Laboratory and Epidemiological Characteristics of *Streptococcus pseudopneumoniae*

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Abstract

The species designation S*treptococcus pseudopneumoniae* has recently been proposed for a streptococcus that is phenotypically and genetically distinct from *Streptococcus pneumoniae* and other viridans streptococci. The key characteristics of *S. pseudopneumoniae* are the absence of a pneumococcal capsule, insolubility in bile, resistance or indeterminate susceptibility to optochin when incubated in 5% CO_2 but susceptible to optochin when incubated in ambient air and a positive reaction with the AccuProbe DNA hybridization test.

In this thesis, the laboratory characteristics and some preliminary clinical associations are described for a collection of sputum isolates of *S. pseudopneumoniae*. *Streptococcus pseudopneumoniae* can be distinguished from *S. pneumoniae* by some key phenotypic characteristics listed above, and also has a positive result with the Binax NOW pneumococcal antigen test. The pneumolysin gene was present in all the isolates.

No high level penicillin resistance was found amongst the strains of *S. pseudopneumoniae* tested but intermediate resistance was detected in 32% of strains. Resistance to erythromycin and tetracycline was greater than that reported for New Zealand strains of *S. pneumoniae* (46% vs 20% and 54% vs 23% respectively). The figures for constitutive clindamycin resistance were the same (17%) but the rate of inducible MLS_{β} resistance was higher for *S. pseudopneumoniae*. In a study of blood culture isolates outside New Zealand in 2004, intermediate penicillin resistance for *S. mitis* was 27% and for erythromycin 50% which compares well with the results obtained in this study.

The clinical significance *S. pseudopneumoniae* is currently unknown but all the strains were isolated from patients who had symptoms of lower respiratory tract infection. 79% of the patients had chronic obstructive pulmonary disease (COPD) and 33% had chest radiographic infiltrates. In a preliminary study with matched control patients who had *S. pneumoniae* isolated from sputum, patients with *S. pseudopneumoniae* infection were more likely to have COPD (OR 5.0, 95% CI) or exacerbation of COPD (OR 6.5, 95% CI). A larger study of 418 adults with and without COPD to investigate the apparent association with COPD was inconclusive but did suggest that there may be an association with smoking. *Streptococcus*

pseudopneumoniae was most commonly isolated from the sputum of participants with stable COPD.

S. pseudopneumoniae was isolated from 5% of the study participants which was higher than that found in the only comparable study, but nasopharyngeal carriage was low (0.7%) and compared well with that of *S. pneumoniae* in adults.

Although it was found that this new species can be differentiated from *S. pneumoniae* in the laboratory, more research is needed to better characterise the members of the *S.mitis* group and the epidemiological role of *S. pseudopneumoniae.*

Preface

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Chapter 1

Introduction

This thesis is focussed on the laboratory detection and characteristics of a newly described viridans group streptococcus named *Streptococcus pseudopneumoniae.* The work arose out of a long standing interest in a group of *Streptococcus pneumoniae* like streptococci that are frequently cultured from lower respiratory specimens. These "atypical pneumococci" share some morphological characteristics with *S. pneumoniae,* but do not meet the formal identification criteria for *S. pneumoniae.*

With the publication of the new species, *S.pseudopneumoniae,* in 2004 (3), we realised that many of the atypical pneumococcal isolates we were observing in sputum specimens at Canterbury Health Laboratories would be identified as this bacterium. Subsequently, we assembled one of the largest collections of this bacterium from routine clinical specimens and the first to be described from outside North America. To date, very little has been published on the laboratory characteristics and clinical relevance of *S. pseudopneumoniae.* This thesis presents new data on the laboratory characteristics and antimicrobial susceptibility patterns of *S. pseudopneumoniae* and some initial observations about the clinical relevance of this bacterium, especially in relation to chronic obstructive airways disease (COPD).

1.1 Historical Background

The α-haemolytic streptococci, also known as viridans streptococci inhabit the human oral cavity and gastrointestinal system as normal flora. They are frequently isolated from clinical specimens but are usually considered to be contaminants. The identification of this generally insignificant group of organisms is difficult in routine laboratories. Consequently isolates are rarely identified to species level and are instead placed into groups.

Streptococcus pneumoniae, the major cause of pneumonia and responsible for millions of deaths annually (123, 124), has been placed into the *Streptococcus mitis* group of viridans streptococci on the basis of 16S rRNA homology. Although ideally identified using serological techniques targeting the capsular polysaccharide, *S. pneumoniae* has in most cases been successfully differentiated from other members of this group by simple tests such as colonial morphology, susceptibility to optochin and bile solubility. Identification by these tests is satisfactory when isolates from sterile sites are tested. However, they can be problematic for isolates from non sterile sites, such as sputa, as results of phenotypic tests such as optochin susceptibility and bile solubility can be ambiguous (87).

In 2004 Arbique et al identified an unusual group of viridans streptococci that resembled *S. pneumoniae* but was shown to be a distinct species on the basis of DNA-DNA homology (3). Although not yet proven to be pathogenic, this new species, *S. pseudopneumoniae* has the potential to be confused with *S. pneumoniae* which could result in incorrect patient management. It is therefore essential that this new species is characterised and can be differentiated from other members of the *S.mitis* group.

1.2 Taxonomy of the streptococci

As a group the streptococci are diverse and subsequently the taxonomy is at best confusing. The earliest attempt to classify the streptococci was in 1903 and was based solely on haemolysis or the effect the bacteria had on blood agar (22, 34). At this time the streptococci were split into 2 groups, those that caused complete haemolysis of the blood creating a clear zone around the colonies. Tests involving fermentation of sugars and tolerance to a variety conditions such as increased temperature or salt concentration were later introduced to aid differentiation.

The first major breakthrough in identification was in 1933 when Lancefield demonstrated the presence of specific carbohydrate group antigens in the β haemolytic species (50, 67). Lancefield groups still play a major role in the identification of streptococci today.

In 1937 Sherman created a scheme that placed the streptococci into 4 groups based on haemolysis, carbohydrate antigen, and tolerance and fermentation tests (50, 67). These groups were:

- The pyogenic division which included the β haemolytic strains with defined Lancefield group antigens (A, B, C, F and G). This group has remained much the same today.
- The lactic division included strains associated with dairy products rather than human infection.
- The enterococci consisted of 4 species of variable haemolytic activity that could tolerate conditions of high pH, high salt concentration and could grow over a range of temperatures between 10°C and 45°C.
- The viridans streptococci.

The fourth group basically consisted of the remaining species that did not fit into any other group. They were not β haemolytic, could not tolerate high salt concentrations or high pH and did not grow at 10°C (34). Some did however produce a greening effect when grown on blood agar and were therefore called the viridans streptococci, “viridis" being the Latin for green. The green effect on sheep blood agar is known as α-haemolysis although this is a misnomer as the red cells are not actually haemolysed(22). This study will be restricted to the viridans streptococci.

1.3 The viridans streptococci

Currently the viridans streptococci are defined as Gram positive cocci that characteristically grow in chains, are catalase negative and do not grow in 6.5% NaCl broth. In addition they produce leucine aminopeptidase positive (LAP) but not pyrrolidonylarylamidase (PYR). There are currently 26 species, including species not yet known to be of human origin, classified as viridans streptococci although many do not have any haemolytic effect on blood agar. On the basis of phenotypic characteristics they are classified into 5 groups (34, 67) which can be differentiated as shown in Table 1 (Page 4).

Table 1. Differentiation of major groups of Viridans streptococci

Group	Esculin hydrolysis	Mannitol fermentation	Sorbitol fermentation	Voges-Proskauer	Arginine dihydrolase
Mutans	+	+	+	+	-[a]
Salivarius	v	-	-	+[b]	-
Anginosus	+	-	-	+	+
Sanginus	+	-	-	v	+
Mitis	-	-	-[c]	-	-[d]
Bovis	+	-[e]	-	+	-

+, positive reaction ≥92% of strains; -, positive reaction ≤ 8% of strains; v, variable reactions positive in 8 to 91% of strains; Positive reaction [a] *S.ratti*, [d] *S.cristatus*, [e] *S.gallolyticus* (*S.bovis 1*); Variable reaction [b]*S.vestibularis*, [c] *S.mitis*.

Briefly these groups are (34):

- The *S. mutans* group which includes *S. mutans* and *S .sobrinus*. They are found in the oral cavity and are most commonly involved in dental caries.

- The *S. salivarius* group. The human members of this group are *S. salivarius, S. vestibularis* and *S. infantarius* which is sometimes placed in the *S. bovis* group due to its variable esculin reaction.

- The *S. sanguini*s group. This group consists of 3 species; *S. sanguinis*, the most common species involved in human infection, *S. parasanguinis* and *S. gordonii*.

- The *S. anginosus* group. This is the most confusing group. It consists of 3 distinct species, *S. anginosus*, *S. constellatus* and *S. intermedius* with a variety of subspecies that have been known historically by the collective species name, *S.milleri*. Whilst most strains are non haemolytic and otherwise fit the definition of the viridans group, some are β haemolytic and may possess any one of 4 Lancefield group antigens (A, C, F or G) or no antigen at all. As a group they are often involved in abscess formation in various sites including the brain, liver and lungs.

- The *S. mitis* group. For many years a poorly defined as a group as there was no standard identification or typing system. Cell wall composition and DNA homology has allowed members of this group to be more accurately described and the group now consists of species of human interest, described in more detail below.

- The *S. bovis* group is closely related but not classified as viridans group streptococcus. They are also referred to as non enterococcal group D streptococci. Like the viridans streptococci, they are a common blood isolate particularly in patients with colon cancer.

1.4 The *S.mitis* group

The lack of a standardised naming system for the *S. mitis* group caused much confusion with many species changing their names over the years. For example *S. mitis* has been known as *S. mitior, S. oralis and S. sanguis* biotype II (6). The term "biotype" describes strains with particular characteristics but has no official taxonomic standing (7). The confusion was made worse by the lack of definite type strains within the groups. For example, the strain accepted as the type strain for the *S.mitis* group was actually a strain of *S .sanguis* which is now known as *S. sanguinis* (6). The group is now much more defined but is still likely to undergo changes as more species are described. For example *S. sinensis* is a recently described species that is closely related to both the *S. mitis* and *S. anginosus* groups (126).

In this study discussion will be restricted to the *S.mitis* group of current human interest that comprises

- *S. mitis*
- *S. oralis*
- *S. cristatus*
- *S. infantis*
- *S. peroris*

On the basis of phenotypic and physiologic tests *S. pseudopneumoniae* and *S. pneumoniae* also belong in this group.

The phylogenetic relationship of this group to other streptococci based on analysis of 16S rRNA gene sequences is shown in Figure 1, (Page7).

Figure 1. Phylogenic relationship of Streptococcus species (34)

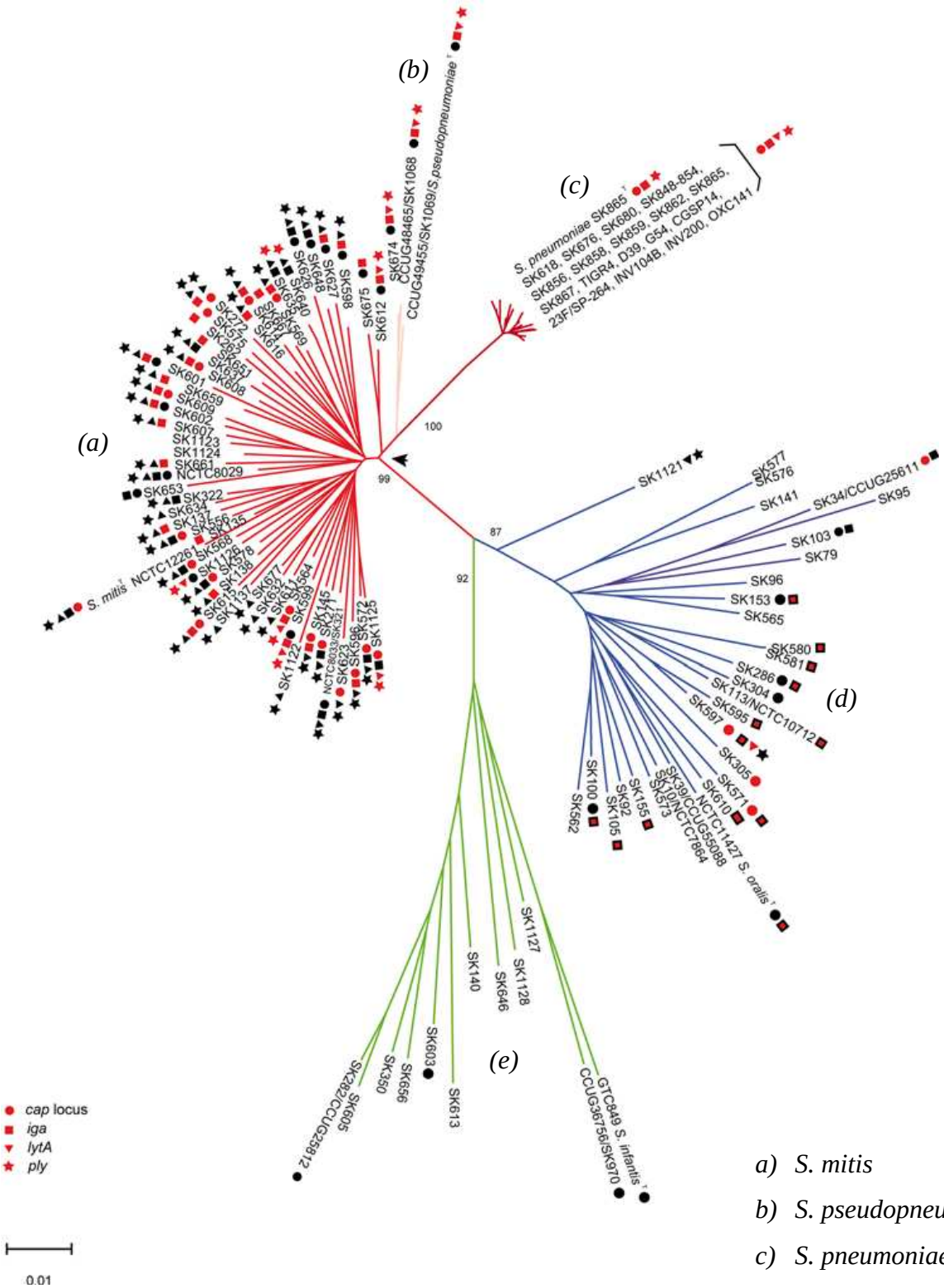
Figure removed for copyright reasons.

Differentiation of the members of the S. *mitis* group is difficult as they fail to utilise many of the substrates used in the biochemical identification kits commonly used in routine laboratories (15, 18). In addition some species are not represented in all databases and others are only represented in very small numbers. This can have a major effect on the accuracy of the identification.

Even the differentiation of *S. pneumoniae* from the other members, once thought to be easily achieved by optochin susceptibility, has become difficult as *S. mitis* and *S. oralis* possess 99% sequence homology with *S. pneumoniae*. The situation has been complicated further by the recognition of "atypical pneumococci" in particular *S. pseudopneumoniae* which also shares 100% sequence homology with *S .pneumoniae, S. mitis* and *S. oralis* and has variable optochin susceptibility (3, 121).

The close relationship of this group of streptococci is illustrated by the work of Kilian et al (64). They sequenced 118 strains that had been identified as *S. pneumoniae, S. mitis, S. oralis* and *S. infantis* using 4 housekeeping genes (ddl,gdh, rpoB and sodA). The alignment of the sequences is shown in Fig 2, (Page 9). All the strains fell into 3 close but distinct clusters, *S. mitis-pneumoniae- pseudopneumoniae* (clusters a,b,c), *S. oralis* (cluster d) and *S. infantis* (cluster e). The largest cluster contained the type strains of *S.pneumoniae* (cluster c), *S. mitis* (cluster a) and the recently recognised *S. pseudopneumoniae* (cluster b). Members of the *S. mitis* group are naturally competent for gentic transformation. This plays a major role in the evolution of species, allowing adaptation to environmental change and exchange of virulence factors such as pneumolysin and autolysin (121). The study by Kilian et al suggests that the *S. mitis* group including *S. pseudopneumoniae* has evolved from *S. pneumoniae* by losing virulence genes and adapting to a commensal lifestyle (64). However, *S. pseudopneumoniae* is so new that it has not yet been fully characterised. As such it does not feature on any databases making reliable identification difficult in routine laboratories. In addition, it has not yet been established whether it is a commensal viridans streptococcus or does play a role in disease.

Figure 2. Phylogenetic tree constructed with the minimum evolution algorithm in MEGA version 4.0 and based on concatenated partial sequences of the house-keeping enzyme genes ddl, gdh, rpoB, and sodA.(64)



a) S. mitis

b) S. pseudopneumoniae

c) S. pneumoniae

d) S. oralis

e) S. infantis

1.5 Clinical significance of the viridans streptococci

The most significant member of the viridans streptococcal group is *S. pneumoniae. S. pneumoniae* is a major pathogen worldwide. It is the most common cause of community acquired pneumonia (CAP) accounting for 500,000 cases of pneumonia each year in the USA. It also accounts for 7 million cases of otitis media, 50,000 cases of bacteraemia and 3,000 cases of meningitis annually (123). It is also a significant cause of other infections such as sinusitis. Although the incidence of invasive disease is changing in developed countries such as the US (15), it remains as a major cause of death in children due to respiratory infection (124).

Apart from *S. pneumoniae,* members of the *S. mitis* group of streptococci are often dismissed as contaminating organisms. They are generally considered to be commensal or normal flora of the oropharyngeal tract but may also originate from gastrointestinal or vaginal sources (91). However, if they are able to penetrate host boundaries they can cause serious infection. For example, viridans streptococci are the most common oral bacteria to cause bacterial endocarditis in native valves (91) and can cause lung abscesses. Infection usually follows oral/dental surgery but is now also been seen as a result of increasingly common practices such as intravenous drug use, tattooing and body piercing (69). The likelihood of such infections is increased if the patient has an underlying condition such as prosthetic heart valves or malignancy.

There is now widespread use of broad spectrum antibiotics, indwelling lines and immunosuppressive drugs. This may extend the life of patients but also puts them at risk of blood infections with organisms such as viridans streptococci, previously thought to be insignificant. In one study involving immunocompetent patients the incidence of viridans streptococcus bacteraemia was 6.9% in adults and 12.3% in children (116). The bacteraemia was generally transient following oral surgery in adults or seizures in children and was associated with low mortality, only patient dying as a direct result of infection. In contrast, in neutropaenic cancer patients the incidence increased to 32% with mortality as high as 12% (49, 103). The most common isolates in bacteraemia are reported to be *S. mitis* and *S. oralis* but these identifications are based on traditional phenotypic methods and biochemical kits that may not be accurate due to incomplete databases (40, 56). The increase in bacteraemia in cancer patients caused by viridans streptococci is due to a number of reasons including their selection as competing flora is

reduced by treatment regimes including use of antibiotics that target Gram negative bacteria (74, 102). Bacteraemia due to viridans streptococci can lead to complications, the most serious of which is viridans streptococcal shock syndrome (VSSS). It occurs in up to 25% of child cancer patients with bacteraemia and has reported mortality rates between 40% and 100%. It is most commonly caused by *S. mitis* (43, 80).

Of particular interest in this study is the association of viridans streptococci with respiratory infection. The presence of viridans streptococci in the oral flora makes their association with pneumonia unclear and there have been few studies to clarify its etiological role. In one study of 71 children, of 13 cases where viridans streptococci were isolated from 2 sets of blood cultures, only 3 had community-acquired pneumonia (44). In the same study viridans streptococci were isolated from a further 45 cases but only from 1 set of blood cultures. In this group 6 had community acquired pneumonia but the lack of repeat cultures suggested the viridans streptococci were contaminants and not the etiological agent. In a later study, viridans streptococci were found to be the second most common isolate, after *S. pneumoniae*, from blood cultures of children with community acquired pneumonia suggesting that viridans streptococci should not be ruled out as cause of pneumonia (37). In a study amongst 1118 adults with community acquired pneumonia, viridans streptococci were isolated from 7 bacteraemic patients and accepted as pathogens or co-pathogens (78). In 3 cases, viridans streptococci were the only organisms implicated in the pneumonia and were accepted as definite viridans streptococcal pneumonia. Of the remaining 4, two were classed as probable and 2 as possible. All the patients had underlying conditions such as COPD, neoplasia and other co-morbidities (78). It is likely that these patients had received treatment such as antibiotics and that the viridans streptococci had become predominant in the oral flora due to selective pressure. The current evidence for viridans streptococci playing pathogenic role in respiratory disease is therefore weak.

Even if the viridans streptococci cannot be shown to be respiratory pathogens, they are important in the management of disease as they have the potential to be misidentified as *S. pneumoniae*. As they tend to be more resistant to antibiotics there is also the potential to over estimate the rate of penicillin resistant *S. pneumoniae* (119). Correct identification is therefore essential.

1.6 A Brief History of the Pneumococcus

In 1875 Klebs saw non-motile “monads” in fluid from the lungs of patients with pneumonia(122). By 1881 Pasteur in France and Sternberg in the USA had independently demonstrated pathogenicity of the organism by injecting rabbits with the saliva from an infected patient Pasteur also managed to isolate the organism from the infected rabbits and went on to report his findings, naming the organism *Microbe septicemique du salive*. Over the years it has had many names including *Micrococcus pasteuri* (Sternberg), Pneumococcus and *Diplococcus pneumoniae* (122). It was placed into the streptococci because it grew in characteristic chains of cocci and given its present name, *Streptococcus pneumoniae*, in 1974 (9).

Since its recognition, the pneumococcus has been studied intensely (4, 122). Some of the important milestones include:

1884: First report of Gram positive cocci in stained sections of lungs (4)

1900: Neufeld noted that *S. pneumoniae* was soluble in bile; a solution once used for therapeutic purposes, and developed the first diagnostic test.

1902: Another early remedy, serum therapy, led to the discovery of the quellung reaction, also by Neufeld. This in turn led to the recognition of the capsular antigen and development of a serotyping system. To date there are 90 known capsular types of *S. pneumoniae*

1911: Morgenroth and Kaufman described the protective effect of optochin in mice experimentally infected with pneumococci and in 1912 they noted the development of in vivo resistance (96). This was confirmed by Moore and Chesney 5 years later (83, 84). It proved to be unsuitable as a treatment but did later become the basis of the most commonly used identification test, optochin susceptibility, as described by Bowers and Jeffries in 1953 (12).

1911: The use of the first pneumococcal vaccine is attributed to Sir Almroth Wright who investigated its efficacy in the gold mines of South Africa (6).

1917: The presence of capsular antigen was demonstrated in serum and urine by Avery and Dubos (118). By the early 1970’s this observation had been used to develop antigen tests to enable rapid diagnosis of pneumonia.

The pneumococcus is still being rigorously investigated using molecular techniques targeting virulence factors and sequencing to aid differentiation from close

relatives such as members of the *S. mitis* group of viridans streptococci. These will be discussed in greater detail later in the chapter.

1.7 Differentiation of *S.pneumoniae* from other Streptococci

As other members of the viridans streptococci are often dismissed as commensal bacteria they are not usually worked up in the laboratory with the same rigour as *S. pneumoniae.* Emphasis has always been placed on being able to differentiate *S. pneumoniae* from the rest of the group.

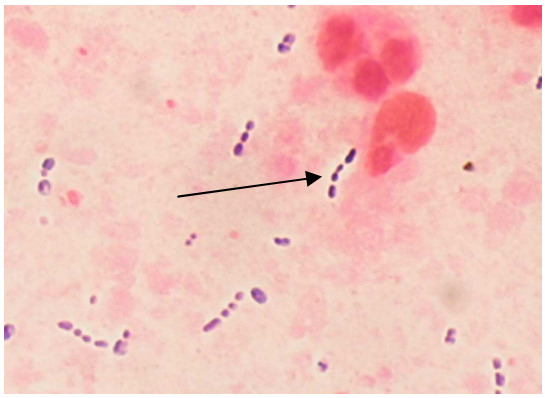
At Canterbury Health Laboratories (CHL) isolates from sterile sites such as blood cultures, that are considered significant, are identified using the API® rapid 32 STREP system.

Streptococcus pneumoniae is differentiated from other viridans streptococci using routinely available phenotypic methods described below. Unfortunately, as more is learned about *S. pneumoniae* and its close relatives, including the recently recognised *S. pseudopneumoniae,* the accuracy of some of these traditional tests is being questioned (3, 63, 105).

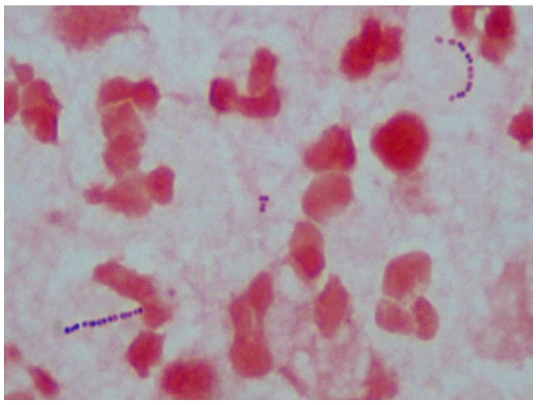
1.7.1 Microscopic Morphology of *S. pneumoniae*

In a Gram stained film of sputum or other clinical specimens *S. pneumoniae* typically appears as elongated Gram positive cocci, known as lanceolate cocci, generally arranged in pairs. A capsule around the cocci is often visible as shown by the arrow in Fig 3.a, (Page 14). Other members of the streptococci tend to appear in the more classical form of chains of cocci without a capsule Fig 3.b, (Page 14).

Figure 3. Microscopic morphology of *S. pneumoniae* and *S. pseudopneumoniae*



a. Encapsulated lanceolate diplococci of *S. pneumoniae*.



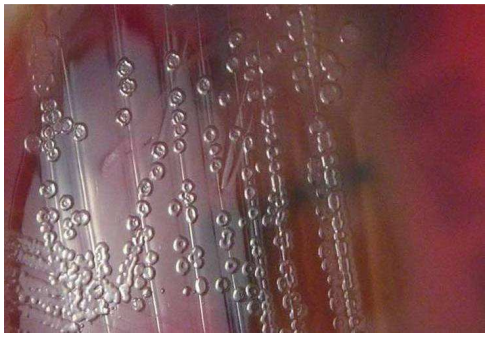
b. Streptococci in chains

1.7.2 Colonial morphology of *S. pneumoniae*

The colonial morphology of pneumococci is very important as it is distinctive. Although large, mucoid colonies are not uncommon; colonies of *S. pneumoniae* after 24 hours incubation are typically 2mm in diameter with an entire edge and a glossy surface. On sheep blood agar the colonies cause greening of the agar known as α - haemolysis. Due to the action of autolysin the colonies typically collapse upon themselves to form the characteristic draughtsmen morphology with raised edges and a depressed centre. The depression of the large, mucoid colonies is not so prominent but collapsed colonies are still distinctive, appearing as a wet sheen on the surface of the agar. These colonies often coalesce or merge into each other producing colonies with an "hourglass" shape. Rarely, non-capsulated strains with rough (R) form colonies are isolated. Mutant rough (R) forms also appear spontaneously among laboratory cultures of the smooth (S) form colonies of capsulated strains (76).

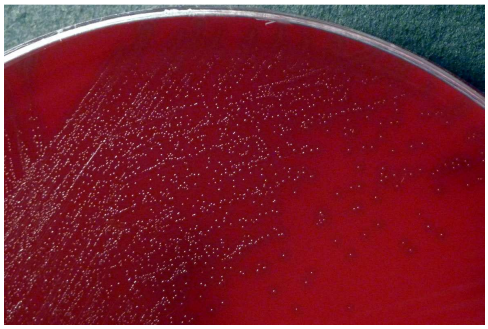
Some of the commonly isolated viridans streptococci are shown in Fig 4, (Page 15) It can be seen that many strains of viridans streptococci other than *S. pneumoniae* appear very similar for example *S.mitis, S. oralis* and *S. parasanguinis* c, d, e. Fig 4, (Page 15). Whilst the distinctive morphology of *S. pneumoniae* is not reliable as a sole means of identification (105), it is an important characteristic and should be considered in any identification scheme.

Figure 4. Colonial morphology on sheep blood agar of commonly isolated viridans streptococci

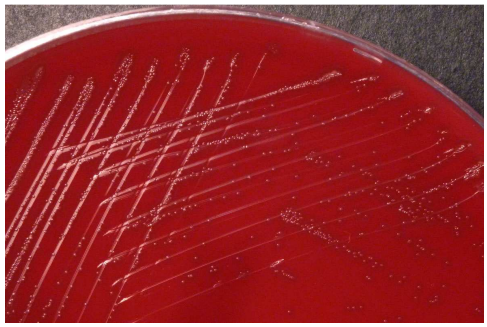


a. Draughtsmen colonies of *S. pneumoniae*

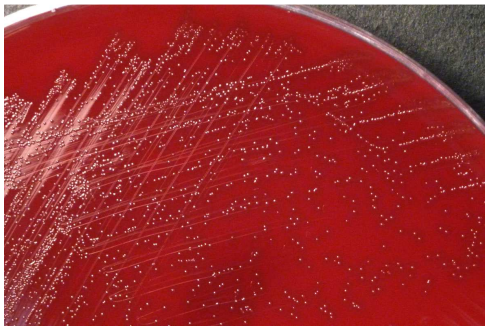
b. Mucoid colonies of *S. pneumoniae*



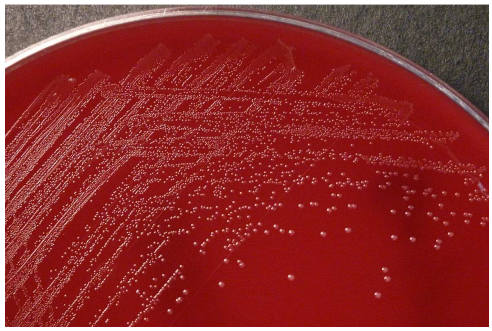
c. *S. mitis*



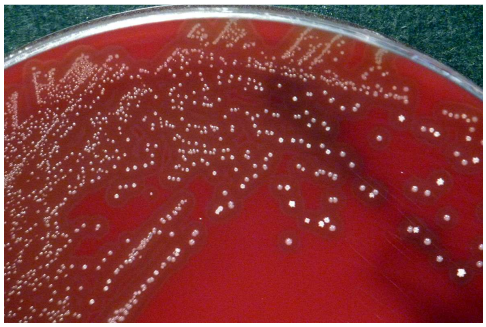
d. *S. oralis*



e. *S. parasanguinis*



f. *S. salivarius*



g. *S. anginosus*

1.7.3 Optochin Susceptibility

Susceptibility to ethylhydrocupreine hydrochloride (optochin) is the test most commonly used to differentiate *S. pneumoniae* from other members of the viridans streptococci.

At the turn of the twentieth century it was found that quinine had some bactericidal effect on pneumococci but it proved to be unsuccessful as a therapeutic agent as it was too toxic at therapeutic doses (122). In 1915, Moore noticed that *S.pneumoniae* was the most sensitive of the streptococci to a quinine derivative, ethylhydrocupreine (84). In 1943 Mørch demonstrated that low concentrations of ethylhydrocupreine hydrochloride on blood agar would inhibit pneumococcal growth (122). By 1953 Bowers and Jeffries had developed the optochin susceptibility as we know it, using a paper disc containing diluted optochin (12). Many laboratories still rely on optochin susceptibility as the sole method of identifying pneumococci today.

Getting the test right is important. The density of inoculum is essential. If it is too light the resulting zone may be too large; too dense and the zone may become too small (77).

Concerns have been raised about the correct medium and appropriate incubation conditions for accurate interpretation of the test. Gardam and Millar looked at 72 clinical isolates confirmed as *S. pneumoniae* by bile solubility and capsular antigen serotyping and 22 isolates confirmed as viridans streptococci by bile solubility and carbohydrate fermentation (42). A standard inoculum (0.5 MacFarland) of each was tested against a 6mm disc containing optochin on sheep blood agar using Columbia (CA), Typticase soy (TSA) and Mueller Hinton (MH) agars as the base. The plates were incubated at 35°C for 24 hours in 5% CO_2 and in ambient air. The viridans streptococci did not produce a zone in either CO_2 or ambient air. The strains of *S. pneumoniae* produced zones in both atmospheres. All the zone sizes were >14mm in ambient air but 15.3% of those on CA and 22.2% of those on MH produced zones <14mm when incubated in CO_2. At the time CLSI criteria for presumptive identification of *S. pneumoniae* included a zone of >14mm for a 6mm disc incubated overnight in 5% CO_2 so these isolates would have been misidentified as viridans streptococci. Incubation in ambient air gave greater discrimination but this was not recommended as it is reported that up to 8% of isolates of *S. pneumoniae* do not grow or grow poorly without

CO_2 (7). This is supported by Arbique et al who recommend testing optochin in CO_2 to avoid selection of non pneumococcal isolates (3).

The new species, *S. pseudopneumoniae*, behaves like the odd strains in Gardam and Millar's study in that it produces variable optochin results i.e. resistant when incubated in 5% CO_2 but susceptible when incubated in ambient air. If the strains had not been serotyped at a reference laboratory it could be suggested that they were in fact strains of *S. pseudopneumoniae.*

At CHL the optochin susceptibility test is currently performed in 2 ways. For preliminary identification a single colony is inoculated onto Columbia sheep blood agar. This is confirmed by inoculating a standard inoculum (0.5 MacFarland) onto Mueller Hinton sheep blood agar. A 6mm optochin disc is applied and the plates incubated for 24 hours at 36°C in an atmosphere containing 5% CO_2. A zone diameter >14mm is considered to be susceptible. Results of both tests are considered when making a final identification.

Optochin works by binding to H^+-ATPase, a membrane bound enzyme found in bacteria that is involved in ion transfer across the plasma membrane (99) . Resistance is the result of point mutations in the 'a' or 'c' subunit of ATPase which effects optochin binding (93, 99). The existence of optochin resistant pneumococci has been noted since the test was developed. Bowers and Jeffries noted that of 695 strains tested, 2 did not show complete susceptibility to optochin but retained bile solubility (12). Resistance to optochin has been reported since 1987 in the USA, Spain and Israel and more recently in Portugal and Brazil (93). Although the exact origin of optochin resistance is unclear, Pikis et al did note a connection between resistance and increased use of other quinine derivatives such as mefloquine for the treatment and prophylaxis of malaria (99). However, this was not confirmed by Cortes et al who did not report an increased incidence of resistance in areas where malaria is endemic (21).

Based on the studies above there is a potential for misidentification if other tests are not employed. At CHL isolates giving zones >6mm and <14mm are confirmed by other tests including, since this study began, optochin susceptibility performed in ambient air and bile solubility.

1.7.4 Bile solubility

Prior to the development of the optochin susceptibility test *S. pneumoniae* was identified by the bile solubility test. Neufeld first noticed that bile was able to dissolve the pneumococcal cell in 1900 and later found that it only worked on freshly isolated virulent strains (122). Bowers and Jeffries used the bile solubility test as the "gold standard" when developing the optochin susceptibility test (12).

The test is based on the triggering of uncontrolled activity of the major lytic enzyme, amidase lytA, in response to treatment with bile (73). Various methodologies, employing concentrations of sodium taurocholate or sodium deoxycholate ranging from 1% (94) to 10% (59) have been suggested for the bile solubility test. The plate method can be performed in 15minutes whereas the tube method can vary from 15minutes to overnight incubation (35, 59, 77). Despite the differences in methodology the test has been associated with a sensitivity and specificity ≥98% (14, 63). However, Arbique noted differences between results obtained for the bile solubility of the same isolates but performed by different technicians in different laboratories using different techniques (2). In addition there have been reports of bile insoluble, optochin susceptible strains. In 1986 Pease et al reported 10 strains of non- capsulated *S. pneumoniae* from eyes that were optochin susceptible but bile insoluble (98).

As stated above, bile solubility is based on triggering the *lyt*A gene, a gene once thought to be characteristic of *S. pneumoniae*. It has however been found in viridans streptococci closely related to *S. pneumoniae* (71) and in 2002 Obregon et al described mutations in the *lyt*A gene of atypical pneumococci that were bile insoluble (94). In this study the atypical strains were found to be insoluble in 1% sodium deoxycholate but soluble in a solution containing 0.1% and when tested with an alternative detergent, Triton X-100, which stimulates amidase activity (24).

Three decades earlier, Lund had reported optochin to be more reliable in differentiating *S. pneumoniae* as it was observed that when smooth, capsulated virulent strains were transformed to rough, non-capsulated avirulent strains they kept their optochin susceptibility but lost their bile solubility (75). On this basis, it could be possible that the new species, *S. pseudopneumoniae*, which is optochin variable and bile insoluble, has evolved from *S. pneumoniae* by losing genes as suggested by Kilian (64).

1.7.5 The Quellung Reaction

The Quellung reaction was first described by Neufeld in 1902 (5). It was originally thought to be a swelling of the capsular polysaccharide hence the name "Quellung", German for swelling. It is in fact the refractive property of the capsule when exposed to homologous antibodies, also known as the capsular precipitin reaction. By 1910 Neufeld and Händel had demonstrated 2 distinct pneumococcal capsular types.

Prior to the discovery of antibiotics, pneumococcal pneumonia was treated by administering type specific capsular antiserum produced in animals. It was therefore necessary to isolate the pneumococcus and determine the capsular type. Over the next 20 years considerable progress was made and by 1929 the Quellung reaction had been used to recognise 32 distinct capsular types of pneumococcus (6). However the reaction was not recognised as a valuable method of identifying pneumococci in clinical specimens until 1931 when Neufeld and Etinger- Tulczynska noted that if homologous serum was added to the mixed cultures isolated from nasal specimens of animals, the pneumococci could be distinguished from other cocci by their capsular swelling. From then on, in addition to being used to type pneumococci, the test was widely used to identify pneumococci, becoming the "gold standard" (4). Serum therapy was abandoned with the introduction of drugs such as sulfapyridine and later penicillin that could be used to treat all cases of pneumonia irrespective of capsular type. As a result, typing serum that had been produced as a necessary part of the therapy ceased to be available commercially. This led to the Quellung reaction being used infrequently for identification purposes. Nowadays it is generally only used in reference laboratories specifically for typing purposes.

The major obstacle to use in routine laboratories is the high cost of the typing sera. However this can be reduced by use of "Omniserum" which contains antibodies to all 90 capsular types currently known. This allows an isolate to be categorised as S. *pneumoniae* or not which is generally adequate for routine treatment purposes.

There have been some reported problems with the use of Omniserum. Not all type specific antibodies are present in high enough titres to make the capsules visible but instead produce agglutination and some R forms may give non specific agglutination with several types (76). Cross reactions with other encapsulated bacteria e.g. *Haemophilus sp*ecies, *Klebsiella species* and *Escherichia coli* (5) have been reported but this is unlikely to be a problem unless the technique is being used directly

on clinical specimens such as sputum. In one study by Holmberg et al (54) 68% of α haemolytic streptococci of clinical interest were found to cross react with Omniserum. Other non pneumococcal protein e.g. C reactive protein has also given false positive results (5). False negative results can also occur if the number of organisms per field is too high. Some pneumococcal types, particularly 3 and 37, produce large amounts of capsular polysaccharide which results in a prozone effect with inhibition of the Quellung reaction (5).

More recently it has been considered the most specific test for the identification of pneumococci. In the study by Arbique et al (3) all 35 of the optochin variable strains and those isolates confirmed as viridans streptococci by other means failed to react with CDC typing sera whereas all the pneumococci did. It is also a very rapid test and although some experience is required is relatively simple to perform. It maybe as Austrian suggests "a neglected technique" that should be re-evaluated in light of the emergence of new species such as *S. pseudopneumoniae.*

1.7.6 Pneumococcal Antigen testing

The Quellung reaction was the only way to rapidly diagnose pneumococcal pneumonia as optochin susceptibility testing and bile solubility testing as described by Facklam involved overnight incubation (35). However, it fell it fell out of use as a routine test as it required the availability of high titre specific antisera and a degree of expertise to produce accurate results.

The presence of pneumococcal capsular antigen (PCA) in the urine and serum of patients with lobar pneumonia had been recognized in 1917(122). However, it was not used a means of rapid diagnosis until 1971 when it was shown that these antigens could be detected in patients with pneumonia and bacteraemia. The method used was counter-current immunoelectrophoresis (32). Patient specimens such as serum, urine or sputolysed and washed sputum were placed in wells cut into agarose mounted on a glass slide immersed in veronal buffer. Omniserum was placed in parallel wells positioned 2mm apart. A current was applied and allowed to run for about 20 minutes. Positive reactions were indicated by lines of precipitation. The test did have some drawbacks.

Cross reactions with some α-haemolytic streptococci were reported in one study (115) although the lines of precipitation were generally weaker than those obtained with *S. pneumoniae* and not considered a problem as long as other tests/morphology were used. In another study the method was shown to have a sensitivity of 40% (14) due to the inability to detect the antigen of types 7 and 14 without special preparation. In addition it was laborious and still required a high degree of expertise to obtain reliable results. It therefore remained as a serotyping tool rather than a routine diagnostic test.

The earliest commercially available rapid pneumococcal antigen tests used slide coagglutination as described by Kronvall in 1972 (68). The test used staphylococci sensitised with Omniserum, at that time containing antibodies to the 83 known capsular antigens, and was found to be in complete agreement with the quellung serotyping performed in parallel.

Over time the sensitized staphylococci were replaced by polystyrene latex beads coated with capsular antibodies (13). This forms the basis of current serotyping systems such as BBL™ Pneumoslide™(1) and Pneumotest-Latex (Statens Serum Institute, Denmark) (113). These tests are rapid, simple and more economical than quellung and have shown good agreement, with sensitivity and specificity of up to 100% for serotyping unequivocal pneumococci. However, cross reactions with *S. mitis* and *S. oralis* have also been reported (13, 86, 113). Slotved et al (113) recommended that the Pneumotest –Latex should only be used for isolates that have been confirmed as pneumococci by other methods such as optochin or bile solubility.

It has long been recognised that pneumococcal antigen is found in urine and is therefore a basis for a rapid diagnostic test. The latex agglutination tests have low sensitivity when used on urine specimens. It has been suggested that this is due to unfavourable conditions in urine such as pH and salt content or may also be due to low concentrations of specific capsular antigens or differences in immune response (31).

To get around the problem different antigens were targeted. The cell wall of all pneumococcal serotypes contains a phosphocholine-containing polysaccharide (PnC) (112). The NOW *S. pneumoniae* urinary antigen test (Binax Inc., Portland, Maine) is an immunochromatographic test designed to detect PnC in urine and CSF. It is reported to have a sensitivity of 80% for urine samples (31, 89) and provides a rapid test that is particularly useful in non bacteraemic pneumonia. Due to the close relationship with other viridans streptococci, particularly *S. mitis,* cross reactions do occur (31, 90). However, the cell wall antigen of the viridans streptococci is not released as readily as

that of *S. pneumoniae* so does not reach the same concentration in urine and the antigen level created by carriers of pneumococci is below the level of detection. In addition, *S. mitis* is not associated with pneumonia and is unlikely to appear in the target population of the test. That is, when used as intended by the manufacturer, the cross reactions are not a problem.

1.7.7 Biochemical Identification

Biochemical identification is based upon the ability of bacterial species to ferment various sugars or produce enzymes that are active on other substrates. Early testing was generally based on fermentation of sugars such as glucose and maltose and these still play a big role in identification schemes today. However, as stated above the members of the viridans streptococci are relatively inert in terms of fermentation of sugars. In early schemes they were generally placed into the viridans group on the basis of green colouration of blood agar or because they didn't fit into any of the other groups.The development of commercial kits made biochemical identification much easier. One of the earliest and most commonly used kits is The API® 20 Strep (bioMérieux, France).

The API® 20 STREP system is based on a gallery made up of 20 cupules containing dehydrated substrates, including sugars to detect fermentation and substrates to detect enzyme activity such as the Vogues –Proskauer (VP) reaction. The cupules are grouped in 3's with each cupule given a value of 1, 2 or 4. After 24 hours incubation at 36°C, positive reactions, as described by the manufacturer, are scored as 1, 2 or 4 accordingly. These scores are totalled to give a score for each set of 3, the maximum being 7. The resulting 7 digit profile number is compared with the database to produce the final identification. Tillotson (117) showed that the API® 20 STREP performed well for the identification of viridans streptococci particularly when used alongside other tests such as colonial morphology and optochin susceptibility as recommended by the manufacturer. However there was a major problem with the study in that it did not include any strains of *S. pneumoniae*.

The API® rapid 32 STREP, used at CHL, is based on the same design but consists of 32 tests that detect preformed enzymes and requires only 4 hours incubation

to achieve identification. A recommendation to use optochin susceptibility is not specifically made by the manufacturer of the API® 20 STREP although it does recommend taking into consideration the patient history, source, morphology and any other tests performed (11). Freney et al (38) showed that it gave good results for the enterococci, *S. anginosus* (*S. milleri*) group and β haemolytic streptococci although it gave no advantage over Lancefield grouping for the latter. Correct identifications were achieved for only 1/3 of viridans streptococci and 1/2 of *S. pneumoniae* without the use of additional tests. When the additional tests were used 64/68 strains of *S. mitis*, *S. oralis* and *S. pneumoniae* were identified correctly (based on available database). A study by Appelbaum (2) compared a similar system, the IDS RapID, with conventional tests and found it correctly identified 88.5% of *S. pneumoniae* and 72.5% of viridans streptococci when used alongside optochin susceptibility as recommended. This dropped to 26.9% and 52.3% respectively when optochin susceptibility was not used.

The major advantage of the API ®rapid 32 STREP system is the speed with which a result is obtained, but the optochin test requires 24 hours incubation reducing the rapidity. As most laboratories use optochin as their primary means of identifying pneumococci, the API ®rapid 32 STREP is only really useful for confirming equivocal optochin results. If such rapid identification was essential to exclude *S. pneumoniae* it would need to be used alongside other tests such as bile solubility or Quellung reaction to increase the accuracy of identification.

Whilst these kits have undoubtedly revolutionised biochemical identification there are some drawbacks. They are relatively expensive when compared to an agar plate plus an optochin disc and can be very subjective. This is particularly true of the API® rapid 32 STREP which can be difficult to read. The major drawback however is the inadequacy of the databases with some species represented only by small numbers or not at all so in some cases a final identification is not possible.

A number of automated identification systems such the BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD) and the Vitek 2 (bioMérieux Vitek, USA) are now available. These systems offer rapid identification and susceptibility without the need for additional tests or addition of reagents. In one study evaluating the Phoenix and the MicroScan Walkaway System(81), 100% and 79.3% of streptococci respectively were identified correctly. The Phoenix also identified 90.9% of *S. pneumoniae* (not tested by MicroScan) and both correctly identified the 2 viridans streptococci included in the trial. The Vitek 2 only identified 6/15 viridans

streptococci correctly (45). Of the remaining 9, seven were misidentified at species level and 2 failed to identify at all. As with the API systems, the major problem is again the inadequacy of the database. Whilst these systems may work well for other streptococci and the enterococci(45, 81), they currently offer no advantage over non automated identification for the viridans group streptococci.

1.7.8 Molecular methods

a) DNA Hybridisation

ACCUPROBE™ (GenProbe®, San Diego, CA) is a commercial nucleic acid hybridization test. It uses a single stranded DNA probe with a chemiluminescent label that is complementary for ribosomal RNA of *S. pneumoniae.* It is designed to identify *S. pneumoniae* isolated from culture (46).

In 1992 Denys and Carey (23) reported that AccuProbe was 100% sensitive and specific when compared with phenotypically identified pneumococcal and non-pneumococcal isolates. In 1998 Mundy et al agreed with the findings for unequivocal isolates but found that of 155 equivocal isolates, 4 that were optochin susceptible failed to identify as pneumococci (87). Kearns et al (62) also found the AccuProbe excellent for unequivocal isolates but reported 5 phenotypically non-pneumococci were identified as *S. pneumoniae.* Whatmore et al (121) also reported that “atypical pneumococci” had been identified as *S. pneumoniae* even though the strains were genetically divergent. Given the current state of the identification of viridans streptococci it was questioned whether the isolates in the studies had been correctly identified prior to being tested with AccuProbe (3).

These studies suggest that AccuProbe is suitable for use on isolates from sterile sites such as blood cultures but not so reliable for isolates from sputum where there is an increased chance of isolating non capsulated, non pneumococcal strains.

b) Detection of virulence factors

Streptococcus pneumoniae has a number of well recognised virulence factors which have been used as targets for identification by PCR.

Pneumolysin (*ply*) is an intracellular pore- forming toxin that can lyse any eukaryotic cell which contains cholesterol in its membrane. It is a major factor in pneumococcal virulence, is possessed by virtually all strains and is the most commonly used target for identification (62, 92, 106). It was originally thought to be a unique character of *S. pneumoniae* but it has been shown that *S. mitis* and other closely related viridans streptococci can harbor the *ply* gene (121).

Autolysins are extracellular virulence factors that work by degrading bacterial peptidoglycan causing cell wall lysis. The major autolysin of *S. pneumoniae* is *lyt*A amidase; also know as N-acetylmuramoyl-L-alanine amidase. Like pneumolysin, it was thought to be characteristic of *S. pneumoniae* and has been used as a target in a number of studies (82, 92, 106, 121). However, it too has been found in other closely related streptococci (71), including members of the *S. mitis* group that are optochin susceptible (8) and in a mutated form in atypical pneumococci that were bile insoluble (94).

Other extracellular virulence factors include pneumococcal surface protein (pspA) and pneumococcal surface antigen (psaA). The surface protein is a choline binding protein important for protection from the host immune system and binding to lactoferrin which plays a role in colonization. The surface antigen is a lipid attached protein involved in the transport of manganese and zinc into the cell. Morrison et al (85) developed an assay to detect all 90 known pneumococcal serotypes containing psaA. However, as with other assays, the gene was found in other viridans streptococci including *S. mitis* and *S. oralis* (60).

Poyart et al (100) obtained clear differentiation of *S. pneumoniae, S. mitis* and S. *oralis* by sequencing the gene encoding the manganese -dependent superoxide dismutase but Arbique et al (3) reported that it did not differentiate *S. pneumoniae* and *S. pseudopneumoniae* .

16S rRNA sequencing has been used with limited success due to the high degree of sequence homology in the *S. mitis* group. However, new targets are being investigated. Westling et al (120) were able to differentiate 131/132 viridans streptococci by sequencing the RNase P RNA gene, *rnp*B. Unfortunately, all the isolates were from blood cultures and did not include *S. pseudopneumoniae.* Sequencing is not a

technique available to most routine laboratories and was not used in this study. It will therefore not be discussed further but may be investigated in the future.

Matrix- associated laser desorption ionization-time of flight (MALDI-tof) is a new technology based on mass spectrometry. In 2007, Friedrichs et al (39) evaluated the system for the rapid identification of viridans streptococci. It successfully identified 99 clinical isolates, 71 as *S. mitis* group, 23 as *S. anginosus* group and 5 as *S. salivarius.* The isolates investigated did not include any that had been identified as *S. pseudopneumoniae* using phenotypic methods. Although the technology looks promising, it may suffer from the same resolution problems as 16s rRNA sequencing(18).

1.7.9 Susceptibility of *S.pneumoniae* and the viridans streptococci

Resistance to β lactam antibiotics among S. *pneumoniae* emerged as a significant problem in the early 1990's. By 2002 resistance to penicillin had reached 35% in the USA (29). A similar trend has been observed with non β lactam antibiotics. The current rates for macrolides, clindamycin, tetracyclines, sulphamethoxazole-trimethoprim and chloramphenicol are ~30%, 9-10%, 15-17%, 30-40% and 12% respectively (25-27, 29, 61). So far the rate of resistance for the fluoroquinolones has remained low at 1-2% (30) although it has suggested that this may change as the use of oral fluoroquinolones increases. The same study also suggests that resistance to other classes of antibiotic may have reached a plateau.

Resistance of streptococci to different classes of antimicrobials is increasing worldwide. In a study of 8 European countries (104) the resistance rates were similar for macrolides (28%), sulphamethoxazole-trimethoprim (33.4%), slightly lower for penicillin (24.6%) and chloramphenicol (8.6%). Resistance to clindamycin was higher (22.6%). So far the rate of resistance for the fluoroquinolones has remained low at 1-2% in both the USA and Europe (30, 104). However, it has been suggested that this may change as the use of oral fluoroquinolones increases and that resistance in other classes

of antibiotic may have reached a plateau (30) . So far there have been no reports of vancomycin resistance(17).

Reduced susceptibility to penicillin and other β-lactams is also increasing amongst the viridans streptococci (61) along with increased resistance to the macrolides and fluoroquinolones (57, 101). It has been observed that amongst the viridans streptococci, *S. mitis* is the most resistant (28).

Resistance to antimicrobials is due to a number of mechanisms (17). Penicillin works by binding to proteins in the cell wall of *S. pneumoniae* and disrupting cell wall synthesis. Resistance is due to altered penicillin binding proteins (PBPs) that have reduced affinity for β- lactam antibiotics. There are 6 different PBPs and the degree of resistance depends upon which PBPs are altered. For example, low level resistance is conferred by changes to PBP2x and PBP2b and high level resistance by PBP1a. Resistance in many clinical isolates is due to changes in only these 3 PBPs.

There are 2 predominant mechanisms that cause macrolide resistance and 1 less common (17, 33). The first modifies the ribosomal target site mediated by methylases encoded by the erm(A) or erm(B) genes. This confers high level resistance to macrolides, lincosamides and streptogramins (MLS_{β} phenotype) which may be constitutive or inducible. Active drug efflux is mediated by the membrane bound efflux protein encoded by mef(A). This confers low level resistance to the 14 and 15 membered macrolides only (M phenotype). Mutations in the 23S rRNA ribosomal protein genes confer resistance to macrolides and streptogramin B antibiotics (MS phenotype) (33).

The fluoroquinolones, particularly the older ones such as ciprofloxacin also exhibit active efflux which removes the drug from the bacterial cell but more commonly resistance is due to point mutations in the DNA gyrase or topoisomerase IV enzymes that are involved in bacterial DNA replication (17, 96).

Chloramphenicol inhibits protein synthesis by targeting peptidyl transferase during translation. Resistance is the result of the chloramphenicol acetyltransfease enzyme production which converts the drug to its derivatives which cannot bind to the target enzyme(17).

Sulphamethoxazole and trimethoprim work by interfering with the folic acid synthesis in the bacterial cell. Resistance is due to alteration of the target enzymes, dihydropteroate reductase and dihydrofolate reductase respectively (17).

The viridans streptococci and *S. pneumoniae* are naturally competent and capable of transferring genetic material (73). There have been reports detailing the transfer of most antibiotic resistance mechanisms from resistant strains of *S. pneumoniae* (16, 20, 57, 58)

As far as we know the susceptibility profile of *S. pseudopneumoniae* has not been studied in any detail. One of the aims of this study is to therefore determine the susceptibility pattern of *S. pseudopneumoniae* and compare it to that of *S. pneumoniae* and other viridans streptococci both in New Zealand and globally.

Whilst collecting isolates from sputum specimens for characterisation it was noted that a number of the "atypical pneumococci" had been initially identified as pneumococci with reduced penicillin susceptibility (personal observation). In 2002, Wester et al (119) reported that misidentification of oral streptococci could be a possible cause of overestimation of resistance among *S. pneumoniae.* The recognition of the new species, *S. pseudopneumoniae*, adds credibility to this idea. The need for correct identification and determination of susceptibility patterns is therefore essential.

1.7.10 Prevalence of *S.pseudopneumoniae*

As *S. pseudopneumoniae* has been so recently recognised there have been no studies to determine the carriage rate of *S. pseudopneumoniae* in adults. Another aim of this study therefore was to determine the prevalence in the Christchurch area. It had also been noted during the initial study to characterise *S. pseudopneumoniae*, that it was disproportionately isolated from patients with chronic obstructive airways disease (COPD). Together with the Canterbury Respiratory Research Group, this apparent association was investigated further to determine whether this observation could be confirmed.

1.8 Outline of the thesis

The overall aim of this thesis is to characterize this recently described species, determine its susceptibility profile and establish whether it plays any role in COPD. The thesis will be presented in 4 parts.

The aim of the first part is to characterize the recently recognized species, *S. pseudopneumoniae* using readily available laboratory techniques including morphology, phenotypic and genotypic methods.

In part 2 the susceptibility profile of *S. pseudopneumoniae* is to be determined by disc susceptibility and MIC (minimum inhibitory concentration) testing. The resulting profile will be compared with data available nationally and globally.

The third part will examine the possible association with COPD and the final part will determine the carriage rate of *S.pseudopneumoniae* and its prevalence in sputum specimens from the Christchurch area.

All the experimental work has been performed by the author apart from the following.

- Ros Podmore and colleagues working in the respiratory section of Canterbury Health Laboratories collected and stored the isolates for characterization.
- Alvin Chua performed the pneumolysin PCR using an in- house method designed by Trevor Anderson.
- Professor David R. Murdoch collated the patient details and performed the statistical analysis in Chapter 5.
- Specimens for the prevalence study were collected and transported to the laboratory by Fiona McCartin of the Canterbury Respiratory Research Group.

Chapter 2

General Methods

2.1 Introduction

The following are some of the key methods used throughout the study using media supplied by Fort Richard (Auckland, NZ). Any deviation from these methods is highlighted in appropriate section.

2.2 Processing of sputum specimens

2.2.1 Gram stain

Smears of sputum samples were prepared by selecting purulent portions of sputum and squashing them between 2 clean glass slides to create a uniformly thick smear. These were stained using a modification of Gram's method;

- Gentian violet for 20 seconds
- Lugol's iodine for 20seconds
- Rinsed with acetone for 5 seconds
- Counterstained with safranin 20 seconds

The smears were examined under lower power (x10) to determine the quality of the specimen. Specimens having >25 leucocytes/low power field (lpf) and <10 epithelial cells/lpf were considered to be of good quality and accepted into the study (111).

2.2.2 Sputum culture

All sputa were processed by inoculating purulent portions onto

- Sheep Blood Agar (SBA) + 6mm optochin 5μg disc (Oxoid, Basingstoke, UK) placed at the junction of the primary inoculum and second streak
- Chocolate Agar + bacitracin 10μg disc
- MacConkey Agar

- CNA (Blood agar+ colistin + nalidixic acid) +optochin disc.

All plates were incubated at 36°C for 48 hours in 5% CO_2. The plates were examined after 24 and 48hours incubation for predominant cultures of α-haemolytic streptococci.

2.3 Storage of Isolates

Isolates of interest were stored at -80°C in beads + serum. Prior to testing they were subcultured twice onto Sheep Blood Agar incubated at 36°C in 5% CO_2 for 24 hours to check for purity.

2.4 Colonial Morphology

Colonial morphology was determined after 24hours incubation on SBA at 36°C in 5% CO_2. Morphology was described on the basis of three criteria;

- Size of colony- small < 1 mm and large >2mm
- Consistency of colony – mucoid, smooth or dry surface
- Appearance – domed or with depressed centres resembling ”draughtsmen”
- Entire or rough edge

Type strains of *S. pneumoniae* and (ATCC 49619) and *S. pseudopneumoniae* NZRM 4311 (ATCC BAA-960) were used for comparison.

2.5 Optochin Susceptibility

Optochin susceptibility was performed by inoculating a single colony of each isolate onto one half of two SBA plates and placing a 6mm optochin disc (5μg) in the centre of the inoculum. One plate was incubated at 36°C in 5% CO_2 for 24hours, the other at 36°C in ambient air for 24 hours.

Zone sizes were measured and interpreted in accordance with ASM criteria

< 14mm = resistant : > 14mm = susceptible (59).

2.6 Bile Solubility

Two methods were used to determine whether isolates were soluble in bile salts (sodium deoxycholate).

2.6.1 Plate Bile Solubility

A drop of 2% sodium deoxycholate solution (Sigma- Aldrich, NZ) was added to well isolated colonies of a 24 hour culture of each isolate on Columbia SBA. The plates were incubated at 36°C for 15 minutes and examined.

Colonies that completely disappeared after incubation were recorded as bile soluble; colonies that remained visible were recorded as bile insoluble.

2.6.2 Tube Bile Solubility

Each isolate was inoculated into 1.5 ml of Phosphate Buffered Saline (PBS) pH6.8 to give a density equivalent to 1.0 McFarland.

A 0.5ml aliquot was transferred to a second tube containing an equal volume of 2% sodium deoxycholate to give a final concentration of 1.0% sodium deoxycholate.

Both tubes were incubated at 36°C and examined for clearing every 30 minutes for up to 2 hours.

Tubes remaining turbid when compared to the negative control (PBS only) were recorded as bile insoluble. Tubes showing significant clearing were recorded as bile soluble (59).

Streptococcus pneumoniae (ATCC 49619) and *S. pseudopneumoniae* NZRM4311 (ATCC BAA-960) were included as positive and negative controls respectively.

2.7 Biochemical Testing

Biochemical testing was performed using the API® rapid ID32 STREP system (bioMérieux, SA, France). Briefly, growth from an overnight anaerobic culture on Columbia blood agar was confirmed as a viridans streptococcus on the basis of α-haemolysis, Gram stain and negative catalase reaction as recommended by the manufacturer for viridans streptococci (11). The growth was harvested using a sterile swab and suspended in 2.0ml of sterile distilled water without additives to give a dense inoculum ≥4.0 McFarland. The manufacturer states that a 48hr culture is preferable for isolates other than the β-haemolytic streptococci and enterococci due to the colony size. At CHL, repeated use of the rapid ID32 STREP has shown that the density of inoculum is the important factor in reliable testing. Where necessary, growth from more than one plate was used to give a sufficient inoculum. After mixing, 55.0µl was dispensed into each of the 32 cupules of the strip. The inoculated strips were incubated at 36°C ± 2°C for a maximum of 4½ hours under aerobic conditions.

Reagents were added to the following tests to reveal results

- Voges –Proskauer: 1 drop of VP A and VP B reagents (5% α- naphthol in ethanol hydroxide and 40% potassium hydroxide respectively)
- Hippurate : 1 drop of ninhydrin reagent
- Tests APPA to GTA: 1 drop of Fast Blue (bioMérieux)

The reactions were compared with the reading chart provided and recorded as positive or negative. The results were read in groups of 3 to produce an 11 digit profile number that was interpreted using the API® data base (Version 3).

2.8 Quellung Reaction (Capsular swelling)

The Quellung reaction was performed using Pneumococcal Omni serum (Statens Serum Institut, Copenhagen, Denmark) at a titre of 1:4 as recommended by the manufacturer using the method described below (22).

A small amount of bacterial growth was taken from a sheep blood agar plate incubated overnight at 36°C in 5% CO_2, mixed with a drop of saline on a clean glass

slide and spread to create a light smear containing approximately 4 organisms per field. If the preparation is too heavy false negative reactions may occur.

The smear was air dried and a small drop of Omni serum was added and spread over the smear. A small drop of filtered 0.3% methylene blue was placed onto a cover slip and inverted over the smear.

Positive and negative controls were prepared in the same way using a wild strain of *S. pneumoniae* and the type strain of *S. pseudopneumoniae* respectively. Quality control strains of *S. pneumoniae* are not used as they lose their capsules over time leading to false negative results.

The smears were examined after 10 minutes under lower power with the condenser set to allow only one third of the light through. The capsules were viewed by switching to oil immersion.

A positive test was indicated by marked swelling of the capsule giving a sharply demarcated halo when compared to the negative control.

A negative test was marked by no difference between the test and control smears.

All smears were re-examined after 30 minutes before being discarded as negative.

2.9 Pneumococcal Antigen test

The NOW™ *S.pneumoniae* Immunochromatographic antigen test (Binax, Portland, ME) detects the C-polysaccharide cell wall antigen (PnC) common to all *S.pneumoniae* strains. The test consists of a hinged book-like device containing a nitrocellulose membrane on which rabbit anti- *S. pneumoniae* antibody is absorbed (sample line). Goat anti-rabbit immunoglobulin G (IgG) is absorbed onto the same membrane as a second (control) line. Rabbit anti- *S. pneumoniae* antibodies are conjugated to colloidal gold particles dried onto an inert fibrous support. Pneumococcal antigen (PnC) binds with the anti- *S. pneumoniae* conjugated antibodies and the resulting complexes are captured by the immobilised anti- *S. pneumoniae* antibodies forming a pink/purple sample line (31).

The test was performed by touching a single, well isolated colony with the swab provided. This was then inserted into the device and 6 drops of reagent were

added to the strip as described in the manufacturer's instructions for QC testing. The device was closed and incubated at room temperature. It was examined after 15 minutes for the presence of pink lines.

The results were interpreted as below

- Control line only– negative, test performing correctly.
- Lines with both control and test – positive result.
- One line with test only – invalid result, control not working.
- No lines – invalid result

2.10 Molecular testing

2.10.1 DNA probe hybridisation

The AccuProbe™ *Streptococcus pneumoniae* culture identification test (Gen-Probe, San Diego, CA) was performed in accordance with the manufacturer's instructions using 18-24 hour isolates grown on SBA. Briefly, a 1mm colony or several smaller colonies were inoculated into 50 µL of lysis reagent. After addition of an equal volume of hybridisation buffer, the tubes were capped, mixed and incubated at 60°C for 15minutes.

300 µL of selection reagent was added to each tube and after mixing they were incubated for a further 5 minutes at 60°C. During the reaction, the DNA probe combines with target organism rRNA to form a labelled DNA-RNA hybrid. The product is detected by a luminometer with a cut off value of 50,000 relative light units. Readings equal to or greater than this value were considered AccuProbe™ positive.

2.10.2 Pneumolysin PCR

The presence of the pneumolysin gene (ply) in extracted DNA was performed by Alvin Chua of the Virology Department, Canterbury Health Laboratories using an in house method (88).

The method used was a modification of a nested PCR assay using the following primers.

First round

- pn1a 5’ -ATTTCTGTAACAGCTACCAACGA-3’
- pn1b 5’ –GAATTCCCTGTCTTTTCAAAGTC-3’

Second round

- pc11a* 5’-CCCACTCTTCTTGCGGTTGA-3’
- pn11b 5’-TGAGCCGTTATTTTTTCATACTG-3’

DNA was extracted using the QIAamp DNA minikit or QIAamp Viral RNA minikit (Qiagen, Hilden, Germany). Five microlitres of extracted DNA was added to 20.0µl of first round reaction mixture.

Amplification was performed on a thermocycler as below:

- 94°C for 15minutes followed by
- 30 cycles of 94°C for 1minute, 55°C for 1minute and 72°C for 1minute.
- A final cycle of 72°C for 5minute and 4°C for 5minute.

The nested round was performed by transferring 1 µl to 24 µl of the amplicon to the second round PCR mixture and amplified as above but cycling only 20 times. The PCR mixtures were analysed using electrophoresis on 2% agarose gels. Visualisation of the amplicon was achieved by using ethidium bromide and UV illumination.

Chapter 3

Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples

3.1 Background

Streptococcus pseudopneumoniae is a recently described streptococcus that is phenotypically and genetically distinct from *Streptococcus pneumoniae* and other members of the viridans streptococci (3). On the basis of DNA-DNA homology this species is a member of the *Streptococcus mitis-oralis* group of viridans streptococci. It is likely that this new species is similar to strains previously described by other workers as atypical pneumococci (94, 121).

During routine work at CHL we had noticed a number of α-haemolytic streptococci from good quality sputum specimens that could not be classed as *S. pneumoniae* on the basis of available phenotypic tests. Concerned that we may be missing something, sequential isolates of *Streptococcus pneumoniae* and other α-haemolytic streptococci that were the predominant isolate from good quality sputum specimens were stored as isolates of interest.

Upon the publication of the work by Arbique et al (3) it was realised that we might hold one of the largest collections of α- haemolytic streptococci known as atypical pneumococci, including the recently recognised *S.pseudopneumoniae*, providing an ideal opportunity to study this species further.

This chapter describes some of the general characteristics of the *S. pseudopneumoniae* isolates in our collection

3.2 Aim

The primary aim of this study was to examine 805 stored isolates to determine the proportion identified as *S. pseudopneumoniae* and to characterise these isolates on

the basis of phenotypic, biochemical and genotypic tests available in the routine laboratory.

As the isolates had been stored sequentially the prevalence of *S.pseudopneumoniae* was also to be investigated in this hospital based study.

The study was approved by the Canterbury Ethics Committee.

3.3 Methods

All tests were performed as described in chapter 2. Any variations are highlighted.

3.3.1 Isolates

All isolates were recovered from good quality sputum specimens based on the Gram stain and were the predominant isolate on culture. The isolates had been collected before the first description of *S. pseudopneumoniae* and were originally classified as either *S. pneumoniae* or α-haemolytic streptococci on the basis of morphology and optochin susceptibility. Bile solubility (plate method) had been tested on some atypical isolates.

From May 2001 to May 2004 sequential isolates were stored at -80°C until 100 “atypical pneumococci” had been collected. In total 805 consecutive isolates were stored. Collection of the isolates was rigorously controlled to maintain the integrity as far as possible. Isolates to be tested were recovered by preliminary culture onto sheep blood agar incubated at 36°C in 5% CO_2 for 24 hours. Cultures were cultured twice prior to testing. If cultures appeared to be mixed, all colonial types were sub cultured and tested.

3.3.2 Identification Methods

Isolates including were recovered and characterised using the following tests as described in Chapter 2.

- Colonial morphology
- Optochin susceptibility in 5%CO_2 and ambient air
- Plate bile solubility
- Tube bile solubility

All isolates showing resistance or having indeterminate susceptibility to optochin in 5% CO_2 (zone size < 14 mm) but were susceptible in ambient air and were bile insoluble were further characterised using the following tests.

- Biochemical profile by API® rapid 32 STREP
- Detection of pneumococcal capsule
- Detection of pneumococcal antigen
- DNA probe hybridisation by a commercial kit (AccuProbe™, GenProbe)
- Pneumolysin (*ply*)PCR

In addition, the original Gram stains were reviewed.

The testing was performed in batches of 100. In the first batch, all the isolates initially identified as *S. pneumoniae* gave unequivocal results in terms of morphology, optochin susceptibility and bile solubility. In the subsequent batches only the strains initially identified as α-haemolytic streptococci other than *S. pneumoniae* were recovered and tested as above.

Streptococcus pneumoniae was defined as having typical morphology, either draughtsmen or mucoid colonies, optochin susceptible in 5% CO_2 and soluble in bile.

Streptococcus pseudopneumoniae was defined as having small, smooth, domed colonies or colonies or small draughtsmen colonies, having variable susceptibility to optochin (resistant in CO_2 but susceptible in ambient air) and bile insoluble.

3.4. Results

A total of 100 α- haemolytic streptococcal isolates and 705 pneumococcal isolates were collected. All 100 atypical strains were tested as outlined above. Only 29 of the typical pneumococci were tested as they gave consistently unequivocal results.

3.4.1 *S. pneumoniae*

A total of 29 isolates of *S.pneumoniae* were tested. All had typical colonial morphology when compared to the type strain, were susceptible to optochin in 5% CO_2 (zone size >14.0m) and were bile soluble. As these isolates consistently gave the expected results no additional phenotypic or genotypic tests were performed on these isolates and no further isolates initially identified as *S.pneumoniae* were tested.

3.4.2 *S. pseudopneumoniae*

Of the 805 consecutive isolates a total of 35 isolates (4%) from 33 patients were identified as *S. pseudopneumoniae*. The colonial morphology of all isolates was similar to that of the type strain. The colonies of *S. pseudopneumoniae* were typically small (≤1 mm) on sheep blood agar after 24hours incubation in 5 % CO_2, domed with entire edges and a smooth, shiny surface. Some strains had depressed centres making them appear as smaller versions of the draughtsmen colonies typical of *S. pneumoniae*.

All isolates were resistant or had indeterminate susceptibility to optochin in CO_2 but were susceptible to optochin in ambient air, were bile insoluble and did not possess a pneumococcal capsule. All isolates gave positive reactions with the NOW™ pneumococcal antigen test and the AccuProbe™ DNA hybridisation test. The pneumolysin gene (*ply*) was detected in 30/35 isolates. The biochemical profiles were

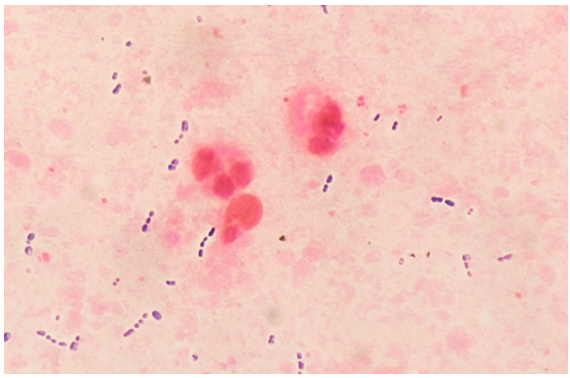
varied but the majority of isolates (25/35) were identified as belonging to the *Streptococcus mitis/oralis* group as did the type strain. All the results are shown in table 2, (Page 42)

Table 2. Characteristics of S. pseudopneumoniae

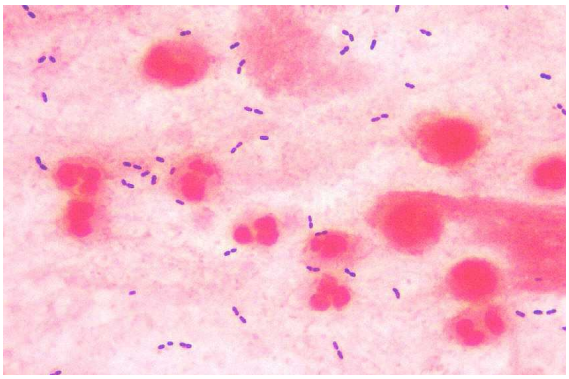
Isolate Number	Optochin zone		Bile soluble	AccuProbe	Ply PCR	NOW Antigen	API rapid 32 STREP	
	CO2	O2					Profile	Identity
22	14	32	-	+	+	+	64032441100	86.7% S. oralis II
28	10	32	-	+	+	+	40012441100	65.5% S. mitis I 33.4% S. oralis II
35	14	24	-	+	+	+	04012541100	73.8% S. mitis I 23.2% S. oralis II
78	14	28	-	+	+	+	24002500000	36.7% Gemella haemolysans
83	12	34	-	+	+	+	44012241100	95.9% S. oralis II
114	10	24	-	+	-	+	44012541100	54.8% S. oralis II 42.5% S. mitis I
134	6	30	-	+	+	+	44132544100	Unacceptable profile
147	6	26	-	+	-	+	04112541100	98.2% S. mitis I
153	12	34	-	+	-	+	44112641100	87.3% S. oralis II
169	13	32	-	+	+	+	44112441100	93.4% S. mitis I
170	13	22	-	+	+	+	64002400000	46.2% S. mitis II
178	8	26	-	+	+	+	44112641100	87.3% S. oralis II
226	10	26	-	+	+	+	04012541100	73.8% S. mitis I 23.2% S. oralis II
288	8	22	-	+	-	+	64036641100	98.3% S. intermedius
369	6	26	-	+	+	+	44012541100	54.8% S. oralis II 42.5% S. mitis I
407	6	26	-	+	+	+	04002500000	48.5% G. haemolysans
418	8	22	-	+	+	+	44112441100	93.4% S. mitis I
431	12	46	-	+	+	+	44012441100	56.1% S. oralis II 43.5% S. mitis I
434	8	22	-	+	+	+	24332441100	96.9% S. sanguis II
441	13	36	-	+	+	+	64112544100	Unacceptable profile
457	10	26	-	+	+	+	64132541100	Unacceptable profile
458	6	24	-	+	+	+	64012560100	Unacceptable profile
474	12	26	-	+	+	+	44012441100	56.1% S. oralis II 43.5% S. mitis I
479	10	28	-	+	-	+	64112441100	56.3% S. mitis I 43.4% S. oralis II
480	6	36	-	+	+	+	04002401100	88.6% G. morbillorum
490	6	36	-	+	+	+	44012441100	56.1% S. oralis II 43.5% S. mitis I
499	10	36	-	+	+	+	44012441100	56.1% S. oralis II 43.5% S. mitis I
554	8	24	-	+	+	+	44112441100	93.4% S. mitis I
589	10	26	-	+	+	+	44002400000	Unacceptable profile
669	9	26	-	+	+	+	44112441100	93.4% S. mitis I
705	10	30	-	+	+	+	44012401100	77.0% S. oralis II
747	6	21	-	+	+	+	44312441100	93.4% S. mitis I
773	8	26	-	+	+	+	44012441100	56.1% S. oralis II 43.5% S. mitis I
785	6	24	-	+	+	+	24012441100	71.4% S. oralis II 20.4% S. mitis I
Reference Strain	13	22	-	+	+	+	44012441100	56.1% S. oralis II 43.5% S. mitis I

On review of the original Gram stain of the sputum specimens, the appearance of *S. pseudopneumoniae* was remarkably similar to that of *S. pneumoniae* i.e. Gram positive lanceolate diplococci. In the Gram stain *S. pneumoniae* often appears to be over decolourised or partially Gram negative, commonly described in this laboratory as "sick". The capsule typical of *S. pneumoniae* is often visible. By comparison the isolates presumptively identified as *S. pseudopneumoniae* appeared to be "healthy". Gram stains showed uniformly Gram positive lanceolate cocci and capsules were not observed. The Gram film from a purulent sputum that grew *S. pseudopneumoniae* as the predominant isolate is shown in Figure 5, (Page 43).

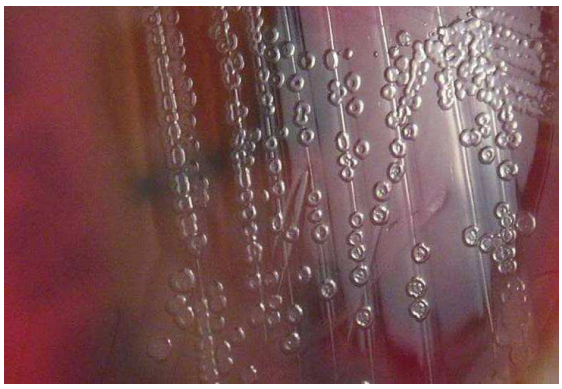
Figure 5. Microscopic and Colonial Morphology of *S. pneumoniae* and *S.pseudopneumoniae*



a. *S. pneumoniae*



b. *S. pseudopneumoniae*

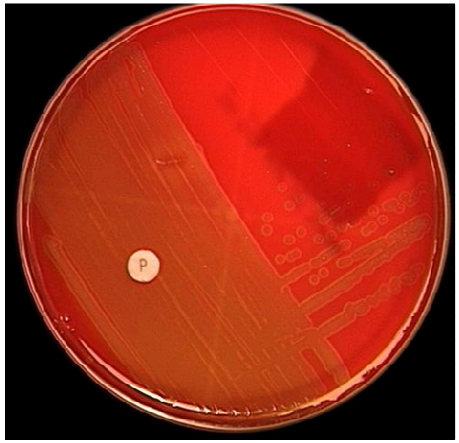


c. *S. pneumoniae*

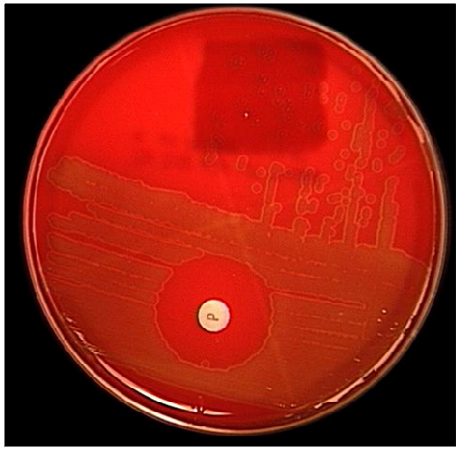
d. *S. pseudopneumoniae*

Figure 6. Phenotypic characteristics of *S. pneumoniae* and *S. pseudopneumoniae*

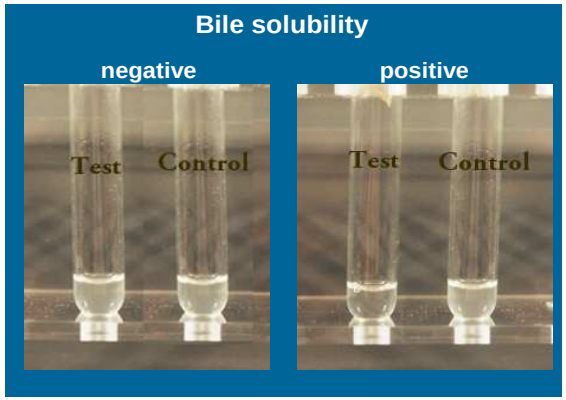
a. *S. pneumoniae* – optochin in CO_2 susceptible.



b. *S. pseudopneumoniae* – optochin resistant in CO2

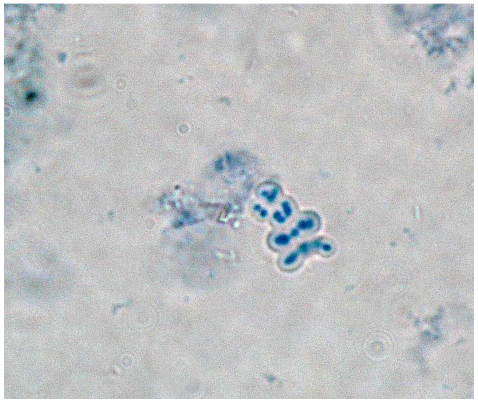


c. *S. pseudopneumoniae* – optochin susceptible in ambient air.

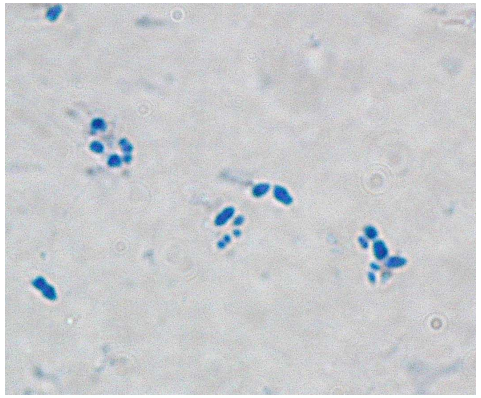


d. *S. pseudopneumoniae* – bile insoluble

S. pneumoniae bile soluble.



e. *S. pneumoniae* – positive quellung reaction.



f. *S. pseudopneumoniae* – negative quellung reaction.

3.5 Discussion

As *S. pseudopneumoniae* has not yet been shown to be of clinical relevance, the major problem at CHL is, at present, correct differentiation from *S pneumoniae*. The aim of this study was to characterise *S. pseudopneumoniae* and determine whether simple phenotypic tests could differentiate it from other members of the viridans streptococci. This study has shown that although *S. pseudopneumoniae* and *S. pneumoniae* share many common features, it is possible to differentiate the two using simple phenotypic tests available in routine laboratories.

The starting point for the differentiation of *S. pneumoniae* from other members of the oral flora is colonial morphology. In this study this proved to be extremely important.

Like most other members of the oral streptococci, *S .pneumoniae* exhibits α-haemolysis, a green effect on sheep blood agar. However, colonial morphology amongst the α-haemolytic streptococci, also known as viridans streptococci, is highly variable in terms of colony size, surface texture and regularity of the colony edge. Amongst the viridans streptococci, *S. pneumoniae* has a distinctive morphology that allows presumptive identification. Typical colonies of *S. pneumoniae* are approximately 2 mm in diameter with entire edges and a very shiny, glossy surface. Due to the action of autolysin, the colonies collapse upon themselves resulting in colonies with central depressions and raised edges that resemble draughtsmen. When large numbers are present the colonies tend to merge or coalesce. Larger, mucoid colonies of *S. pneumoniae* which are also distinctive do occur. They also collapse on themselves but tend to form a flat “sheen” on the surface of the agar, the raised edges typical of draughtsmen not always being so apparent.

By contrast, the colonies of *S. pseudopneumoniae* are smaller than those of *S. pneumoniae* even after 48 hours incubation. Some do appear as small draughtsmen that coalesce which can be misleading. With hindsight, it is probable that strains of *S. pseudopneumoniae* had been erroneously reported as *S. pneumoniae* by CHL prior to this study. However, in this study the colonies never reached the same size as the colonies of *S. pneumoniae*. The colonies are also more matt in appearance, this being most evident when large numbers are present such as on susceptibility test plates. They

do not however have dry, rough surfaces or irregular edges commonly seen amongst other viridans streptococci.

The most commonly used test to confirm the identity of *S. pneumoniae* is susceptibility to optochin. At CHL an optochin disc is applied to the primary inoculum of sputum test plates, the aim being to provide an early aid to diagnosis. However this has created problems. Even when a heavy growth of oropharyngeal flora is present, an apparent zone of susceptibility may be visible around the optochin disc. The exact size of this non standardised zone, particularly if it is on the borderline of 14 mm can be easily misinterpreted. Problems may arise if any zone to optochin is interpreted as a presumptive identification of *S. pneumoniae* without considering the colonial morphology and a preliminary report of pneumococcal infection is issued.

Even when used as an identification test this apparently simple test can be influenced by the test medium, density of inoculum and incubation conditions used. At CHL the test is performed initially by inoculating a single colony onto Columbia sheep blood agar which was shown by Gardam and Millar to produce smaller zones than other media particularly when combined with a large inoculum and incubation in CO_2 (41). The test is confirmed using a standardised (0.5 MacFarland) inoculum on Mueller Hinton sheep blood agar. When optochin was first used as a means of differentiating *S. pneumoniae* the test was performed in ambient air but studies showed that 8% of *S. pneumoniae* strains failed to grow in ambient air (6). As a result the test is now routinely performed in an atmosphere containing 5% CO_2. In this study only 2 isolates failed to grow in ambient air but there were marked differences in the results obtained under the different conditions.

When incubated in CO_2 all the isolates identified as *S. pneumoniae* had zone diameters >14mm when tested against optochin, the mean zone size being 20mm. There was no appreciable increase in zone size when incubated in ambient air. In contrast, all the isolates presumptively identified as *S. pseudopneumoniae* had zone diameters $\leq$14mm in CO_2 (32/35 being between 6mm and 13mm) but had greatly increased zone diameters when incubated in ambient air. These zones ranged from 21mm (increased from 6mm) to 46mm (increased from 12mm). Overall increases ranging from 9mm to 34mm were observed.

Many different methods have been described for the determination of bile solubility. Despite the differences in methodology, bile susceptibility testing is associated with a sensitivity exceeding 98% and a specificity of 100% (9, 24). Although

all the isolates initially identified as *S. pneumoniae* on the basis of colonial morphology and optochin susceptibility were soluble in bile salts by both methods, bile solubility amongst the α-haemolytic streptococci tested was harder to establish particularly by the plate method. One of the major problems encountered was that even strains of α-haemolytic streptococci known to have originated from a single colony often appeared to be mixed. On occasions some of the colonies disappeared completely whilst others remained visible making interpretation difficult (personal observation). Whilst methods and guidelines for interpretation vary, i.e. clearing vs. significant clearing, the inclusion of insoluble control strains made interpretation of the tube test easier. Although it takes longer to perform, the tube method for determination of bile solubility is now the method of choice at CHL. However, the method is still subjective and does need to be reviewed and standardised. Particular areas to be reviewed include the use of phosphate buffered saline rather than isotonic saline, density of the inoculum, concentration of bile salts and possible use of an indicator to help visualise the endpoint as has been used in some methods (77).

Obregon et al showed that whilst *S. pseudopneumoniae* was insoluble in bile salts at a concentration ≥ 1.0% it was soluble at lower concentrations i.e. 0.1% due to the mutations in the L-amidase gene (94). This is a potential characteristic that may be used in addition to variable optochin susceptibility to identify *S. pneumoniae* in the routine laboratory.

In the study by Arbique (3), the biochemical profile of *S. pseudopneumoniae* was determined using the API 20 STREP system. The method gave varied identifications and proved to be of little use. Although *S. pseudopneumoniae* is not included in the database, the API® rapid 32Strep system, which is currently in use at CHL, was substituted in this study. The aim was to determine how closely the isolates resembled other viridans streptococci and determine whether a particular test or tests, not included in the API 20 STREP system, may present as a method of distinguishing it from other species.

The API profiles were varied but 25/35 strains were identified as belonging to the *S. mitis/oralis* group on the basis of the current API database (version 3), the most common profile being 44012441100 as shown in the results Table 4, (Page 49).

The percentage of positive reactions for *S. pneumoniae, S. pseudopneumoniae* all the study strains and the most common profile is shown in table 4, (Page 49).

On the basis of these results, the test most likely to be helpful in differentiating *S. pneumoniae* from *S. pseudopneumoniae* is alkaline phosphatase activity although others as shown in Table 3, (Page 48) may also be useful if used together with other characteristics such as variable optochin susceptibility.

Table 3. Tests useful for differentiation of S.pneumoniae and S.pseudopneumoniae

	% positive	
Test	*S.pneumoniae*	*S.pseudopneumoniae*
PAL	1	100
αGAL	84	0
RAF	84	0
TRE*	100	0

*Included on basis of the most common profile obtained for study isolates.

However, the percentages for *S. pseudopneumoniae* are based only on the results for 2 strains including the same control strain used in this study (55). Interestingly, in the Hoshini study, both strains of *S. pseudopneumoniae* were negative for fermentation of pullulan. By comparison the control strain used here and 83% of test strains were positive for pullulan fermentation as are most strains of *S. mitis* and *S. oralis*. The control strain of *S. pneumoniae* used in this study gave an unacceptable profile and failed to identify. The strain used (ATTC 49619) was a routinely used laboratory strain subcultured on multiple occasions and as such may have adapted to laboratory conditions. With hindsight a freshly subcultured strain may have been more appropriate. The results were no help in distinguishing *S. pseudopneumoniae* from *S. mitis* or *S. oralis*, both of the latter species giving positive results for all tests to varying degrees.

Table 4. Characteristics of α- haemolytic streptococci as determined by the API rapid ID 32 Strep system.

	%POSITIVE																															
	ADH	βGLU	βGAR	βGUR	αGAL	PAL	RIB	MAN	SOR	LAC	TRE	RAF	VP	APPA	βGAL	PYR	βNAG	GTA	HIP	GLY	PUL	MAL	MEL	MLZ	SAC	LAR	DAR	MβD	TAG	βMAN	CDEX	URE
Study Isolates (n=35)	0	31	77	0	0	98	33	5	0	86	14	0	0	100	3	31	8	100	0	0	83	83	0	0	86	0	0	0	0	0	0	0
Most common profile (n=6)	0	0	100	0	0	100	0	0	0	100	0	0	0	100	0	0	0	100	0	0	100	100	0	0	100	0	0	0	0	0	0	0
*S.pneumoniae**	26	26	88	1	84	1	0	0	0	99	95	84	1	99	2	23	74	90	1	1	74	79	5	0	98	1	0	14	2	0	0	0
*S.pseudo - pneumoniae***	0	50	100	0	0	100	50	50	0	50	50	0	0	100	0	0	0	50	0	0	0	50	0	0	50	0	0	0	50	0	0	0

ADH, arginine dihydrolase; βGLU , β- glucosidase; βGAR, β-galactosidase(*p*-nitophenyl-β-D-galactopyranoside as substrate); βGUR, β-glucuronidase; αGAL, α-galactosidase; PAL, alkaline phosphatase; VP, Voges-Proskauer; APPA, alanine-phenylalanine- -proline- arylamidase; βGAL, β-galactosidase(2-naphthyl-β-galactopyranoside as substrate);PYR, pyrrolidonyl arylamidase; βNAG, *N*- acetyl- β-glucosaminidase; GTA, glycl-tryptophan arylamidase; HIP, hippurate hydrolysis; βMAN, β-mannosidase; MβD, methyl-β-D-glucopyranoside; URE, urease; RIB, MAN, SOR, LAC, TRE, RAF, GLY, PUL, MAL, MEL, MLZ, SAC, LAR, DAR, TAG, CDEX-fermentation of ribose, mannitol, sorbitol, lactose, trehalose, raffinose, glycogen, pullulan, maltose, melibiose, sucrose, L-arabinose, D-arabitol, tagatose and cyclodextrin respectively.

* based on API database
** based on 2 strains (55)

The Quellung reaction was easier to perform than expected, the only real problem being the density of the inoculum. If it is too heavy, i.e. too many organisms on the slide, the capsular swelling can be inhibited leading to false negative results (5). Once the technique was established the test gave good clear cut results, all study isolates failing to exhibit capsular swelling indicating a lack of pneumococcal capsular antigens. Although good for differentiating *S. pneumoniae* and *S. pseudopneumoniae*, it is however expensive in terms of reagents and would not be financially viable in a routine laboratory that may only be testing a few isolates.

In the study by Arbique (3) the AccuProbe, pneumolysin PCR and coagglutination (antigen) tests were positive for all strains. These tests were therefore included in this study to help select appropriate strains for investigation and to highlight the similarity between *S. pneumoniae* and *S. pseudopneumoniae*. The coagglutination test was replaced by the Binax NOW antigen test as it was the test in use at CHL and had previously been shown to give a positive reaction with *S.mitis* (90)

All the isolates of *S. pseudopneumoniae* produced a positive result with the NOW *S. pneumoniae* antigen test, indicating that the cell wall polysaccharide antigen is shared between the species. Whilst this highlights the similarity of the two species, it is unlikely to cause a problem as the NOW antigen test is designed to detect pneumococcal antigen in CSF and urine, fluids not likely to contain viridans streptococci other than *S. pneumoniae.* It does however have implications for other pneumococcal antigen tests that can be used on other specimens. For example, the Wellcogen latex test (Remel, Europe Ltd) is used at CHL on sputa that have equivocal Gram stains i.e. those films demonstrating organisms that may or may not be *S. pneumoniae.* Positive antigen results, however strong, have been used to qualify equivocal Gram films and give an early, if presumptive, diagnosis of pneumococcal infection. In light of the similarity of *S. pneumoniae* and *S. pseudopneumoniae* in the Gram stain and the sharing of the cell wall antigen, this test may not be as reliable as once thought. It is therefore recommended that this test should only be used as an aid to laboratory isolation and not as a diagnostic tool when dealing with non sterile respiratory specimens.

As in previous studies (3, 121) all the isolates gave positive reactions with the AccuProbe. These results suggest that the AccuProbe cannot reliably distinguish *S pneumoniae* from *S. pseudopneumoniae* and again highlights the similarity between the two.

Arbique et al also reported that the pneumolysin gene (*ply*), thought to be unique to *S. pneumoniae* was also possessed by *S. pseudopneumoniae* (3).

In this study 5 isolates gave a negative result. On review it was found that although they did not resemble *S. pneumoniae* and were bile insoluble, they consistently produced zones to optochin when incubated in CO_2. Biochemical testing identified 4/5 as being *S. mitis/oralis.* All 5 fitted the profile in all other aspects. The negative results may have been due to technical error and with hindsight should have been repeated. Alternatively, these strains may represent another subset of atypical *S. pneumoniae* or *S.mitis* that warrant further investigation.

3.6 Conclusions

S. pseudopneumoniae is a new species that, whilst very closely resembling *S. mitis, S. oralis* and *S. pneumoniae,* is genetically distinct. It may be defined as below.

S. pseudopneumoniae is α haemolytic on sheep blood agar. The colonies are small, approximately 1mm in diameter after 24 hours incubation in 5% CO_2, with entire edges. The colonies have a smooth, shiny, surface and may be domed or form small draughtsmen like colonies that coalesce. It is characterized by having variable optochin susceptibility, resistant in 5% CO_2 and susceptible on ambient air. It is insoluble in 1% sodium deoxycholate and lacks the pneumococcal capsule. It gives a positive reaction with the Binax NOW antigen test and AccuProbe ™ DNA Hybridization test. Most strains possess the pneumolysin (ply) gene.

S. pneumoniae is also α haemolytic on sheep blood agar but the colonies are larger after 24 hours incubation in CO_2 particularly in the case of mucoid strains. The edges are entire and the colonies typically collapse to form draughtsmen like colonies that coalesce. Mucoid strains collapse to form flat patches on the surface of the agar. The colonies have a very shiny surface that is much glossier than that of
S. pseudopneumoniae. Streptococcus pneumoniae is susceptible to optochin in both CO_2 and ambient air, is soluble in 1% sodium deoxycholate and possesses the pneumococcal capsule. It gives a positive reaction with the Binax NOW antigen test, AccuProbe DNA Hybridization test. All strains possess the pneumolysin (ply) gene.

From personal observation, *S. mitis* and *S. oralis* form colonies similar in size or slightly larger than those of *S. pseudopneumoniae*. The colonies have entire edges and a smooth surface after 24 hours incubation in CO_2. They do not coalesce. The colonies of *S. oralis* have a waxy texture when touched with an inoculating loop. They are resistant to optochin and bile insoluble. Their reactions with the NOW antigen test, AccuProbe and pneumolysin PCR are unknown.

As the pathogenicity of *S. pseudopneumoniae* has not been established the major problem is, at present, being able to differentiate it reliably from *S .pneumoniae*. This study shows that this can be achieved by taking careful note of colonial morphology and performing the optochin test in CO_2. All isolates having atypical morphology and/or reduced susceptibility to optochin should be confirmed by optochin susceptibility in ambient air and bile solubility. Biochemical testing is of limited value as the database does not yet include *S. pseudopneumoniae* so cannot give a reliable identification and commercial molecular tests cannot at present differentiate the species.

The study has some limitations, primarily that all the isolates were collected from patients attending one tertiary health care centre in Christchurch. The sample may therefore not be a true reflection of the amount of *S. pseudopneumoniae* in the general community. Further studies would be required confirm these findings.

However, *S. pseudopneumoniae* isolates have been described in North America and it likely that some of the atypical strains reported from Europe are the same species (24, 94, 121).

Chapter 4

Antimicrobial susceptibility profile of *Streptococcus pseudopneumoniae*

4.1 Introduction

Streptococcus pseudopneumoniae is a recently described member of the *Streptococcus mitis/oralis* group of viridians streptococci that share some characteristics with *Streptococcus pneumoniae* (3, 121).The clinical relevance of *S. pseudopneumoniae* has not yet been established. As yet, the antibiotic susceptibility profile of *S. pseudopneumoniae* has not been reported in detail.

The aim of this study was to determine the antibiotic susceptibility profile of a large number of *S. pseudopneumoniae* isolates recovered from clinical specimens.

4.2 Methods

4.2.1 Isolates

Ninety five isolates of *S. pseudopneumoniae* that had been collected between 2000 and 2007 were studied. These included the 35 strains that had been characterised in the original study (Chapter 3) and 60 that were subsequently collected. All had been isolated from consecutive good quality sputum specimens (>25 leucocytes and ≤10 squamous epithelial cells/x 100 field) showing a Gram stain and culture predominance. Only one isolate per patient was included. The isolates were assigned to the species on the basis of phenotypic characteristics as previously described in Chapter 3. In addition *S.pseudopneumoniae* NZRM4311 (ATCCBAA-960) and *S.pneumoniae* ATCC 49619 were included as control strains.

4.3 Susceptibility testing

All susceptibility testing was performed in accordance with CLSI guidelines (19) using Mueller- Hinton susceptibility test media plus sheep blood and an inoculum equivalent to 0.5 McFarland prepared in Mueller Hinton Broth (Oxoid, Basingstoke, UK).

Results were interpreted using Zone Diameter and MIC Interpretive Standards for Streptococcus spp. Viridans Group. (Appendix 1)

4.3.1 Disc diffusion susceptibility test

The following antibiotic impregnated discs (Oxoid, Basingstoke, UK) were applied to seeded plates in accordance with Canterbury Health Laboratories testing procedures.

- Oxacillin 10µg (surrogate disc for penicillin)
- Erythromycin15µg
- Tetracycline (30µg)
- Sulphamethoxazole/trimethoprim
- Vancomycin 30µg

Plates were incubated at 36 °C for 24 hours, zone diameters recorded and interpreted as susceptible or resistant according to CLSI guidelines (19)

4.3.2 Broth Microdilution test

Minimum inhibitory concentrations (MIC's) were determined by broth dilution using the MicroScan Micro STREP *plus* 1 system (Dade Behring, West Sacramento, CA).The MIC's for penicillin, ampicillin, ceftriaxone, cefotaxime, cefepime,

meropenem, chloramphenicol, clindamycin, erythromycin, azithromycin, tetracycline, vancomycin, gatifloxacin, levofloxacin and sulphamethoxazole/trimethoprim were determined. Susceptibility breakpoints were based on CLSI guidelines (19)

A standard inoculum was prepared in 3.0ml saline (0.9%) to give a turbidity equivalent to that of 0.5 McFarland. After vortexing 0.1ml was added to 25ml of Mueller- Hinton broth supplemented with 3% laked blood, mixed, and used to inoculate the antibiotic panels using the Renok inoculator in accordance with the manufacturer's instructions.

After incubation in ambient air for 20-24hrs at 35°C the plates were wiped to remove condensation and examined for growth using a concave mirrored plate reader. Only panels showing growth in the control wells were read.

Growth was determined by haze throughout a well, a button in the centre of the well or a fine granular growth throughout the well. The MIC for an antibiotic was recorded as the first clear well in a series of dilutions.

4.3.3 MIC determination by E-Test

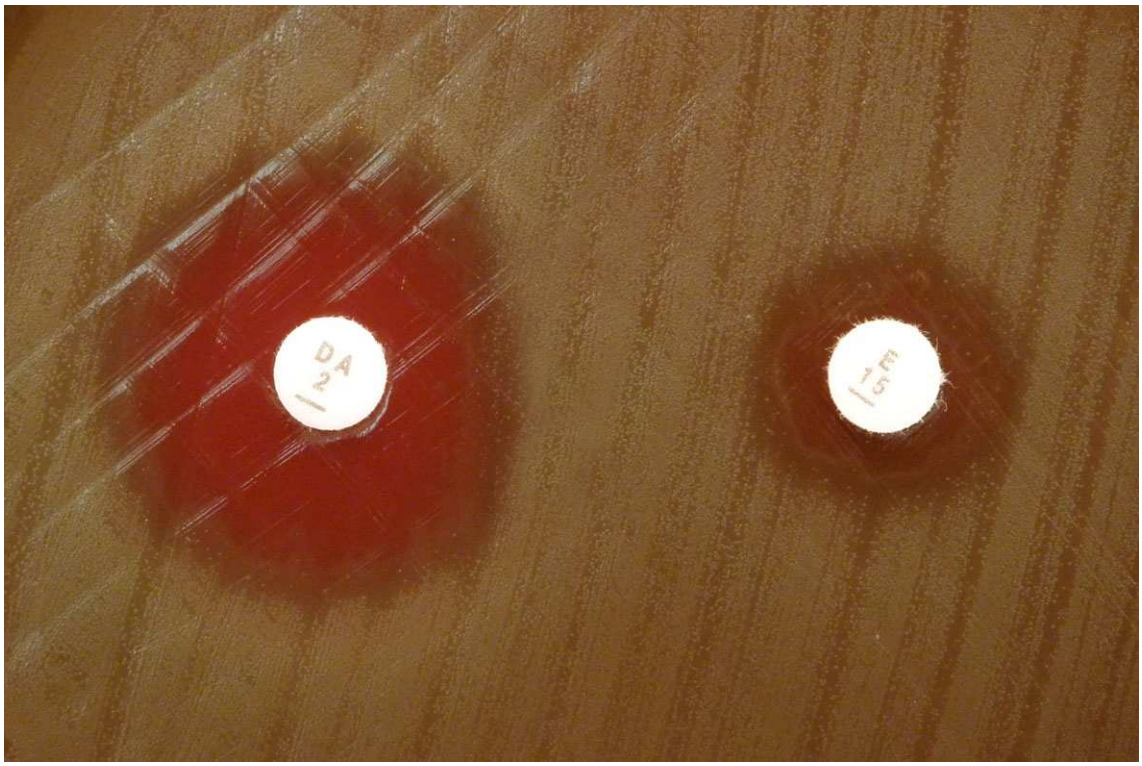
Strains showing MIC's greater than the highest dilution included on the MicroScan panel were retested using E-Test (AB BIODISK, Solna, Sweden) to determine the precise MIC. The plastic strips are impregnated with specific antibiotics to create a gradient, the lowest concentration being at the bottom of the strip and the highest at the top. The antibiotics diffuse into the medium and inhibit of growth of susceptible organisms resulting in an elliptical zone, the size of which is dependant on degree of resistance present. The point at which growth crosses the gradient is taken to be the MIC i.e. the minimum concentration that inhibits growth. If growth is inhibited below 1μg/ml the absolute figure is recorded. For MIC's greater than 1μg/ml the MIC is recorded as the next doubling dilution as in traditional methods.

A standard inoculum (0.5 McFarland) was prepared for each strain in Mueller-Hinton broth. This was used to inoculate the surface of STA +sheep blood (Fort Richard, Auckland, and NZ) to give confluent growth and appropriate E-Test strips were applied. The plates were incubated at 35°C in 5% CO_2 for 18-24 hrs and read in accordance with the manufacturer's instructions.

4.3.4 MLS_{β} detection

An additional plate was inoculated for strains showing resistance to erythromycin but susceptibility to clindamycin to determine the presence of macrolide-lincosamide-streptogramin β resistance (MLS_{β} phenotype). Discs containing erythromycin (15μg) and clindamycin (2μg) were placed 20 mm apart on the plate. After incubation the plates were examined for flattening of the clindamycin zone, a 'D-zone' indicative of the phenotype. (Fig7, Page 56)

Figure 7. Inducible clindamycin resistance in *S. pseudopneumoniae*



4.4 Results

The MIC's for the 15 antibiotics are shown in table 5, (Page 58).

No isolate was resistant to the β lactam antibiotics, although about one third showed reduced susceptibility to penicillin and ampicillin. On preliminary disc testing all isolates demonstrating reduced susceptibility exhibited penicillin resistance based on

the use of oxacillin (1μg disc) suggesting that disc testing for penicillin susceptibility is, as for other members of the viridians group, unreliable.

About one third of isolates were resistant to macrolides and almost half were resistant to tetracycline. Overall 71% showed reduced susceptibility to tetracycline and/or macrolide antibiotics (erythromycin and azithromycin).

True resistance to the macrolide antibiotics alone (M phenotype) occurred in 7 isolates. An additional isolate was resistant to clindamycin (intrinsic MLSβ resistance).

Inducible MLS_{β} resistance was noted in 3/28 erythromycin resistant, clindamycin susceptible isolates.

Reduced susceptibility to erythromycin or clindamycin alone occurred on only 3 occasions. In each case the MIC was within one dilution difference from susceptible and possibly due to technical error.

All azithromycin resistant isolates were resistant to erythromycin suggesting that resistance could be inferred.

No resistance was observed for the fluoroquinolones or vancomycin.

Table 5. In vitro activity of antimicrobial agents against *S.pseudopneumoniae*

	MIC μg /ml				
Antimicrobial agent	Range	50%	90%	% reduced [a] susceptibility	% resistant
Penicillin	≤0.03 – 2.0	<0.03	1.0	32.0	0.0
Ampicillin	≤0.06 – 4.0	<0.06	2.0	31.0	0.0
Cefotaxime	≤0.25 – 2.0	<0.25	0.5	0.0	0.0
Ceftriaxone	≤0.25 – 1.0	<0.25	0.5	0.0	0.0
Cefepime	≤0.25 – 1.0	<0.25	1.0	0.0	0.0
Meropenem	≤0.06 – 0.5	<0.06	0.5	0.0	0.0
Erythromycin [b]	≤0.06- ≥256	0.25	≥256	46.0	35.0
Azithromycin	≤0.25- ≥256	<0.25	≥256	41.0	32.0
Clindamycin	≤0.06- ≥256	<0.06	≥256	17.0	16.0
Gatifloxacin	≤0.12 – 0.5	0.25	0.25	0.0	0.0
Levofloxacin	≤0.25 – 1.0	1.0	1.0	0.0	0.0
Tetracycline	≤0.5 – 24.0	4.0	4.0	54.0	45.0
Vancomycin	≤0.12 – 0.5	0.25	0.5	0.0	0.0
Chloramphenicol	≤1.0 - ≥256	2.0	2.0	2.0	2.0
Sulphamethoxazole	≤0.5 - ≥2.0	<0.5	>2.0	-	-
Trimethoprim	-	-	-	-	-

[a] Clinical and Laboratory Standards Institute (19) – No breakpoints established by CLSI

[b] Phenotypic MLSβ resistance exhibited by 3/28 isolates with reduced susceptibility

4.5 Discussion

This is believed to be the first systematic evaluation of the antimicrobial susceptibility pattern of *S. pseudopneumoniae*.

The key findings of this study were decreased resistance to penicillin but high level resistance was not detected. Forty six per cent of strains showed decreased susceptibility to macrolide antibiotics with 35 % having high level resistance. Inducible MLS_{β}

resistance was noted in 10% of erythromycin resistant strains. Increased resistance to tetracycline was also noted in 54% of strains with 45% showing high level resistance.

In general, susceptibilities of viridans streptococci of oral origin are not reported and it is therefore difficult to compare our results. In one European study the level of resistance for all antibiotics other than levofloxacin was lower than that seen in this study (107). Resistance to levofloxacin was 1.9% whereas no resistance was noted in this study. A second European study showed similar results but also showed true resistance to penicillin (2.3%) (70). In studies of Asia-Pacific isolates the level of resistance to erythromycin, clindamycin, tetracycline and chloramphenicol compare well to those found here although once again resistance to levofloxacin and penicillin was higher (3.1% and 9.3% respectively) (10, 48). When compared to resistance among New Zealand isolates of *S. pneumoniae* a similar pattern was seen. The level of resistance demonstrated to the classes of antibiotic investigated was higher for *S. pseudopneumoniae* except in the case of high level penicillin resistance (53).

In a 2008 study penicillin resistance among New Zealand strains of *S. pneumoniae* isolated from sputum was reported to be 20.2% (52). This figure is based on oral treatment guidelines which are lower than those for invasive disease. No high level resistance was found amongst the strains of *S. pseudopneumoniae* tested but intermediate resistance was detected in 32% of strains. However, in retrospect, the guidelines for invasive disease were used in the study. If interpreted using the same guidelines penicillin resistance amongst *S. pseudopneumoniae* may have been higher than that for *S. pneumoniae*. For the macrolides antibiotics and tetracycline resistance amongst *S. pseudopneumoniae* was higher than for *S. pneumoniae* (46% vs 20% and 54% vs 23% respectively). The figures for constitutive clindamycin were the same (17%) but the rate of inducible MLS_{β} resistance was higher for *S. pseudopneumoniae*.

In a 1996 study penicillin resistance amongst 219 strains of *S. mitis* was only 16% and tetracycline resistance was 29% indicating that resistance has increased dramatically(28). Resistance for erythromycin and chloramphenicol was the same as found in this study (45% and 2% respectively). In that study *S. mitis* was the most resistant of the strains investigated. In a study of blood culture isolates in 2004(114), intermediate penicillin resistance for *S. mitis* was 27% and for erythromycin was 50% which compares well with the results obtained in this study.

Table 6 (page 60) shows how the results obtained in this study compare with those obtained for 264 isolates of viridans streptococci, including 42 isolates of *S. mitis*, in the 2009 LEADER Program (36).

Table 6. Comparison of reduced susceptibility/resistance amongst viridans streptococci and S.pseudopneumoniae

Antibiotic	**Viridans streptococci**		***S. pseudopneumoniae***	
	% Reduced Susceptibility	% Resistant	% Reduced Susceptibility	% Resistant
Penicillin	22.7	3.0	32.0	0.0
Tetracycline	39.4	36.4	54.0	45.0
Erythromycin	53.8	50.8	46.0	35.0
Clindamycin	11.4	9.1	17.0	16.0
Vancomycin	0.4	0.0	0.0	0.0
Ceftriaxone	8.3	3.8	0.0	0.0

Again these figures compare well, the only major differences being the true resistance to penicillin and reduced susceptibility and resistance to ceftriaxone seen amongst the viridans streptococci. These differences may be explained by the inclusion of viridans streptococci other than members of the *S. mitis* group.

There are limitations to this study. In all the studies used for comparison the number of isolates studied was larger, collected from different patient populations and included a number of different species. In this study all the isolates were collected from respiratory specimens from patients attending one tertiary care centre in one geographic area of New Zealand. According to the ESR report (52), penicillin resistance was higher amongst patients from Christchurch. As such they may not be representative of isolates found in the wider community or other regions of the world. Further studies are needed from other geographic regions and, possibly from other sample types in order to better clarify the antibiotic susceptibility profile of *S. pseudopneumoniae.*

However, the results obtained here have helped in the identification of *S. pneumoniae* at CHL. As a result of this work, the bile solubility test is performed on all isolates provisionally identified as *S. pneumoniae* that demonstrate resistance to penicillin, particularly when combined with small colony size and resistance to erythromycin and/or tetracycline, before releasing a report of penicillin resistant *S. pneumoniae*. The additional testing results in a delay of up to 2 hours but avoids the issue of an incorrect report that may have a significant effect on patient management.

Chapter 5

Association of *S. pseudopneumoniae* with COPD

5.1 Background

During the study period it was noted that *S. pseudopneumoniae* had been isolated from one patient on 3 separate occasions over 17 months. This raised the possibility that the isolates may be associated with the clinical presentation of this patient. On review of the clinical history it was discovered that the patient suffered from COPD (chronic obstructive pulmonary disease) and on reflection it was noted that several of the isolates of *S. pseudopneumoniae* came from patients with COPD.

This study examines the potential association between the isolation of *S. pseudopneumoniae* and COPD.

5.2 Methods

A case control study was conducted to determine the association between the presence of *S. pseudopneumoniae* in sputum and the presence of COPD. The cases were 33 consecutive patients who had *S. pseudopneumoniae* isolated from sputum at CHL in Chapter 3. One patient featured in the study on 3 separate occasions but only 1 incident was included in this study. For comparison, two control patients, matched for age and sex, were obtained for each case. The controls were identified from the same database by selecting the next two patients chronologically after the case that were of the same gender and within 5 years of age but did not have *S. pseudopneumoniae* isolated from sputum. The same data as for the cases was collected from the control group. Mantel-Haenszel odds ratios were calculated for comparisons between cases and controls.

Demographic, clinical and laboratory characteristics were obtained for all patients who had *S. pseudopneumoniae* isolated from sputum by review of clinical

records. The same data were collected for the controls and included smoking history, antibiotic use, chest radiograph changes and outcome.

The study was approved by the Canterbury Ethics Committee.

5.3 Results

Clinical, laboratory and treatment data were obtained for 33 patients with positive sputum culture for *S. pseudopneumoniae.* The results are presented in table 7 (page 64).

In 10 patients *Haemophilus influenzae* was also isolated but S. *pseudopneumoniae* was the predominant isolate. In 2 of the patients with mixed infections *Moraxella catarrhalis* and *Pseudomonas aeruginosa* were also isolated (1 case of each). The index patient had 3 positive sputum cultures over 17 months. The isolates had identical phenotypic and genotypic characteristics except for some minor differences in the Rapid ID 32 Strep profiles as shown in the result table in chapter 2. These differences made no difference to the final identification of the isolates, all being identified as belonging to the *S. mitis/oralis* group. Consequently only data related to the first isolation were analysed.

The median age of the patients was 68 years, the range being 15 to 89 years. Nineteen patients were male (58%) and 30 (91%) were inpatients. All patients had lower respiratory tract symptoms with cough and 26 patients had a history of COPD. Exacerbation of COPD was the primary reason for presentation in 17 (52%) cases.

Spirometry data was available in 17 cases. Four patients were of severity II, 5 of severity III and 8 of severity IV according to the GOLD criteria (47). Where data were available, 9/31(29%) had peripheral leucocyte counts >11 x 10^9/l and only 3/30 (10%) had a temperature >37.5°C on presentation.

All the control patients had positive sputum cultures for *S. pneumoniae,* 20 (30%) mixed with other respiratory pathogens predominantly *H. influenzae* and *M. catarrhalis*.

A comparison of the clinical features of cases and controls is shown in Table 8, (Page 65)

Table 7. Characteristics of patients with *S. pseudopneumoniae* isolated from sputum

Patient No.	Age (yrs)	Sex	Clinical Presentation	Current or Ex-smoker	Chest X-ray Infiltrate[a]	Other Bacteria in Sputum	Antibiotic Treatment[b]	Outcome
1	64	F	Exacerbation of COPD	+	-	*H. influenzae*	Amox/clav	Improved
2	82	M	Motor neurone disease, COPD	+	+		None	Improved
3	63	M	Cough, ischemic heart disease, diabetes	-	+		Amox/clav	Readmitted pneumonia
4	63	M	Amaurosis fugax, cough, COPD	+	-		None	Improved
5	89	F	Lobar pneumonia, COPD	+	+		Amoxicillin	Improved
6	80	M	Ischemic heart disease, cough, COPD	+	+		None	Readmitted pneumonia
7	51	F	Exacerbation of COPD	+	-		Ciprofloxacin	Improved
8	15	M	Bronchiectasis	-	-	*H. influenzae*	Cefuroxime	Improved
9	78	F	Pubic rami fractures, cough, COPD	+	-	*H. influenzae*	Amox/clav	Improved
10	57	M	Exacerbation of COPD	+	+		Amox/clav	Improved
11	78	M	Fractures, cough, COPD	+	+		Amox/clav	Improved
12	86	M	Exacerbation of COPD	+	-	*H. influenzae*	Amoxicillin	Improved
13	78	M	Exacerbation of COPD	+	+		Clarithromycin	Improved
14	77	M	Exacerbation of COPD	+	-		Erythromycin	Improved
15	62	M	Exacerbation of COPD	+	ND	*H. influenzae*	Amox/clav	Improved
16	55	F	Ischemic heart disease, cough	+	-		None	Improved
17	81	F	Exacerbation of COPD	+	-		Amoxicillin	Improved
18	68	M	Exacerbation of COPD	+	-	*H. influenzae*	Amoxicillin	Improved
19	52	F	Bronchiectasis	-	-	*H. influenzae*	Amoxicillin	Improved
20	77	M	Exacerbation of COPD	+	+	*H. influenzae*	Roxythromycin	Improved
21	28	F	Asthma	+	ND		Unknown	Unknown
22	74	M	Lung cancer, COPD	+	-	*H. influenzae M. catarrhalis*	None	Improved
23	69	F	Exacerbation of COPD	+	-		Amox/clav	Improved
24	53	M	Bronchiectasis	+	ND		None	Improved
25	50	F	Exacerbation of COPD	+	+		Amox/clav	Improved
26	61	F	Exacerbation of COPD	+	-	*H. influenzae P. aeruginosa*	Amox/clav	Improved
27	77	M	Renal cell carcinoma, COPD	+	-		Roxythromycin	Improved
28	53	M	Asthma, COPD	+	-		Unknown	Unknown
29	66	F	Exacerbation of COPD	+	-		None	Improved
30	72	M	Exacerbation of COPD	+	-		Ciprofloxacin	Improved
31	74	F	Exacerbation of COPD	+	-		None	Improved
32	75	M	Prostate cancer, cough	+	+		Amox/clav	Improved
33	63	F	Exacerbation of COPD	+	-		Amox/clav	Improved

[a]ND = not done; Amoxclav= Amoxicillin+Clavulanic acid

Table 8. Clinical features of patients who had *S. pseudopneumoniae* cultured from sputum compared with control patients who had *S. pneumoniae* cultured from sputum

	Cases[a]	Controls[a]	Odds ratio (95%CI)		P value
History of COPD	26/33 (79%)	36/66 (55%)	5.00	(1.67 to 20.11)	0.002
Exacerbation of COPD	17/33 (52%)	12/66 (18%)	6.50	(2.61 to 16.20)	0.0001
History of bronchiectasis	3/33 (9%)	4/66 (6%)	1.50	(0.36 to 7.23)	0.75
History of asthma	2/33 (6%)	4/66 (6%)	1.00	(0.07 to 13.80)	1.00
Current or ex-smokers	30/33 (91%)	50/64 (78%)	3.00	(0.91 to 12.76)	0.08
Chest radiographic infiltrates	10/30 (33%)	29/54 (54%)	0.45	(0.18 to 1.03)	0.06

[a]Data are number with characteristic/number with available information (%)

5.4 Discussion

It can be difficult to determine whether a micro-organism is a respiratory pathogen or whether it is simply a respiratory tract colonizer. This is especially so in the setting of COPD where lower respiratory tract colonization with potential pathogens may occur even during times of clinical stability (108). In this study, all strains of *S. pseudopneumoniae* were isolated as the predominant or only micro-organism from good quality purulent sputum samples obtained from patients with lower respiratory tract symptoms. Furthermore, in all cases the sputum smear Gram stain results indicated the presence of *Streptococcus species* as the predominant bacteria. These findings are difficult to ignore and provide supporting evidence of a potential pathogenic role of *S. pseudopneumoniae*. In addition, approximately one third of these patients had chest radiographic infiltrates and one third had peripheral leucocytosis. However, all these findings do not constitute definitive evidence of the pathogenic potential of *S. pseudopneumoniae* and more data are required to support this notion. It is interesting to note that about one quarter of patients with *S. pseudopneumoniae* in sputum was not treated with antibiotics and all but one of these patients clinically improved.

The preliminary data indicate that the isolation of *S. pseudopneumoniae* from sputum was associated with both a history of COPD and exacerbation of COPD. Over

three quarters of cases had documented COPD and for at least half of all cases exacerbation of COPD was the primary reason for seeking medical attention. Due to the large proportion of cases with a history of COPD, a case-control study was performed to determine whether this result was a true association or whether it just reflected the background rate of COPD among patients who submit sputum samples to our tertiary hospital laboratory. The findings of this study indicate that COPD was significantly more common among patients with *S. pseudopneumoniae* isolated from sputum than among the control group of patients who did not have *S. pseudopneumoniae* isolated from sputum.

This study has several limitations. It was performed in one tertiary centre in Christchurch and may not reflect the true picture in other areas of New Zealand or globally. The patient information was retrospectively retrieved from clinical records. Some information from the time of sputum collection was sparse, limiting the ability to obtain a detailed clinical picture for some patients. The number of patients with COPD and other respiratory diseases may be underestimated owing to missing information.

The nature of the data from this study is preliminary. To better characterize the potential role of *S. pseudopneumoniae* in exacerbation of COPD it will be essential to examine COPD patients with exacerbations compared to those who do not with regard to isolation of this organism. Ideally, it would be important to follow a cohort of patients with COPD and examine them during both exacerbations and during times of clinical stability. The appearance of *S. pseudopneumoniae* or a new strain of *S. pseudopneumoniae* during exacerbations would support the widely accepted hypothesis that acquisition of a new strain of bacteria plays a causative role in exacerbation of COPD (109). Further work also needs to focus on the natural habitat of *S. pseudopneumoniae*, the epidemiology of *S. pseudopneumoniae* colonization, and the role of *S. pseudopneumoniae* in infections outside the setting of COPD, including pneumonia.

S. pseudopneumoniae isolates have now been described from North America and New Zealand, and it is likely that some of the so-called atypical pneumococci reported from Europe (24, 94, 121) are the same species. Increased awareness of this species will help better determine its prevalence and clinical importance. Preliminary data from this study prompted further research to characterize the role of *S. pseudopneumoniae* in COPD.

Chapter 6

Prevalence of *Streptococcus pseudopneumoniae*

6.1 Background

Chronic obstructive pulmonary disease (COPD) is characterised by chronic airway inflammation and progressive deterioration of lung function. In 90% of cases it is directly attributable to smoking. In New Zealand it is estimated that 15% of adults >45 years old have COPD and it is the 4th most common cause of death after cancer, heart disease and stroke (79). The disease is characterised by acute exacerbations leading to inflammatory tissue damage of the airway. Approximately 50% of exacerbations are caused by bacterial pathogens. These pathogens may also colonise the damaged tissue during periods of stable COPD (95, 108).

The findings presented in Chapter 5 provide some evidence that isolation of *S. pseudopneumoniae* in sputum is associated with COPD. To further investigate this association and to determine the prevalence of *S. pseudopneumoniae* carriage, a cross sectional study was performed involving adults with and without COPD from the Christchurch community.

6.2 Methods

6.2.1 Participants

The aim was to recruit a large group of adults with and without COPD and with varying smoking histories. Respiratory specimens from all participants would then be cultured for *S. pseudopneumoniae* in order to determine the prevalence in this population and to determine whether isolation varied with COPD and smoking status.

Participants were all aged over 40 years and were excluded if any of the following were present:

- Receipt of any antibiotics in the previous 6 weeks
- Presence of any other lung disease e.g. pulmonary fibrosis, cystic fibrosis, lung cancer
- Taking regular corticosteroids
- Presence of an immune suppressed condition e.g. HIV, haematological malignancy

All participants were enrolled from either the Canterbury Respiratory Research Group volunteer databases, through respiratory outpatient clinics or through advertisements. All were resident in the Christchurch area. In addition, to examine *S. pseudopneumoniae* isolation during COPD exacerbations, sequential patients admitted to Christchurch Hospital with COPD exacerbations were recruited. The intention was to recruit 600 participants in total.

On recruitment the participants were assigned to groups according to their COPD status. Patient demographic information was recorded together with smoking history and antibiotic use. The permission of GP's was obtained for all participants who were identified by a unique study number only.

The study was approved by the Canterbury Ethics Committee. Informed consent was obtained from all participants before enrolment in the study.

6.2.2 Definitions

Current smoking was defined as smoking at the time of enrolment with a >10 pack-year history.

Ex- smokers had a >10 pack-year history but had been abstinent for >6weeks.

Those who had never smoked had a <5 pack-year history.

COPD was defined by the GOLD (Global Initiative for Chronic Obstructive Lung Disease) criteria (47)

6.2.3 Specimens

Throat and nasopharyngeal swabs were collected swabs collected from all patients. Sputum specimens were collected from as many acute COPD patients as possible presenting at Christchurch Hospital and from other participants with COPD who expectorated sputum. All specimens were collected under the supervision of trained nursing staff. Specimens were hand delivered to the laboratory immediately after collection to avoid refrigeration which can damage respiratory pathogens.

6.2.4 Microbiology

1. Throat swabs

Throat swabs were inoculated onto

- Sheep blood agar(SBA)

The plates were streaked and an optochin disc placed at the junction of the primary inoculum and first streak. Growth was examined after 24 and 48 hours incubation in 5% CO_2 at 36^oC

2. Nasopharyngeal swabs

Nasopharyngeal swabs were inoculated onto

- Sheep blood agar + optochin disc
- Chocolate agar + bacitracin 10unit disc

Growth was examined after 24 and 48 hours incubation in 5% CO_2 at 36°C.

3. Sputum

All sputum specimens were examined and described on the basis of macroscopic appearance.

Gram stained smears were prepared to determine the quality of each sputum specimen according to the criteria described in Chapter 2.

Purulent portions were inoculated onto:

- Sheep blood agar + optochin disc
- Chocolate agar + bacitracin 10unit disc
- MacConkey agar
- Sheep blood agar + colistin and nalidixic acid (CNA)+optochin disc

Growth was examined after 24 and 48 hours incubation in 5% CO_2 at 36°C.

Preliminary identification of bacterial isolates currently recognised as respiratory pathogens i.e. *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* was made on the basis of colonial morphology. All α haemolytic streptococci that were predominant on culture were tested irrespective of colonial morphology. Any other isolates of possible clinical significance e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Aspergillus fumigatus* were also recorded if predominant on culture. Isolates were confirmed as below.

Streptococcus pneumoniae and Streptococcus pseudopneumoniae

Streptococcus pneumoniae and *S.pseudopneumoniae* were identified on the basis of the phenotypic characteristics described in Chapter 2.

Haemophilus influenzae

Colonies colonially resembling *Haemophilus species* were confirmed as *H. influenzae* by inoculating suspect colonies onto Columbia Agar to produce confluent growth and placing growth factor discs (Oxoid, Basingstoke, UK) onto the plate

- X(haemin)
- V (NAD)
- X+V

Growth around the X+V disc only after 24 hours incubation in 5%CO_2 confirmed the isolate as *H.influenzae*

Moraxella catarrhalis

M. catarrhalis was identified by phenotypic/biochemical characteristics

- positive oxidase reaction
- being able to push the colony across the agar(push test)
- Positive butyrate esterase (BE) test (Remel, Lenexa, KS)

6.3 Results

A total of 418 participants were enrolled into the study. Overall, the female: male ratio of participants was in favour of males, the ratio being 196 female participants (47%) and 222 male participants (53%).

Approximately half the participants were ex smokers. The number of current smokers and those who had never smoked were similar (24% and 23% respectively).

The number of patients who presented with acute exacerbation of COPD was 40 (9.6%). Of the remaining participants 140 had stable COPD (33.5%) and 238 had normal lung function (57%). The distribution of participants with COPD /smoking history is shown in Figure 8, (Page 72).

Table 9 (page 72) shows the demographic and clinical characteristics of the participants. The age range of the participants was 47 years, the youngest being 40 years and the oldest 87 years old. The median age of all participants was 63 years with an interquartile range (IQR) of 51-75 (data not shown)

Figure 8. Distribution of participants by COPD status / Smoking history

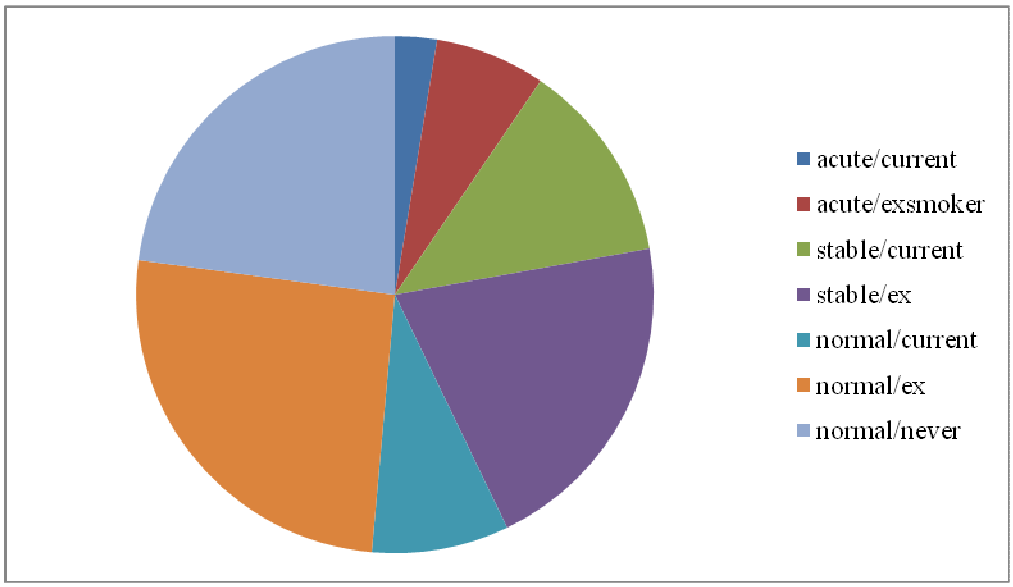


Table 9. Demographic and clinical characteristics of participants

	ACUTE COPD	STABLE COPD	NO COPD	TOTAL
n =	40	140	238	418
age(median)	71	68	66	-
IQR	65-75	61-75	55.5-72.5	-
Female (%)	19 (47.5%)	60 (42.8 %)	117(49%)	196(46.9%)
Smoker	11 (27.5%)	53 (38%)	35 (15%)	99 (24%)
Ex smoker	29 (72.5%)	87 (62%)	108 (45%)	224 (53%)
Antibiotics	23 (57.5%)	0 (0%)	2 (0.84%)	25 (5.98%)

Table 10. Carriage rate of isolates from nasopharyngeal swabs, throat swabs and sputum in participants with and without COPD. (Number/ specimen (%))

Species	Specimen	Acute COPD	Stable COPD	No COPD Current/ ex smoker	No COPD Never smoked	Total
		n=40	n=140	n=138	n=100	n=418
S.pseudopneumoniae	Any	2/40(1.7)	12/140(8.5)	9/138 (6.5)	0	23/418 (5.5)
	NP	1/38 (2.5)	1/140 (0.7)	1/135 (0.7)	0	3/418 (0.7)
	TS	0	2 /140(1.4)	6/136 (4.4)	0	8/418 (1.9)
	SPT	1/35(2.8)	9 /52(17.3)	2/18 (11)	0	12/418(2.8)
S.pneumoniae	Any	7/40 (17.5)	1/140 (0.7)	1/138(0.7)	0	9/418 (2.1)
	NP	1/38 (2.6)	0	1/135(0.7)	0	2/418 (0.5)
	TS	1/39(2.6)	0	0	0	1/418(.24)
	SPT	5/35 (14.2)	1/52 (1.9)	0	0	6/418(1.4)
H.influenzae	Any	3/40 (7.5)	9/140 (6.4)	8 /138(5.8)	1/100(1)	21/418(5.)
	NP	0	0	2/135 (1.4)	1/100 (1)	3/418 (0.7)
	TS	0	0	0	0	0
	SPT	3/35 (8.5)	9/52(17.3)	6/18 (33.3)	0	18/418 (4.3)
M.catarrhalis	Any	2/40 (5.0)	8/140 (5.7)	4/138 (2.8)	2/100 (2)	16/418 (3.8)
	NP	1/38 (2.6)	6/140 (4.3	3/135(2.2)	2/100 (2)	12/418 (2.8)
	TS	0	0	0	0	0
	SPT	1/35(2.6)	2/52 (3.8)	1/18(5.5)	0	4/418 (0.95)

NP=Nasopharyngeal swab; TS=Throat swab; SPT=Sputum

Table 10 (page 73) shows the number of respiratory pathogens isolated from each specimen type for patients with and without COPD.

Overall, *S. pseudopneumoniae* was isolated on 23 occasions from 418 participants (5.5%). Twelve of the isolates were from 140 participants with stable COPD (8.6%), and 2 from 40 participants with acute COPD (5.0%). The remaining 9 isolates were from 138 current or ex-smokers without COPD (6.5%). On 1 occasion it

was isolated together with *S. pneumoniae* and on another with *S. pneumoniae* and *H. influenzae* (data not shown). *Streptococcus pseudopneumoniae* was only isolated from participants who had a history of smoking (4 current and 15 ex smokers). It was not isolated from any of the participants who had never smoked.

Of the recognised respiratory pathogens, *H.influenzae* was the most common isolate. Overall, it was isolated on 21 occasions (5.0%). It was isolated from 3/40 participants with acute COPD (7.5%), 9/140 participants with stable COPD (6.4%) and from 8/138 current or ex-smokers with normal lung function (5.8%). It was only isolated from 1/100 participants who had never smoked (1.0%). This was from the nasopharynx only and probably indicated colonisation rather than infection.

In total, *Moraxella catarrhalis* was isolated from 16/418 participants (3.8%). It was isolated from 8/140 participants with stable COPD (5.7%), 2/40 participants with acute COPD (5.0%) and 4/138 current or ex-smokers without COPD (2.8%). Like *H. influenzae*, it was only isolated in 2/100 of those who had never smoked. Again these were from the nasopharynx only and probably represented colonisation.

S. pneumoniae was isolated on 9 occasions in total (2.1%), 7 being isolated from patients with acute COPD (17.5%). The carriage rate for this group was adjusted to 12.5% as 3 of the isolates were from 1 participant. It was isolated only once from participants with stable COPD and current or ex-smokers with normal lung function (0.7%). Like *S. pseudopneumoniae* it was not isolated from participants who had never smoked.

A number of other isolates were recovered, the majority being from participants with acute exacerbations. These were predominately Gram negative bacilli including *Escherichia coli, Pseudomonas aeruginosa and Stenotrophomonas maltophilia* but also included *Staphylococcus aureus, Candida albicans* and *Aspergillus fumigatus*. In the groups with stable COPD and normal lung function the only other isolate was *S.aureus,* isolated exclusively from the nasopharynx (data not shown)

Except in the case of *M. catarrhalis*, sputum was the best specimen for the recovery of respiratory pathogens. Twelve of the 23 isolates of *S. pseudopneumoniae* (52%), 6/9 isolates of *S. pneumoniae* (66.6%) and 18/21 isolates of *H. influenzae* (86%) were isolated from sputum specimens. *M. catarrhalis* was only isolated from sputum on 4/16 occasions (25%).

The nasopharyngeal swab was the best specimen for the recovery of *M.catarrhalis*, 12/16 isolates (75%). *S. pneumoniae* was isolated from the nasopharynx of 2 participants (0.5%) and *S. pseudopneumoniae* and *H. influenzae* were both isolated 3 times (0.7%).

Only *S. pseudopneumoniae* and *S.pneumoniae* were recovered from throat swabs; 8 (1.9%) and 1 (0.24%) respectively.

Overall 63% of all isolates were from sputum specimens, 25% from nasopharyngeal swabs and 12% from throat swabs.

Over half of the participants in the acute COPD group were receiving antibiotic therapy at the time of testing, the most commonly prescribed antibiotic being amoxycillin/clavulanic acid. Both patients from whom *S. pseudopneumoniae* was isolated were being treated with this drug. The reason why *S. pseudopneumoniae* was isolated from these participants is unclear as no high level resistance was detected in the earlier study (Chapter 4). Only 2 participants in the non acute group had received antibiotics, both being treated with amoxycillin/clavulanic acid. Respiratory pathogens were not recovered from either participant.

6.4 Discussion

There were 2 major findings in this study. The most interesting finding was that *S. pseudopneumoniae* was not isolated from anyone who had never smoked which suggests that there may be an association with smoking history. Secondly,

S. pseudopneumoniae was isolated from 5.0% of all specimens from all participants but the nasopharyngeal carriage rate was the same as other respiratory pathogens in adults including *S. pneumoniae*.

The results obtained show that it is most prevalent in people with stable COPD (8.5%) followed by those who have no lung disease but are current or ex-smokers (6.5%).

The other respiratory pathogens were also more commonly isolated from current and past smokers than from those who have never smoked. Recovery of pathogens was very low amongst the latter, *H. influenzae* being isolated on one occasion and *M. catarrhalis* on two occasions. These were isolated from the nasopharynx of healthy participants with normal lung function suggesting colonisation rather than infection.

The majority of isolates were recovered from participants in the 60-69 and 70-79 year age groups but this may simply be due to the increased numbers of participants in these groups, particularly ex smokers with stable COPD (data not shown).

Streptococcus pseudopneumoniae was more likely to be isolated from sputum followed by nasopharyngeal swab and throat swab (11, 8, 4). This fits well with other studies that showed that sputum was the best non sterile respiratory site for the recovery of pathogens (72). That study also stated that the value of sputum culture in establishing a bacterial cause of lower respiratory tract infections depends on how specimens are collected and processed and whether a predominant morphotype was seen in the Gram film. All sputum specimens processed at CHL are checked for quality prior to culture and are rejected if of poor quality. This increases the likelihood that isolates are significant. It also recommends that upper respiratory tract specimens such as nasopharyngeal swabs should not be used for diagnosing lower respiratory tract infection as infection cannot be distinguished from carriage. In this study specimens were collected by trained nursing staff and poor quality sputum specimens based on the criteria described in chapter 2 were rejected. This increased the likelihood that the respiratory pathogens isolated, including *S. pseudopneumoniae* were of clinical significance. Upper respiratory swabs were collected so that the carriage rate of potential pathogens could be investigated.

Respiratory pathogens are isolated from sputum in 40-60% of acute exacerbations of COPD(108). In this study, the isolation rate from sputum including *S. pseudopneumoniae* was lower at 25%. Other isolates that were of uncertain significance (*Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Stenotrophomonas maltophilia* and yeasts and fungi) were isolated on 9 occasions from sputum of patients with acute exacerbations (data not shown). If these isolates are included, the isolation rate for this group of patients' increases to 47.5%.

Of the recognised respiratory pathogens the most common isolate was *H. influenzae* particularly amongst stable COPD patients. This is in keeping with other studies (32, 44, 45, 71, 82) that have found non-typeable *H. influenzae* to be the most prevalent bacterial pathogen in COPD globally, being isolated in up to 50% of cases (108). In these studies the prevalence of other commonly isolated pathogens including *S. pneumoniae*, *M. catarrhalis* and *Pseudomonas aeruginosa* varied. For example, in 2 studies by Ko et.al. involving patients with acute exacerbations (65, 66), *Ps. aeruginosa* was more commonly isolated than *S. pneumoniae.* Conversely, in a British study by Patel et.al. looking at both colonisation and acute exacerbation (97) *S. pneumoniae* was more commonly isolated. This is not surprising as it recognised that Gram negative bacilli are associated with severe lower respiratory disease whereas *S. pneumoniae* and other Gram positive cocci are more commonly found in mild disease (110, 125).

The isolation rate of *M. catarrhalis* also varies according to the group being studied. In the studies by Ko et. al.(65, 66) the isolation rate was between 3% and 4% whereas studies by Parameswaran et al (95) and Patel et al (97) report isolation rates of 10% and 20% respectively. A metaanalysis by Sethi (108) of 14 trials found that when all patients with COPD are taken into account the overall isolation rates of bacterial pathogens are:

- *H.influenzae* 13-50%
- *M. catarrhalis* 9-21%
- *S. pneumoniae* 7-26%
- *Ps. aeruginosa* 1-13%
- Enterobacteriaecae 3-19%
- *H. parainfluenzae* 2-32%
- *S. aureus* 1-20%

In this study the isolation rate of pathogens in sputum from patients with acute exacerbations was lower than those shown above and *S. pneumoniae* was the most common isolate (12.5%). This was surprising as this is the group with exacerbations severe enough to require admission. As such, a greater number of Gram negative pathogens would be expected. However, the number of participants was small (40) so the results may not be statistically valid.

All the bacterial pathogens can colonise the airways without causing overt disease. COPD is characterised by acute exacerbations and periods of colonisation often referred to as a "vicious circle"(110). After the initial damage to the airways by tobacco smoke, bacteria colonise the lung and damage the tissue causing inflammation. Each exacerbation is capable of bringing in new colonising strains that can cause more damage and leave the host susceptible to further exacerbations (95).

In the group with stable COPD, *H. influenzae* was isolated from 6.4% of sputum specimens. As this group did not have an acute exacerbation it could be assumed that this represents colonisation silently causing damage to the lungs. *S. pseudopneumoniae* was isolated at the same rate. It has not yet been shown to be pathogenic but it is known that it possesses some genes, e.g. the pneumolysin gene, thought to be unique to *S. pneumoniae* (3). It may well have other genes for enzymes or antigens that can cause damage to the tissue and therefore have an indirect role in infection.

The carriage rate of all species was low, *M. catarrhalis* being the most common isolate (2.8% of all participants). The carriage rate for *S. pseudopneumoniae* and *H. influenzae* was 0.7% and for *S. pneumoniae* was 0.5%. This compares well with carriage rates in adults throughout the world which are as low as 2.0%, 3.0% and 1.0% for *M. catarrhalis, H. influenzae* and *S. pneumoniae* respectively (41).

On the basis of the original hypothesis more isolates of *S. pseudopneumoniae* were anticipated in the group having acute exacerbations of COPD. The lack of isolates prompted the question why was the prevalence so low?

One factor that may be influencing the isolation of *S. pseudopneumoniae* is the use of antibiotics. A number of isolates other than recognised respiratory pathogens were recovered from patients with acute exacerbations only. These included coliform bacilli, *Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Candida species and*

Aspergillus fumigatus (data not shown). These species are commonly isolated from patients with COPD, particularly in severe disease, and may be causing infection or represent overgrowth flora following antibiotic therapy. Many of these bacterial species exhibit multiple resistances to antibiotics and have the potential to overgrow more slowly growing species. To prevent the overgrowth of potential *S. pseudopneumoniae* colonies a selective medium was used to inhibit growth of unwanted species. In this study CNA was used as it has been shown in this laboratory to be the optimum selective medium. The isolation rate of *S. pseudopneumoniae* was still lower than expected in this group.

Many strains of *S. pseudopneumoniae* exhibit resistance to antibiotics such as erythromycin, tetracycline and sulphamethoxazole/trimethoprim as described in Chapter 4. COPD patients are commonly prescribed a number of antibiotics on an empiric basis. When the antibiotic history of the group was examined it was found that 23/40 patients in group A were on antibiotics at the time of sampling. Of these, 4 patients had received sulphamethoxazole/trimethoprim (1), tetracycline (2) and erythromycin +tetracycline (1) to which *S. pseudopneumoniae* has shown resistance (chapter 4). The remaining patients had received amoxycillin/clavulanic, amoxycillin and / or a quinolone. So far resistance to these antibiotics has not been documented in New Zealand and studies in other countries have not yet been undertaken. The low isolation rate of *S. pseudopneumoniae* in this group could therefore possibly be attributed to the use of these antibiotics prior to hospital admission.

There are some limitations to this study. Firstly, the number of participants in some groups was small so the results may not be statistically valid. The original aim was to recruit 100 participants into each group but this proved to be difficult particularly for acute COPD, stable COPD and “healthy smokers”. Secondly, all the participants were >40 years of age and they were all from one geographical area. The study intentionally targeted COPD patients attending one tertiary medical centre and most were recruited via the hospital database. Children were not included in the sample as there is no information on the carriage of *S. pseudopneumoniae* in this group. As a result, the sample may not be representative of the general population and the results may not reflect the true carriage rate.

The results of this study were not conclusive but did show an association with smoking. As COPD is a condition almost exclusively associated with smoking it could

be inferred that *S. pseudopneumoniae* is playing some role in the disease process. That we only isolated *S. pseudopneumoniae* from those with a history of smoking does suggest that smoking creates changes in the lungs that predispose to colonisation. Additionally, although other studies have not been carried out on *S. pseudopneumoniae* specifically, increased antimicrobial resistance has been documented amongst other viridans streptococci (48, 57). The potential for colonisation and opportunistic infection following antibiotic therapy does exist. Further studies on larger numbers of participants from other geographic areas are required to clarify any association of *Streptococcus pseudopneumoniae* with COPD. Studies also need to be undertaken to establish any potential virulence factors that may allow *S. pseudopneumoniae* to become pathogenic in this group of patients.

Chapter 7

Discussion

There were a number of key findings in this study.

The first major finding was the characterization of *S. pseudopneumoniae*. The results of this study confirmed the findings of Arbique (3) who described *S. pseudopneumoniae* as an α haemolytic streptococcus that exhibits variable optochin susceptibility, is bile insoluble, is positive by the GenProbe AccuProbe Pneumococcus test and possesses the pneumolysin *(ply)* gene.

The second finding was that this new species can be recognised in the routine laboratory using some basic phenotypic tests. Biochemical testing and the use of genotypic techniques were of little value as this new species is not currently included in test databases.

The recognition of *S. pneumoniae* in the primary Gram film has long been a useful diagnostic tool. Unfortunately, the microscopic morphology of *S. pseudopneumoniae* has proved to be remarkably similar to that of *S. pneumoniae*. The only noticeable difference is that the cocci of *S. pseudopneumoniae* appear a little healthier. The lanceolate diplococci of *S. pneumoniae* often stain unevenly giving a motley Gram variable appearance or lose their Gram positivity completely. These cells are referred to at Canterbury Health Laboratories as “sick”. By comparison the lanceolate diplococci of *S. pseudopneumoniae* stain evenly Gram positive. In some ways this is an unhelpful characteristic as it can lessen the confidence with which *S. pneumoniae* is reported in primary Gram films.

The colonial morphology was more helpful and its value should not be overlooked when differentiating *S. pseudopneumoniae* and *S. pneumoniae*. Although the colonies of *S. pseudopneumoniae* may appear as “draughtsmen” colonies typical of *S. pneumoniae;* they never achieve the same size. In addition, the colonies of *S. pneumoniae* have a rich, glossy surface that was not exhibited by any of the strains identified as *S. pseudopneumoniae*. The latter difference is particularly noticeable when the growth is confluent such as on susceptibility plates. The overall surface has a much more matt appearance when compared to a similar growth of *S. pneumoniae*. These

colonial characteristics now form an important part of the identification of *S.pneumoniae* at Canterbury Health Laboratories and are particularly important when combined with optochin susceptibility.

There has, in the past, been a tendency to see a zone of inhibition around an optochin disc and report the isolate as *S. pneumoniae* without any regard for the size of the zone. At Canterbury Health Laboratories, as in many other laboratories, a 6mm disc containing 5ug of optochin is used. For an isolate to be susceptible the zone of inhibition has to be ≥14 mm when incubated in CO_2. Like the isolates of *S pseudopneumoniae* first described by Arbique (3) all the strains had zones <14 mm, the majority had zone sizes between 8mm and 13mm. Zone sizes at the upper limit can be hard to determine by eye and it is recommended that they should always be measured.

As recommended by other studies, isolates that are equivocal on the basis of morphology and optochin susceptibility need to be confirmed by an additional method. The bile solubility test is reported to be more sensitive than optochin susceptibility for the identification of *S. pneumoniae* (14) and is the test used at Canterbury Health Laboratories. However, from personal experience it isn't the easiest test to perform. There are too many methods and the interpretation is subjective. Obregon et al (94) reported that *S. pseudopneumoniae* is insoluble in a solution of 1% bile salts but soluble in 0.1% bile salts and in Triton X-100, an alternative detergent. This is one of the areas for future investigation.

Another major finding is that *S. pseudopneumoniae* is and probably always has been a relatively common isolate from sputum specimens in Canterbury. In this particular study a total of 805 isolates of *S. pneumoniae* or α haemolytic streptococcus that were the predominant organism from consecutive good quality sputa were collected over a 3 year period. Collection of the isolates was well monitored so the integrity of the study population was maintained. On the basis of phenotypic and genotypic testing 35 of these isolates were identified as *S. pseudopneumoniae*. This represents a prevalence of approximately 1/30 which is higher than that found by Harf-Monteil (51). The prevalence in Christchurch could be higher as not all the isolates of *S. pneumoniae* were tested. Only the first 100 isolates were fully investigated. Within those first 100 isolates, the species initially identified as *S. pneumoniae* gave unequivocal results with all phenotypic tests. Subsequent isolates that had been stored as S. *pneumoniae* were accepted as such and only the remaining α haemolytic streptococcus other than *S. pneumoniae* were identified further. With hindsight, particularly after investigating the

susceptibility patterns, it is possible that some of the isolates had been misidentified as *S. pneumoniae as* suggested by Wester et al (119) which could increase the prevalence and the rate of antimicrobial resistance. This could also have contributed to the higher penicillin resistance noted by ESR in the Christchurch region (52). Whilst this work was being carried out, another study to determine the prevalence of *S. pseudopneumoniae* in sputum was published (51).The prevalence was found to be much lower (1/120), possibly due to differences in the study population.

Of particular interest was the apparent association of *S. pseudopneumoniae* with COPD discovered during the characterization of the species. It was disappointing that this was not confirmed by the prevalence study. However, it was only isolated from smokers or ex-smokers and most commonly from those with stable COPD. As COPD is almost exclusively related to smoking there may still be an association. It is possible that *S. pseudopneumoniae* is playing a role in the inflammatory tissue damage that occurs in the stable, colonization phase and has yet to be identified.

Another key finding was the susceptibility pattern of *S. pseudopneumoniae*. Overall, it is more resistant than *S. pneumoniae,* the resistance pattern being more comparable to that of *S. mitis* in other areas of the world (33, 58, 107, 114). Although susceptibility testing of non pneumococcal isolates from non sterile sources, such as sputum, is not undertaken at Canterbury Health Laboratories, the findings have been very important. Oxacillin discs are added to the primary sputum culture plates to give an early indication of possible penicillin resistance. A number of isolates appearing to be penicillin resistant pneumococci have been later identified as *S. pseudopneumoniae* (personal observation). Consequently, the morphology of any isolate presumptively resistant to penicillin is therefore carefully examined before being reported to clinicians. Similarly, resistance to erythromycin and/or tetracycline is common amongst *S. pseudopneumoniae*. If present, the identification should be confirmed to avoid overestimation of resistant strains of *S. pneumoniae.* With hindsight it is felt that some of the resistant strains of *S. pneumoniae* that were collected but not confirmed in the original study (Chapter 3) could actually be *S. pseudopneumoniae* and do warrant future attention. If this is the case, it could explain the higher rate of resistance in Canterbury as reported by ESR(52).

As a result of the study there is a heightened awareness of *S. pseudopneumoniae* at Canterbury Health Laboratories and it is regularly isolated on the bench suggesting that it has always been there. At one time, small colonies that resembled the draughtsmen of pneumococci were called “squitty pneumos” and would have been reported as pneumococci if the optochin appeared to be susceptible. Now such colonies are checked more thoroughly.

Streptococcus pseudopneumoniae is not reported as an individual isolate as the significance is still unknown but it is rarely erroneously reported as *S. pneumoniae* either.

There were some limitations to this study. Specific limitations have been detailed in the preceding chapters but in general, all the participants were all from one geographical area (Christchurch) and attended one tertiary health care centre. As such the sample population may not be representative of New Zealand or other regions of the world. In addition the isolates were all from sputum specimens and may not reflect the true distribution within the community.

Future work

The first area to be addressed is the bile solubility test. The test needs to be standardized and adapted so that it can more reliably differentiate *S. pseudopneumoniae* and *S. pneumoniae,* possibly by using different concentrations of bile salts or alternative detergents.

The biochemical identification of *S.pseudopneumoniae* also needs to be investigated further using different methodologies. The rapid ID 32 STREP identified some tests that may be useful in the differentiation of *S. pseudopneumoniae* and *S. pneumoniae*. The reliability of these tests needs to be confirmed. The aim is to find or develop a simple, rapid test that can be used in the routine laboratory.

The remaining isolates in the original collection need to be investigated to determine whether any of them really are *S. pseudopneumoniae.* Since the study began a number of isolates that do not fit the description of either *S. pseudopneumoniae* or S.

pneumoniae has been isolated during routine work e.g. optochin susceptible strains in CO_2 that do not resemble *S. pneumoniae* and are bile insoluble. Some have been identified as *S.mitis* by rapid ID 32 STREP. These may be atypical strains of *S. mitis* or possibly represent another subset of the *S. mitis* group and need to be characterized.

Further research is needed to further differentiate the members of the *S. mitis* group of viridans streptococci. This could involve molecular techniques including sequencing using different targets such as rpoA or rnpB and multi locus sequencing (MLST). As automated identification systems, possibly including MALDI-tof, are being introduced to Canterbury Health Laboratories the opportunity to learn more about this new species will arise.

The presence of pneumococcal virulence factors such as pneumolysin in the isolates of *S. pseudopneumoniae* suggests that it may have some role in respiratory disease. This needs to be investigated further probably using molecular techniques.

The role of *S. pseudopneumoniae* in COPD also needs to be investigated further. As previously discussed there were limitations to the study. Current or past history of smoking, an activity associated with COPD, and antibiotic use were the only factors considered. A much larger, possibly multicentre, study is required. Additional information about other factors such as passive smoking, use of the pneumococcal vaccine and alcohol use may provide valuable information. It would also be interesting to investigate any association with respiratory viruses.

The original study set out to characterize *S. pseudopneumoniae* and find a means to differentiate it from *S. pneumoniae* in the routine laboratory. It has been successful on both counts. We now know its characteristics and can confidently identify it in the laboratory. It also opened opportunities for other studies along the way. As a result of the original work to characterize *S. pseudopneumoniae* we have also learned about its susceptibility profile, its prevalence in the Canterbury region and of its possible role in the pathology of COPD.

This is a newly recognized species and already we have learned a great deal. There is bound to be much more to learn about *S. pseudopneumoniae* and its' close relatives in the future.

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Appendix 1

Table 2H-2
Streptococcus spp.
Viridans Group
M02 and M07

Table 2H-2. Zone Diameter and MIC Interpretive Standards for *Streptococcus* spp. Viridans Group

Testing Conditions

Medium:	Disk diffusion: MHA with 5% sheep blood Broth dilution: CAMHB with LHB (2.5 to 5% v/v); the CAMHB should be supplemented to 50 µg/mL calcium for daptomycin (see M07-A8 for instructions for preparation of LHB) Agar dilution MHA with sheep blood (5% v/v) (when testing a sulfonamide, LHB should be used); agar dilution has not been validated for daptomycin
Inoculum:	Direct colony suspension, equivalent to a 0.5 McFarland standard
Incubation:	35 ± 2 °C; Disk diffusion: 5% CO_2; 20 to 24 hours Dilution methods: ambient air; 20 to 24 hours (CO_2 if necessary for growth with agar dilution)

Minimal QC Recommendations (See Table 3A for acceptable QC ranges.)

Streptococcus pneumoniae ATCC® 49619

General Comments

(1) For disk diffusion, measure the diameter of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Measure the zones from the upper surface of the agar illuminated with reflected light, with the cover removed. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth.

(2) Small-colony-forming beta-hemolytic strains with Group A, C, F, or G antigens (*S. anginosus*, previously termed *"S. milleri"*) are considered part of the viridans group, and interpretive criteria for the viridans group should be used.

(3) Interpretive criteria for streptococci other than *S. pneumoniae* are proposed based on population distributions of various species, pharmacokinetics of the antimicrobial agents, previously published literature, and the clinical experience of certain members of the subcommittee. Systematically collected clinical data were not available for review with many of the compounds in the group.

(4) For some organism/antimicrobial agent combinations, the absence or rare occurrence of resistant strains precludes defining any results categories other than "susceptible." For strains yielding results suggestive of a "nonsusceptible" category, organism identification and antimicrobial susceptibility test results should be confirmed. Subsequently, the isolates should be saved and submitted to a reference laboratory that will confirm results using a CLSI reference dilution method.

Table 2H-2. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Breakpoints, nearest whole mm			MIC Interpretive Standard (µg/mL)			Comments
			S	I	R	S	I	R	
PENICILLINS (5) A streptococcal isolate that is susceptible to penicillin can be considered susceptible to ampicillin, amoxicillin, amoxicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefazolin, cefdinir, cefepime, cefprozil, cefotaxime, ceftibuten (Group A streptococci only), ceftriaxone, cefuroxime, cefpodoxime, ceftizoxime, cephradine, cephalothin, cephapirin, ertapenem, imipenem, loracarbef, and meropenem for approved indications, and need not be tested against those agents.									
A	Penicillin	-	-	-	-	≤ 0.12	0.25-2	≥ 4	(6) **Disk testing is not reliable for testing penicillin and ampicillin.** (7) Viridans streptococci isolated from normally sterile body sites (eg, CSF, blood, bone) should be tested for penicillin susceptibility using an MIC method. (8) ***Rx:*** Penicillin- or ampicillin-intermediate isolates may require combined therapy with an aminoglycoside for bactericidal action.
A	Ampicillin					≤ 0.25	0.5-4	≥ 8	
CEPHEMS (PARENTERAL) (Including cephalosporins I, II, III, and IV. Please refer to Glossary I.)									
B	Cefepime	30 µg	≥ 24	22-23	≤ 21	≤ 1	2	≥ 4	See comment (5).
B	Cefotaxime	30 µg	≥ 28	26-27	≤ 25	≤ 1	2	≥ 4	
B	Ceftriaxone	30 µg	≥ 27	25-26	≤ 24	≤ 1	2	≥ 4	
CARBAPENEMS									
O	Ertapenem	-	-	-	-	≤ 1	-	-	See comment (4).
O	Meropenem	-	-	-	-	≤ 0.5	-	-	See comment (4).
GLYCOPEPTIDES									
B	Vancomycin	30 µg	≥ 17	-	-	≤ 1	-	-	See comment (4).
LIPOPEPTIDES									
O	Daptomycin	-	-	-	-	≤ 1	-	-	**(9) Disk testing is not reliable for testing daptomycin.** See comment (4).
MACROLIDES (10) Susceptibility and resistance to azithromycin, clarithromycin, and dirithromycin can be predicted by testing erythromycin. (11) Not routinely reported on isolates from the urinary tract.									
C	Erythromycin	15 µg	≥ 21	16-20	≤ 15	≤ 0.25	0.5	≥ 1	
O	Azithromycin	15 µg	≥ 18	14-17	≤ 13	≤ 0.5	1	≥ 2	
O	Clarithromycin	15 µg	≥ 21	17-20	≤ 16	≤ 0.25	0.5	≥ 1	
O	Dirithromycin	15 µg	≥ 18	14-17	≤ 13	≤ 0.5	1	≥ 2	
TETRACYCLINES									
O	Tetracycline	30 µg	≥ 23	19-22	≤ 18	≤ 2	4	≥ 8	(12) Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.
FLUOROQUINOLONES									
O	Levofloxacin	5 µg	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8	
C	Ofloxacin	5 µg	≥ 16	13-15	≤ 12	≤ 2	4	≥ 8	
O	Gatifloxacin	5 µg	≥ 21	18-20	≤ 17	≤ 1	2	≥ 4	
O	Grepafloxacin	5 µg	≥ 19	16-18	≤ 15	≤ 0.5	1	≥ 2	
O	Trovafloxacin	10 µg	≥ 19	16-18	≤ 15	≤ 1	2	≥ 4	

Table 2H-2
Streptococcus spp.
Viridans Group
M02 and M07

Table 2H-2. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Breakpoints, nearest whole mm			MIC Interpretive Standard (µg/mL)			Comments
			S	I	R	S	I	R	
PHENICOLS									
C	Chloramphenicol	30 µg	≥ 21	18-20	≤ 17	≤ 4	8	≥ 16	See comment (11).
LINCOSAMIDES									
C	Clindamycin	2 µg	≥ 19	16-18	≤ 15	≤ 0.25	0.5	≥ 1	See comment (11).
STREPTOGRAMINS									
O	Quinupristin-dalfopristin	15 µg	≥ 19	16-18	≤ 15	≤ 1	2	≥ 4	
OXAZOLIDINONES									
C	Linezolid	30 µg	≥ 21	-	-	≤ 2	-	-	See comment (4).

Appendix 2

The attached publications have arisen from this study:

"Characteristics of *Streptococcus pseudopneumoniae* Isolated from Purulent Sputum Samples."
Elaine R. Keith, Roslyn G. Podmore, Trevor P. Anderson, and David R. Murdoch. Microbiology Unit, Canterbury Health Laboratories, Christchurch, New Zealand, and Department of Pathology, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand.

"Antimicrobial Susceptibility Profile of *Streptococcus pseudopneumoniae* Isolated from Sputum."
Elaine R. Keith and David R. Murdoch.
Microbiology Unit, Canterbury Health Laboratories, Christchurch, New Zealand, and Department of Pathology, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand.

"Characterisation of *Streptococcus pseudopneumoniae* from Respiratory Specimens in Christchurch, New Zealand." (Poster presentation)
Elaine Keith, Microbiology Unit, Canterbury Health Laboratories, Christchurch, New Zealand.