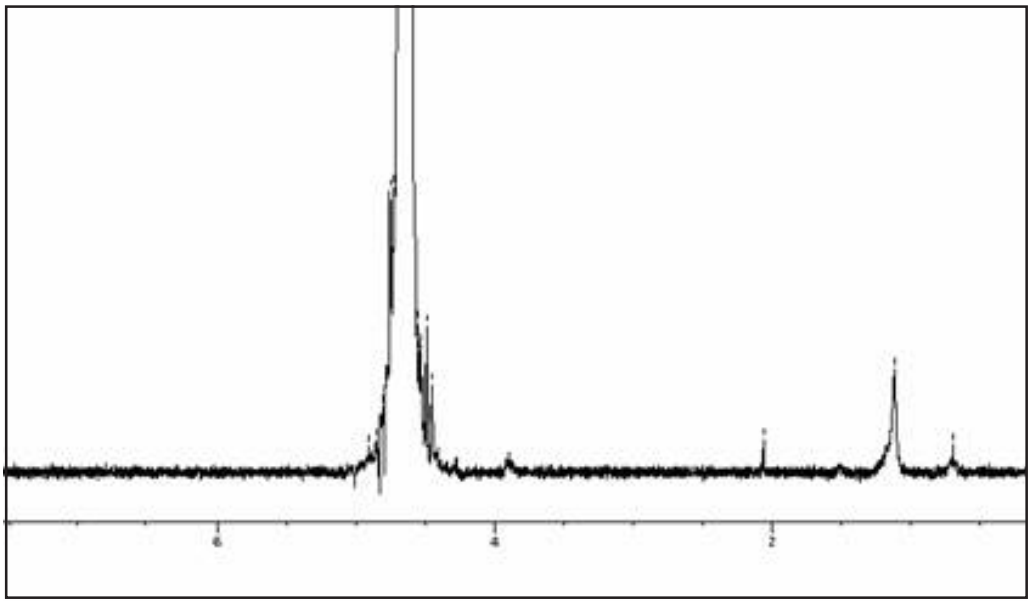
The spectral regions 0.5 to 5.0 ppm were compared for the resistant and susceptible strain. Typical spectra obtained are shown in Figures 2(a) and 2(b). These spectra show the specific characteristic resonances of the cell wall extracted from strains of penicillin-resistant and -susceptible *S. pneumoniae*, respectively. Visually, the spectra in Figures 2(a) and 2(b) appear similar but with differences in the intensity and the frequency of the peak. Detailed spectral analysis identified four significant regions, representing different metabolites (Table 3). Regions of chemical shift at 2.06, 1.12, and 0.75 ppm was found in both the susceptible and resistant strain. The presence of the peak at 0.75 ppm is characteristic of the presence of valine, leucine, and isoleucine residues whereas the presence of a peak at 1.12 ppm is characteristic of a methyl group in these amino acid residues which forms long chain fatty acids. The presence of the peak at 2.06 ppm is characteristic of amino acids such as isoleucine, glutamine, glutamate, methionine, polyamine, and N-acetyl compounds. An additional peak is observed in the cell wall composition of the resistant strain at a region with a chemical shift at 3.89 ppm. This peak is characteristic of amino acids, betaine, glycerol phosphorylcholine, glycerol phosphoethanolamine, ethanolamine, glycerol, and glycerol-3-phosphate.

DISCUSSION

The *murMN* genes exhibit the mosaicsm phenomena, similar to the PBPs in pneumococci[18,19], and are responsible for the variations in the catalytic function of the PBPs.[13,20] Previous studies have shown that the amount of *murM* alleles indicates branching of the muropeptides.[20] The branching of the muropeptides causes modifications to the cell wall structure, hence causing reduced affinity for drug binding. The *MurM* protein has also been reported to play a role in the maintenance of the PBP genes. Therefore, indirectly the branching muropeptides could cause an interruption in the function of the PBP transpeptidase enzyme in catalyzing the polymerization and cross linking of



(a) Strain R98 (penicillin-resistant strain).



(b) Strain S676 (penicillin-susceptible strain).

Figures 2(a) and 2(b). Typical spectra of the Proton Nuclear Magnetic Resonance (NMR) Spectroscopy.

Table 3. Chemical shift of the Proton Nuclear Magnetic Resonance (NMR) analysis.

No	PPM	Strain				Metabolites with resonance
		R98		S676		
		Intensity	Frequency	Intensity	Frequency	
1	3.89	0.01533	1052.521	Not Identified	-	Amino acid (non-specific), betaine, glycerol phosphorylcholine, glycerol phosphoethanolamine, ethanolamine, glycerol, glycerol-3-phosphate
2	2.06	0.01559	556.656	0.02282	557.949	Isoleucine,glutamine, glutamate, methionine, polyamine, N-acetyl coumpounds
3	1.12	0.05882	300.811	0.07196	302.775	Methyl group in valine, leucine, isoleucine, terminal methyl groups in long chain fatty acids.
4	0.75	0.01903	187.396	0.01911	188.706	Valine, leucine, isoleucine

ppm: parts per minute, R98: penicillin-resistant strain, S676: penicillin-susceptible strain

peptidoglycans during the cell wall formation. In our study, the additional *murM* allele in the penicillin-resistant strain could be the cause of reduced binding affinity to the drug. The higher divergence of the penicillin-susceptible strain, compared to the *murM* variant allele might be due to its difference in the genetic background of the strain. The published *murMB* variant alleles[13] that have been reported are of the strain Pen6 origin, which is a penicillin-resistant strain. However, the function of this allele should be investigated in order to conclude its role in the development of penicillin resistance in *S. pneumoniae*.

The FTIR analysis showed that the functional group detected at 3,299 wave number/cm, representing the aromatic structure, was only detected in the susceptible strain. These data suggest the lack of branching of the muropeptides in the susceptible strain whereas the absence of an aromatic structure suggests branching of the peptides into smaller structures. The higher transmittance value in the susceptible strain also postulates the presence of molecular vibration of a larger mass as compared to the resistant strain, which had smaller vibrational energy.

Pneumococci require choline residues for the structure of the cell wall. However, it can also be replaced by ethanolamine, which is a component of the pneumococci teichoic acid (muropeptides). Previous studies have shown that *S. pneumoniae* becomes resistant to autolysis-inducing agents in the presence of ethanolamine.[21] This was also observed in our study, as the 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. This suggests that the presence of the ethonolamine component has reduced the autolytic activity of the strain, causing it to be more tolerant to penicillin. The amino acid composition 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 structure and branching of the cell wall may be the cause of reduced affinity of the cell wall to β-lactam drugs. However, the variation caused by the different serotypes of strains may also contribute to the frequency of divergence of the gene. Other variations of the genetic background such as the source of isolation, horizontal transfer of genes, and environmental stress may also have a role to play in the variation of the structure of the cell walls of these strains. Variations occurring at the cell wall may be an adaptive response of the organism towards environmental changes for survival. To further elucidate, and confirm the role of the *murM* and *murN* genes more strains need to be sequenced in order to study the distribution and evolution of the *murM* and *murN* genes. It would be an added advantage to quantitate the amount of cell wall components in order to investigate further the specific role of these components towards the development of penicillin resistance.

In conclusion, penicillin resistance in *S. pneumoniae* is conferred by many contributing factors such as the variation of the PBPs, the *murMN* operon and the composition of the cell wall, of which form part of the physical structure of the cell wall region. The variations in these factors causes modification in the cell wall structure, which may lead to decreased binding capacity to penicillins and other β-lactam drugs.

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