Staphylococcus haemolyticus: A NOVEL METHOD FOR SPECIES IDENTIFICATION BASED ON THE HSP60 GENE AND A MODEL FOR VANCOMYCIN RESISTANCE

by

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(Department of Experimental Medicine)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1998
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Department of EXPERIMENTAL MEDICINE

The University of British Columbia
Vancouver, Canada

Date 30TH APRIL 1998
ABSTRACT

*S. haemolyticus* is emerging as an important nosocomial pathogen. In spite of its increasing importance, the exact incidence of infections is still unknown due to the lack of accurate identification methods. Therefore, part of this thesis involves the evaluation of a novel identification method for *S. haemolyticus* based on the HSP60 gene. The HSP60 gene is ubiquitous and highly conserved among *staphylococci*, but still contains species-specific signature sequences which may be useful for species identification of *staphylococci*. To evaluate the specificity and sensitivity of the HSP60 method, HSP60 probes were generated from reference strains of *S. haemolyticus* (ATCC29970) and *S. epidermidis* (ATCC14990) for use in dot blot hybridization reactions with a collection of 66 consecutive bacteremic isolates of CNS, previously identified by the Microscan method and confirmed by BCCDC. The hybridization results were further compared to the API Staph method. The results were as follows:

<table>
<thead>
<tr>
<th>Reference Isolate (No.)</th>
<th>HSP60 Method</th>
<th>Microscan Method</th>
<th>API Method</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>S. haemolyticus</em></td>
<td><em>S. epidermidis</em></td>
<td>Others</td>
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<td>1</td>
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<td><em>S. haemolyticus</em> (32)</td>
<td>31</td>
<td>1</td>
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<td>22</td>
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<td>10</td>
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<td>18</td>
<td>0</td>
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<tr>
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<tr>
<td></td>
<td>1</td>
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<td></td>
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<td>0</td>
<td>13</td>
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<tr>
<td>Unidentifiable (2)</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
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The results were as follows:
The HSP60 method was highly accurate for the species identification of both \textit{S. haemolyticus} and \textit{S. epidermidis}, while the Microscan method lacked specificity and the API method lacked sensitivity. Therefore the HSP60 gene is an excellent target for the species identification of \textit{S. haemolyticus}. The second part of the thesis involved elucidating the mechanism of vancomycin resistance in \textit{S. haemolyticus}, for which little is known. The incidence of vancomycin resistance was examined among the 66 isolates of CNS mentioned earlier. All but one isolate (B7786), a \textit{S. haemolyticus}, was sensitive to vancomycin (MIC below 2 ug/ml). This isolate displayed intermediate resistance to vancomycin (MIC 4ug/ml). Population analysis of B7786, however, yielded a highly resistant and stable subpopulation (128G3) (MIC 32 ug/ml; selection frequency \(10^{-7}\)). The resistant phenotype was characterized by comparing its biochemical, antibiotic, cytoplasmic and cell membrane proteins and vancomycin binding profiles with the parent isolate. While no significant differences were revealed in the biochemical and cytoplasmic protein profiles of 128G5 compared to its parent, two membrane proteins, 44 kDa and 30 kDa, were observed to be downregulated and upregulated, respectively. The antibiotic profiles with ceftriazone, cefotaxime, tobramycin and amikacin were also dramatically decreased in 128G5; pointing to an altered cell membrane. Furthermore, competition studies of 128G5 with isoglutaminy1-D-alanine-D-alanine demonstrated a 16-fold increase in its vancomycin MIC with no effect on the parent; suggesting an altered resistant cell wall. Scatchard plots from saturation binding studies with \(^{125}\text{I}-\text{labeled vancomycin confirmed that 128G5 had an altered cell wall with 4-fold more receptors than B7786 (23,790 nM vs 5,504nM per 10^7 cells). However, only one type of receptors with similar affinity constants (Kd approximately 1 nM) for both the resistant phenotype
and the parent were found. SEM and TEM revealed the reason for the greater number of receptors. The resistant cells had 60% larger diameters, four-fold thicker cell walls and poorly divided daughter cells with apparent missing splitting systems compared to the parent. Taken together, these data suggest that the resistant phenotype is associated with a defect in cell wall division, synthesis and turnover. We postulate that this defect results in an abnormally thickened cell wall with abundant cell wall material which can “mop” up vancomycin in the medium, reduce its availability to the active sites near the cytoplasmic membrane and therefore confer vancomycin resistance.
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ARA</td>
<td>Arabinose</td>
</tr>
<tr>
<td>AAR</td>
<td>Arginine arylamidase</td>
</tr>
<tr>
<td>ADH</td>
<td>Arginine dihydrolase</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>Amk</td>
<td>Amikacin</td>
</tr>
<tr>
<td>AM</td>
<td>N-acetylmuramyl-L-alanine amidase</td>
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
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<td>Arg</td>
<td>Arginine</td>
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<td>Asn</td>
<td>Asparagine</td>
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<tr>
<td>Az</td>
<td>Azlocillin</td>
</tr>
<tr>
<td>BE</td>
<td>40% Bile Esculin</td>
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<tr>
<td>Cefo</td>
<td>Cefotaxime</td>
</tr>
<tr>
<td>Cefz</td>
<td>Cefozulin</td>
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<tr>
<td>Ceft</td>
<td>Ceftriaxone</td>
</tr>
<tr>
<td>Celo</td>
<td>Cellulose</td>
</tr>
<tr>
<td>Cip</td>
<td>Ciprofloxacin</td>
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<tr>
<td>CNS</td>
<td>Coagulase-negative <em>staphylococci</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CV</td>
<td>Crystal Violet</td>
</tr>
<tr>
<td>DAP</td>
<td>Meso-diaminopimelic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>High performance chemiluminescence detection film</td>
</tr>
<tr>
<td>FRU</td>
<td>Fructose</td>
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<tr>
<td>GAL</td>
<td>Galactose</td>
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<tr>
<td>β-GAL</td>
<td>β-galactosidase</td>
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<tr>
<td>GL</td>
<td>endo-β-N-acetylglicosaminidase</td>
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<tr>
<td>β-GLR</td>
<td>β-Glucoronidase</td>
</tr>
<tr>
<td>GLU</td>
<td>Glucose</td>
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<tr>
<td>IDX</td>
<td>Indoxyl Phosphatase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LAC</td>
<td>Lactose</td>
</tr>
<tr>
<td>LCDC</td>
<td>National Center for Disease Control (Ottawa)</td>
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<tr>
<td>MAL</td>
<td>Maltose</td>
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<tr>
<td>MAN</td>
<td>Mannitol</td>
</tr>
<tr>
<td>MDG</td>
<td>α-methyl-D-glucoside</td>
</tr>
<tr>
<td>Me</td>
<td>Methicillin</td>
</tr>
<tr>
<td>MEL</td>
<td>D-Melibiose</td>
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MHB  
Mueller-Hinton Broth

MgCl₂  
Magnesium chloride

MNE  
Mannose

MS  
Micrococcus screening

NAG  
N-acetylglucosamine

dNTP  
deoxynucleoside triphosphate

NCCLS  
National Committee for Clinical and Laboratory Standards

NIT  
Nitrate reductase

NOV  
Novobiocin

OD  
Optical Density

ORN  
Ornithine

OPT  
Optochin

PAL  
Alkaline phosphatase

PBP  
Penicillin-binding proteins

Pen G  
Penicillin G

PGR  
PNP-β-D-Glucuronide

PGT  
PNP-β-D-Galactopyranoside

PHO  
Phosphatase

PYA  
Pyrrolidonyl arylmidase

PYR  
L-Pyrrolidonyl-β-Naphthylamide

TRE  
Trehalose
<table>
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<td>RAF</td>
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<tr>
<td>RIB</td>
<td>Ribose</td>
</tr>
<tr>
<td>SAC</td>
<td>Sucrose</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tm</td>
<td>Tobramycin</td>
</tr>
<tr>
<td>TUR</td>
<td>Turanose</td>
</tr>
<tr>
<td>URE</td>
<td>Urease</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>Va</td>
<td>Vancomycin</td>
</tr>
<tr>
<td>I(^{125})-Va</td>
<td>Iodinated vancomycin</td>
</tr>
<tr>
<td>VHHSC</td>
<td>Vancouver Hospital and Health Sciences Center</td>
</tr>
<tr>
<td>VP</td>
<td>Voges-Proskauer</td>
</tr>
<tr>
<td>XLT</td>
<td>Xylitol</td>
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<td>XYL</td>
<td>Xylose</td>
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Bacterial Isolates

ATCC 29213  Staphylococcus aureus (S. aureus)
ATCC 12600  Staphylococcus aureus
ATCC 29970  Staphylococcus haemolyticus (S. haemolyticus)
ATCC 14990  Staphylococcus epidermidis (S. epidermidis)
8523        Staphylococcus saprophyticus (S. saprophyticus)
EF97038     Enterococcus faecium (VanA) (E. faecium)
EF97051     Enterococcus faecalis (VanB) (E. faecalis)
118BA8      Clinical Staphylococcus haemolyticus (S. haemolyticus)
4G1         First generation S. haemolyticus subpopulation from 118BA8 passaged in 4 ug/ml vancomycin
8G1         First generation S. haemolyticus subpopulation derived from 118BA8 passaged in 8 ug/ml vancomycin
16G1        First generation S. haemolyticus subpopulation derived from 118BA8 passaged in 16 ug/ml vancomycin
32G2        Second generation S. haemolyticus subpopulation derived from 16G1 subpopulation passaged in 32 ug/ml vancomycin
64G2        Second generation S. haemolyticus subpopulation derived from 16G1 subpopulation passaged in 64 ug/ml vancomycin
128G332G2   Third generation S. haemolyticus subpopulation derived from 32G2
subpopulation passaged in 128 ug/ml vancomycin

128G₃₆₄G₂  Third generation *S. haemolyticus* subpopulation derived from 64G₂ subpopulation passaged in 128 ug/ml vancomycin

128G₄  Fourth generation *S. haemolyticus* subpopulation derived from 128G₃₆₄G₂ subpopulation passaged in 128 ug/ml vancomycin

128G₅  Fifth generation *S. haemolyticus* subpopulation derived from 128G₄ subpopulation passaged in 128 ug/ml vancomycin
ACKNOWLEDGEMENTS

This thesis is dedicated to my parents, especially my mother, for their support and encouragement throughout my time in graduate school. I am also most grateful to Dr. Anthony Chow for his advice and guidance and to all who have contributed and helped me in my project. Thank you.
**Staphylococcus haemolyticus and the Rationale of the Current Thesis**

*Staphylococcus haemolyticus* (*S. haemolyticus*) is a gram-positive coccus bacterium belonging to the genus *Staphylococcus*. Members of this genus exist in various cellular forms: singly, paired, tetrad, short chains or irregular clusters. The name “*Staphylococcus*” was derived from the word “Staphyle”, meaning bunch of grapes (Ogston, A. 1883). This genus was first described as nonmotile, nonspore forming, and catalase-positive (with the exception of *S. saprophyticus* and *S. aureus* subsp. *anaerobius*) (Rosenbach, F.J., 1884). They are also non-encapsulated (or have limited capsule formation).

1. Coagulase-negative *staphylococci* versus *Staphylococcus aureus*

The genus *Staphylococcus* can be further divided into two “families”: the coagulase-negative *staphylococci* (CNS) and *S. aureus*. Clinically, members of the CNS family are differentiated from *S. aureus* mainly by the absence of an extracellular staphylocoagulase, a free coagulase, which reacts with prothrombin to convert fibrinogen to fibrin (Murray P.R. et al 1995). However, not all members of the CNS are coagulase-negative; some strains of *S. hyicus* and *S. intermedius* also produce staphylocoagulase. For the purposes of this thesis all *Staphylococci* which are not *S. aureus* will be regarded as CNS. Presently, there are more discriminatory characteristics than the coagulase reaction for use in the classification of *Staphylococci*, and these will be discussed later in the review.
2. *S. haemolyticus*: a commensal of the human body

The CNS family is composed of at least 27 species and 3 subspecies; 13 of its members, including *S. haemolyticus*, are indigenous to humans (Murray, P.R. et al, 1995; Bannerman, T.L. et al, 1991; Chesneau, O. et al, 1993; Hajek, V. et al, 1992; Igimi, S. et al, 1990; Kloos, W.E. et al, 1991; Tanasupawat, S. et al 1992; Webster, J.A. et al, 1994). Among the CNS, *S. haemolyticus* is the second most encountered species in clinical infections (Neumeister, B. et al 1995; Gill, V.J. et al 1983; Marsik, F.J. et al, 1982). It is a common opportunistic pathogen found on the skin and mucous membranes of humans, mainly in the axillae, inguinal, perineal areas and the anterior nares (Kloos W.E. 1994). As *S. haemolyticus* is part of the normal flora of the human skin and mucous membranes, for nearly a century since its initial isolation, it was thought to be a harmless and non-pathogenic commensal of the human body. Recognized diseases caused by *S. haemolyticus* and other CNS prior to 1958 were few, mostly in patients with infective endocarditis associated with rheumatic valvular heart disease. If *S. haemolyticus* isolates were cultured from blood or infected tissue, they were frequently not considered as the causative agent of disease, but merely as contaminants.
3. Clinical Significance of *S. haemolyticus*

In the last two decades, *S. haemolyticus* associated with infections among immunocompromised patients has been isolated with increasing frequency. Such infections have increased to the point that their implication as causative agents of disease can no longer be denied. They are now recognized to be the etiological agents of such diseases as bacteremia, prosthetic valve endocarditis, peritonitis, deep wound infections, and many others (Martin, M.A. et al, 1989; Gill, V.J., 1983; Leighton, P.M. et al, 1986; Ponce de Leon et al, 1986; Shlaes, D.M., 1993; Kleeman, K.T. et al, 1993; Kloos, W.E. et al, 1994; Low, D.E. et al, 1992; Pfaller, M.A. et al, 1988; Veach, L.A. et al, 1990). The increase in frequency of *S. haemolyticus* infections is paralleled by the increased use of implantable devices (Kloos, W.E. et al 1994). It seems that the use of temporary or permanently implanted devices, like central venous catheters, prosthetic cardiac valves and such, have created a pathogenic niche for *S. haemolyticus* (Beachy, E.H., 1981). It has been suggested that the first step in foreign-body related infections involves the adhesion of the *Staphylococci* to the biomaterial (often made of silastic) followed by the production of slime which is poorly permeable to antibiotics (Kloos, W.E. et al, 1994; Hamilton-Miller, J.M. et al, 1993). This primary area of infection can then become a source for the seeding of bacteria to other parts of the body. An example is the use of intravenous catheters. The use of intravenous catheters has been one of the major causes of bloodstream infections with *S. haemolyticus*. The infected catheter allows for the introduction of the bacteria into the patients’ bloodstream, which often results in bacteremia.
By the mid-1980s, CNS had emerged as the leading pathogen responsible for nosocomial bacteremia due to the increased use of such prosthetic devices. Reports by the National Nosocomial Infection Surveillance program in 1988 indicated that CNS was responsible for up to 25% of all nosocomial bloodstream infections (Horan, T. et al, 1988). Presently, CNS was found to cause nosocomial septicemia among 3.3 newborns per 1000 line births in 1994, a significant increase from 2.3 cases per 1000 live births in 1981 (Kallman J., et al 1997). The infections caused by CNS were relatively serious, in one report 64% of the patients with CNS associated bacteremia were in critical care units versus 41% with other bloodstream infections (P<0.05) (Stillman, R.I. et al, 1987). These findings further point to the increasing significance of *S. haemolyticus* and its CNS relatives as nosocomial pathogens.

Besides bacteremia, CNS is also reported to be the most common cause of peritonitis among patients undergoing continuous ambulatory peritoneal dialysis (CAPD) (Chan, M.K. et al, 1981).

In addition to the rising frequency of CNS-associated infections, *S. haemolyticus* and certain members of the CNS family have attracted the interest of clinicians and scientists because of increasing difficulties in treating infections caused by these bacteria. Studies have shown that *S. haemolyticus* is resistant to a wide spectrum of antistaphylococcal antibiotics, including methicillin, a widely used antistaphylococcal drug (Woods, G. L. et al, 1988; Marsik, F.J. 1982; Degener, J.E. et al, 1994; Tripodi, M.F. et al 1994). These observations are further validated by surveys on *S. haemolyticus* and CNS infections, which have reported that as high as 35% to 85% of isolates associated with infections are resistant to the

Furthermore, *S. haemolyticus* has one of the widest spectrum of antibiotic resistance with the highest minimum inhibitory concentrations, amongst *Staphylococci* (Hamilton-Miller et al, 1985). In a hospital neonatal nursery in New Dehli, a *S. haemolyticus* isolate with three different resistance patterns causing infections among 22 neonates was described (Mehta, G. et al, 1997). This strain was resistant to penicillin, methicillin, gentamicin, erythromycin, chloramphenicol and tetracycline. Moreover, the *S. haemolyticus* strain exhibited a higher frequency of resistance to antibiotics than either *S. aureus* or *S. epidermidis* isolated from the unit at the same time. Froggat (1989) reported similar findings from a survey of nosocomial isolates of *S. haemolyticus*. He showed that 77% of 70 isolates of nosocomial *S. haemolyticus* were resistant to three or more antistaphylococcal agents and 41% to five or six agents. This wide spectrum of antibiotic resistance has made *S. haemolyticus* infections increasingly difficult to treat. Presently, vancomycin, a powerful drug against gram-positive organisms, has become the drug of choice for treating such infections. Although effective in the treatment of *Staphylococci* related infections for over 30 years, resistance to vancomycin has now been observed in *S. haemolyticus* (Schwalbe, R. et al 1987; Veach, L.A. et al 1990; Aubert, G. et al 1990).

Despite the emerging significance of this pathogen, the exact incidence of *S. haemolyticus* associated infections remains relatively unknown. The reason is that the current identification methods, based on the phenotypic differences between species of *Staphylococci*, are not discriminating enough for the species identification of all isolates of *S. haemolyticus* (Grant, C.E. et al 1994; Giger, O. et al 1984; Crouch, S.F. et al 1987). There is
an urgent need for more accurate and comprehensive identification systems that are able to accurately identify all strains of *S. haemolyticus* as well as other members of the CNS family; the delineation of the CNS family will aid clinical epidemiological studies, diagnosis and management of serious CNS infections.

Furthermore, accurate identification of *S. haemolyticus* will be important for the management of vancomycin resistant strains. Vancomycin resistance in *Staphylococci* has been mostly confined to this species; an observation that is of great concern to the medical community as many strains of *S. haemolyticus* are already resistant to multiple antibiotics, including methicillin. It is, therefore, of prime importance to understand the mechanisms of vancomycin resistance so as to manage and control vancomycin resistant *S. haemolyticus*. So far, little is known about vancomycin resistance in *Staphylococci* and a model of resistance is required.

The present thesis covers both areas of concern with the introduction of a novel method for the species identification of *S. haemolyticus*, and a model of vancomycin resistance in *Staphylococci* based on the case study of several laboratory-derived strains of *S. haemolyticus*.

The novel method for the species identification of *S. haemolyticus* involves the use of the heat shock protein 60 kDa (HSP60) gene. This gene is an excellent target for use in the species-specific identification of *S. haemolyticus* as it is a highly conserved, single copy, essential gene with species-specific signatures for all *Staphylococci* isolates. In this study, the species-specificity of the HSP60 gene for *S. haemolyticus* was tested for multiple clinical isolates (36 isolates) of *S. haemolyticus* associated with nosocomial bacteremia. These
isolates were previously identified by the Microscan method (Baxter, West Sacramento, California). In addition to the testing of a new method for the species identification of S. haemolyticus, a model for vancomycin resistance was also proposed in this thesis. The model was based on experiments and observations made from several laboratory-derived subpopulations of vancomycin resistant S. haemolyticus (MIC 32 ug/ml). The parent isolate, 118BA8 was intermediately resistant to vancomycin (MIC 4ug/ml) and was from the collection of S. haemolyticus used to test the HSP60 probes.

The approach, methods and results for the HSP60 identification method of S. haemolyticus and the model for vancomycin resistance will be presented as two separate sections - Sections I and II, respectively.
Section I

Species Identification of *S. haemolyticus* with HSP60 DNA Probes

Compared to Current Phenotypic Methods
Chapter 1

Introduction

1. Speciation of CNS based on phenotypic characteristics

Baird and Parker (1963) proposed one of the first classification schemes for members of the *Staphylococcus* family. According to this classification scheme, the genus *Staphylococci* is subdivided into six biotypes (Baird and Parker et al 1963, 1964) based on certain physiological and biochemical characteristics. Biotype I consists of coagulase-positive *Staphylococci*, such as *S. aureus*, and biotypes II - VI were known simply as “*S. epidermidis*”. Previously, the nomenclature *S. epidermidis* was used to refer to any *Staphylococci* which were not *S. aureus*. Presently, the name *S. epidermidis* is used to describe a specific group of CNS. The descriptions of the six biotypes are summarized in Table 1. This scheme was later modified such that the *S. epidermidis* subgroups consisted of only 4 biotypes and four new subgroups which were initially thought to belong to the genus *Micrococcus*, were added to the scheme and named *S. saprophyticus* (Baird and Parker 1974). However, later on the Baird and Parker schema ran into problems because the subgroups of CNS categorized according to the scheme, thought to be homogenous, were later found to consist of more than one group. This scheme was, therefore, found to be inadequate for the classification of *Staphylococci*. 
Table 1. Baird and Parker's classification scheme for *Staphylococci*

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<th>Subgroup</th>
<th>Coagulase Test</th>
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<th>Acetoin</th>
<th>Phosphatase</th>
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</table>

1.1. The Kloos and Schleifer's scheme for the classification for *Staphylococci*

Another scheme for the classification of *Staphylococci* was introduced by Kloos and Schleifer at around the same period (1967), and was more comprehensive for the speciation of CNS. It permitted the speciation of the CNS family into 9 different groups according to their morphological and physiological characteristics, antibiotic susceptibility patterns and cell wall compositions (Kloos, W.E., 1967). This schema was modified several years later (Kloos, W.E. et al, 1975) with the addition of more discriminating tests to speciate the CNS to an even greater level. It has, today, become the reference method against which other methods for the species identification of CNS is compared (Giger, O. et al, 1984; Kloos, W.E. et al, 1994).
The modified Kloos and Schleifer scheme categorizes the CNS according to the following phenotypic characteristics: colony morphology, coagulase production, oxygen requirements, hemolysin production, resistance to certain antibiotics, various enzymatic activities and certain carbohydrate fermentation reactions. The specific tests used are summarized in Table 2. Initially, this classification scheme identified only 10 species of *Staphylococci*; today it is able to speciate *Staphylococci* into 27 species and three subspecies.

1.2. Rapid methods for the identification of *Staphylococci*

The Kloos and Schleifer scheme for identifying *Staphylococci* requires the use of specialized media which are tedious to prepare, and relatively long incubation times. Even with the recent modifications by Kloos to simplify the scheme for use in the routine identification of human *Staphylococcus* species, the procedure is still cumbersome (Kloos, W.E. et al 1975). Currently, faster and less tedious alternatives to the conventional method of Kloos and Schleifer are available (Grant, C.E., 1994; Perl, T.M., 1994). These commercial systems are miniaturized and simplified versions of the Kloos and Schleifer identification method. They are generally comprised of a range of dehydrated substrates carried in strips or panels that are reconstituted by the addition of a few drops of bacterial suspension. Incubation times range from 5 to 24 hours, a shorter duration than the conventional method. The more popular systems include the API Staph (API BioMerieux SA, Lyon, France), Staph-Ident (Analytab, Plainsview, NY, USA), Staphzyme (Rosco Diagnostica) and Minitek Gram-positive Set (Becton-Dickinson Microbiology Systems, Cockeysville, Md)
identification kits. There are also commercial identification systems which are automated to further reduce labor intensiveness, an example is the autoSCAN-W/A system which inoculates and reads Microscan Pos Combo Type 5 panels and Microscan Pos RAPID POS ID panels (Baxter, Healthcare Corp., Microscan division, West Sacramento, California). The automated systems are, however, very expensive.

The identification method currently used by the Vancouver Hospital and Health Science Center to identify CNS to the species level is the Microscan Pos RAPID POS ID panels with the automated autoSCAN-W/A system (Baxter, Healthcare Corp., Microscan division, West Sacramento, California). This method involves the use of 18 major biochemical tests and 7 additional tests (used in the case of low percentage probabilities) for the speciation of CNS. The 18 major biochemical tests are: CV, MS, NIT, VP, BE, PYR, URE, β-GLR, IDX, AP, AAR, β-GAL, resistance to NOV and OPT, and carbohydrate fermentations of RAF, LAC, TRE and MNE (see list of abbreviations on p.viii). The Microscan Pos RAPID POS ID panels containing the substrates and fluorometric indicators are inoculated and read by the autoSCAN-W/A system and the results interpreted with the aid of a computerized database. A report of the identification of the organism can be obtained in as little as 2 hours of incubation with the panels.
Table 2. Kloos and Schleifer's Classification Scheme for *Staphylococci* (Adapted from
the Manual of Clinical Microbiology, sixth edition; Murray, P.R. et al 1995; p.282-298)

<table>
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<th>Species</th>
<th>Colony size (large)</th>
<th>Colony pigment</th>
<th>Anaerobic growth</th>
<th>Aerobic growth</th>
<th>Saprophytogenase</th>
<th>Clumping factor</th>
<th>Heat-stable nuclease</th>
<th>Hemolysis</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Alkaline phosphatase</th>
<th>Pyrophosphorylase</th>
<th>Ornithine decarboxylase</th>
<th>Lysine</th>
<th>α-Galactosidase</th>
<th>β-Glucuronidase</th>
<th>β-Galactosidase</th>
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* Symbols (unless otherwise indicated): +, 90% or more strains positive; ±, 90% or more strains weakly positive; -, 90% or more strains negative; d, 1 to 89% of strains positive; ND, not determined; ( ), delayed reaction.

* Positive is defined as the visual detection of carotenoid pigments (e.g., yellow, yellow-orange, or orange) during colony development at normal incubation or room temperatures. Pigments may be enhanced by the addition of milk, fat, glycerol monostearate, or soaps to P agar.

* Anaerobic growth in a semisolid thioglycolate medium (61). Symbols: +, moderate or heavy growth down tube within 18 to 24 h; ±, heavier growth in the upper portion and weaker growth in the lower, anaerobic portion of tube; -, no visible growth within 48 h, but by 72 to 96 h very weak diffuse growth or a few scattered, small colonies may be observed in the lower portion of tube; ( ), delayed growth appearing within 24 to 72 h, sometimes noted as large discrete colonies in the lower portion of tube.

* On P agar or bovine, sheep, or human blood agar at 34 to 37°C, *S. equorum* grows slowly at 35 to 37°C; its optimum growth temperature is 30°C. The anaerobic species *S. saccharolyticus* and subspecies *S. aureus* subsp. *anaerobius* grow very slowly in the presence of air. Aerobic growth may be increased slightly by subculture in the presence of air. *S. aureus* subsp. *anaerobius* requires the addition of blood, serum, or egg yolk for growth on primary isolation medium. *S. auricularis* and *S. lentus* produce just detectable colonies on P agar or blood agar in 24 to 36 h, and these remain very small (1 to 2 mm in diameter).
### Characteristic

<table>
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<td><em>S. lentus</em></td>
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^1 Detected in rabbit or human plasma (slide coagulase test). Human plasma is preferred for the detection of clumping factor with *S. lugdunensis* and *S. schleiferi*. Latex agglutination is somewhat less reliable for detection of clumping factor or fibrinogen affinity factor in *S. lugdunensis*.

^2 Hemolysis on bovine blood agar (58). Symbols: +, wide zone of hemolysis within 24 to 36 h; (+), delayed moderate to wide zone of hemolysis within 48 to 72 h; (d), no or delayed hemolysis; —, no or only very narrow zone (≤1 mm) of hemolysis within 72 h. Some of the strains designated negative may produce a slight greening or browning of blood agar.

^3 Catalase and cytochrome synthesis cannot be induced in *S. aureus subsp. anaerobius* by the addition of H2O2 or hemin to the culture medium.

^4 Catalase can be induced in *S. saccharolyticus* by hemin supplementation. In this species, cytochromes a and b are present in small quantities.

^5 Determined by the modified oxidase test of Faller and Schleifer (32) used to detect the presence of cytochrome c.

^6 Pyrrolidonyl arylamidase, urease, ß-glucosidase, ß-glucosidase, ß-galactosidase, and arginine utilization are characteristics that have been determined primarily by commercial rapid-identification test systems (e.g., API Staph-Ident, DMS Staph Trac, ATB 32 Staph, Baxter-MicroScan Pos Combo Type 3 panel and RAPID POS ID panel, Becton Dickinson Minitek Gram-Positive Set, and Vitek Systems Gram-Positive Identification Card).

^7 Positive is defined as an MIC of ≥0.6 μg/ml or a growth inhibition zone diameter of ≥16 mm with a 5-μg novobiocin disk.

^8 Positive is defined as a growth inhibition zone diameter of <10 mm with a 300-U polymyxin B disk.

^9 Alkaline phosphatase activity is negative for approximately 6 to 15% of strains of *S. epidermidis*, depending on the population sampled. A low but significant number of clinical isolates have been phosphatase negative.
The identification of the microorganism is derived from a biotype number generated by assigning a weighted numerical value to all positive reactions. As shown below, positive tests in the top horizontal row get a value of 4, positive tests in the middle row a value of 2, and positive tests in the bottom row a value of 1. The biotype is determined by adding the values for each vertical column.

CV - NOV - VP - BE - PGT + Lac -
MS + PGR - OPT + PYR - URE + TRE +
NIT - DX - PHO - ARG - RAF - MNS -

Biotype number: 2 0 2 0 6 2

Biotype numbers are listed in numerical order. Each biotype number is followed by the percent probability for the organism identification. All possible species for each biotype number up to a probability level of 99.9% to 100.0% are listed. The percent probability of each identification is derived from a list of organism based upon the percent of positive reactions for each biochemical test. Five or less organisms with the highest probability with a predetermined cut off value are evaluated for the percent probability. This determination is based upon summing the frequencies and then dividing each organism’s probability by the sum total (Biotype Codebook for Aerobic Gram-positive Organisms).

95 - 99.9% Identification: most probable
90 - 94.9% Identification: very probable
85 – 89.9% Identification: probable

60 – 84.9% Identification: low selectivity

1. Recheck and confirm reactions
2. Check organism purity
3. Retest organism on new panel
4. Set up additional confirmatory tests

Below 60% Identification: questionable

1.3. Limitations of species identification based on phenotypic characteristics

There are several limitations associated with the identification of *S. haemolyticus* by identification methods based on phenotypic characteristics. The first limitation is that a significant number of strains of CNS, particularly strains of *S. haemolyticus*, have been reported to have variant or atypical phenotypic characteristics. In a study of pathogenic CNS other than *S. epidermidis*, Fleurette J. (1987) reported many isolates belonging to the *S. haemolyticus/S. hominis/S. warneri* group that expressed weak and/or variable biochemical reactions which did not fit into any typical identification scheme described by Kloos and Schleifer. Three of such isolates were also present in this study. Similar variant strains of CNS were also reported by other investigators such as Kloos W.E (1994) and Hedin G. (1994). The former documented a phosphatase-negative variant strain of *S. epidermidis*, and the latter phosphatase-positive variants of *S. haemolyticus*. The phosphate-negative variant strains of *S. epidermidis* are often misidentified as *Staphylococcus hominis* *(S. hominis)*,
The second limitation associated with phenotypic identification methods is the ambiguity that often results from the subjective interpretation of colorimetric biochemical tests. The majority of the phenotypic tests are based on colorimetric changes and when the results are weak, it can be difficult to determine if a test is truly positive or negative. As mentioned above, the phenotypic speciation of *S. haemolyticus*, *S. hominis*, and *S. warneri* are especially difficult, partially due to test results which are difficult to interpret. *S. haemolyticus* is only differentiated from *S. hominis* and *S. warneri* by three definitive biochemical tests (Table 2) (Kloos, W.E. et al 1975). If even one of them is misinterpreted, the identity of that isolate cannot be accurately ascertained.

Other limitations include the imprecise genetic characterization of the bacteria based on their phenotypic characteristics and the limited range of phenotypic tests which can differentiate some species from others. For example, *S. hominis* and *S. haemolyticus* are distinguished from each other by only two tests, the acidification of lactose or mannitol (Kloos, W.E. et al 1975). Both false-positive and false-negative results will lead to the misidentification of the two species of CNS. As a result of these limitations in the laboratory-based identification techniques for *S. haemolyticus*, the exact incidence of infections associated with this organism is unknown. An alternative method of speciation based on the genotype of the organism may be a preferable approach for the establishment of the true incidence of infections.

In a genotypic identification method, the choice of gene target used for identifying the bacteria is of utmost importance. A good gene target should be one that is universally present in all strains of *Staphylococci* and highly conserved with sufficient...
variability to allow for species-specific sequences. The gene should ideally be less prone to mutations. An example of such a gene target is the heat shock protein 60 kDa gene.

2. The Staphylococcal Heat Shock Protein (HSP) 60 KDa Gene

The staphylococcal heat shock protein (HSP) 60 kDa gene belongs to a large family of genes which encode proteins known as the heat shock proteins. Heat shock proteins are found universal in all living organisms and are among the most conserved proteins and genetic elements presently known. HSP genes have recognizable homology that cross the boundaries of the procaryotic, eucaryotic and archaebacterial kingdom (Poncho, N.A. et al 1996; Kaufman S.H. et al 1991; Bonay, M. et al 1994; Ingolia, T.D. et al 1982).

2.1. Function of Heat Shock Proteins

Heat shock proteins are proteins with synthetic rates that are dramatically affected (i.e. increased and decreased by 10 – 50 fold) by upward or downward shifts in the growth temperature of the organism. Heat shock proteins’ synthesis are also affected by such compounds as ethanol, nalidixic acid and X-rays, all of which are deleterious to the cells (Sahu, G.K. et al 1994; Suzuki et al 1998). When induced, they have been shown to confer tolerance to extreme temperatures and the deleterious compounds mentioned (Laszlo, A. 1992; Nakamura, K. et al 1991; Li, G.C. et al, 1982). Mutants with an inability to upregulate the synthesis of HSP in the presence of such deleterious compounds are killed at lower
concentrations than their parent cells. The mechanism by which these proteins confer their protection remains unknown. Although HSPs are found in larger amounts when induced by temperature changes, they are present at basal levels in the cell at normal growth temperatures, and are essential for growth. They have been implicated in the major processes of macromolecule synthesis, modification and assembly and shown to bind transiently and noncovalently to nascent polypeptides and unfolded or assembled proteins. In so doing, they aid in protein biogenesis by blocking nonproductive protein-protein interactions, mediate the folding of proteins into their native state by sequestering folding intermediates, and therefore allow the concerted folding by domains and assembly of oligomers (Craig, E.A. et al, 1993; Basharov, M.A. et al 1997). Therefore, HSPs have also been termed molecular chaperonins as they accompany proteins through various stages of assembly and synthesis (Nieba-Axman S.E. et al 1997).

2.2. The Heat Shock Protein 60 kDa gene as a Target for Species Identification of *Staphylococci*

The HSP that is of interest to this thesis is the heat shock protein 60 kDa. The HSP60 gene is an essential, single-copied gene which is less prone to mutations (Craig, E.A. et al, 1993). It has been shown to be ubiquitous and highly conserved among *Staphylococci* species and yet each gene contains signature sequences which are species and even genus specific (Goh, S.H. 1996). These properties make the HSP60 gene a potentially ideal target for speciating the CNS family in general and for the species identification of *S. haemolyticus*
in particular.

Previous studies (Goh, S.H. et al, 1996) using the HSP60 gene as a target for speciation demonstrated that DNA probes generated from a 600 bp region of the HSP60 gene by a set of universal degenerate primers from 6 reference species of *Staphylococci* (*S. aureus, S. epidermidis, S. haemolyticus, S. lugdunensis, S. saprophyticus, and S. schleiferi*) could be used for the speciation of *Staphylococci*. The species-specific probes correctly identified all isolates of a set of 58 reference and clinical isolates of *Staphylococcus* and non-*Staphylococcus* species. Furthermore, the 6 probes did not cross-hybridize with each other. This work was further enhanced by a more recent publication (Goh, S.H., et al, 1997) that demonstrated the accurate speciation of 40 known *Staphylococcus* isolates with HSP60 gene probes from 34 species of *Staphylococcus* species and subspecies. The entire set of HSP60 DNA probes was species-specific except for *S. intermedius* and *S. delphini*, which cross-hybridized with each other. The probes were able to correctly identify 36 of 40 staphylococcal isolates. The four remaining strains were *S. hyicus* of bovine origin which failed to hybridize to DNA from the *S. hyicus* target strain, which was of porcine origin, or to any other *Staphylococcus* species. However, DNA from these four strains cross-hybridized with one another. DNA sequence data suggested that there was some genetic divergence between the *S. hyicus* of the target strain and the four *S. hyicus* of bovine origin. Phenotypically, these two groups of *S. hyicus* could not be differentiated from each other. However, at the genetic level they were distinct from one another and should be designated as two different subspecies. The limitations of the phenotypic system of identification and the advantages of the genotypic HSP60 method is once again clearly demonstrated. These results
further validate the usefulness of the HSP60 gene as a target for the speciation and taxonomic classification of coagulase-negative *staphylococci*, including *S. haemolyticus*.

3. Aims of the current study: The testing of the specificity and accuracy of the HSP60 gene for the species identification of multiple isolates of *S. haemolyticus* compared to two phenotypic methods of identification commonly used in the diagnostic laboratories (Microscan and API Staph)

Although studies on the specificity of probes from reference species of *Staphylococcus* have been tested on a relatively wide range of CNS species, the testing of these probes on multiple isolates of each species has not been carried out. Therefore, the HSP60 method of identification is based on an untested assumption that the HSP60 DNA probes generated from reference strains are specific for all isolates within any given species. In the present study, this assumption was tested using the *S. haemolyticus* species. The specificity and accuracy of the HSP60 gene probe from a reference strain of *S. haemolyticus* (ATCC29970) was tested on 36 isolates of bacteremic *S. haemolyticus* as well as 30 other species of CNS collected from the Clinical Medical Microbiology Laboratory of Vancouver Hospital and Health Sciences Center. The 30 other species of CNS were isolated consecutively with the *S. haemolyticus* isolates. All 66 isolates were previously identified according to the MicroScan method. A second probe was generated from *S. epidermidis* and
tested on the same collection of 66 isolates as a control. Discrepancies between the HSP60 method of identification and the Microscan were resolved by further identification according to the method of Kloos and Schleifer (1975), which was carried out by the BCCDC as well as LCDC. The combined results of the Microscan method with corrections by the BCCDC and LCDC laboratories were considered as the reference standard. The results from the hybridization studies were further compared to the API Staph (Biomerieux, Lyon, France), a widely used commercially available rapid method for the speciation of *Staphylococci*. 
Chapter 2

Materials and Methods

2.1. Approach

The HSP60 method for the species identification of *S. haemolyticus* was tested by generating HSP60 DNA probes using a set of universal degenerate primers designed specifically to amplify by PCR a 600-bp portion of the gene. These amplified products were labeled with digoxigenin-dUTP, and used to probe a collection of 66 isolates of CNS and six control isolates (*S. haemolyticus* ATCC29970, *S. epidermidis* ATCC14990, *S. aureus* ATCC12600 and ATCC29213, *E. faecium* EF91038, *E. faecalis* EF97058) in dot blot hybridization reactions. The hybridization reactions and washings were carried out under highly stringent conditions.

2.2. Materials and methods

2.2.1. Bacterial isolates and growth conditions

The bacterial isolates used in the study consisted of 6 reference strains of various *Staphylococcus* species and *Enterococcus* species obtained from the American Type Culture
Collection and other sources. For this study, all bacteremic *S. haemolyticus* isolates and those from deep tissue infections (36 isolates) cultured in 1994 by the Clinical Medical Microbiology Laboratory of the Vancouver Hospital and Health Science Centers, Vancouver, B.C., were chosen for this study. As negative controls, other CNS species, which were isolated consecutively with the *S. haemolyticus* isolates (on the same day or the date closest to the isolation of *S. haemolyticus*), were also picked for testing. As an additional negative control, a DNA probe was generated from *S. epidermidis* and used to probe the same isolates. These clinical isolates were previously identified with the Microscan method. All isolates were grown on Mueller Hinton Agar plates. The clinical isolates that gave discrepant results when probed with the 600-bp HSP60 probes were coded and sent to the provincial laboratory of the BCCDC, Vancouver, for re-identification according to the method of Kloos and Schleifer (1975). The battery of tests used by the BCCDC included carbohydrate fermentation of ARA, CELO, FRU, GAL, LAC, MAL, MAN, MNE, RAF, RIB, SAC, TRE, TUR, XLT and XYL; testing of enzymatic activities for ARG, NIT, ORN and URE; miscellaneous tests included tests for coagulase, Staphaurex, resistance to BAC and NOV and gram stain. Isolates which could not be identified by BCCDC were submitted to LCDC in Ottawa for further testing.
2.2.1. PCR amplification of the HSP60 gene

PCR products of 600-bp were obtained from the isolates by the colony PCR method. A single colony was picked from the agar plate and boiled in 20 ul of water. Following that, the bacterial cell suspension was centrifuged for 5 minutes at 6000 x g and 10 ul was taken from the tubes and mixed with PCR reaction mixture for PCR amplification. The PCR amplification reactions of the reference strains and the coded isolates were carried out under identical conditions that have been described previously (Goh, S.H. et al, 1996). Briefly, the PCR mixture contained, in a final concentration, 50 mM KCL, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 200 uM (each) dNTP, 10 ul supernatant from water boiled with bacteria, 2 U of Taq polymerase (GIBCO) and, 0.5 ug of each degenerate HSP60 primers in a final volume of 100ul of distilled water. The sequences of the 5' and 3' HSP60 primers, designated H279 and H280, were:

(H279) 5'-GAATTCGAIIIIGCIGGIGA(TC)GGIACIAIACIAC-3'
(H280) 5'-CGCGGGATCC(TC)(TG)(TC)(TG)ITCICC(AG)AAICCIIGGIGC(TC)TT-3'

Inosine (I) was used to reduce the degeneracy of the primers. The thermal cycling conditions were 3 minutes at 95°C, 2 minutes at 37°C, and 5 minutes at 72°C for 40 cycles. The last cycle was for 10 minutes at 72°C. After PCR, 5 ul of PCR products was visualized on 2% agarose gel and photographed under UV light after ethidium bromide staining.
2.2.3. Optimization of the amount of genomic DNA for use in PCR

Template DNA from bacterial isolates was obtained for PCR by boiling a single colony in 20 ul of distilled water. The optimal volume of the supernatant from the boiled mixture for PCR was determined by carrying out PCR with various volumes of the supernatant. The volumes used were 5 ul, 7.5 ul, 10 ul, and 15 ul.

2.2.4. Purification and digoxigenin labeling of HSP60 PCR products for use as DNA probes

The 600-bp PCR products amplified from genomic DNA from S. haemolyticus (ATCC 29970), and S. epidermidis (ATCC 14490) were purified by the QIA Quick-spin PCR Purification Kit (Qiagen), according to manufacturer’s instructions. The samples were visualized on a 2% agarose gel to ensure the presence of only a single band of 600-bp PCR product. The PCR products were labeled for use as DNA probes with digoxigenin-11-dUTP according to the standard random prime method of Boehringer Mannheim. Briefly, the template DNA was denatured by heating at 95°C for 10 minutes and subsequently cooled on ice. To a microfuge tube, placed on ice, 25 ng of denatured DNA, 2 ul DIG DNA Labeling Mix (10 x; consist of 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM DIG-dUTP, pH6.5), 2 ul Hexanucleotide Mix (10 x) and 1 ul Klenow enzyme (labeling grade; 2 U) were added. The mixture was incubated overnight at 37°C and 5 ul of the mixture was analyzed on 2% agarose to visualize the products.
2.2.5. Dot blot hybridization reactions

PCR products from isolates that were to be identified were purified with QIA Quick-spin PCR Purification Kit (Qiagen) and dot blotted onto nylon membranes after denaturation with 0.4M NaOH. The DNA was fixed onto the membranes by exposure to UV light for 2 minutes on the transilluminator. After the template DNA was fixed onto the filter, they were probed with the HSP60 probes in hybridization experiments. The filters were pre-hybridized at 42⁰C in 50% formamide, 5 x SSC (1 x SSC consist of 0.15 M NaCl plus 0.015 M sodium citrate), 2% Boehringer Mannheim blocking reagent, 0.1% N-lauryl sarcosine, 0.02% SDS for at least 1 hour 45 minutes. Hybridization with the same pre-hybridization buffer and digoxigenin probes was allowed to proceed overnight at 42⁰C. After hybridization, the membranes were washed sequentially with 2 x SSC and 0.1% SDS (wt/vol) twice for 15 minutes at room temperatures and then with 0.1 x SSC, 0.1% SDS twice for 15 minutes at 68⁰C. Visualization was by autoradiography with high performance chemiluminescence film (ECL) (Amersham Life Science, Buckinghamshire). All signals which gave similar background to signal ratio as the positive controls were considered positive while those with ratios similar to the negative controls were considered negative for the hybridization reaction.
2.2.6. Determination of the optimal amount of template DNA that gives the strongest positive signal and the lowest non-specific binding with the negative control

The amount of DNA to be dot blotted on the nylon membrane for hybridization reactions with the DNA probe should give a strong clean signal with low non-specificity. Different concentrations (5 ng, 10 ng, 20 ng) of DNA were dot blotted onto the nylon membrane and tested with a fixed concentration of probe to give the strongest positive signal with a positive control (for the *S. haemolyticus* probe, the positive control would be *S. haemolyticus* ATCC29970 PCR products) and the lowest or no background signal for the negative control (PCR products from *S. epidermidis* ATCC14490).

2.2.7. Optimization of dot blot exposure time to ECL film

The amount of time required for the exposure of the dot blots to the ECL films was determined with a dot blot containing a fixed concentration (determined in the prior optimization experiments) of template DNA and incubated with a pre-determined concentration of HSP60 DNA probes. The resulting dot blot was exposed to ECL film for lengths of 5 minutes, 10 minutes and, 20 minutes.

2.2.8. API Staph identification system

The API Staph (BioMerieux SA, Lyon, France) commercial kit comprises of a strip
containing dehydrated test substrates in individual microtubes. The tests were performed by adding to each tube an aliquot of API Staph Medium that had been inoculated with the strain to be studied. The bacterial suspension was adjusted to a visual density comparable to a 0.5 MacFarland standard. The strip was then incubated for 18 - 24 hours at 35 - 37° C after which the results were read and interpreted according to the criteria provided with the kit. The biochemical tests are colorimetric tests; for example, the ability to ferment various carbohydrates is detected by the production of acid during the fermentation process which then changes the color of the pH indicator from one color (red) to the other (yellow).

The identification was facilitated by the use of the API STAPH Analytical Profile Index. The identification obtained is based on the classification of Kloos and Schleifer (1975). The biochemical and enzymatic tests included in the kit are given in Table 3. A comparison of the biochemical tests for the three phenotypic methods BCCDC (reference laboratory method, Microscan method and API Staph) used in this thesis are given in Table 3.
The sensitivity, specificity, positive and negative predictive values and accuracy of an identification method for the identification of a particular species of CNS was calculated according to the diagram above. ‘A’ is the number of positive identifications (if the systems were being tested for their ability to identify *S. haemolyticus*, a positive identification would be an isolate identified as a *S. haemolyticus* and a negative identification would be an identification other then *S. haemolyticus*) which were in agreement for both the reference method and the method being tested. ‘B’ is the number of positive identifications for the method being tested which were negative according to the reference method and vice versa for ‘C’. Finally, ‘D’ is the total number of negative identifications which were in agreement for both methods.
The formulas for calculating the sensitivity, specificity, positive and negative predictive values and accuracy of an identification method for the identification of a particular species of CNS is shown below:

Sensitivity \( \frac{A}{A+C} \)

Specificity \( \frac{D}{B+D} \)

Positive predictive value \( \frac{A}{A+B} \)

Negative predictive value \( \frac{C}{C+D} \)

Accuracy \( \frac{A+D}{\text{total no. of isolates tested}} \)
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Table 3. The panels of biochemical tests included in three systems for the identification of Staphylococci

(+) represents a positive reaction with the test and (-) a negative reaction.
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<th>β-GLR</th>
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</table>

Table 3. The panels of biochemical tests included in three systems for the identification of *Staphylococci* (Con’t)

((+)) represents a positive reaction to the test and (-) a negative reaction)
Chapter 3

Results

3.1. The optimal amount of genomic DNA that gave specific PCR amplification products

The PCR results showed that 10 ul of supernatant contained sufficient extracted DNA to give good amplification results and hence this volume of template DNA was used for all PCRs (Figure 1). Furthermore, all 66 clinical isolates and reference strains gave good amplification results with the H279 and H280 primers. Figure 2 displays a 2% agarose gel with PCR products from 7 clinical isolates and a reference strain of S. aureus (ATCC12600).

3.2. The optimal amount of template DNA for dot blot hybridization experiments

10 ng of PCR products dot blotted onto the filters for hybridization reactions gave the lowest background and strongest signal compared to the other two concentrations, hence this amount of PCR product was used for all dot blot hybridization experiments. Figure 3 shows the results of a hybridization experiment with various concentrations of the positive and negative controls.
3.3. The optimal autoradiography exposure time

The optimal exposure time was chosen to be the time that gave a low background to signal ratio. A comparison of the three different exposure times is shown in Figure 4. The optimal exposure time was determined to be 10 minutes.
Figure 1. PCR results with various amounts of template DNA. Template DNA was obtained by boiling a single colony *S. haemolyticus* in 20 ul of distilled water. (a) 15 ul (b) 10 ul (c) 7.5 ul and (d) 5 ul of supernatant from the boiled mixture.
Figure 2. PCR amplifications of the HSP60 gene of seven clinical isolates of CNS and a reference strain of *S. aureus* (ATCC12600). (a) 100-bp DNA ladder and (b) *S. aureus* reference strain (ATCC12600)
Figure 3. A dot blot filter containing three different amount of template DNA (20 ng, 10 ng and 5 ng) which had been probed with a *S. haemolyticus* probe. The template DNA were from *S. haemolyticus* and *S. epidermidis*. *S. epidermidis* PCR products are in column A and *S. haemolyticus* are in column B.
Figure 4. Results of different lengths of exposure times of ECL film to a dot blot hybridization filter hybridized with the HSP60 *S. haemolyticus* probe.
Figure 5. A sample of a dot blot hybridized with the *S. haemolyticus* probe. The dot blot contains PCR products from the collection of 66 clinical isolates, *S. haemolyticus* ATCC29970 and *S. epidermidis* ATCC14990.
3.4. Dot blot hybridization results with clinical and reference isolates

The results of the dot blot hybridization of the sixty-six clinical CNS isolates and six reference isolates of Staphylococcus (4) and non-staphylococcal species (2) with the two staphylococcus species HSP60 probes are summarized in Table 6. A sample of a dot blot probed with a S. haemolyticus probe is shown in Figure 5. The control isolates, E. faecalis and E. faecium did not give positive hybridization signals with the S. haemolyticus probes or with the S. epidermidis probe. Furthermore, the S. haemolyticus probe did not cross-hybridize with the 600-bp product from the reference species of S. epidermidis (ATCC 14490) and only did so weakly with the two reference species of S. aureus (ATCC 29213 and 12600).

The results for the hybridization experiments of the S. haemolyticus probe with the 66 clinical staphylococcal isolates showed 88% (58 out of 66) agreement with the Microscan method. The eight isolates that did not agree with the Microscan identification included six false-negatives (identified as S. haemolyticus by the Microscan method but did not hybridize with the S. haemolyticus probe) and two false-positives (identified as non-S. haemolyticus isolates but hybridized with the S. haemolyticus probe) (Table 4).
Table 4. Re-identification results of the isolates with discordant identifications by the *S. haemolyticus* probe and the Microscan method

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Microscan</th>
<th>Re-identification</th>
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<td></td>
<td></td>
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<td><em>S. haemolyticus</em></td>
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<td><em>S. haemolyticus</em></td>
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</tr>
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<td><em>S. haemolyticus</em></td>
<td><em>S. haemolyticus</em></td>
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</tr>
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<td>B10464-1</td>
<td><em>S. haemolyticus</em></td>
<td><em>S. epidermidis</em></td>
<td>-</td>
</tr>
<tr>
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<td><em>S. haemolyticus</em></td>
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</tr>
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<td><em>S. epidermidis</em></td>
<td><em>S. epidermidis</em></td>
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</table>

In addition to the eight discordant identifications of the *S. haemolyticus* probe, there were also discordant identifications between the *S. epidermidis* probe and the Microscan method. The results for the hybridization studies of the *S. epidermidis* probe with the 66 clinical staphylococcal isolates showed 89% (59 out of 66) agreement with the identification by the VHHSC Diagnostic Microbiology Laboratory. The seven discordant isolates consisted of four false-negatives and three false-positives (Table 5).
Table 5. Re-identification results of isolates with discordant identifications by the *S. epidermidis* probe and the Microscan method

<table>
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<th>Isolate no.</th>
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<td><em>S. epidermidis</em></td>
<td>Mix of <em>S. epidermidis</em> and unidentifiable bacteria</td>
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Table 6. The identification results of the HSP60 hybridization method and the reference method

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3.5. Reference laboratory (Ottawa) identification results

There were in total eleven isolates that provided discordant results between the Microscan and the HSP60 method (although there were a total of 15 discordant identifications between the two probes with the Microscan method, four isolates were discordant for both probes). These isolates were coded and sent for re-identification at the BCCDC Provincial Health Laboratory according to the method of Kloos and Schleifer (1975). The reference laboratory was not able to identify two of the discordant isolates and had to send them to the LCDC National Health Laboratory in Ottawa for further identification (Table 5 and 6).

a. Re-identification of isolates with discordant identification results between the *S. haemolyticus* probe and the Microscan method

With respect to the six apparent “false-negatives” with the *S. haemolyticus* probe, three of them were subsequently re-identified to be true negatives while two isolates were unidentifiable to the species level even when submitted for further re-identification by the LCDC in Ottawa. The three apparent “false-negatives” were found to be in fact two isolates of *S. epidermidis* and one isolate of *S. lugdunensis*. The two *S. epidermidis* isolates also hybridized with the *S. epidermidis* probe while the *S. lugdunensis* did not hybridize with either probe.

With respect to the two apparent “false-positives”, one isolate was re-identified to be
a true *S. haemolyticus* while the latter was still re-identified to be *S. epidermidis* despite its hybridization with the *S. haemolyticus* probe and lack of hybridization with the *S. epidermidis* probe.

b. Re-identification of isolates with discordant identification results between the *S. epidermidis* probe and the Microscan method

With respect to the four apparent "false-negatives" identified by the *S. epidermidis* probe, three of them were in fact true negatives as the three isolates were re-identified to be a *S. haemolyticus*, a gram-negative bacilli and a mixed culture of an unidentifiable bacteria with a *S. epidermidis*, respectively (Table 5 and 6). The mixed culture isolate CF285, did not probe positive with the *S. epidermidis* probe probably because the unidentifiable bacteria was the one picked for the hybridization studies.

In like manner, all but one of the three apparent "false-positive" identifications were subsequently re-identified to be indeed true-positives. The *S. capitis* and *S. haemolyticus* were re-identified to be indeed *S. epidermidis*. Although isolate B6358 was not identified as a *S. epidermidis*, it was not identified to belong to any other species either. The biochemical profile it exhibited with the panel of tests described by the conventional method of Kloos and Bannerman (1994) did not fit the existing profiles and therefore its identity could not be ascertained.
3.6. API Staph identification results

The results of the API Staph identification method are summarized in Table 7. Of the 32 isolates of CNS identified to be *S. haemolyticus* in the reference set (i.e. the identification results by the Microscan method which was corrected by BCCDC and LCDC), only 22 (68.75%) were identified by the API commercial kit as *S. haemolyticus*. The results for the identification of *S. epidermidis* were more sensitive. Out of the 19 isolates which were identified to be *S. epidermidis* by the reference method, 16 of them (84%) were identified correctly by the API commercial kit. The results of the three identification systems are summarized in Table 7. The sensitivity, specificity, positive and negative predictive values and the accuracy of the probes, the Microscan and API method were calculated and summarized below (Table 8).
Table 7. The results of the HSP60 hybridization, Microscan and API identification systems

<table>
<thead>
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<th>Species</th>
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<td>2</td>
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<th>Species</th>
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<td>31</td>
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<td>S. epidermidis</td>
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<table>
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<th>Species</th>
<th>Reference re-identification of Microscan results</th>
<th>API Staph method</th>
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<tr>
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Table 8. The sensitivity, specificity, positive and negative predictive values and accuracy of the HSP60 hybridization, Microscan and API method compared to the reference identifications

<table>
<thead>
<tr>
<th></th>
<th>HSP60 Method (%)</th>
<th>Microscan (%)</th>
<th>API (%)</th>
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<tr>
<td></td>
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<td>S. epidermidis</td>
<td>S. haemolyticus</td>
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<tr>
<td>Specificity</td>
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<td>98</td>
<td>85</td>
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<tr>
<td>Positive predictive value</td>
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<tr>
<td>Negative predictive value</td>
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<tr>
<td>Accuracy</td>
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Chapter 4

Discussion

The observation that the API commercial kit misidentified 10 of 32 (31%) isolates of
*S. haemolyticus* as non-*S. haemolyticus*, and the Microscan misidentified 5 of 34 (15%) non-*S. haemolyticus* isolates as *S. haemolyticus* and one of 32 isolates of *S. haemolyticus* as a non-*S. haemolyticus* isolate further supports the notion that these clinical diagnostic systems are not accurate for the identification of *S. haemolyticus*. These systems are generally known to be more accurate for the identification of *S. epidermidis*, and less for the identification of other species of *Staphylococci*, like *S. haemolyticus*. Because *S. epidermidis* and *S. saprophyticus* were previously the best-documented opportunistic pathogens, many clinical laboratories have restricted complete identification of CNS to these two species only. Hence commercial identification kits were tailored to meet these needs. Other members of the CNS group, like *S. haemolyticus*, were only routinely speciated if they were found associated with serious infections, such as deep infections or endocarditis (Fleurette, J. 1987).

Several problems have been encountered with the use of phenotypic based identification methods. The most serious one is the presence of a significant number of strains of *S. haemolyticus* species which express variant or atypical phenotypic characteristics. Fleurette J. et al (1987) reported he had encountered difficulties in the species identification of the *S. haemolyticus/S. hominis/S. warneri* group of *Staphylococci* using phenotypic identification schemes because of weak or variable biochemical test results.
Hedin G. (1994) also reported problems with the identification of *S. haemolyticus* because of variants from the typical biochemical profiles; he reported a phosphatase-positive variant. In this present study, three of such strains were encountered. They exhibited atypical biochemical profiles and were unidentifiable to the species level by the classification scheme of Kloos and Schleifer (1975). To achieve a higher degree of sensitivity for the identification of *S. haemolyticus*, a nucleic-acid based approach may be better. This present study presents a novel genotypic method using the heat shock 60 kDa protein (HSP60) gene as a target for the species identification of *S. haemolyticus*.

Previous studies in our laboratory with the use of the HSP60 gene as a target for the speciation of *Staphylococci* have been most encouraging. It was shown that six HSP60 DNA probes, generated with the use of a set of universal degenerate primers, from 6 reference species of *Staphylococci* (*S. aureus*, *S. haemolyticus*, *S. epidermidis*, *S. schleiferi*, *S. saprophyticus*, *S. lugdunensis*), correctly identified a set of 58 *Staphylococcus* and non-*Staphylococcus* species. Goh, S.H. (1997) recently published another paper on the speciation of 40 known *Staphylococcus* isolates with the use of HSP60 DNA probes from 34 different species and sub-species of *Staphylococci* by a reverse hybridization method. The HSP60 method was able to correctly speciate 36 of 40 isolates; the remaining four *S. hyicus* species which did not hybridize with the *S. hyicus* probe may belong to a different subspecies from the ATCC reference strain.

Although the HSP60 method of identification has been tested with many different species of *Staphylococci*, the HSP60 DNA probes have not been tested with multiple clinical isolates of each species. In this study, extensive testing of two of these probes, the *S.*
haemolyticus and S. epidermidis HSP60 probes, with multiple isolates of S. haemolyticus and S. epidermidis were performed. The results which ensued from the study demonstrated that the 600-bp HSP60 DNA probes were specific for all isolates within a given species. The HSP60 probes consistently identified isolates to the correct species level. Furthermore, all isolates that probed positive with the S. haemolyticus probe did not hybridize with the S. epidermidis probe. An observation which further validates our previous data that demonstrated the presence of species-specific signature sequences within the HSP60 gene, and therefore DNA probes generated from these sequences from different species were unlikely to cross-hybridize with each other.

Secondly, the S. haemolyticus HSP60 DNA probe correctly identified all but one isolate of S. haemolyticus and the S. epidermidis probe correctly identified all but one isolate of S. epidermidis. These results strongly suggest that the HSP60 gene is conserved within all strains of S. haemolyticus and S. epidermidis, and therefore has great potential for use as a target for species identification of S. haemolyticus and S. epidermidis.

Furthermore, the HSP60 probes proved to have better positive and negative predictive values for the identification of S. haemolyticus and S. epidermidis than the Microscan method and was far more sensitive in identifying both species than the API method (Table 8).

A possible explanation for the apparently false-negative data for isolate BI 1152, could be that this particular isolate belongs to a different subspecies of S. haemolyticus from that of the reference strain. There is a possibility that different subspecies of S. haemolyticus strains exists but are very similar phenotypically. However, at the genetic level they may contain enough variation in the HSP60 gene that a DNA probe generated from one
subspecies strain does not hybridize with another subspecies. To test this hypothesis, the 600-bp portion of the HSP60 gene of *S. haemolyticus* isolate B11152 should be cloned and sequenced, and a comparison made with sequences from the reference strain for determination of sequence homology and identity. A DNA probe can also be amplified from this particular isolate and used to further screen a larger sample of *S. haemolyticus* from various sources to determine if similar subspecies exists. Another possibility is that the reference method, which is based on phenotypic characteristics, actually misidentified this isolate.

In the paper by Goh et al (1997), a group of four isolates of *S. hyicus*, which did not hybridize with the probe from the reference strain of *S. hyicus* was discovered. However, when the DNA probe was generated from one of the four isolates, it hybridized with the three others. DNA sequence analysis of the four isolates suggested that these putative *S. hyicus* isolates belonged to the same subspecies which was different from the reference strain. This further demonstrates that phenotypic characteristics may not be sufficiently discriminating to allow accurate differentiation between species and subspecies and that the taxonomic classification of *Staphylococcus* according to a phenotypic schema has limited value.

Phenotypically, isolate B6424 resembles a *S. epidermidis* isolate but yet did not hybridize with the *S. epidermidis* probe and instead hybridized with the *S. haemolyticus* probe. A possible explanation for such an observation is that the 600-bp region of the HSP60 gene flanked by the universal degenerate primers did not contain sufficiently conserved species-specific signature sequences so that this genotypic system cannot differentiate between *S. haemolyticus* and this putative *S. epidermidis* species. However, if this were true,
cross-hybridization of the isolate with both probes would be observed. As it is, not only was there lack of cross-hybridization of this isolate with both probes, but all isolates which hybridized with one probe, did not cross-hybridize with the other. These results demonstrate that the discordance between the two types of identification systems is not due to the choice of genotypic target. Previous studies with the *S. haemolyticus* and *S. epidermidis* probes have also demonstrated that the *S. haemolyticus* and *S. epidermidis* HSP60 DNA do not cross-hybridize with each other (Goh, S.H. et al, 1996). Furthermore, sequence analysis of this 600-bp region of the HSP60 gene in our laboratory has shown that within this highly conserved region of the HSP60 gene, there are species-specific signature sequences.

Another possible explanation for the discrepancy between the phenotypic method and the HSP method is the potential problem of contamination of the sample with DNA from a *S. haemolyticus*. However, this possibility is very small, for the hybridization test was repeated and both times the results were identical. A more likely explanation is that this is in fact a strain of *S. haemolyticus* which the phenotypic method has misidentified as *S. epidermidis*. It is likely that some strains of *S. haemolyticus* are indistinguishable from *S. epidermidis* using the phenotypic tests upon which the reference and Microscan methods are based. The phenotypic identification scheme has only five out of 20 biochemical tests that can definitively differentiate *S. epidermidis* from *S. haemolyticus*. The tests are based on the enzymes pyrrolidonyl arylamidase, urease, D-trehalose, N-acetylglucosamine, and the carbohydrate fermentation test of D-mannose. It is a possibility that this small number of tests may be inadequate for differentiating all species of *S. haemolyticus* from *S. epidermidis*. Furthermore, of these five tests, only four were included in the panel of tests used by the
reference laboratory to speciate isolate B6424. With regards to the five definitive tests for the
differentiation of *S. epidermidis* from *S. haemolyticus*, the typical species-specific profile for
*S. haemolyticus* and *S. epidermidis* is not applicable to all *S. haemolyticus* strains. The reason
for this is that for each test, the typical species yield a representative result in only 98% of the
isolates. Therefore, it is possible that this one isolate, B6242, is in fact a *S. haemolyticus* and
not a *S. epidermidis*, in which case, this result would further support the contention that
phenotypic methods are inadequate for the accurate speciation of *Staphylococci* to the species
level. The isolate B6242 could be further re-identified by DNA-DNA hybridization studies
with genomic DNA of reference isolates to confirm the HSP60 identification results.

In conclusion, the results of the HSP60 identification method in this study strongly
support the findings of previous studies which showed that the region of the HSP60 gene
flanked by the universal degenerate primers has highly conserved species-specific signature
sequences. The results also support the assumption that these signature sequences are well
conserved for all isolates within the *S. haemolyticus* and *S. epidermidis* species. Moreover,
DNA sequencing data of the HSP60 gene of these two species and other species and
subspecies of *Staphylococci* in our laboratory have further demonstrated the utility of the
HSP60 gene for taxonomical classification and phylogenetic analysis of the CNS
(Unpublished data). Strains of different species of CNS share only 71 to 93% DNA sequence
identity while those of different subspecies display at least 92% sequence similarity. Finally,
all strains of the same genus share at least 70% DNA sequence identity. Therefore, the
HSP60 method is useful for the species identification of *Staphylococcus haemolyticus* and
*Staphylococcus epidermidis.*
Section II

A Model for Vancomycin Resistance in *S. haemolyticus*
Chapter 1

Introduction

*S. haemolyticus* is emerging as an important organism to study, not only because it is emerging as an important nosocomial pathogen, but also because it exhibits a wide spectrum of antibiotic resistance. Amongst *Staphylococci*, it has the widest spectrum of antibiotic resistance demonstrating the highest degree of resistance (Hamilton-Miller et al, 1985; Mehta, G. et al, 1997). On account of this, vancomycin has become a drug of choice for treating *S. haemolyticus* associated infections. However, resistance to vancomycin has now been observed in *S. haemolyticus* with an alarming frequency.

1.1. History of *S. haemolyticus* resistance to vancomycin

Schwalbe R. et al (1987) reported the first clinical isolates of *S. haemolyticus* with decreased susceptibility to vancomycin in 1987. *S. haemolyticus* isolates with stepwise increase in minimum inhibitory concentrations (MIC) to vancomycin (MIC of 1 ug/ml to 8 ug/ml) were cultured from the peritoneal fluid of a patient undergoing continuous ambulatory peritoneal dialysis (CAPD). An MIC of 8 ug/ml was reached by the final isolate cultured. According to the National Committee for Clinical and Laboratory Standards (NCCLS), this isolate exhibited intermediate resistance to vancomycin (intermediate resistant MIC range is...
from 4 to 16 ug/ml). As a result of infections with these isolates, treatment of the patient with vancomycin alone was not adequate and additional antibiotics, rifampin and tobramycin, were required.

Since then, other cases describing treatment failure due to intermediate vancomycin resistant or truly resistant *S. haemolyticus* have been reported. One such case report describes the isolation of a teicoplanin resistant, vancomycin intermediate resistant strain of *S. haemolyticus* from a patient receiving teicoplanin therapy for a prosthetic knee joint infection (Aubert, G. et al, 1990). Yet another describes an elderly leukemic patient with a *S. haemolyticus*, which also demonstrated an MIC of 8 ug/ml to vancomycin.

Most of the vancomycin resistant *Staphylococci* isolates so far have been *S. haemolyticus*. It seems that this particular species of *Staphylococci* has an innate ability to acquire or develop resistance to vancomycin during therapy. This observation is further validated by a report from Froggat *et al* (1989). After surveying a large population of nosocomial *S. haemolyticus* isolates, he found that 77% of the isolates (36 of 47 isolates) were multiply resistant to more than three antibiotics, and 61% (29 of 47) expressed intermediate levels of resistance to vancomycin (MIC>6.25 ug/ml). Even though most of the *S. haemolyticus* strains demonstrated only intermediate resistance, they will prove to be a problem clinically, because exposure to vancomycin may cause the eventual selection of truly resistant organisms which will render the drug ineffective for continued treatment of the disease.

This ability of *S. haemolyticus* to develop stepwise resistance to vancomycin is further supported by *in vitro* selection studies. *S. haemolyticus* acquires stepwise resistance to
vancomycin with relative ease when grown in the presence of subinhibitory concentrations of vancomycin. An example is the report by Schwalbe et al (1987) in which a clinical *S. haemolyticus* isolate was cultured from peritoneal fluid showing an intermediate level of vancomycin resistance (MIC 8ug/ml). When grown in the presence of subinhibitory concentrations of vancomycin, it was able to acquire a much higher MIC of 128 ug/ml to vancomycin. Herwaldt, L. (1991) also showed that vancomycin resistant strains of *S. haemolyticus* were easily selected from susceptible isolates *in vitro* by broth or agar methods. This ability to acquire vancomycin resistance with relative ease either *in vivo* or *in vitro* has not been observed in other CNS, including *S. epidermidis*. *S. epidermidis*, under *in vitro* selection, is able to acquire resistance to vancomycin and other glycopeptides, but it has a three-fold lower selection frequency and resistant or intermediately resistant isolates are rarely isolated in the clinical setting (Herwaldt, L., 1991).

Although glycopeptide resistance has mainly been seen in *S. haemolyticus*, it is however not restricted to this species. Recently, three different clinical isolates of *S. aureus* expressing intermediate resistance to vancomycin have been reported in Japan and the United States (Hiramatsu, K. et al, 1997; MMWR, 1997). The appearance of glycopeptide resistance in these pathogens is of great concern as many *S. aureus* isolates are already resistant to other antistaphylococcal drugs, leaving vancomycin as the only chemotherapeutic agent. Two approaches can be undertaken to control this situation.

Firstly, the development of decreased susceptibility to vancomycin is observed to parallel the increase in antibiotic exposure. The use of vancomycin was reported to have increased 17-fold from the year 1981 to 1988 at the University of Iowa College of Medicine.
(1,158 g to 17,384g) (Herwaldt, L. et al, 1991), and similar reports have been published by other medical centers (Horan, T., 1988, Fletcher C. V., 1986, Nightingale J., 1987). Frequent and indiscriminate use of vancomycin would only increase the exposure to these organisms, providing them the opportunity to evolve and develop resistance. There needs to be a stricter policy for the controlled use of vancomycin. Secondly, there needs to be a better understanding of the possible mechanisms conferring vancomycin resistance in Staphylococci, so that effective strategies can be developed to overcome this problem. This is the focus of the present thesis.

1.2. Vancomycin

Vancomycin is an antibiotic which was first isolated in 1956 by McCormick et al from cultures of Amycolatopsis orientalis (previously known as Norcadia orientalis and Streptomyces orientalis). This vancomycin-producing bacterium was discovered during a large-screening program aimed at the identification of antibiotics with high-antistaphylococcal activity. It was found in the soil samples from the Borneo jungles.

Vancomycin is the first member of the family known as “glycopeptide antibiotics” or as the “glycopeptide antibiotics of the vancomycin group” (Cheung, R.F. et al, 1986). Other members of the glycopeptide antibiotic family include teicoplanin, actinoidin, and ristocetin (Katrukha, G.S. et al, 1986). Vancomycin has powerful bactericidal activity against most gram-positive bacteria. However, associated with the use of this glycopeptide
are severe side effects (i.e. ototoxicity and nephrotoxicity) and tedious administration requirements. Vancomycin must be administered by the intravenous route for the treatment of systemic infections. Consequently its use was not widespread until the advent of methicillin resistant *Staphylococci* (Cheung, R.P.F. et al, 1986). Presently, vancomycin is the drug of choice for the treatment of serious deep-seated infections caused by methicillin-resistant *Staphylococci*.

Previously, resistance to vancomycin was thought to be virtually impossible because the action of the drug was against the end product of a biochemical pathway, rather than the enzymes or the precursors of the pathway. For almost 30 years, this notion seemed to be true. However, within the last decade, vancomycin resistance has been increasingly seen, first amongst *Enterococci*, and then *Staphylococci* (particularly in strains of *S. haemolyticus*).

1.2.1. The structure of vancomycin

All glycopeptide antibiotics contain a linear heptapeptide in which, at least, 5 amino acid residues, common to all glycopeptides, are benzenic rings joined to each other to form a triphenylether moiety and a diphenyl group (Nagarajan R., 1991). A structural representation of the glycopeptide antibiotics is shown in Figure 6. The first amino acid from the C-terminus is a dihydroxyphenylglycine. The second and sixth are substituted tyrosines and the third and fourth p-hydroxyphenylglycines. The substitutions on the aromatic rings are often hydroxyl or methyl groups, sugars and/or chlorine atoms. For vancomycin, the sugar
substituents are glucose and vancosamine or epi-vancosamine, with a few exceptions of olivose and eremosamine.

The degree and type of substitution of these rings confer a wide variation of physico-chemical characteristics to the drug. Analogs of vancomycin, like M43E (Nagarajan, R. et al, 1988) which carry substituents other than those found on vancomycin have been shown to have decreased bactericidal activity. Experiments studying the role of chlorine substituents in vancomycin have demonstrated a direct relationship between the chlorination of the drug and bactericidal activity (Allen, N.E. et al, 1997).

The fifth and seventh amino acid residues are different depending on the type of glycopeptide antibiotic. Vancomycin and its related analogs are characterized by an asparagine and a leucine residue in the fifth and seventh amino acid position respectively.

Vancomycin acts on Staphylococci by interacting with the bacterial cell wall, specifically the N-terminus D-alanine-D-alanine of the pentapeptides. The antibiotic, once bound sterically, inhibits at least three enzymes involved in cell wall biosynthesis: transglycosylase, transpeptidase, and D, D-carboxypeptidase (Figure 7). The function of each enzyme is given in detail below together with a brief description of the pathways involved in cell wall structure and biosynthesis.
Figure 6. A structural representation of glycopeptide antibiotics (adapted from Antibacterial Activities and Mode of Action of Vancomycin and Related Glycopeptides; Nagarajan R.; Antimicrobial Agents and Chemotherapy 1991, 35:605-609)
Figure 7. The cell wall biosynthetic enzymes inhibited by vancomycin

(A) TRANSPEPTIDASE AND CARBOXYPEPTIDASE


Carboxypeptidase

Ala

Glu

DAP

Ala

Gly

Gly

Gly

Gly

GlySer

Gly

Gly

Gly

Ala

DAP

Glu

Ala

Transpeptidase

Ala

Ala

DAP

Gly

Gly

Gly

Gly

Nascent peptidoglyca

Carboxypeptidase

Ala

Glu

DAP

Ala

Ala

Ala

Ala

Ala

66
1.3. Structure of cell wall

The cell wall is a single, complex, macromolecule that serves, firstly, as an envelope to protect the protoplast from lysis due to the high osmotic pressure generated within the cytoplasm by low molecular weight compounds required in metabolic and biosynthetic processes. Secondly, the cell wall is important for maintaining the shape of the cell. The cell wall of *Staphylococci* is made up of two main components: peptidoglycan and teichoic or teichuronic acid.

Peptidoglycan is a polymer, which is unique to both the gram-positive and gram-negative bacteria. The peptidoglycan is the component of the cell wall which confers rigidity to the cell wall, and is responsible for maintaining the wall integrity. Studies involving the enzymatic degradation of peptidoglycan by the treatment with lysozyme, an N-acetylmuramidase, demonstrated the loss of rigidity of the bacterial cell wall, leading to cell lysis and death. However, if the cells were in an environment that afforded osmotic support, they would assume a spherical shape and remain viable.

The cell wall peptidoglycan is not only important to the integrity and viability of bacteria, it is also important as a target for the bactericidal activity of many antibiotics. One of these is vancomycin and its related group of the glycopeptide antibiotics.

Vancomycin attacks the cell by binding to the N-terminus of pentapeptides of the peptidoglycan (Ward, J.B. et al, 1974; Johnston, L.S. et al 1975). The amino acid sequence of these pentapeptides is:
L-Alanine (or glycine or L-serine) - D-glutamic acid - L-R₃ - D-Alanine - D-Alanine.

The R₃ residue is species-specific; in *S. haemolyticus*, it is meso-diaminopimelic acid (DAP). The N-terminus D-alanine-D-alanine dipeptide is the target of vancomycin (Barna, J.C. et al 1984; Williams, D.H. et al 1977).

Pentapeptides are substituents of N-acetylmuramic acids (MurNAc), an important component of the peptidoglycan; the other is N-acetylglucosamine (GlcNAc). The syntheses of both of these compounds are as follows: GlcNAc is synthesized from UTP and GlcNAc-1-P to give MurNAc, some of which are converted to GlcNAc in a two step reaction. Synthesis of MurNAc is followed by the addition of three amino acids, L-Alanine (or glycine or L-serine) - D-glutamic acid – meso - DAP to the precursor by enzymes specific for both the nucleotide and amino acids (Ito, E. 1966). The last two amino acids, D-alanine-D-alanine, are synthesized as two molecules of D-alanine by the action of D-alanyl: D-alanine synthetase, and added onto the nucleotide tripeptide in a reaction catalyzed by the enzyme UDP-MurNAc-L-ala-D-glu-meso-DAP:D-ala-D-ala ligase. Vancomycin resistance in *Enterococci* is due to novel D-alanyl:D-alanine synthetases and UDP-MurNAc-L-ala-D-glu-meso-DAP:D-ala-D-ala ligases which make a depsipeptide, D-alanyl-D-lactate, in place of the D-alanyl-D-alanine dipeptide. These depsipeptides have low affinity for vancomycin.

N-acetyl muramic acids are linked to N-acetylglucosamines when the two precursors are transferred sequentially to carriers in the membrane (Anderson, J.S. et al., 1967) resulting in the formation of a disaccharide-pentapeptide derivative of the carrier. The
carrier is undecaprenyl alcohol (Higashi et al 1967). The additions of amino acids, which form the cross-bridges between tetrapeptides, are generally done at this stage. GlcNAc and MurNAc are joined together by beta-1, 4- linkages, in an alternating fashion forming long oligosaccharide chains (up to 500 disaccharide units in length) which make up the backbone of the peptidoglycan. Polymerization of these subunits are believed to occur via the transfer of a growing nascent peptidoglycan chain from its carrier in the membrane to the NAM of a new disaccharide-pentapeptide subunit (Ward, J.B. et al 1973, 1974). An enzyme called the transglycosylase catalyzes this reaction (Figure 7).

The nascent peptidoglycan is added into the cell wall by reactions called transpeptidation (Keglevic et al, 1974; Mirelman et al, 1974; Tynecka et al, 1975). Transpeptidation involves the formation of a new peptide bond between the penultimate D-alanyl residue of one pentapeptide chain and the substituent amino acid chain (cross-bridge) of the meso-DAP residue in the other peptide (Figure 7). The cross-bridges are made up of 5 - 12 amino acid residues. In the process of the bond formation, the transpeptidase cleaves the peptide bond between the two D-alanyl residues in the pentapeptide.

In addition to the transpeptidase, another enzyme has been purified and characterized which exhibits the ability to catalyze the removal of D-alanine from free pentapeptide chains in the wall without catalyzing the cross-linking reaction. The enzyme is known as the D, D-carboxypeptidase. When cell fragments were studied, it was found that although most muramic acid peptide substituents were tetrapeptides, they were not all cross-linked. This observation supports the hypothesis of the function of the D,D-carboxypeptidase (Figure 7).
Vancomycin binds to the same target as the transpeptidases and the D,D-carboxypeptidases (Figure 7). Therefore, it inhibits both reactions by hindering the enzymes from binding onto their substrate. When the transpeptidase is inhibited from carrying out cross-bridge formation, nascent peptidoglycan strands cannot be added onto the cell wall. Keglevic et al (1974) proved that transpeptidation reaction is essential for the attachment of the nascent peptidoglycan to the pre-existing cell wall by inhibiting transpeptidation activity. As the result of such an inhibition, the nascent peptidoglycan is secreted into the medium.

Besides the inhibition of the addition of new cell wall material, vancomycin affects the strength and rigidity of the peptidoglycan. Although a single cross-linking event is sufficient to attach the nascent peptidoglycan to the rest of the pre-existing cell wall, additional cross-linking is required to impart rigidity to the structure. Once the nascent peptidoglycan is covalently linked to the cell wall, additional cross-linking occurs (Fordham et al, 1974). When the formation of such cross-links is inhibited, the resulting structure is weak.

Vancomycin has also been shown to inhibit the transglycosylase enzyme (Anderson, J. S. et al, 1965). It is believed that the glycopeptide molecule is so big that once bound onto the N-terminal of the pentapeptide of cell membrane bound precursors, it sterically hinders the transglycosylase from binding onto the sugar to carry out polymerization reactions.

Furthermore, although the addition of nascent peptidoglycan to the "old" cell wall is prevented, the turnover of the cell wall by autolytic enzymes is still occurring. As a result, the cell wall is slowly being broken down without new materials being added, this may
eventually lead to cell death. However, the action of killing is still not clear. It could be due to the accumulation of the cell wall precursors which leads to the disruption of cellular functions or it could be due to autolysin liberation which would lead to cell lysis, or a combination of both effects.

The turnover of the staphylococcal cell wall is carried out by a variety of autolytic enzymes, including extracellular hydrolases such as amidases, glucosaminidases and endopeptidases (PBP4 in *S. aureus*) (Koyama, T. et al, 1977; Sugai, M. et al, 1990). These enzymes are involved in nicking connections within the cell wall between N-acetylmuramic acid and N-acetylglucosamine as well as the cross-bridges between peptides. These enzymes are not only involved in cell wall turnover, they are also responsible for breaking down the cell wall between fully formed daughter cells to allow them to separate. Two of the major autolytic enzymes in *S. aureus* are a 51 kDa endo-β-N-acetylglucosaminidase (GL) and a 62 kDa N-acetylmuramyl-L-alanine amidase (AM) (Oshida, T. et al, 1995). The 51 kDa GL and 62 kDa AM from the culture supernatant and cell extract have been observed to disperse cell clusters (Sugai, M. et al 1995; Sugai, M. et al 1994). Vancomycin has no effect on these enzymes; in fact, they may aid the antibiotic in destroying the cell.

Because vancomycin and its related class of antibiotics act on the end-product and substrate of an enzymatic reaction rather than an enzyme or other macromolecules in the biosynthetic pathway, it was thought that resistance to it was not possible. For more than twenty years, it seemed that this theory was true, until the recent emergence of vancomycin and teicoplanin resistant *Enterococcus* and *Staphylococci.*
Figure 8. The binding of vancomycin to the pentapeptide target (adapted from Antibacterial Activities and Modes of Action of Vancomycin and Related Glycopeptides; Nagarajan R. 1991, 35:605-609)
1.4. Mechanisms of vancomycin resistance in *Enterococcus*.

There are at least 5 different types of glycopeptide resistance in *Enterococci* (VRE) which are distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin. The 4 main types of resistance are known as VanA, VanB, VanC, and VanD. The mechanism of the different types of resistance are slightly different but mostly involve the synthesis of a modified cell wall precursor terminating in an amino acid with a lower affinity for the glycopeptides.

1.4.1. VanA

VanA glycopeptide resistance manifests as an inducible high level resistance to both vancomycin (MICs > 64 ug/ml) and teicoplanin (MICs > 16 ug/ml) (Allen, N.E. et al 1995; Nicas, T.I. et al, 1989; Shales, D.M. et al, 1989a, 1989b; Handwerger, S. et al 1990). It has been primarily described in strains of *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*). Resistance is inducible by low-levels of vancomycin and teicoplanin.

Current knowledge of the VanA class of resistance is based mainly on studies of a cluster of genes on a 10,851-bp transposon (Tn1546) of *E. faecium* (Evers, S. et al 1996). The cluster of genes associated with VanA-resistance consists of 7 operons known as vanR, vanS, vanH, vanA, vanX, vanY and vanZ. These genes are found on Tn1546 which usually
resides on a plasmid; therefore the VanA type of glycopeptide resistance is transferable (Handwerger, S. et al 1995). Nobel W. C. (1992) showed that this plasmid is transferable in vitro and on the skin of mice to *S. aureus*.

The mechanism of the VanA type of glycopeptide resistance involves the synthesis of cell wall precursors, which terminate with D-lactate instead of D-alanine; such precursors have significantly lower affinities for vancomycin and teicoplanin than the normal dipeptide products (Handwerger, S. et al, 1992; Billot-klein, D. et al, 1992; Messer, J. et al 1992). This type of resistance also ensures that normal precursors with the D-alanyl-D-alanine dipeptides are quickly reduced to the tetrapeptide form by a dipeptidase and a D, D-carboxypeptidase which are synthesized solely for this purpose (Arthur, M. et al 1994; Gutmann, l. et al, 1992).

The functions of the proteins encoded by the clusters of genes on Tn1546 are as follows: VanR and VanS comprise the two-component regulatory system of the resistance mechanism, whose function is to regulate the synthesis of VanH, VanA and VanX at the transcription level (Lai, M.H. et al 1996; Arthur, M., 1997).

The vanHAX gene cluster is primarily responsible for the synthesis of the pentadepsipeptide terminating with D-alanine-D-lactate. The VanH dehydrogenase reduces pyruvate to D-lactate, the substrate for VanA ligase, which is used by the ligase to synthesize depsipeptide D-alanyl-D-lactate instead of the normal dipeptide D-alanyl-D-alánine. Although the resistant bacterium is able to synthesize cell wall precursors terminating with D-lactate, the normal synthetic pathway is still operative in making some precursors terminating with the D-alanyl-D-alanine dipeptides. To eliminate these glycopeptide targets,
VanX, a D, D-dipeptidase lacking activity against D-alanine-D-lactate hydrolyzes pools of the dipeptide D-alanyl-D-alanine produced by the native enterococcal ligase (Reynolds, P.E., 1994).

The VanY protein is another D, D-carboxypeptidase that cleaves the D-alanine terminal peptide from the normal pentapeptide. It contributes modestly to vancomycin resistance but is not essential for the trait (Arthur, M. et al 1996). The function of VanZ is unknown; however, it modestly increases the MICs of teicoplanin but not of vancomycin. It is also not essential for the expression of the VanA phenotype (Arthur, M. et al 1996, Arthur, M. et al 1995)

1.4.2. VanB

The VanB-type glycopeptide resistance is characterized by the variable expression of resistance to vancomycin (MICs ranging from 4 to 1000 ug/ml) but sensitivity to teicoplanin. This type of resistance is commonly found among E. faecium and E. faecalis and is an inducible trait. The cluster of genes responsible for the VanB-type of glycopeptide resistance is similar to those of the VanA-type of resistance (Gold, H.S. et al, 1993). They are responsible for synthesizing cell wall precursors terminating with a D-lactate; resulting in a decreased affinity of vancomycin for the cell wall of the bacteria (Billot-Klein et al, 1996).

The VanB ligase is structurally and functionally similar to the VanA ligase (76 % amino acid identity) (Meziane-Cherif, D. et al, 1994). The VanB cluster of genes is also found on a transposon, designated Tn1547, which in turn is located on a larger conjugative
chromosomal element (90 - 250 kb). Therefore, vanB resistance can also be transferred conjugatively among different species of Enterococci.

Genes analogous to their vanA resistance counterparts are designated vanH_B, vanX_B, vanY_B, vanR_B, and vanS_B (Baptista, M. et al 1996). Levels of VanX_B D,D-carboxypeptidase activity correlate with levels of vancomycin resistance (Aurthur, M., 1996). VanHAX and VanH_BA_BX_B are highly homologous to each other (approximately 70%) but the VanRS and VanY proteins are less homologous to their VanA counterparts. The VanR_BS_B system seems to be insensitive to induction by teicoplanin (Aurthur, M. et al, 1996, Baptista, M. 1996) and there is no gene counterpart of vanZ in this class of glycopeptide resistance. VanY_B is only found in some strains and its position in the gene cluster differs from that in the class A resistant gene cluster (Aurthur, M. et al 1996, Ever, S. et al 1996).

1.4.3. VanC

The VanC phenotype of resistance in Enterococci is an intrinsic property of Enterococcus gallinarum (E. gallinarum) and Enterococcus casseliflavus (E. casseliflavus). VanC resistant Enterococci are constitutively resistant to low-levels of vancomycin and teicoplanin, and are characterized by the synthesis of peptidoglycan precursors ending with D-ala-D-Ser or a D-Asn (Reynolds, P.E. et al, 1994; Park, I.S. et al, 1997). Vancomycin presumably has lower affinity for the depsipeptide D-alanine-D-serine than for D-alanine-D-alanine. VanC resistance in E. gallinarum is known as VanC1 and E. casseliflavus as VanC2. The vanC-2 abnormal ligase gene of E. casseliflavus shows 66% nucleotide sequence
homology with vanC-1 ligases (Patel, R. et al, 1998).

While the depsipeptide is being produced by the mutant ligase, normal dipeptides of D-alanine-D-alanine are also being produced (Reynolds, P.E., 1994). These dipeptides are kept in low amounts because of D, D-carboxypeptidase and D,D-dipeptidase activities analogous to those of VanA and VanB strains previously described. The balance between normal and abnormal peptidoglycan synthesis is the determining factor for the level of vancomycin resistance. The VanC type of resistance is both inducible and constitutive (Sahm, D.F., 1995).

Recently, vanA genes have been found in *E. gallinarum* and *E. casseliflavus* conferring high levels of resistance (MIC > 256 ug/ml) to vancomycin and teicoplanin (Dutka-Malen S. et al, 1994) suggesting the possibility of transfer of these resistance genes to different microorganisms.

1.4.4. VanD

A fourth type of glycopeptide resistance is VanD resistance. The expression of VanD resistance is characterized by constitutive high level resistance to vancomycin (MIC 64 ug/ml) and low-level resistance to teicoplanin (MIC 4 ug/ml). This form of resistance is presently observed only in *E. faecium* and involves the synthesis of cell wall precursors terminating with D-lactate. The VanD D-alanine: D-alanine ligase has been amplified, cloned and sequenced by Perichon, B. et al (1997). It exhibited 69% sequence identity with VanA and VanB and 43% identity with VanC. It seems to be distinct from the vanA and vanB genes (Gold, H.S. et al, 1993). The rest of the genes involved in vancomycin resistance have
not yet been cloned.

Glycopeptide resistance is also present in other genus of bacteria such as *Lactobacillus casei*, *Pediococcus pentosaceus*, and *Leuconostoc mesenteroides*. These organisms are naturally resistant to glycopeptides. The genes involved in conferring vancomycin resistance are distinct from those in *Enterococcus*, although UDP-N-acetyl muramyl tetrapeptide-D-lactate has been detected in these species (Handwerger, S. et al, 1994; Billot-Klein et al, 1994). DNA from these organisms does not hybridize with resistance gene probes prepared from VRE (Billiot-Klein D. et al, 1994).

Although glycopeptide resistance in *Enterococcus* is well studied and relatively well-elucidated, the mechanisms of vancomycin resistance in *Staphylococci* remain relatively unknown.

1.5. Mechanism of vancomycin resistance in *Staphylococci* differs from *Enterococci*

*Staphylococci*, like Enterococci, have several glycopeptide resistant phenotypes. Presently, there are mainly two phenotypes which have appeared in the clinical setting - *Staphylococcus* isolates resistant to vancomycin and teicoplanin and isolates which are resistant to teicoplanin but susceptible to vancomycin. Unlike *Enterococci*, the ligases involved in the synthesis of the novel depsipeptides appear not to be involved in glycopeptide resistance among *Staphylococci*. A 39 kDa cytoplasmic NAD$^+$-dependent D-lactate dehydrogenase (D-nLDH) associated with vancomycin and teicoplanin low-level resistance
was isolated from a strain of *S. aureus* (523k), derived *in vitro* from a glycopeptide susceptible strain (523) (Shlaes, D.M. et al, 1994). It was found that this protein (D-nLDH) is related to the NAD\(^+\)-dependent D-lactate dehydrogenases, VanA and VanH, which are responsible for synthesizing the depsipeptide D-alanyl-D-lactate that confers glycopeptide resistance to *Enterococci* strains. However, when D-nLDH was overproduced in 523k and high performance liquid chromatography analysis was carried out on the muropeptide and cell wall of the mutant, no production of D-lactate-containing peptidoglycan precursors was observed. Furthermore, susceptibility testing of D-nLDH inactivated mutants of 523k, produced by insertional mutagenesis, demonstrated that *S. aureus* D-nLDH is not essential for glycopeptide resistance in this isolate (Boyle-Vavra, S. et al 1993).

In a glycopeptide resistant isolate of *S. haemolyticus* (18-88) which was derived *in vitro* from a susceptible strain, only 1.7% of the total identifiable UDP-muramyl precursors were found to terminate with a D-lactate (Billot-Klein, D. et al 1996). Furthermore, disaccharide-tetrapeptide-D-lactate was undetectable as a monomer or an acceptor for the transpeptidation reaction. This amount of depsiprecursors is relatively small compared to that found in glycopeptide resistant *Enterococci* and it is unclear if such small amounts could confer the glycopeptide resistance observed in isolate 18-88. In *Enterococci*, the amount of depsiprecursors was at least 10% of the total UDP-muramyl-pentapeptides to give a 4-fold increase in MIC to vancomycin (Billot-Klein et al, 1992). Similar results obtained from the amino acid analysis of the cell wall of other strains of glycopeptide resistant *S. haemolyticus* and *S. epidermidis* have also demonstrated insignificant differences between the teicoplanin sensitive and resistant strains and the absence of D-lactate within the
These results strongly suggest that the mechanism of glycopeptide resistance in *Staphylococci* differs from that in *Enterococci*, and may not be related to the synthesis of peptidoglycan precursors with an altered affinity for glycopeptides. The mechanism for glycopeptide resistance in *Staphylococci* therefore remains to be elucidated.

1.5.1. Biochemical and phenotypic changes associated with glycopeptide resistant *Staphylococcus*

Only a few clinical and laboratory-derived glycopeptide resistant isolates of *S. aureus*, *S. haemolyticus* and *S. epidermidis* have been studied to date. Several interesting biochemical and phenotypic changes have been demonstrated in the glycopeptide resistant *Staphylococci*.

Resistant cells have slower growth rates than their susceptible parents. In broth, they often yield a less turbid culture; and on agar, they have smaller colony sizes. Such morphological characteristics are indicators of chromosomal mutations, supporting the possibility that glycopeptide resistance in *Staphylococci* is constitutive, rather than by the acquisition of exogenous genetic material. Besides displaying lower growth rates and smaller colony sizes, glycopeptide resistant staphylococcal cells also display a decreased susceptibility to peptidoglycan hydrolases such as lysostaphin, exhibits thicker cell wall, and altered cell membrane-associated proteins, particularly penicillin binding protein (PBP) 2 (Shlaes, D.M. et al, 1993; Mainardi, J.L. et al 1995; Moreira, B. et al, 1997).
1.5.2. Decreased susceptibility to lysostaphin

Resistance to lysostaphin may be due to two possibilities. The first possibility involves changes in the target of the lysostaphin – cross-bridges. The cell wall structures and UDP-muramyl precursors of a glycopeptide sensitive isolate of *S. haemolyticus* and its vancomycin resistant (MIC 16 ug/ml) and teicoplanin resistant (32 ug/ml) derivative (*in vitro*) were studied by high-performance liquid chromatography coupled with mass spectrometry (Billot-Klein et al 1997). It was discovered that the cross-bridges in the resistant derivative contained more serines than those in the susceptible parent. The glycopeptide sensitive *S. haemolyticus* strain contained only one serine per cross-bridge and the amino acid sequences of their cross-bridges were as follows: Gly<sub>2</sub>SerGly<sub>2</sub> and AlaGlySerGly<sub>2</sub>. Cross-bridges in the glycopeptide resistant derivative, however, were mostly GlySer<sub>2</sub>Gly<sub>2</sub> with an occasional AlaGlySer<sub>2</sub>Gly cross-bridge. Although the author was only able to study up to half of the muropeptides of the isolates, their data suggests the possibility that as much as 13.6% of the muropeptides from resistant strains may contain these altered cross-bridges.

The presence of serines in the cross-bridges of *S. haemolyticus* strains has made this group of bacteria more resistant to lysis by lysostaphin, as the substrate preferred by lysostaphin is pentaglycine. With additional serines in the cross-bridges of the resistant *S. haemolyticus*, it is not surprising that the resistant isolates display an even lower susceptibility to lysis by lysostaphin than the parent isolate. However, the precise relationship
of the altered cross-bridges with glycopeptide resistance is unclear.

Such an alteration in the amino acid composition of the cross-bridges may simply be a secondary effect of mutations which confer glycopeptide resistance to the host. These mutations may have affected the regulatory elements important for cross-bridge synthesis.

Alternatively, the alteration of the composition of the cross-bridges may be a reflection of the general disruption of peptidoglycan synthesis observed in many resistant mutant cells. The causal relationship of such altered cross-bridges to glycopeptide resistance still needs to be established.

In some resistant isolates, there is a direct correlation between the decrease in susceptibility to lysostaphin with glycopeptide resistance. However, neither compositional nor structural changes in the peptidoglycan are observed in these isolates; only an increase in the cell wall thickness is seen. Therefore a second explanation for the decrease in susceptibility to lysostaphin in glycopeptide resistant isolates may be an increase in the thickness of the cell wall. Such an increase in thickness would mean that there is more material for the enzyme to “chew” through before the cells are lysed by lysostaphin.

1.5.3. Cell wall thickness and glycopeptide resistance

The increase in cell wall thickness among glycopeptide resistant Staphylococci has been frequently reported (Biavasco, F., et al, 1991; O’Hare, M.D., et al 1992; Sanyal, D. et al 1993; Siedradzki, K. et al, 1996). Robert Daum et al (1992) reported an increase in the mean diameter of the cell wall of a vancomycin and teicoplanin resistant laboratory-derived
S. aureus strain compared to its sensitive parent strain. Another report by O’Hare, M.D. et al (1992) also noted a similar effect on the cell wall of a strain of S. haemolyticus which was incubated in the presence of teicoplanin.

In addition to the thickening of the cell wall, other cell wall anomalies were sometimes observed in resistant cells. In the case of a S. haemolyticus isolate incubated in the presence of vancomycin (Bivasco F., 1991; Sanyal, D. et al 1993) the thickening of the cell wall was irregular and cytoplasmic blebs were also seen on the periphery of the cells, surrounded by a protruding membrane.

The increase in cell wall thickness observed with the acquisition of glycopeptide resistance maybe an important contributor to such resistance in Staphylococci. An interesting phenomenon regarding cell wall thickness was observed in an isolate of S. aureus known as VM reported by Sieradski K. (1996). It appears that this particular strain of resistant S. aureus could “turn on or off” its machinery for synthesizing more cell wall materials. When the resistant cells were incubated in subinhibitory concentrations of vancomycin, their cell walls were thickened and loose, but under normal conditions of growth, they looked identical to their susceptible parents. The difference in cell wall thickness of the resistant cells incubated in subinhibitory concentrations of vancomycin was demonstrated by transmission electronmicroscopy (TEM). These electronmicrographs showed that the glycopeptide resistant cells were surrounded by thick and irregular cell walls while VM cells incubated without vancomycin resembled the sensitive parent strain. Furthermore, TEM showed that when VM cells were grown in the presence of vancomycin, most of the cells did not split after cell division despite fully formed septa, resulting in units comprising of at least three
incompletely divided cells. TEM of the VM isolate incubated with and without vancomycin and the parent strain are shown in Figure 9.
Figure 9. TEM of a *S. aureus* isolate and its vancomycin resistant derivative VM incubated in the presence and absence of vancomycin. (a) Vancomycin sensitive parent (b) VM incubated in vancomycin free media and (c) VM incubated the presence of vancomycin. (Adapted from Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant *Staphylococcus aureus*, Sieradzki K. et al.; Journal of Bacteriology, 1997, 179:2557-2566)
Accompanying the increase in cell wall thickness in VM was an immediate inhibition of cell wall turnover in the presence of subinhibitory concentrations of vancomycin, possibly explaining how this isolate acquired a thickened cell wall. When the concentration of vancomycin in the media dropped below 0.1 ug/ml, cell wall turnover immediately reverted to the rate similar to that observed in the absence of vancomycin. The reversion of cell wall turnover was accompanied by a burst in viable titer, indicating the division of the daughter cells which were previously attached to the mother cell. Both of the above characteristics were reversible when vancomycin was withdrawn.

The most interesting characteristic of isolate VM is not the increase in thickness of its cell wall but its ability to absorb twice as much vancomycin (500 ug of vancomycin per mg of cell wall) from the media as its sensitive parent (235 ug of cell wall per mg of cell wall). Intact wall suspensions of VM were able to bind twice as much vancomycin per mg of cell wall than the susceptible parent (500 ug of vancomycin per mg of cell wall versus 235 ug). The ability of resistant *S. aureus* VM to retain vancomycin appears to be aided by the presence of teichoic and teichuronic acid and/or the quarternary structure of the intact wall because upon treatment with hydroflouric acid, half the binding capacity of the cell wall was lost. Incubation of cell wall with hydroflouric acid removes the teichoic and teichuronic acid from the walls. The cell walls from the parent isolate were similarly treated with no observable changes to its vancomycin binding capacity.

The model proposed by the authors for the mechanism of vancomycin resistance in *S. aureus* Vm is that the cell wall structure of the bacteria is altered such that it allows “capture” of the glycopeptide molecules at the periphery of the cells away from the sites of
cell wall biosynthesis, thus minimizing the bactericidal effect of vancomycin on this isolate. This model probably works as follows: the addition of vancomycin causes the cell wall turnover to come to a complete halt resulting in the retention of cell wall material around the periphery of the cell and inhibition of the separation of daughter cells at the end of cell division. This phenomenon successively contributes to the deposition of increasing amounts of cell wall material which is capable of binding and entrapping vancomycin at the periphery of the cell wall, thus hindering access of vancomycin to the plasma membrane where the sites of cell wall synthesis are. Whether this model serves as an accurate representation of glycopeptide resistance in all species of *Staphylococci* is yet to be determined. The possibility that *S. haemolyticus* selected in the presence of subinhibitory concentrations of vancomycin may develop a similar mechanism of glycopeptide resistance will be examined in the research reported here.

1.5.4. Cell membrane proteins changes associated with vancomycin resistance

Another phenotypic characteristic associated with vancomycin resistant *Staphylococci* is an altered cell membrane protein profile. The 1-dimensional SDS-PAGE profiles of cell membrane proteins extracted from glycopeptide sensitive and resistant strains have shown differences which have been consistently observed among different resistant and susceptible pairs of isolates (Sieradzki, K. et al 1997; Daum, R.S., 1992; Mainardi, J.L., 1995; O’Hare et al 1992; Shlaes, D.M. et al 1993). Some of the differences include the discovery of novel proteins approximately 35 to 39 kDa in molecular mass, and the increased
production of the penicillin binding proteins (PBP) and decreased production of other proteins.

As mentioned previously, a novel 39 kDa cell membrane-associated protein was found in a strain of laboratory-derived teicoplanin resistant *S. aureus* isolate. When this protein was subjected to N-terminal sequencing, amino acid sequence homology to the VanA ligase responsible for high-level glycopeptide resistance in *Enterococci* was demonstrated. However, further study by overproduction of the protein in the sensitive parent strain and insertional inactivation of the gene in resistant cells showed no effect on teicoplanin resistance. Therefore, it was concluded that this particular protein was not essential for teicoplanin resistance.

Another group of proteins that has been associated with glycopeptide resistance is the PBPs. There has been several reports on the detection of greater amounts of PBPs, especially PBP2, a transpeptidase, in glycopeptide resistant staphylococcal isolates. In a clinical isolate of *S. aureus* with low-level teicoplanin resistance, an increase in PBP2 was found compared to epidemiologically related susceptible isolates. Similar high-level PBP2 production was detected in intermediate vancomycin (MIC 8 ug/ml) and methicillin resistant *S. aureus* clinical isolates in Japan which resulted in failure of treatment with vancomycin (Hiramatsu, K. et al, 1995). Such an observation suggests that low-level vancomycin resistant *S. aureus* isolates in the clinical setting have similar phenotypic characteristics to laboratory-derived resistant mutants and that the mechanism of resistance in the latter may be clinically relevant.

The strong correlation between increased PBP2 production and low-level
vancomycin and teicoplanin resistance is further demonstrated by the study of several laboratory-derived \textit{S. aureus} isolates. Beatriz Moreira (1997) selected several strains of resistant isolates from various parent clinical isolates by incubation in subinhibitory concentrations of either vancomycin or teicoplanin. PBP2 was assayed by penicillin-binding assays as well as western blotting while the degree of peptidoglycan polymerization was determined by enzymatic digestion of the cell wall followed by reverse phase high-performance liquid chromatography. The results showed a strong correlation between increased production of PBP2 and low-level vancomycin and teicoplanin resistance in these laboratory-derived \textit{S. aureus} isolates. However, there was no consistent association between the degree of cross-linking in the peptidoglycan and either PBP production, vancomycin resistance or lysostaphin susceptibility. Two of the resistant isolates 1714s (MIC to vancomycin 32 \text{ug/ml}, teicoplanin 8 \text{ug/ml}) and 1715w (MIC to vancomycin 16 \text{ug/ml}, teicoplanin 16 \text{ug/ml}) had a decrease in polymerization of their peptidoglycan while a third isolate 523k (MIC to vancomycin 8 \text{ug/ml}, MIC teicoplanin 8 \text{ug/ml}) had a higher degree of cross-linking. Despite the decrease in peptidoglycan polymerization of the two isolates, they were less susceptible to lysostaphin lysis than their parent strains. In the case of these mutants, a decrease in their susceptibility to lysostaphin lysis was probably due to an increase in the thickness of the cell wall.

PBPs are cell membrane-associated proteins of bacterial cells, and are involved in the synthesis of the cell wall. There are at least 6 types of PBPs in \textit{S. aureus} and 4 types in \textit{S. haemolyticus}. Their functions include transpeptidation and carboxypeptidation, reactions mentioned earlier in the section under cell wall synthesis. The function of PBP2 is believed
to be transpeptidation. Transpeptidation is a reaction that cross-links the peptidoglycan. These enzymes are named PBPs because they bind penicillin and can be easily detected by penicillin-binding assays using radiolabelled penicillin. Because PBPs share the same substrate as the glycopeptides, the D-alanyl-D-alanine termini of the pentapeptide precursor, glycopeptide resistance could result from competition by PBPs either through increasing the production or binding affinity of their substrates. The strong correlation observed by Beatriz Moreira between PBP2 production and glycopeptide resistance further supports this concept. Increasing the production of a PBP might increase the concentration of glycopeptide that is needed to interfere with the interaction between a PBP and the D-alanyl-D-alanine substrate during peptidoglycan synthesis. However, there still needs to be more direct evidence to establish the relationship between PBP2 and glycopeptide resistance.

Alternatively, PBPs may play an indirect role in glycopeptide resistance through coregulation with other protein(s) which are responsible for glycopeptide resistance. For example, a cluster of genes called the DCW gene cluster, which carries genes responsible for cell division in \textit{S. aureus}, has a lone PBP gene in it known as the \textit{pbpA} gene (Pucci, M.J. et al 1997). This PBP gene encodes PBP1, a penicillin-binding protein which is involved in the transpeptidation of peptidoglycan. Although the function of PBP1 differs from the rest of the cell division genes, its production is coregulated with them. If a mutation occurs in the regulatory element of this gene cluster, it would also affect the production of PBP1. Thus, further investigation is required to clarify the precise relationship between PBPs and glycopeptide resistance.
1.6. Aims of this thesis: establishing the relationship of certain phenotypic characteristics to vancomycin resistance and development of a model for vancomycin resistance in *S. haemolyticus*

There appears to be several characteristics which are associated with the resistant phenotype, but not all may be essential for the presence of resistance. The precise relationship between these phenotypic characteristics and glycopeptide resistance needs to be further clarified.

This present study reports the characterization of a series of vancomycin and teicoplanin resistant *S. haemolyticus* isolates which were derived from *in vitro* selection experiments. The aim of this thesis is to establish a stronger relationship between the phenotypic changes observed in vancomycin resistant phenotype and the possible mechanisms of resistance. The functional and structural changes associated with these vancomycin resistant strains of *S. haemolyticus* were examined in detail and a model for vancomycin resistance was proposed from the results obtained. Other aspects of the resistant cells were also examined. However, due to time limitations, a more comprehensive characterization of these aspects must be left to future investigations.
Chapter 2

Experimental Approach

In order to improve our understanding of the nature of glycopeptide resistance in
*S. haemolyticus*, laboratory-derived vancomycin resistant subpopulations of *S. haemolyticus*
were selected for and the following phenomena examined:

i) the cytoplasmic and cell membrane-associated protein profiles of the resistant and
suscptible isolates;

ii) the biochemical profiles of resistant and susceptible subpopulations;

iii) the effect of decreased susceptibility to vancomycin on the *in vitro* activity of other
classes of antibiotics;

iv) the binding characteristics of the susceptible and resistant subpopulations to
vancomycin;

v) morphologic differences in the surface and cellular structures of susceptible and
resistant subpopulations studied by scanning and transmission electronmicroscopy
Chapter 3

Materials and Methods

3.1. MIC agar dilution methods

A survey of the trend in vancomycin susceptibility among the 66 CNS isolates obtained for the HSP60 identification study was carried out using the agar dilution method in accordance with the NCCLS guidelines. The goal of the survey was to screen for isolates that might have decreased susceptibility to vancomycin in order to characterize the mechanism of vancomycin resistance. The MICs of the eight laboratory-derived resistant subpopulations (see below) to a panel of antibiotics were also determined by agar dilution. The classes and types of antibiotics tested are summarized in Table 9. Changes in the susceptibilities of the resistant cells to different classes of antibiotics, which target different areas of a cell, may suggest how the cells have adapted to the presence of lethal concentrations of vancomycin.
Table 9. The antibiotics used for characterizing vancomycin resistant subpopulations and their mechanisms of action

<table>
<thead>
<tr>
<th>Class</th>
<th>Name of Antibiotics Tested For</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactams</td>
<td>Methicillin (Me)</td>
<td>Inhibits a number of bacterial enzymes, namely penicillin-binding proteins (PBP), that are involved in cell wall synthesis.</td>
</tr>
<tr>
<td></td>
<td>Penicillin G (Pen G)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ampicillin (Amp)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Azlocillin (Az)</td>
<td></td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Cefazolin (Cefz)</td>
<td>Binds to PBPs thereby interfering with the synthesis of the peptidoglycan. May induce bactericidal effects by triggering autolytic enzymes</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime (Cefo)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone (Ceft)</td>
<td></td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Vancomycin (Va)</td>
<td>Inhibits peptidoglycan synthesis in bacterial cell wall by complexing with the D-ala-D-ala portion of the cell wall precursor</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Amikacin (Amk)</td>
<td>Inhibits bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit. The aminoglycoside bound ribosome then becomes unavailable for protein synthesis, thereby leading to cell death.</td>
</tr>
<tr>
<td></td>
<td>Tobramycin (Tm)</td>
<td>Uptake: An aerobic energy-dependent uptake enables the aminoglycoside to reach the intracellular ribosomes. The bacterial uptake is further facilitated in the presence of inhibitors of bacterial cell wall synthesis such as β-lactams and vancomycin</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin (Cip)</td>
<td>Inhibits the bacterial enzyme, DNA gyrase, and thereby inhibiting DNA replication leading to cell death</td>
</tr>
</tbody>
</table>
Three to four colonies were picked from overnight BHI agar plates and inoculated into falcon tubes containing 5 ml of MHB for overnight culture in a shaking water bath at 37°C. The overnight culture was diluted two-fold and incubated till logarithmic phase was achieved. When the culture reached logarithmic phase (as determined by optical density), it was diluted to give a concentration of 10^6 CFU/ml. Three drops of the bacterial suspension were applied to the wells of a Steer’s replicator and inoculated onto agar plates containing two-fold dilutions of antibiotics (final inoculum of 10^4 CFU/spot). The plates were allowed to dry and then incubated for 24 hours at 37°C in air.

The MIC was determined to be the lowest concentration of the antimicrobial agent that completely inhibited growth, and the presence of a single colony or a faint haze caused by the inoculum was disregarded (NCCLS Document M7-T2, volume 8, number 8).

3.2. Population analysis of isolate 118BA8

The *S. haemolyticus* clinical isolate with an MIC to vancomycin of 4 ug/ml, selected from the collection of 66 CNS, was chosen for the selection of vancomycin resistant subpopulations according to the pour plate procedure modified from that of Schwalbe at al (1981). The isolate was streaked onto Brain Heart Infusion (BHI) agar (Difco) and incubated overnight at 37°C. A single colony was picked from the plate and used to inoculate a falcon tube containing 5 ml of MHB. The tube was incubated in a shaking waterbath overnight at 37°C and then diluted three-fold and re-incubated in the shaking waterbath until the exponential phase was reached as measured by optical density. The exponential phase culture
was further diluted with MHB to yield a suspension of $1 \times 10^8$ CFU/ml (0.5 MacFarland standard). One ml of the culture was inoculated into 20 ml of molten agar (50°C) which contained predetermined concentrations of vancomycin. The mixture was vortexed and poured immediately into sterile petri plates and allowed to solidify. The agar plates were incubated at 37°C for 48 hours. The above procedure was repeated five times, each time picking the colony that grew in the plate with the highest concentration of vancomycin. The selection frequency of resistant subpopulations of *S. haemolyticus* was calculated as the negative log of (CFU on BHI-agar plates containing vancomycin)/(CFU on plates without vancomycin).

3.3. Cytoplasmic and membrane protein profiles

The cytoplasmic and cell membrane protein profiles of the resistant and susceptible isolates were examined by 1-dimensional SDS-PAGE.

Bacterial cultures were prepared for the analysis of cytoplasmic and cell membrane protein profiles as follows: single colonies from BHI agar plates were picked and used to inoculate a glass flask containing 250 ml of sterile MHB for overnight incubation at 37°C with aeration. The next day the culture was spun down in a Beckman centrifuge J2-21 (Beckman, California) at 6000 x g for 10 minutes. The supernatant was discarded and the cell pellet washed with Tris buffer (pH7.5) to remove all traces of broth. The pellet was resuspended in 2 ml of Tris buffer with 100 units of lysostaphin and incubated for two to three hours at 37°C. After enzymatic digestion of the cell walls, the culture was sonicated on
ice until the culture appeared homogeneous.

Cell lysis was followed by further centrifugation in Beckman Optima™ TLX Ultracentrifuge (Beckman, California) for 30 min, 100 000 x g at 4°C to collect the membrane-enriched portion. The resultant supernatant containing cytoplasmic proteins was decanted into a separate tube and the pellet containing membrane enriched fractions was washed with Tris buffer (pH 7.5) twice before being dissolved in the same buffer. Protein concentration was quantitated with the Bradford assay (BioRad) and boiled in sample buffer before separation on a 12% SDS-PAGE. Visualization of proteins was done with silver staining (Biorad).

Densometric analysis was carried out on the SDS-PAGE gels to determine which bands differed in density between the vancomycin susceptible and resistant subpopulations. The density of individual lanes of protein was scanned and read and the total protein density ratio of the parent to the resistant subpopulations was calculated. In like manner, the density ratios of comparable bands of proteins from the parent and the resistant isolate were also calculated and those that gave ratios which were higher or lower than the total protein ratios were designated as proteins affected by the acquisition of vancomycin resistance.
3.4. Biochemical profiles according to API Staph strips

The sensitive and resistant isolates were tested with a range of biochemical and enzymatic tests using API Staph (API Biomerieux). Description of the API Staph system was provided earlier in Section I, under Materials and Methods. Bacterial cultures were prepared in the same way as mentioned for the antibiotic tests. The strips were inoculated and used according to manufacturer’s instructions (previously described in Section I, page 28-29).

3.6. Competition studies with an alternate glycopeptide target

Changes in the vancomycin binding ability of the resistant cells compared to their sensitive parent were established by competition studies with an alternative glycopeptide target (Lys-isoglutamyl-D-alanyl-D-alanine). Competition studies involved the incubation of vancomycin in the presence of a cell bound target (cell wall) and an alternative free glycopeptide target (Lys-isoglutamyl-D-Alanyl-D-alanine). Then, the ability of the free target to compete with the cell bound target for vancomycin was assayed by observing the bactericidal effect on the cells. The rationale of the experiment was that resistant isolates may change the binding properties of the peptidoglycan, either structurally or by compositional changes, such that there may be:

(1) a decrease in the affinity of the peptidoglycan target for vancomycin,
(2) a decrease in the number of peptidoglycan targets,
(3) a decrease in the accessibility of vancomycin to the target.
If such changes occurred in the peptidoglycan of the resistant cells, then their peptidoglycan would be a poorer competitor than that of the sensitive cells for vancomycin in the presence of the alternative glycopeptide target.

Staphylococcal strains were grown on agar plates at 37°C overnight. A single colony was then picked to inoculate a tube of MHB for overnight incubation at 37°C with shaking. The bacterial suspension was diluted five-fold and incubated until log phase (determined by OD) before dilution to a suspension visually comparable to 0.5 MacFarland standard. The culture was further diluted 100-fold to give a concentration of $10^6$ CFU/ml. Microtiter wells containing predetermined concentrations of vancomycin and alternative glycopeptide were inoculated with 50 ul bacterial suspension (Figure 10). The microtiter plates were incubated for 18 to 24 hours and bacterial growth was determined using Elisa reader MR 5000 (Dynatech, Virginia).
Figure 10. An illustration of a 96 well plate used for the competition studies. The molar ratio of the competitor to vancomycin used was 1.5:1

**Bacterial strains**

<table>
<thead>
<tr>
<th>Vancomycin concentration (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>128G</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Growth</td>
</tr>
</tbody>
</table>

Legend: ////////// represents bacterial growth

For strain designations (4G₁ etc) see Bacterial Isolates, p.xvii

3.6. Binding characteristics of vancomycin to resistant and parent cells

To further study the binding characteristics of the resistant strains and parent strain, saturation binding experiments with radiolabeled vancomycin were carried out. These
experiments measured equilibrium binding concentrations of the radioligand and the results were analyzed to establish a relationship between binding and ligand concentration in order to determine the number of sites, designated as Bmax and ligand affinity, Kd.

3.6.1. Radioligand binding and the law of mass action

The law of mass action is the basis on which radioligand binding experiments are analyzed. This law can be represented by a simple equation:

\[
\begin{align*}
\text{Ligand} + \text{Receptor} & \rightarrow \text{Ligand-Receptor} \\
& \quad \text{\textbullet} k_{on} \quad \text{\textbullet} k_{off}
\end{align*}
\]

K_{on} is the association rate constant (M⁻¹·min⁻¹) and k_{off} (min⁻¹) is the dissociation rate constant.

According to the law of mass action, binding occurs at random and is affected only by the concentrations of ligand and receptor molecules and their association constant. Therefore, the number of binding events per unit of time equals [Ligand]·[Receptor]·k_{on} and once bound, they remain together for a random amount of time. The number of dissociation events per unit time is then determined by the concentration of bound molecules and their dissociation constant and equals [Ligand-Receptor]·k_{off}. The probability of dissociation is the same at every instant of time; the receptor does not know how long it has been bound to the ligand.
At equilibrium, the rate at which new ligand-receptor complexes are formed equals the rate at which the old complexes dissociate. The equation is:

\[ [\text{Ligand}][\text{Receptor}]k_{\text{on}} = [\text{Ligand-Receptor}]k_{\text{off}} \]

The equation is rearranged to define the equilibrium constant \( K_d \) (M):

\[
\frac{[\text{Ligand}] \cdot [\text{Receptor}]}{[\text{Ligand} - \text{Receptor}]} = \frac{k_{\text{off}}}{k_{\text{on}}} = K_d
\]

**Legend:**

[\text{Ligand} - \text{Receptor}] : Receptor and ligand complex

This equation defines the meaning of \( K_d \), which is the concentration of ligand that will bind to half the receptors at equilibrium. That is, when ligand occupies half the receptors, \([\text{Receptor}] = [\text{Ligand-receptor}]\); therefore, \( K_d = [\text{Ligand}] \). Hence the equilibrium constant can be used to measure ligand affinity for the receptor, as the higher the ligand’s affinity for the receptor, the lower the concentration of ligand required to occupy half the receptors present and vice versa.

### 3.6.2. Analysis of saturation radioligand binding data

These analyses are dependent on several assumptions:
(1) Binding had proceeded to equilibrium (based on law of mass action).

(2) Only a small fraction of radioligand has bound (the amount of free ligand is almost identical to the concentration added)

(3) The binding of ligand to one receptor does not affect the affinity of another binding

(4) Binding is reversible

Therefore, in this present study, optimization experiments were carried out to determine incubation times required to reach equilibrium for the highest concentration of ligand required to give saturation of binding with the lowest amount of receptors.

In saturation radioligand binding analysis, nonspecific binding of radioligand has to be accounted for. In this present study, nonspecific binding was determined by measuring radioligand binding in the presence of saturating levels of unlabeled vancomycin which block all binding sites. Under such conditions, the unlabeled drug would have bound up all receptors leaving the radioligand to bind to nonspecific sites. Nonspecific binding was measured for every concentration of radioligand concentration used in the saturation experiments and the nonspecific binding was subtracted from the total binding at that concentration to calculate the specific binding to receptors.

3.6.3. Using nonlinear regression to determine Bmax and Kd

The goal of nonlinear regression is to fit a model to the data and in this thesis, the program GraphPad Prism version 2.01 (Graph Pad Intuitive Software for Science, User’s
Guide) was used to perform these analyses. The program finds the best fit values of the variables in the model which could be interpreted scientifically. Nonlinear regression work is performed by fitting data to any equation that defines Y as a function of X for one or more variables. It finds the values of those variables that generate the curve that comes closest to the data; therefore its goal is to minimize the sum of squares of the data points from the curve.

3.6.4. Models for saturation binding experiments

There are two models for binding studies: one site binding and two site binding. If there are more than one type of receptors on the cells, Prism cannot do the analysis. Fortunately, for the current study, there was only one type of receptors. One site binding (hyperbola) is defined by the equation:

\[ Y = \frac{B_{\text{max}} \cdot X}{K_d + X} \]

Y is specific binding, Bmax is the specific binding at saturation, X is the concentration of radioligands added and Kd is the concentration of ligands where half of all receptors were bound.
The curve looks like the one below.

![Saturation Binding Isotherm](image)

Two binding site equation:

\[ Y = \frac{B_{max1} \cdot X}{Kd1 + X} + \frac{B_{max2} \cdot X}{Kd2 + X} \]

This equation is an extension of the one site binding curve. It defines the binding of a ligand to two receptors with different affinities.

Results obtained from binding experiments in this study were analyzed by the Prism software using the two model system and nonlinear regression. The total number of receptors, Bmax, on the last generation resistant subpopulation 128G5 and the parent cells were calculated from the concentration of radioligand which saturated all the receptors, and Kd was calculated from the concentration of radioligands that bound to half of receptors present.

By comparing the Kd and Bmax of the resistant subpopulations and sensitive
parent, we can determine if the target had been altered in terms of numbers, affinity or accessibility.

3.7. Iodination of vancomycin for binding experiments

Iodination of vancomycin was done by the chloramine-T method as previously described (Fong, K.L. et al, 1981). Briefly, 10 μl of vancomycin (50 μg/ml) and chloramine-T (5 mg/ml) was incubated with 5 mCi of radiolabeled sodium iodide (ICN) in 20 μl of borate buffer (pH 7.5) for one minute. The reaction was stopped with 10 μl of potassium iodide (10 mg/ml) and 10 μl of sodium metabisulfite (12 mg/ml). The final reaction mixture was diluted with 200 μl of 0.01M ammonium acetate, pH 6.0, and applied to a carboxymethylcellulose column (carboxymethylcellulose was packed into a 10ml falcon pipette) (Whatman) packed in the same buffer. The carboxymethylcellulose was prepared beforehand according to manufacturer’s instructions. The washing of the column and final elution steps were carried out as previously described (Fong, K.L. et al, 1981). The column was washed with at least 4 bed volumes of wash buffer (0.01 M ammonium acetate, pH 6.0) before 10 μl of wash was counted in the gamma counter (Searle, Model 1185, California). When the counts of the washes were as low as background (40 cpm), the elution of the column was carried out. Radiolabeled vancomycin was eluted with 0.05 M ammonium acetate, pH 6.5. Eluate was collected in sample volumes of 1 ml. The elution profile was followed closely by subjecting 10 μl of the eluate for scintillation counting in the gamma counter from every sample of 1 ml collected. Samples with counts per minute (CPM) above
150 000 cpm were collected and kept. The specific activity of the labeled vancomycin was approximately 0.68 mCi/ug of protein.

3.8. Effect of iodination on the binding function of vancomycin

To determine if $^{125}$I-labeled vancomycin had retained its ability to specifically bind *Staphylococci* cells, $10^7$ *S. haemolyticus* 118BA8 cells were incubated with a fixed concentration of $^{125}$I-vancomycin in the presence of various concentrations of unlabeled drugs. The various concentrations of unlabeled drug versus $^{125}$I-labeled drug used are given in Table 9. The incubation time was arbitrarily picked to be 45 minutes. Vancomycin bound cells were separated from free $^{125}$I-vancomycin by centrifuging (6000 x g) in an oil mixture (Dibutyl Phthalate and Dicotyl Phthalate in 1.1:1 ratio) for 7 minutes. Binding was read in terms of counts per minute (CPM).

Table 10. Concentrations of labeled and unlabeled vancomycin incubated with $10^7$ cells

<table>
<thead>
<tr>
<th>Cell Numbers</th>
<th>Va-$^{125}$I (uM)</th>
<th>Va (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^7$</td>
<td>$1.91 \times 10^{-7}$</td>
<td>$7.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^7$</td>
<td>$1.91 \times 10^{-7}$</td>
<td>$3.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^7$</td>
<td>$1.91 \times 10^{-7}$</td>
<td>$1.91 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^7$</td>
<td>$1.91 \times 10^{-7}$</td>
<td>0</td>
</tr>
</tbody>
</table>
3.9. Length of incubation required for labeled vancomycin binding to achieve binding equilibrium

To determine the length of time required for equilibrium to be achieved, parent cells (118BA8) were incubated in MHB with $1.53 \times 10^{-6}$ uM of labeled vancomycin for 5 min, 15 min, and 45 min. Nonspecific binding was tested for each time point in separate tubes containing $1 \times 10^{-5}$ uM unlabeled vancomycin in addition to the labeled vancomycin. The results for total binding obtained were normalized for nonspecific binding to give specific binding. Cell bound vancomycin was separated from free labeled vancomycin as described for the previous experiment. Resistant isolates were similarly tested; however, lower concentrations of labeled vancomycin was used ($1.91 \times 10^{-7}$ uM) in order to conserve labeled vancomycin for further tests.

3.10. Saturation binding experiments with radiolabeled vancomycin

Saturation binding experiments were carried out with $10^7$ cells of the parent isolate (118BA8) and resistant (128G5) cells. The cells were incubated with two fold-increasing doses of labeled vancomycin beginning with the lowest concentration of $9.5 \times 10^8$ uM and highest of $2.27 \times 10^3$ uM. The incubation time chosen was 30 minutes as by this time the ligand binding receptors had come to equilibrium.
3.11. Transmission and scanning electron microscopy

*S. haemolyticus* subpopulations were fixed with 2.5% glutaraldehyde (final concentration) in 0.1M sodium cacodylate buffer for half an hour. Washing followed fixation with 0.1M sodium cacodylate buffer three times for durations of 5 minutes. Cells were stained with osmium tetroxide 1% (final concentration) in 0.1M sodium cacodylate buffer for half an hour. Then cells were spun down (6000 x g), supernatant discarded and the cell pellet rinsed with water once. Enbloc staining was carried out with 2% uranyl acetate for the portion of cells prepared for TEM. The portion of cells prepared for SEM did not require enbloc staining. Staining was followed by dehydration in stepwise increases of ethanol of 70% to 100%, each step requiring 5 minutes of reaction time. Cells prepared for SEM were harvested for critical point drying in the critical point dryer (Balzers CTD 020, Liechtenstein), while cells for TEM were embedded in spurr resin (BioRad), polymerized and cut.

The equipment used for scanning electronmicroscopy was the Hitachi S41000 (Hitachi, Japan) and its magnification ranged from 100 x to 100 000 x. The confocal microscope used for carrying out transmission electronmicroscopy was the BioRad 600 (BioRad, England). The BioRad 600 has a magnification ranging from 100 x to 80 000 x.
Chapter 4

Results

4.1. One *S. haemolyticus* isolate, 118BA8, among 66 clinical isolates of CNS
demonstrated decreased susceptibility to vancomycin (MIC < 4 ug/ml)

Antimicrobial susceptibility testing by agar dilution method showed that all but one
of the 66 CNS isolates were sensitive to vancomycin (MIC < 2ug/ml). The single isolate
(118BA8) which displayed an MIC of 4ug/ml to vancomycin was *S. haemolyticus*
demonstrating an intermediate level of resistance to vancomycin.

All but one isolate of the CNS collection was also shown to be sensitive to
teiocoplanin according to NCCLS standards. The one isolate which was resistant (MIC 64
ug/ml) was the same *S. haemolyticus*, 118BA8, which was also intermediately resistant to
vancomycin.

Figures 11 and 12 are summaries of the results of the vancomycin and teicoplanin
susceptibility studies. No difference was found in the MIC₅₀ and MIC₉₀ of vancomycin
between *S. haemolyticus*, *S. epidermidis* and other species of CNS. The MIC₅₀ and MIC₉₀ of
vancomycin for all three groups was 1 ug /ml and 2 ug/ml (Table 11), respectively. However,
the MIC₅₀ and MIC₉₀ of teicoplanin were significantly higher for *S. haemolyticus* compared
to the other two groups (Table 11). *S. haemolyticus* isolates had MIC₅₀ and MIC₉₀ to
teicoplanin of 8 ug/ml and 32 ug/ml respectively. For *S. epidermidis*, the MIC\textsubscript{50} and MIC\textsubscript{90} were both 4 ug/ml. For the other CNS species, the MIC\textsubscript{50} was 2 ug/ml and the MIC\textsubscript{90} was 4 ug/ml.

The MICs for teicoplanin were distributed over a wider range compared to vancomycin, and in *S. haemolyticus* there was a bimodal distribution (Figure 11 and 12). *S. haemolyticus* isolates appear to be distributed around two peaks, one at MIC 4 ug/ml and the other at MIC 32 ug/ml. This effect was not seen with the *S. epidermidis* isolates nor with all the other species of CNS.

Table 11. The vancomycin MIC\textsubscript{50} and MIC\textsubscript{90} of the 66 isolates of CNS

<table>
<thead>
<tr>
<th></th>
<th>MIC\textsubscript{50} (ug/ml)</th>
<th>MIC\textsubscript{90} (ug/ml)</th>
</tr>
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<tr>
<td><strong>Vancomycin:</strong></td>
<td></td>
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<tr>
<td><em>S. haemolyticus</em> (n=32)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>S. epidermidis</em> (n=19)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Other CNS species (n=15)</td>
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<td>2</td>
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<tr>
<td><strong>Teicoplanin:</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>S. haemolyticus</em> (n=32)</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td><em>S. epidermidis</em> (n=19)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Other CNS species (n=15)</td>
<td>2</td>
<td>4</td>
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</table>
Collectively, the *S. haemolyticus* isolates did not demonstrate higher MICs to vancomycin compared to the other species of *Staphylococci* or to *S. epidermidis*. The MIC$_{50}$ and MIC$_{90}$ of both groups were similar. However, the *S. haemolyticus* isolates demonstrated higher MIC to teicoplanin than *S. epidermidis* and other species of *Staphylococci*.

4.2. *In vitro* selection of vancomycin resistant *S. haemolyticus* subpopulations from isolate 118BA8

*S. haemolyticus* isolate 118BA8 was studied for the stepwise selection of high level resistance to vancomycin by incubation in MHB agar containing various concentrations of vancomycin, ranging from 4 ug/ml to 128 ug/ml. Two colonies with MICs to vancomycin of 32 ug/ml were easily selected after the first passage through vancomycin containing agar. Further passaging of one of these isolates in higher concentrations of vancomycin, did not result in an increase in the MIC to vancomycin. The selection frequency of the first two vancomycin resistant colonies was 1 out of $10^7$ cells. The ease with which the first subpopulation of vancomycin resistant *S. haemolyticus* was selected suggests that there were resistant subpopulations of cells which were pre-existing in isolate 118BA8 and the ratio of sensitive to resistant cells was approximately 1 to $10^7$. Due to the low number of resistant cells in the parent isolate 118BA8, these cells may have evaded detection by agar and broth dilution as the inoculum size used by these methods was only $10^4$ CFU/ml.

After the fifth passage through vancomycin containing agar, a subpopulation which grew on the agar plate containing the highest concentration of vancomycin (128ug/ml) was
randomly picked from the plate and passaged five times through vancomycin free media to ensure that the expression of resistance was stable. The resistant cells retained their resistance to vancomycin and did not revert to the sensitive phenotype.

Colonies which were picked for re-passaging through higher concentrations of vancomycin were also collected for further characterization. Figure 13 shows the flowchart for the selection procedure. Table 12 contains a summary of the results of the selection procedure, the isolates’ MIC to vancomycin and their selection frequencies.
Figure 11. The frequency distribution of the vancomycin MICs of the collection of 66 clinical isolates of CNS

- S. haemolyticus
- S. epidermidis
- Other species of CNS

Vancomycin MIC (µg/ml)

No. of isolates

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>S. haemolyticus</th>
<th>S. epidermidis</th>
<th>Other species of CNS</th>
</tr>
</thead>
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<td></td>
<td></td>
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<td>4</td>
<td></td>
<td><strong>10</strong></td>
<td></td>
</tr>
</tbody>
</table>
Figure 12. The frequency distribution of teicoplanin MICs of the collection of 66 clinical isolates of CNS
Figure 13. Flowchart depicting the passaging of subpopulations and their MICs to vancomycin

Parent (118BA8)
(MIC < 4 ug/ml)

Subpopulation 16 G₁
(MIC < 32 ug/ml)

Subpopulation 32 G₂
(MIC < 32 ug/ml)

Subpopulation 128G₃32
(MIC < 32 ug/ml)

Subpopulation 64G₂
(MIC < 32 ug/ml)

Subpopulation 128G₃64G₂
(MIC < 32 ug/ml)

Subpopulation 128G₄
(MIC < 64 ug/ml)

Subpopulation 128 G₅
(MIC < 32 ug/ml)
Table 12. The MICs and selection frequencies of the *S. haemolyticus* subpopulations

<table>
<thead>
<tr>
<th>Name of subpopulation</th>
<th>Vancomycin concentration of agar during passage (ug/ml)</th>
<th>Selection frequency (10^{-x})</th>
<th>MIC vancomycin (ug/ml)</th>
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<tr>
<td>4G1</td>
<td>4</td>
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<td>32G2</td>
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<td>5</td>
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<tr>
<td>64G2</td>
<td>64</td>
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<td>32</td>
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<td>128G332G2</td>
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<td>128G364G2</td>
<td>128</td>
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<td>32</td>
</tr>
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<td>128G4</td>
<td>128</td>
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</tr>
<tr>
<td>128G5</td>
<td>128</td>
<td>&gt;5</td>
<td>32-64</td>
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</table>
4.3. Altered MICs to β-lactams, aminoglycosides and quinolones in parallel with increase in vancomycin resistance

Changes in the susceptibilities to other classes of antibiotics in vancomycin resistant isolates were investigated. The antibiotic profiles of the 9 resistant subpopulations and the parent isolate were determined by agar dilution; drug concentrations used ranged from 0.0625 to 128 ug/ml.

Susceptibilities to five antibiotics were apparently affected by the acquisition of vancomycin resistance. The antibiotics affected were cefotaxime, ceftriaxone, tobramycin, amikacin and ciprofloxacin (Table 13).

The MICs of certain drugs including ceftriaxone and cefotaxime were re-tested with a wider range of antibiotic concentrations (0.0625 ug/ml to 1024 ug/ml) when significantly different susceptible patterns were observed between the vancomycin sensitive parent, and the laboratory-derived resistant isolates.

An interesting phenomenon was observed with cefotaxime and ceftriaxone. It appears that high level resistance to these drugs were lost in parallel to the acquisition of vancomycin resistance. The parent isolate was highly resistant to both cefotaxime (512 ug/ml) and ceftriaxone (1024 ug/ml); however, a three-fold decrease in the MIC to cefotaxime (128 ug/ml) and six-fold decrease to ceftriaxone (32 ug/ml) was observed with the first resistant colony, 16 G1. The MICs for both drugs were subsequently maintained at this lower concentration for all except the last subpopulation, 128G5. Apparently, 128G5 was slightly more sensitive than the rest with MICs to cefotaxime and ceftriaxone at 64 ug/ml (four-fold
decrease) and 4 ug/ml (eight-fold decrease).

A similar discordant pattern in sensitivity to the aminoglycosides, tobramycin and amikacin, and to the quinolone, ciprofloxacin, was observed between the vancomycin sensitive parent and its resistant derivatives. Although isolate 118BA8 was not resistant to tobramycin and amikacin, subsequent passage of cells through vancomycin containing media selected for cells that were "hypersensitive" to the aminoglycosides. Minimum inhibitory concentrations to tobramycin and amikacin decreased from 4 ug/ml and 1 ug/ml respectively, in isolate 118BA8, to less than 0.0625ug/ml (at least five-fold decrease) and 0.25 ug/ml (two-fold decrease) for both drugs, respectively. Similarly, MICs to ciprofloxacin decreased more than 8-fold from an MIC of greater than 128 ug/ml in the parent to 16 ug/ml in isolate 128G5.

Aminoglycosides act on ribosomes within the cell, therefore, the cell membrane is a barrier which aminoglycosides must overcome. The "hypersensitive" response seen in the vancomycin resistant cells may be due to changes in the cell membranes resulting in the loss of selective permeability and ineffectiveness as a barrier against the aminoglycosides. Alternatively, the so-called "leakiness" of the cell membrane may simply be caused by an increase of proteins involved in the transport of molecules which are structurally related to tobramycin and amikacin, resulting in the cotransport of these deleterious molecules into the cell. A similar situation could be occurring with ciprofloxacin.
Table 13. The parent and resistant subpopulations’ MICs for various antibiotics

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<th>Cloxa</th>
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<th>Cefz</th>
<th>Cefo</th>
<th>Ceft</th>
<th>Pip</th>
<th>Az</th>
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<tr>
<td>118BA8</td>
<td>4</td>
<td>64</td>
<td>&gt;128</td>
<td>&gt;128</td>
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<td>&gt;128</td>
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4.4. The carbohydrate metabolism and various enzymatic activities of resistant and parent cells are similar

The biochemical profiles, limited to those defined by the API STAPH tests, of vancomycin resistant and sensitive isolates were compared for differences. The aim of this comparison was to correlate any differences between the two phenotypes with vancomycin resistance. Although extensive work cannot be done to characterize these differences in the present thesis, these findings serve as preliminary work for future experiments.

The enzymatic activities and carbohydrate fermentation abilities of the resistant subpopulations were not significantly different from their parent (Table 14). This observation was also found by other investigators (Biavasco, F. et al, 1991). The acquisition of vancomycin resistance did not affect these biochemical pathways significantly. Comparison of the biochemical profiles of the isolate 118BA8 with its 9 subpopulations simply demonstrated that they were phenotypically similar and were all *S. haemolyticus*. 
Table 14. Biochemical test results of parent and resistant subpopulations

<table>
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<tr>
<th>Tests</th>
<th>Parent</th>
<th>4G₁</th>
<th>8G₁</th>
<th>16G₁</th>
<th>32G₂</th>
<th>64G₂</th>
<th>128G₃32</th>
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4.5. Differences observed in the amounts of various proteins from cell membranes of resistant subpopulations compared to the parent

Cytoplasmic and cell membrane-associated protein profiles of vancomycin resistant and parent isolates were compared in order to correlate any changes with the vancomycin resistant phenotype.

The cytoplasmic protein profile of the resistant isolate 128G5 was similar to the parent isolate when analyzed by 1-dimensional SDS-PAGE, reflecting the lack of change observed in the biochemical profiles of their subpopulations (Figure 14a). Differences associated with vancomycin resistance were, however, seen in the cell membrane-enriched protein fractions. Increases in some protein bands and decreases in others were observed. Unlike the work involving the characterization of a laboratory-derived strain of *S. aureus* 523k (Daum, R.S. et al, 1992) no novel protein of 35 - 39 kDa was observed in the resistant populations of *S. haemolyticus* 128G5.

Acquisition of resistance to vancomycin seems to largely affect the cell membrane proteins more than the cytoplasmic proteins, further suggesting that resistance to vancomycin generally involves the cell wall and cell membrane components. However, the identity and characteristics of these proteins associated with resistance have yet to be ascertained. Future work should focus on the isolation and characterization of these membrane-associated proteins and establishing a relationship between their function and resistance.

The membrane-associated proteins affected by the acquisition of vancomycin
resistance were quantitated by densitometric analysis. The expressions of two membrane proteins (29 kDa and 33 kDa) appeared to have been upregulated in the resistant cells by 19% and 56% respectively, while another protein (44 kDa) appeared to have been downregulated by 33% (Table 15). The densities of two other bands (99 kDa and 71 kDa) were also measured. The densities of these two bands were similar for both the resistant subpopulation and the parent isolate (Table 15).

Table 15. The mean ratios of the density of protein bands between the parent isolate and the resistant subpopulation 128G₅ *

<table>
<thead>
<tr>
<th>Protein Band (kDa)</th>
<th>Experiment I</th>
<th>Experiment II</th>
<th>Experiment III</th>
<th>Mean ratio (n=3)</th>
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<td>1.21</td>
<td>1.19</td>
<td>1.118</td>
<td>1.19</td>
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*These ratios have been normalized against the total protein density ratios of parent to resistant isolate 128G₅ for each experiment.
Figure 14. Cell membrane and cytoplasmic protein profiles of parent isolate 188BA8 and the resistant derivative 128G5. ‘P’ represents the parent isolate and ‘G5’ the resistant derivative.
4.6. Competition Studies with alternate glycopeptide target detect changes in the cell wall of resistant subpopulations

Competition studies with the alternative glycopeptide, L-Lys-isoglutamyl-L-Lys-D-alanine-D-alanine, were carried out to determine if the cell wall of resistant cells were altered and bound vancomycin less effectively than sensitive cells. This change was measured by the ability of the cells to compete with the alternative glycopeptide for vancomycin. Once bound by vancomycin, the cell was assumed to undergo stasis or death. Therefore, if the cells could compete effectively with the glycopeptide, its MIC would remain virtually unaffected. However, if the efficiency of vancomycin binding to the cell was decreased or increased in the presence of the competitor, the MIC to vancomycin would be correspondingly increased or decreased respectively.

As predicted, when the vancomycin sensitive parent, isolate 118BA8, and a reference isolate \textit{S. aureus} ATCC29213 (vancomycin MIC < 2ug/ml) were incubated with lethal concentrations of vancomycin in the presence of the alternative glycopeptide in the molar ratio 1 to 1.5, their MIC to vancomycin was not significantly affected (two-fold difference) (Figure 15). However, when resistant cells, such as \textit{E. faecium} 97005 with VanB class of resistance, was incubated with vancomycin in the presence of these alternative peptides, their MICs rose by more than 16-fold. Similarly, the laboratory-derived resistant subpopulations showed a general increase in their MICs in the presence of the alternative peptide; the earlier subpopulations demonstrated three to six fold increases while later generations rose to greater than 16-fold. This data indicates that the peptidoglycan of the
resistant isolates had changed such that they were either not competing as effectively with the alternative peptide or the presence of the peptide aided the cells in resistance to vancomycin. These findings support the hypothesis that vancomycin resistance is related to changes in the cell wall. The nature of such changes is further clarified in the subsequent experiments described below.

4.7. Iodination does not effect binding ability of vancomycin to cells

Labeled vancomycin had to be tested to determine if its binding ability was affected by the iodination reaction since an important assumption of saturation binding experiments is that the labeled and unlabeled drug are able to bind the receptors in a similar fashion.

The iodination process should not alter the molecular structure of vancomycin such that it loses its binding capacity for the cell wall. The binding of labeled or hot vancomycin was tested in the presence of unlabeled or cold drug. This experiment showed that unlabeled vancomycin was able to effectively compete with labeled vancomycin and decrease the binding of labeled vancomycin to the cells. The results are shown in Figure 16.
Figure 15. The effect of an alternative glycopeptide target on the vancomycin MICs of 118BA8 and its resistant derivatives.

\[ \text{Log}_2 \left( \frac{\text{MIC}_{Va^+}}{\text{MIC}_{Va}} \right) \]

*S. haemolyticus* 118BA8 and laboratory-derived vancomycin resistant subpopulations
Figure 16. $^{125}$-Labeled vancomycin binding to parent cells ($10^7$) in the presence of different concentrations of unlabeled vancomycin.
4.8. Length of incubation time determined for binding assay with resistant and parent isolates (118BA8) in order to reach equilibrium

The length of time should allow for binding to come to an equilibrium in order to determine the total number of receptors, Bmax, on the cell surface and vancomycin’s binding affinity to these receptors, Kd.

The shortest time required for binding to reach an equilibrium state for the parent, 118BA8, was 15 minutes as the total binding of labeled vancomycin with $10^7$ cells remained unchanged from 15 to 45 minutes (Figure 17). Binding had also come to an equilibrium by 5 minutes in the resistant isolate, 128G5. Since equilibrium of binding was reached by 5 minutes, any time point can be chosen at or after that time. An incubation time of 30 minutes was chosen for saturation binding studies as this length of time allowed for the experiment to be carried out at a comfortable pace.
Figure 17. Results of incubating fixed concentrations of labeled vancomycin with resistant and parent cells for different lengths of time. (A) Parent isolate (B) Resistant subpopulation, 128G₅

(A) Parent Cells

![Parent Cells Graph](image)

Length of incubation time (minutes)

(B) Resistant cells (128G₅)

![Resistant cells Graph](image)

Length of incubation time (minutes)
4.9. Saturation binding experiments show four-fold more receptors in vancomycin resistant isolates compared to the susceptible parent

The total number of receptors per $10^7$ cells of resistant and parent cells were determined from saturation binding experiments to be 5,504 nM (standard error of 740.5 nM) and 23,790 nM (standard error 2,279 nM) respectively. Therefore, binding studies showed that the resistant subpopulation had at least four-fold more receptors than the parent isolate. For both isolates, there was only one type of receptors as can be seen in the Scatchard plots in Figure 18.

Despite the greater number of receptors in the resistant isolate, the affinity constants (Kd) of these receptors for vancomycin (1.11 nM per receptor) were similar to those on the parent isolate (1.33 nM per receptor).
Saturation binding isotherm of vancomycin to parent isolate (118BA8)

A.

Scatchard plot of $^{125}$-labeled vancomycin binding to parent (118BA8)

B.
Saturation binding isotherm of vancomycin to resistant subpopulation 128G₅

Scatchard plot of vancomycin binding to resistant subpopulation 128G₅
Saturation binding isotherm of vancomycin to parent isolate (118BA8) and resistant subpopulation (128G₅).

Figure 18. Results of saturation binding experiments with parent isolate, 118BA8 and resistant isolate G5. (A) Saturation curve of parent (B) Scatchard plot of parent isolate (C) Saturation curve of 128G₅ (D) Scatchard plot of 128G₅ (E) Saturation binding curves of parent and 128G₅.
4.10. Scanning and transmission electron microscopy showed marked differences in cell division and cell wall structure of resistant cells compared to parent

Scanning transmission electron micrographs showed several distinct differences between the parent isolate and resistant subpopulation 128G5 (Figure 19). Firstly, the resistant cells were significantly larger than the parent cells (longer by one and half times). Some of the resistant cells seen at magnification 50,000 x were measured to be 1.3 to 1.6 um in length and 1.0 to 0.9 um in width; while at the same magnification, the parent cells were uniformly 0.9 um in length and 0.7 um in width. These measurements were confirmed by additional studies of many other cells from each isolate at lower magnifications.

Secondly, the cell surface of resistant cells was much rougher than the parent cells. The cell wall of the resistance cells appeared to be more amorphous and looser, probably due to changes involving cell wall biosynthesis (Figure 19).

Thirdly, the resistant cells developed shapes quite unlike those of the parent cells. Parent cells were generally round, smooth and if in cell division, looked like two spheres attached to each other. However, resistant cells were often seen in odd shapes. For example, some were bone-shaped, while others looked like they were in packs of four cells which did not separate properly during cell division. The transmission electron micrographs displayed characteristics similar to these observations by SEM (Figure 20).

Transmission electron micrographs demonstrated that the resistant cells were indeed “bigger” when compared to sensitive cells at the same magnification. They were “bigger” because the resistant cells were often found in packs of threes, fours or even more,
with fully formed septa but remained attached to one another solely by the cell wall.

Furthermore, the resistant cell walls were at least four times thicker than the sensitive cells. This observation supports the results from the binding assays which demonstrated that resistant cells had four-fold more receptors.
Figure 19. SEM pictures of parent and resistant subpopulation, 128G5. (A) and (B) Resistant cells (C) Parent cells
Figure 20. Cross-sections of resistant and sensitive cells viewed by the transmission electronmicroscope (a) Vancomycin sensitive cells (b) Resistant cells
Chapter 5

Discussion

Vancomycin resistance is appearing in *Staphylococci* with an alarming frequency, especially among *S. haemolyticus* (Schwalbe, R. et al, 1987; Herwaldt, L. et al 1991; Veach, L.A. et al, 1990; Hiratmatsu, K. et al, 1997; Johnson, A.P. et al, 1990). Such isolates have resulted in treatment failures substantially prolonging patient suffering and even causing mortality. Presently, little is known about the mechanisms of vancomycin resistance in *Staphylococci*, although many phenotypic changes associated with resistance have been observed. These phenotypic features associated with vancomycin resistance may not be essential or causally related to resistance. Hence, the characteristics essential for the manifestation of vancomycin resistance in *Staphylococci* and their mechanism for resistance further study.

This present study describes the characterization of a series of laboratory-derived vancomycin resistant subpopulations of *S. haemolyticus*. The aim of this thesis is to establish a stronger relationship between the phenotypic changes associated with vancomycin resistance and their contribution to the mechanism of vancomycin resistance. The binding characteristics to vancomycin and the structural changes of the peptidoglycan cell wall associated with vancomycin resistant strains of *S. haemolyticus* were examined in detail and a model for vancomycin resistance was proposed from these results.

However, before the study could begin, vancomycin resistant isolates had to be
obtained. The vancomycin susceptibility patterns among the collection of 66 coagulase-negative *Staphylococci* consisting of 32 isolates of *S. haemolyticus*, 19 isolates of *S. epidermidis* and 15 other CNS species were determined by agar dilution. The purpose for this experiment was two-fold: to determine the vancomycin susceptibility trend of contemporary CNS isolates from patients hospitalized at the Vancouver Health Sciences Center (VHHSC), and to find a suitable isolate with an elevated MIC to vancomycin for the present project.

Among the 66 CNS isolates, only one showed decreased susceptibility to vancomycin. This isolate, 118BA8, was a *S. haemolyticus* with an MIC to vancomycin of 4 ug/ml. According to NCCLS, such an isolate was considered to express an intermediate level of vancomycin resistance. The remaining CNS isolates, including all *S. epidermidis* isolates, had vancomycin MICs of less than 2 ug/ml.

Although the susceptibility of *S. haemolyticus* to vancomycin was not much different from the other CNS (Figure 11), *S. haemolyticus* appeared to be more resistant to teicoplanin than other species of *Staphylococci*, including *S. epidermidis* (Table 11 and Figure 12). This observation reflects those publications by other investigators (Jones, et al 1989; Goldstein, F.W. et al 1990; Maugein, J. et al 1990; Bannerman, T.L. et al 1991; Ena, J. et al, 1993; Tripodi, M.F. et al 1994; Cercenado, E. et al, 1996; Cunningham, R. et al 1997; Krcmery, V. Jr. et al 1997; Spanik, S. et al 1997). One study reported that 50% of their *S. haemolyticus* isolates were intermediately or truly resistant to teicoplanin (Bannerman, T.L. et al, 1991) while another reported teicoplanin MIC$_{90}$ of 64 ug/ml for their *S. haemolyticus* isolates (Ena, J. et al, 1993). Neither studies encountered vancomycin resistant *Staphylococci*. The precise reason for the difference in susceptibilities to teicoplanin and
vancomycin in CNS is unknown, although the results suggest a possible difference in the mode of action of teicoplanin compared to vancomycin. These results also suggest that the efficacy of teicoplanin in treating CNS infections especially *S. haemolyticus* may not be optimal and it should be used cautiously in chemotherapy.

The frequency distribution of the MICs of teicoplanin is bimodal for *S. haemolyticus*. There seem to be two populations of *S. haemolyticus*, those that are sensitive to the antibiotic (MIC 8 ug/ml) and those that are expressing intermediate resistance (MIC > 16 ug/ml). It is conceivable that the isolates with higher teicoplanin MIC values (32 ug/ml) represent those with heterogenous subpopulations of cells that have different MICs to teicoplanin. *S. haemolyticus* isolates which display intermediate resistance to the glycopeptides often possess such subpopulations (Schwalbe, R. et al, 1987; Seravin, V. et al 1994; Cunningham, R. et al 1997). The phenomenon of heteroresistance to antibiotics in *Staphylococci* has been well-documented for such antibiotics as methicillin, and it appears that a similar mode of resistance may be occurring with the glycopeptides.

Heteroresistance to vancomycin has also been observed in a *S. aureus* isolate from Japan (Hiratsuma et al, 1997). This strain of *S. aureus* (MRSA Mu3) was isolated from the sputum of a patient with pneumonia treated with 12 days of vancomycin. The isolate was shown to contain sub-clones of cells (1 in 10^6 cells) showing MICs to vancomycin from 2 to 9 ug/ml (Hiratmatsu, et al, 1997). The “collective” MIC of the isolate was 3 ug/ml. This type of vancomycin resistance was dubbed “heterogenous” resistance and the strain as “hetero VISA”.

In our study, the *S. haemolyticus* isolate, 118BA8, also demonstrated
heterogenous subpopulations similar to those of S. aureus. In like manner, this S.
haemolyticus isolate consisted of cells exhibiting heteroresistance to vancomycin. Such
vancomycin resistant subpopulations were selected for at the frequency of one per $10^7$ cells
(MIC 32 ug/ml. Comparatively, the selection frequency of the vancomycin resistant
subpopulations from isolate 118BA8 is relatively low since other authors have reported
selection frequencies as high as $10^{2.08}$ cells (Herwaldt, et al, 1991; Marnardi, J.L. et al,
1995).

Furthermore, the unique ability of S. haemolyticus and other CNS to select for
higher levels of glycopeptide resistance is not confined to the laboratory but has been
observed in in vivo situations. Clinicians have been reporting treatment failure with
vancomycin probably due to the selection of subpopulations exhibiting stepwise increases in
heteroresistance to the glycopeptides among the CNS may be more prevalent than expected
and may warrant further study to determine the mechanisms behind the ability of such
isolates to give rise to a heterogenous subpopulations of cells.

Nine subpopulations of vancomycin resistant S. haemolyticus cells were derived
from the initial intermediately resistant isolate. The last generation of vancomycin resistant
subpopulation 128G5 was demonstrated to be stable and homogenous in its expression of
vancomycin resistance after many passages through media with and without the antibiotic.

Vancomycin acts at the level of the cell wall. It binds to the N-terminus of
pentapeptides ending with D-alanyl-D-alanine. The receptors on the wall eventually become
saturated and the vancomycin molecules bind specifically to the pentapeptide precursors
external to the cell membrane where the antibiotic can affect the biosynthesis of the cell. Resistance to vancomycin, therefore, would probably arise by alterations of the cell wall or cell membrane, rather than sites within the cell. This notion is supported partially by the lack of differences between the biochemical profiles of the vancomycin resistant subpopulations and intermediately resistant parent isolate in this study (Table 14). The biochemical profiles of the resistant isolates, comprised of enzymatic reactions and carbohydrate fermentation reactions, seemed unaffected by the acquisition of vancomycin resistance and were very similar to the more sensitive parent isolate. In addition, cytoplasmic protein profiles of resistant cells were identical to their sensitive parent. Differences, however, were observed between the profiles of membrane-associated proteins of the resistant subpopulation and the more sensitive parent isolate. At least three separate protein bands were present in differing amounts in the resistant isolate compared to the parent. These differences suggest that the acquisition of vancomycin resistance was accompanied by changes at the cell wall or cell membrane level rather than at the cytoplasmic level. This observation was further supported by our studies with an alternative glycopeptide target.

The MICs of vancomycin sensitive isolates, such as *S. aureus* ATCC29213, and our isolate 118BA8 were shown to be largely unaffected by the presence of the alternative peptide (Figure 15). In contrast, VanB resistant *E. faecalis* EF97005 demonstrated a 16-fold increase in vancomycin MIC in the presence of the alternative glycopeptide target. Similarly, resistant *S. haemolyticus* subpopulations displayed a marked increase in vancomycin MIC when tested in the presence of the alternative target. The subpopulations from the earlier generations were less affected by the peptides (four-fold increase in vancomycin MIC) than
the later generations (at least 16-fold increase in vancomycin MIC). Such an observation may be caused by two reasons. The first is that the resistant cell walls were altered such that they were not competing effectively with the alternative glycopeptide target. The second is that the alternative peptide could have augmented resistance to vancomycin in the subpopulations by a different mechanism.

Binding assays with iodinated vancomycin suggested that the latter possibility was more likely. In fact, resistant cells bound more vancomycin than the parent cells. Before the binding assays were carried out, we demonstrated that the iodination process per se did not affect the binding capacity of the labeled vancomycin. We also determined the incubation times required for vancomycin binding to reach equilibrium for both the parent and resistant strain. The duration chosen in subsequent studies was 30 minutes. Following all the preparative work, saturation binding experiments were carried out to determine the number of receptors present per cell, the types of receptors present and the affinity constants of these receptors for vancomycin. For the saturation binding experiments, only the resistant subpopulation 128G5, was selected for testing.

The binding experiments showed that only one type of receptor was present on both the parent and resistant cells (Figure 18). The affinity constant of the receptors on the parent cells (1.3 nM per receptor) were similar to those on the resistant cells (1.1 nM per receptor) for binding vancomycin. However, the 128G5 cells had four-fold more receptors than the sensitive parent cells. This observation was further supported by TEM and SEM studies which showed that many of the resistant cells had cell walls which were four times thicker than cell walls from parent cells.
Previous work by Sieradski, K. (1996), with a highly vancomycin resistant mutant strain of *S. aureus* (MIC 100 ug/ml) demonstrated a similar phenomenon. When the mutant *S. aureus* was pre-incubated in the presence of subinhibitory concentrations of vancomycin, the cell wall turnover was inhibited and the cells ceased dividing despite fully formed septa. When seen under TEM, the cells were found in packs of threes or fours surrounded by a thick cell wall. The capacity of these resistant mutants to bind vancomycin was also elevated. They could bind twice the amount of vancomycin compared to their sensitive parent. The mechanism of vancomycin resistance postulated for this mutant was that the cell had an altered cell wall structure which allowed for the “capture” of vancomycin at the periphery of the cells distant from the sites of cell wall biosynthesis, thus minimizing the bactericidal action of vancomycin.

Our isolate of *S. haemolyticus* 128G5 may be demonstrating a similar mechanism of vancomycin resistance. Thus, the resistance in this isolate may be mediated by the presence of a large excess of cell wall receptors that result in the trapping of vancomycin at the periphery of the cell wall away from the cell membrane where the drug can act to kill the cell. These receptors may also take up vancomycin from the media and therefore reduce the amount of free glycopeptide molecules available for binding to other cells. If a large enough inoculum of such vancomycin resistant cells occur, they could act collectively by binding up the free antibiotic, and reduce the availability of the drug to sensitive cells.

A similar hypothesis on the mechanism of vancomycin resistance in *Staphylococci* was also suggested by Reynolds, P.E. et al (1992). In his investigations of two strains of teicoplanin resistant, vancomycin sensitive CNS isolates, he found no difference in
their teicoplanin binding capacity despite the presence of a thickened cell wall in the
teicoplanin resistant phenotype. He suggested that the majority of glycopeptide molecules
were bound non-specifically to the thick cell wall at sites far removed from the site of cell
wall polymerization, therefore protecting these sites from the bactericidal activity of the
antibiotic.

A difference was noted between the *S. aureus* VM and *S. haemolyticus* 128G5 in
that the *S. aureus* only exhibited an altered cell wall phenotype when pre-incubated with
subinhibitory concentrations of vancomycin. However, in the absence of vancomycin, they
looked identical to their sensitive parent. *S. haemolyticus* 128G5 exhibited an altered cell wall
phenotype even in vancomycin-free media.

The inability of cell division between fully formed cells suggest that
subpopulation 128G5 may have a genetic defect in the separation and postfissional movement
of 128G5 cells after cell division. Two aspects of cell division may have been affected by the
acquisition of vancomycin resistance. The first is the cell division autolysins. Two major
autolysins involved in cell division are a 51 kDa endo-β-N-acetylglucosaminidase (GL) and a
62 kDa N-acetylmuramyl-L-alanine amidase (AM). These enzymes are responsible for
nicking the cell wall to free fully formed daughter cells, and to facilitate cell wall turnover.
Mutants with little or no 51 kDa GL and 62 kDa AM activity grow in clusters (Sugai, M. et
al, 1994). Moreover, the 51 kDa GL and the 62 kDa AM from the culture supernatant and
cell extract have been observed to disperse cell clusters (Sugai, M. et al 1995; Sugai, M. et al
1994).

The inactivation or downregulation of enzymes like GL and AM would also
account for the thickened cell walls observed with 128G5, as these enzymes may also be involved in cell wall turnover.

In further support of this hypothesis, there have been many publications describing vancomycin resistant *Staphylococci* with decreased autolytic activity which are normally induced by these same enzymes (Siedrazki, et al 1996). An insertional *S.aureus* mutant RUSAL2 (*alt::TN551*) was described to have defective production of GL and AM proteins which resulted in the inability of the cells to undergo autolysis. Therefore, the thickening of the cell wall is probably due to the down regulation of enzymes involved in the autolytic process of the cell wall.

The second aspect of cell division which may have been defective is the cell splitting system. The splitting system of a cell is the plane of weakness between two fully formed daughter cells and is responsible for facilitating the separation of the cells. Without it, cells cannot separate properly (Giesbrecht, P. et al 1997). Most cells of isolate 128G5 have thickened cross walls with the splitting system missing. The absence of this system maybe one of the reason for the poor cell division between the daughter cells.

Besides the cell wall structural changes observed in isolate 128G5, the acquisition of vancomycin resistance seems to have affected the susceptibility profile to other classes of antibiotics as well. The vancomycin resistant subpopulations showed an increase in susceptibility to at least two β-lactams (ceftriaxone and cefotaxime), a floroquinolone (ciprofloxacin), and two aminoglycosides (amikacin and tobramycin). The trend in the changes of the MICs between the parent (118BA8) and resistant subpopulations to these three classes of antibiotics was very similar. A five to six log₂ decrease in MIC was observed.
between the parent isolate, 118BA8, and the first generation resistant subpopulation 16G₁. Subsequent resistant generations were not markedly different from subpopulation 16G₁ except for a slightly higher sensitivity by 128G₅ to the antibiotics. This observation suggests that the mutations which were conferring resistance to vancomycin were pre-existing in the parent isolate 118BA8, but only manifested when selected for by vancomycin. It is postulated that when isolate 118BA8 was passaged through vancomycin, there were a small number of cells with defects in genes encoding autolytic enzymes, such as 51 kDa endo-β-N-acetylglucosaminidase (GL) and 62 kDa N-acetylglucosaminidase (AM) and that these cells were selected and resulted in the emergence of resistant subpopulations. This could explain why the mutants in this study do not need to be pre-incubated with sub-inhibitory concentrations of vancomycin to express the resistant phenotype.

Not only were the vancomycin resistant subpopulations not resistant to the aminoglycosides, their MICs decreased significantly, demonstrating at least a six log₂ decrease in MIC from 4 ug/ml to less than 0.0625 ug/ml for tobramycin and a two log₂ decrease for amikacin. Loss of a plasmid carrying aminoglycoside resistant genes is not plausible, as the cells were not resistant. A more likely explanation would be that the permeability of the cell membranes of resistance cells were altered. Alteration of the permeability of the cell membrane may be associated with an increase in transport proteins which co-transport the aminoglycosides across the cell membrane together with essential molecules needed by the cells. Similar events may be occurring with ciprofloxacin. This postulation may explain the increase in one of the bands of the cell membrane protein profiles of the resistant subpopulation. Alternatively, such a characteristic may not be
associated with glycopeptide resistance, but may be a secondary effect of the mutations
which conferred resistance to vancomycin. However, the nature of such an alteration and its
relationship to vancomycin resistance remains to be clarified.

These results are potentially important for the therapy of serious Staphylococcal
infections, especially those associated with methicillin resistance. A combination of
vancomycin and an aminoglycoside could be used to avoid selecting for vancomycin
resistant subpopulations.

The effect on the susceptibility to cefotaxime and ceftriaxone demonstrated by the
vancomycin resistant cells could also be related to the increased expression of certain
membrane-bound proteins such as the PBPs. Such a notion could explain the increase in
certain proteins observed by 1-dimensional SDS-PAGE in association with the increased
susceptibility to some β-lactams which bind to such proteins. Moreira B. et al (1997)
correlated the increase in PBP2 to vancomycin resistance in \textit{S. aureus}. She postulated that
such an increase in PBP2 might contribute to vancomycin resistance in \textit{S. aureus} as such
proteins would compete with vancomycin for the N-terminus of pentapeptide precursors for
transpeptidation, and therefore prevent vancomycin molecules from binding to the cell wall.
However, this does not fit with the observations from the vancomycin resistant populations
of \textit{S. haemolyticus} in the current study, since these cells bound better to vancomycin, not less
when compared to the parent cells.

Another possibility is that PBP2 production was upregulated to takeover the
enzymatic reactions normally carried out by other PBPs which were in turn downregulated.
These PBPs, which may have been downregulated, would be those involved in the turnover
of the cell wall, like endopeptidases. Such a response by the cell would thus enhance the cell wall thickening process, and confer resistance to vancomycin. This phenomenon has been observed in *Staphylococci* resistance to cephalosporins where PBP4, an unessential PBP, is upregulated (Chambers, H.F. et al 1990; Rossi, L. et al 1985; Chambers, H.F. et al 1994; Murray T. et al 1997; Murray, T. et al, 1996; Popham D.L. et al, 1994; Selakovitch-Chenu L. et al, 1993). Resistance to a wide spectrum of cephalosporins was found to be mediated through the downregulation of certain PBPs, while upregulating the production of other PBPs. The upregulation and down-regulation of these polypeptides could account for the observed differences in the expression of cell membrane-associated proteins demonstrated by our vancomycin resistant subpopulations.

Similar to the observations in *S. aureus* isolates with low-level glycopeptide resistance (Shlaes, D.M., 1993, Brunet, F. et al 1994; O'Hare, M.D. et al 1992), the resistant subpopulation of *S. haemolyticus* 128G₅ also showed changes in several protein bands when analyzed by SDS-PAGE. Two of the proteins (29 kDa and 33 kDa) appeared to have been upregulated in the resistant sub-clone, while another (44 kDa) appeared to have been downregulated. It is possible that the increased and decreased protein bands, as mentioned above, maybe related to the overproduction of certain proteins involved in cell wall biosynthesis or its regulation and the decreased amounts may be involved in the turnover of the cell wall and cell division. However, the hypothesis put forth remains to be confirmed and the biochemical nature of these proteins and their contribution to vancomycin resistance have still to be determined. It would also be beneficial to carry out an extensive study of the functions and nature of the various PBPs of *S. haemolyticus*. Presently, little is known about
the PBPs of *S. haemolyticus* or other species of CNS and most of the knowledge about staphylococcal PBPs have been acquired with *S. aureus*. However, each bacterial species has its own assortment of PBPs and there is only a very weak relationship between identically numbered PBPs of two difference species of *Staphylococci* (Domanski, T.L. et al, 1997; Hakenbeck R. et al, 1991; Henze U. U. et al, 1996).

Alternatively, the characteristics mentioned above may simply reflect secondary effects of mutations conferring glycopeptide resistance. Types of mutations which may have such pleotropic effects could involve regulatory genes on gene clusters, such as the DCW gene cluster, which is responsible for cell division in *S. aureus*. This gene cluster also contains a lone PBP gene known as the pbpA gene (Pucci, M.J. et al 1997). This PBP gene encodes PBP1, a penicillin-binding protein involved in the transpeptidation of peptidoglycan. Although the function of PBP1 differs from the rest of the cell division genes, its production is coregulated with them. If a mutation occurred in the regulatory element of this gene cluster, it would also effect the production of PBP1. Therefore, there is a possibility that the mutations involved with vancomycin resistance may affect regulatory elements of biosynthetic and cell division genes in this strain of *S. haemolyticus*.

In conclusion, a model of vancomycin resistance was proposed in this thesis, based on observations made on a *S. haemolyticus* resistant isolate 128G5. This model involves the mopping up of vancomycin by a thickened cell wall, which has at least four-fold more glycopeptide receptors than the sensitive parent isolate. Such a response would allow the cells to trap more vancomycin in the periphery of the thickened cell wall away from peptidoglycan biosynthetic sites, therefore conferring some resistance to vancomycin.
Such resistant cells may exist as inherent entities in the parent population with a
downregulation could be
selected during subsequent exposure to vancomycin.

Some of the other phenotypic characteristics observed to be associated with
vancomycin resistance, such as the upregulation and downregulation of certain cell
membrane proteins, and the increased susceptibility to certain β-lactams, aminoglycosides
and quinolones, and defective cell division, still require further characterization before their
contribution to the development of vancomycin resistance can be better delineated.

However, these characteristics all seem to point towards a relationship between
the components of cell wall biosynthesis and maintenance and the development of
vancomycin resistance. These components may include the PBPS which are involved in cell
wall synthesis and turnover, and the autolytic enzymes GL and AM which are involved in
cell division and cell wall turnover.
Overall Summary of Studies Involving *S. haemolyticus*

Speciation and Vancomycin Resistance

The HSP60 method for the species identification of *S. haemolyticus* and *S. epidermidis* was evaluated using a collection of 66 clinical isolates of CNS. These isolates were previously identified by the Microscan method. There were 9 discordant identification results between the Microscan and the HSP60 method. Re-identification of these isolates showed that there was only one false-positive and false-negative identification by the HSP60 method for *S. haemolyticus*. One was a *S. epidermidis* isolate which hybridized with the *S. haemolyticus* DNA probe, and the other was a *S. haemolyticus* isolate which did not hybridize with either probe.

This collection of CNS was used in studies pertaining to vancomycin resistance in *Staphylococci*. A single *S. haemolyticus* isolate, 118BA8, displayed an intermediate level of resistance to vancomycin. Resistant subpopulations were obtained from this isolate by selection in the presence of vancomycin and a model for vancomycin resistance was proposed based on the observations from these studies.

The model for vancomycin resistance describes resistant cells with thick cell walls with the ability to bind vancomycin at the periphery of the cell wall away from cell wall biosynthetic sites. This effect firstly decreases the drug levels in the medium to tolerable levels, and secondly, available vancomycin is bound at the periphery of the cell wall and hindered from accessing the active sites of cell wall biosynthesis. The thickened cell wall.
may be the results of defective or downregulated cell wall turnover mechanisms involving autolytic enzymes such as GL and AM. Other features associated with the resistant phenotype include changes in the cell membrane protein profiles with the upregulation of two proteins (29 kDa and 33 kDa) and downregulation of a 44 kDa protein. These phenotypic characteristics require further study to ascertain their precise relationship and contribution to the mechanism of vancomycin resistance in this strain of *S. haemolyticus*. 
Future Studies

Future studies would include the characterization of penicillin-binding proteins of *S. haemolyticus* and the effect of these polypeptides on the susceptibilities to cephalosporins and vancomycin. Insights from these studies would allow for a better understanding of the relationship between PBPs, cephalosporin susceptibilities and vancomycin resistance. Moreover, the PBP profiles of the resistant isolates should also be compared to those of the parent isolate. This comparison would allow a better correlation between changes in PBP production and vancomycin resistance. The contribution of the identified PBPs to vancomycin resistance can be further studied using insertional knockouts of non-essential PBPs.

Experiments assaying the effect of autolytic enzymes from the supernatant of the reference strain of *S. haemolyticus* on the morphology of poorly divided resistant cells should be performed. If the resistant cells were not dividing due to a defect or downregulation of the autolytic enzyme pathway, the addition of these enzymes to the cell clusters would reverse this phenotype. Autolytic enzyme profiles can be prepared from resistant cells by zymography with heat killed *S. haemolyticus* cells. Such an analysis would show the exact autolytic enzyme being downregulated. Further characterization of the autolytic enzymes from the resistant subpopulations may provide deeper insights into the components involved in cell division in sensitive and resistant populations of *S. haemolyticus*. These studies can ultimately lead to studies on the genetic level linking vancomycin resistance and cell
division.

Further work needs to be done to isolate and characterize the proteins which were either upregulated or downregulated in the vancomycin resistant subpopulation. Purification and overproduction of such proteins in a susceptible host may give direct evidence for their importance to vancomycin resistance.
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