Declaration

The experimental work described in this thesis was conducted under the supervision of Professor Keith Klugman and Dr. Anthony Smith, in the Respiratory and Meningeal Pathogens Research Unit of the National Institute for Communicable Diseases, Johannesburg.

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Nicole Wolter

_____ day of _____ 2007

Abstract

Streptococcus pneumoniae is a leading cause of pneumonia, bacteremia, meningitis, otitis media and sinusitis, and is responsible for significant morbidity and mortality worldwide. The burden of pneumococcal disease has been greatly impacted by the high prevalence of HIV, especially in developing countries. Macrolides are commonly used for the treatment of pneumococcal infections with the resulting effect of increasing resistance. Pneumococci develop resistance to macrolides predominantly by two mechanisms; target modification and drug efflux. Target modification occurs through the acquisition of an *erm*(B) gene (MLS_B phenotype) or through ribosomal mutation, and drug efflux occurs through the acquisition of a *mef*(A) gene (M phenotype). Alternative protein synthesis-inhibiting antibiotics such as linezolid and telithromycin have been developed in response to the increasing level of antibiotic resistance. In this study, novel mechanisms of resistance to protein synthesis-inhibiting antibiotics, and the current prevalence and epidemiology of macrolide resistance in South Africa were investigated.

Two clinical isolates of *S. pneumoniae* resistant to macrolides, linezolid and chloramphenicol were identified in the PROTEKT surveillance study and the ABCs program of the CDC. The isolates were found to each contain a 6 bp deletion, resulting in the deletion of two amino acids from a highly conserved region of ribosomal protein L4 ($_{64}PWRQ_{67}$ to $_{64}P\_Q_{67}$ and $_{67}QKGT_{70}$ to $_{67}Q\_T_{70}$). The genes encoding the mutant ribosomal proteins transformed susceptible strain R6 to macrolide, linezolid and chloramphenicol resistance, proving that the

deletions conferred the resistance on the isolates, and indicating that these antibiotics share a common binding site. Growth studies of the R6 transformants showed increased mass doubling times, suggesting that the L4 mutations were associated with a fitness cost, but the original strains showed evidence of fitness compensation. The L4 mutations in these isolates represent a novel mechanism of cross-resistance to macrolides, linezolid and chloramphenicol.

A macrolide-resistant clinical isolate of *S. pneumoniae* with mutations in 23S rRNA showed a heterogeneous phenotype and genotype. A mutant gene encoding 23S rRNA from this isolate transformed susceptible strain R6 to resistance. Transformants displayed similar heterogeneity to the isolate. Culture of resistant strain R6 in the presence of antibiotic maintained resistance, however culture of the strain in the absence of antibiotic pressure resulted in a reversion to susceptibility. By DNA sequencing, gene conversion was shown to occur between the wild-type and mutant 23S rRNA alleles. Growth studies indicated that the resistant phenotype was associated with a fitness cost. Therefore, under antibiotic selective pressure alleles converted to the mutant form, and in the absence of selective pressure alleles reverted to wild-type, in order to regain fitness. Through gene conversion the pneumococcus has the ability to rapidly adapt to the environment, with implications for susceptibility testing and patient treatment.

A rare clinical isolate of *S. pneumoniae*, highly resistant to telithromycin, was received from the Canadian Bacterial Surveillance Network and was investigated for the mechanism of resistance. The isolate was found to contain an *erm*(B) gene

with a truncated control peptide, as well as a mutant ribosomal protein L4, containing a number of mutations. Transformation of susceptible strain PC13, containing a wild-type *erm*(B) gene, with the mutant *erm*(B) gene decreased the susceptibility of PC13 to telithromycin, but did not confer high-level resistance. Transformation of PC13 with the mutant L4 gene or a fragment of the L4 gene containing only the $_{69}$GTG$_{71}$ to TPS mutation, conferred high-level resistance on PC13. In contrast, transformation of R6, which did not contain an *erm*(B) gene, with the L4 gene or L4 fragment only conferred reduced telithromycin susceptibility. High-level telithromycin resistance was therefore conferred by a combination of an *erm*(B) gene with a $_{69}$GTG$_{71}$ to TPS mutation in a highly conserved region of ribosomal protein L4. The combination of mechanisms inhibited the binding of telithromycin to the ribosome, whereas neither mechanism individually was sufficient.

A telithromycin-resistant clinical isolate of *S. pneumoniae* was received from the PROTEKT surveillance study and was investigated for the resistance mechanism. The isolate was found to contain a 136 bp deletion in the regulatory region of *erm*(B). This mutant gene was shown, by transformation studies, to confer resistance on susceptible strain PC13. Expression of *erm*(B) on the transcriptional level was quantified by real-time reverse transcription PCR. In the presence of erythromycin and telithromycin, *erm*(B) expression was significantly higher in the mutant PC13 strain than the wild-type strain. Growth studies showed that the mutant PC13 strain had a shorter lag phase than the wild-type strain in the presence of erythromycin. Telithromycin resistance was conferred by the mutant

erm(B) gene that was expressed at a higher level than the wild-type gene, most likely resulting in higher ribosomal methylation levels sufficient to hinder telithromycin binding.

Macrolide resistance in invasive pneumococcal disease in South Africa for the period 2000 to 2005 was investigated through a national laboratory-based surveillance system. Viable isolates (n=15982) collected during the six-year period were phenotypically characterised, by determination of MICs and serotyping. Two hundred and sixty random isolates from 2005 were genotypically screened for the presence of *erm*(B) and *mef*(A). Macrolide resistance increased significantly from 9% in 2000 to 14% in 2005. Resistant isolates were received from all provinces of South Africa, with Gauteng and the Western Cape having the highest incidence. Serotype 14 was the most common macrolide-resistant serotype and 96% of macrolide-resistant isolates in 2005 were serotypes included in the 7-valent pneumococcal conjugate vaccine and serotype 6A. Macrolide resistance was significantly higher in children <5 than in individuals 5 years and older. The majority of strains (75%) over the six-year period displayed the MLS_B phenotype. Of the 260 strains genotypically screened, 57% were positive for *erm*(B), 27% were positive for *mef*(A), 15% contained both *erm*(B) and *mef*(A), and 1% were negative for both genes and were found to contain ribosomal mutations. Eighty percent of isolates containing both *erm*(B) and *mef*(A) were serotype 19F and were found to be clonal by PFGE and MLST. These multidrug-resistant isolates were related to the Taiwan^{19F}-14 global clone.

Many protein synthesis-inhibiting antibiotics share overlapping binding sites on the large ribosomal subunit. Alterations in 23S rRNA and ribosomal proteins L4 and L22, within the binding pocket, confer resistance and often cross-resistance to many of these antibiotics. The ability of the pneumococcus to develop resistance and the global spread of resistant strains highlights the importance of monitoring resistance levels and understanding resistance mechanisms.

For my brother,

Rodney Dean Edmondson

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List of Abbreviations

A	-	alanine
ABCs	-	Active Bacterial Core surveillance
AIDS	-	acquired immune deficiency syndrome
AZM	-	azithromycin
BHI	-	brain heart infusion
bp	-	base pair
C	-	cysteine
CAP	-	community-acquired pneumonia
CARTIs	-	community-acquired respiratory tract infections
CAT	-	chloramphenicol acetyltransferase
CDC	-	Centers for Disease Control and Prevention
cDNA	-	complementary DNA
CHL	-	chloramphenicol
CLI	-	clindamycin
CLR	-	clarithromycin
CLSI	-	Clinical and Laboratory Standards Institute
CSF	-	cerebrospinal fluid
CSP	-	competence-stimulating polypeptide
CTAB	-	hexadecyl trimethyl-ammonium bromide
D	-	aspartic acid
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytidine triphosphate

dGTP	-	deoxyguanosine triphosphate
dH_2O	-	deionised water
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
dTTP	-	deoxythymidine triphosphate
E	-	glutamic acid
EC	-	Eastern Cape
EDTA	-	ethylenediaminetetra-acetic acid
ERY	-	erythromycin
F	-	phenylalanine
FS	-	Free State
G	-	glycine
GA	-	Gauteng
H	-	histidine
HIV	-	human immunodeficiency virus
hrs	-	hours
I	-	isoleucine
IPD	-	invasive pneumococcal disease
K	-	lysine
KZ	-	KwaZulu-Natal
L	-	leucine
L4Fr	-	fragment of the L4 gene housing a $_{69}GTG_{71}$ to TPS mutation
LP	-	Limpopo

LZD	-	linezolid
M	-	methionine
mega	-	macrolide efflux genetic assembly
MHA	-	mueller-hinton agar
MIC	-	minimum inhibitory concentration
min	-	minutes
ml	-	millilitre
MLS_B	-	macrolide-lincosamide-streptogramin B
MLST	-	multilocus sequence typing
MP	-	Mpumalanga
mRNA	-	messenger RNA
MRSA	-	methicillin-resistant *Staphylococcus aureus*
mt	-	mutant
N	-	asparagine
NC	-	Northern Cape
ng	-	nanogram
NW	-	North-West
OD	-	optical density
P	-	proline
PC13	-	pneumococcal clone 13
PCR	-	polymerase chain reaction
PCV7	-	7-valent pneumococcal conjugate vaccine
PEN	-	penicillin
PFGE	-	pulsed-field gel electrophoresis

PMEN	-	Pneumococcal Molecular Epidemiology Network
PROTEKT	-	Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin
PsaA	-	pneumococcal surface adhesin A
PspA	-	pneumococcal surface protein A
PspC	-	pneumococcal surface protein C
Q	-	glutamine
Q-D	-	quinupristin-dalfopristin
R	-	arginine
rDNA	-	ribosomal DNA
RMPRU	-	Respiratory and Meningeal Pathogens Research Unit
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
rRNA	-	ribosomal RNA
RT-PCR	-	reverse transcription polymerase chain reaction
s	-	seconds
S	-	serine
S-B	-	streptogramin B
SD	-	Shine-Dalgarno sequence
SDS	-	sodium dodecyl sulphate
SEM	-	standard error of the mean
ST	-	sequence type
T	-	threonine
TAE	-	tris-acetate-EDTA

TBE	-	tris-borate-EDTA
TE	-	tris-EDTA
TEL	-	telithromycin
TET	-	tetracycline
TSB	-	tryptone soya broth
tRNA	-	transfer RNA
UK	-	United Kingdom
UPGMA	-	unweighted pair group method with arithmetic averages
US	-	United States of America
UV	-	ultraviolet
V	-	valine
W	-	tryptophan
WC	-	Western Cape
wt	-	wild-type
Y	-	tyrosine
μg	-	microgram
μl	-	microlitre
μM	-	micromolar
χ^2	-	chi-square

Publications

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