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PROMPT CLINICAL IDENTIFICATION AND CHARACTERIZATION OF VIRULENT *STREPTOCOCCUS PNEUMONIAE*

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ABSTRACT

Streptococcus pneumoniae is the leading cause of pneumonia among people with weak immune systems. Since the organism shares major biochemical characteristics with other organisms of its genetic lineage, means of prompt clinical identification is quite challenging. Colony morphology, optochin, and bile solubility tests are considered the gold standard for the biochemical identification of *S. pneumoniae.* However, they are time-consuming and open to contamination. Other forms of identification such as DNA-DNA hybridization, 16S rRNA, and *rpoB* housekeeping genes have been developed as efficient methods for identification and novel strain assignment but could be expensive and time-consuming. Urine serology offers little hope because of the possibility of the false positive test caused by the pneumococcal vaccine in immunized patients and also the presence of a pneumococcal C-polysaccharide-like compound secreted by *S. mitis.* The antigenic C-polysaccharide has also been detected in the urine of patients several days after treatment and recovery from the infection. For efficient discrimination between infections due to *S. pneumoniae* and nasopharyngeal colonization, the putative proteinase maturation protein A (PpmA) should be exploited.

KEYWORDS: *Streptococcus pneumoniae,* identification, vaccine, serotype, virulence.

INTRODUCTION

Streptococcus pneumoniae is one of the most important human pathogens implicated in several clinical conditions; ranging from mild fever and cough to severe organ damage and death. Upon entrance into a suitable host, the organism tends to thrive asymptomatically by

colonizing the nasopharynx of the respiratory system.[1] However, in the presence of predisposing factors, the pathogen would find its way into other organs of the body where it causes disease conditions such as pneumonia. Pneumococcal infection is among the leading causes of morbidity and mortality in children below 5 years, immunocompromised patients, and elderly people globally.[2] The pathogen has diverse virulent factors that help it to penetrate the tissues of the host, find attachment, and escape arrays of host immunological responses.[3]

Despite the pathogen accounting for 27 % of global pneumonia, means of prompt clinical identification and characterization have been quite challenging. For instance, only 20% to 25 % of all pneumonia cases caused by *S. pneumoniae* are positive in blood culture.[4] Inaccurate identification of *S. pneumoniae* greatly reduces the chance of proper treatment and duration of patient recovery. Furthermore, for efficient formulation and distribution of pneumococcal conjugate vaccines, proper identification and serotyping of *S. pneumoniae* is of utmost importance since conjugate vaccines are based on the most prevalent serotypes.[5]

Identification of *Streptococcus pneumoniae* from closely related organisms such as *Streptococcus mitis* and *Streptococcus pseudopneumoniae* poses a strong challenge for clinical research. Previously, some phenotypic markers such as capsule production, susceptibility to optochin, and bile solubility were believed to be sufficient in the characterization of *Streptococcus pneumoniae*. However, some of these phylogenetically related organisms share most of these characteristics with *S. pneumonia*.[3]

One of the key factors in deciphering the immunology and epidemiology of *S. pneumoniae* is a comparative analysis of immune responses of pneumococcal proteins of clinical isolates. Despite the development of the seven-valent pneumococcal conjugate vaccine (PCV7), which has led to the vaccination of millions of people across the globe; infections due to *S. pneumoniae* persist as some of the major causes of infant morbidity and mortality.[3] Proper identification of *S. pneumoniae* in a biological sample is very important since the organism may likely share a habitat with other organisms belonging to its genetic lineage. Proper identification of the isolates would expose the prevalent serotypes and inform future vaccination programs.[6]

Table 1: Comparison of *S. pneumoniae* and its viridans relatives based on some phenotypic markers.

***Streptococcus* spp**	**Associated clinical conditions**	**Phenotypic markers**		
		Capsule production	**Susceptibility to optochin**	**Bile solubility**
S. pneumoniae	Pneumonia, meningitis, septicemia, otitis media	Positive	Susceptible	Soluble
S. mitis, S. sanguis, S. bovis, S. salivarius, S. oralis, S. gordonii	Endocarditis, dental plaque	Negative	Resistant	Insoluble
S. pseudopneumoniae, S. mutans, S. anginosus	Chronic obstructive pulmonary disease (COPD), respiratory tract infection (RTI)	Negative	Resistant	Insoluble

Colony morphology of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram-positive, non-motile, non-spore-forming, and facultative anaerobic diplococci often seen as short chains or single cocci in blood culture. They are alpha-hemolytic in blood agar, appearing grayish or mucoidal and translucent when viewed with an X40 lens after 18 – 24 hours incubation in an anaerobic jar containing carbon (IV) oxide. They grow at the optimal temperature of 35 – 37 ^{O}C on blood or chocolate agar.[2,7] Their alpha-hemolytic properties help to differentiate them from *Streptococcus pyogenes* and other opportunistic commensals of the throat which produce hemolysin O and S that are able to break blood cells completely (beta-hemolysis). *S. pneumoniae* and its closely related *Streptococcus viridans* partially reduce erythrocytes in any given media (alpha-hemolysis) by the production of hydrogen peroxide which oxidizes the iron in hemoglobin to methemoglobin that can be identified by its green pigmentation.[4] To differentiate, *S. pneumoniae* from the viridians, extended incubation is required. *S. pneumoniae* colonies appear flattened and depressed when incubated for 24 – 48 hours, unlike viridians that remain raised after 48 hours of incubation.[1]

Epidemiology of *Streptococcus pneumoniae*

S. pneumoniae occurs in most countries of the world with high morbidity and mortality rates in children below 5 years, immunocompromised persons, and adults above 65 years. Recent studies show that *S. pneumoniae* is estimated to have a colonization rate of 27 – 65 % of children below 5 years in the USA alone.[8–10] This value could be higher in developing countries due to low standard of living, poor healthcare system, and near absence of

verifiable data on the epidemiology of the organism. Disease conditions associated with *S. pneumoniae* are pneumonia, meningitis, otitis media, sinusitis, bacteremia, and septicemia.[1] Pneumonia is basically categorized into two depending on the location where the infection is contracted according to the Centers for Disease Control and Prevention. Community-acquired pneumonia (CAP) is contracted from any environment that is not a hospital while healthcare-associated pneumoniae (HAP) is contracted within the hospital environment. *S. pneumoniae* is the major cause of CAP globally. Although the use of PCV7 for vaccination programs has led to a reduction in CAP due to pneumonia, the infection continues to account for major causes of death among children below 5 years.[12]

Table 2: Common causes of community-acquired pneumonia.

Causative agent	Predisposing factors	Estimated percentage distribution in the respiratory tract of children
S. pnuemoniae	Weak immunity	37.8 %[13]
Haemophilus influenza	Bacterial superinfection,	20 – 30 %[14]
Staphylococcus aureus	Cystic fibrosis, AIDS, lung infection	30 %[15]
Atypical bacteria (*Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella spp.*)	Lung infection, weak immune cells, smoking	42.4 %[16]
Viruses (Influenza virus, Adenoviruses, Metapneumovirus, Coronaviruses)	Seasonal influenza, weak immunity	8.9 %[16]
Fungi (*Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis*)	Compromised immunity, bacterial superinfection	35.7 %[17]
Pseudomonas aeruginosa	AIDS, cystic fibrosis, neutropenia	7.1 – 7.3 %[18]

Virulence of *Streptococcus pneumoniae*

S. pneumoniae has a number of protective molecules that enable their survival in a host cell. All the structural polysaccharides and proteins that provide attachment, hinder immune activation, disrupt antibiotics and damage host cells constitute virulent factors of the organism.[19,20] *S. pneumoniae* is covered by a protective polysaccharide capsule, which plays a vital role as a virulent factor of the organism. Polysaccharide capsule is a shell-like structure that houses *S. pneumoniae*. It provides attachment to the organism upon entrance into a suitable host cell. It prevents pattern recognition by receptor cells and complement proteins, and helps evade neutrophils and other cells of innate immunity.[21] The

polysaccharide capsule is polymorphic in nature and forms the basis of *S. pneumoniae* serotyping. As vaccines and antibiotics continue to exert lethal.[22] pressure on the cell wall of *S. pneumoniae*, the polysaccharide capsule can undergo recombination. Polysaccharide capsules disrupt clearance by serotype switch. This occurs when there is a mutation within polysaccharide synthesis genes. This possibility of serotype switch is a great challenge to already existing pneumococcal vaccines.[22]

About 30 % of clinical isolates of *S. pneumoniae* possess a hair-like structure called pilli.[23] These organelles provide attachment and help in the colonization of the nasopharynx and other organs of the airway. It also helps the organism evade immune cells and is capable of inducing tumor necrosis factor, which is an agent of inflammatory response. This ensures the spread of the infection from one host to another.[22,24]

After cell lysis, *S. pneumoniae* releases toxins called pneumolysin, which forms pores in the cell membrane of the host.[11,24] Pneumolysin is a strong virulent factor because of the numerous roles it plays in inhibiting mucus clearance and promoting host cell lysis.[25] It promotes inflammation and helps in the transmission of the organism from one cell to another. It adversely interferes with complement cells, and phagocytes and also regulates the production of cytokines.[20]

Other virulent factors include pneumococcal surface protein, hydrogen peroxide, and autolysin Pneumococcal surface proteins provide attachment and help the pathogen evade the complement system. Hydrogen peroxide is secreted to fight off competition with other bacteria. It also attacks and destroys host DNA. Autolysin degrades the cell wall of the bacteria and helps to release toxins, which in turn promote nasopharyngeal colonization.[25]

Upon infection of a suitable host, *S. pneumoniae* is able to acquire new genetic materials through transformation and recombination. These dispensable genes have not been implicated in microbial cell growth. However, they enable the evolution of the pathogen and their survival within the host.[24] The resulting genetic variations are responsible for major phenotypic characteristics such as antibiotic resistance. *S. pneumoniae* can also pick fragments of DNA and integrate the same into their genome through a process called allele replacement. This process compensates for its inability to repair damaged DNA.[26,27]

Immunological Variations of *Streptococcus pneumoniae*

Microbial populations are averse to unfavorable environmental conditions. Upon introduction of antibiotics, the organisms enter into the lag phase and are eventually cleared off if the environment remains hostile. However, some bacterial infections persist after the application of potent antimicrobial agents.[6] These are not unconnected to the ability of the organisms to evade or delay the lethality effect of antimicrobial agents and resist clearance. The human body is equipped with self-defense mechanisms. They have the innate ability to detect and destroy pathogens, preventing them from causing any disease condition upon entrance into the body.[28] The array of molecular mechanisms explored by pathogens to continuously modify their immunogenic molecules and persist in the body is called antigenic variation.[29] *S. pneumoniae* is able to explore diverse genetic machinery to persist in the immune cells, enabling their own transmissions to other suitable hosts.[3,20,30] Most microbial antigens are oligopeptide chains. Therefore, any alterations of the gene that expresses the oligopeptide lead to modification of the amino acid sequence at immune cells' target sites. Such alteration creates a population of microbial cells that are unrecognizable to adaptive immune cells.[20]

Biochemical Identification of *Streptococcus pneumoniae*

The first possible step in the identification of *S. pneumoniae* in a biological sample is through colony morphology on blood agar plate (BAP). *S. pneumoniae* appears flattened and depressed at the centre on 24–48 hours BAP while the viridans streptococci remain raised on a 48 hours BAP. The colony that appeared depressed at the centre is then transferred to a glass slide with a sterilized wire loop for Gram staining. *S. pneumoniae* is able to yield positive to Gram staining because of their thick cell wall, which retains chemical dye and emits purple colour under a light microscope.[31]

To differentiate Gram-positive cocci, a catalase test is carried out. Catalase is an enzyme that breaks down hydrogen peroxide to yield water and oxygen bubbles. This particular enzyme is absent in *S. pneumoniae*, therefore, the organism is catalase-negative. Caution must be applied in transferring *S. pneumoniae* isolates to a glass slide to avoid contamination with blood agar, which could lead to a false positive reaction.[32]

Another important test in the biochemical characterization of *S. pneumoniae* is the optochin susceptibility test. It is a technique for the presumptive differentiation of *S. pneumoniae* from other alpha-hemolytic streptococci. At a very high dilution, ethylhydrocupreine hydrochloride – a chemical agent of optochin, which is a derivative of hydroquinine – is able

to inhibit *S. pneumoniae*. When a 6mm in diameter of 5µm optochin disc is used, a zone of inhibition greater than 14mm is considered susceptible otherwise is regarded as non-susceptible. However, to completely rule out the presence of *S. pneumoniae* in optochin non-susceptible isolate, a bile solubility test is required.[33,34]

The presence of amidase, an autolytic enzyme in *S. pneumoniae* helps to differentiate the bacterium from other alpha-hemolytic streptococci using bile solubility test. The bile salt (sodium deoxycholate) is able to lower the surface tension between the medium and cell membrane of the bacterium upon incubation with the isolate. This creates an optimal condition for intracellular amidase to vigorously lyse *S. pneumoniae* and clear the broth. Other streptococci that do not produce amidase remain turbid upon incubation with the bile salt and are considered negative.[35]

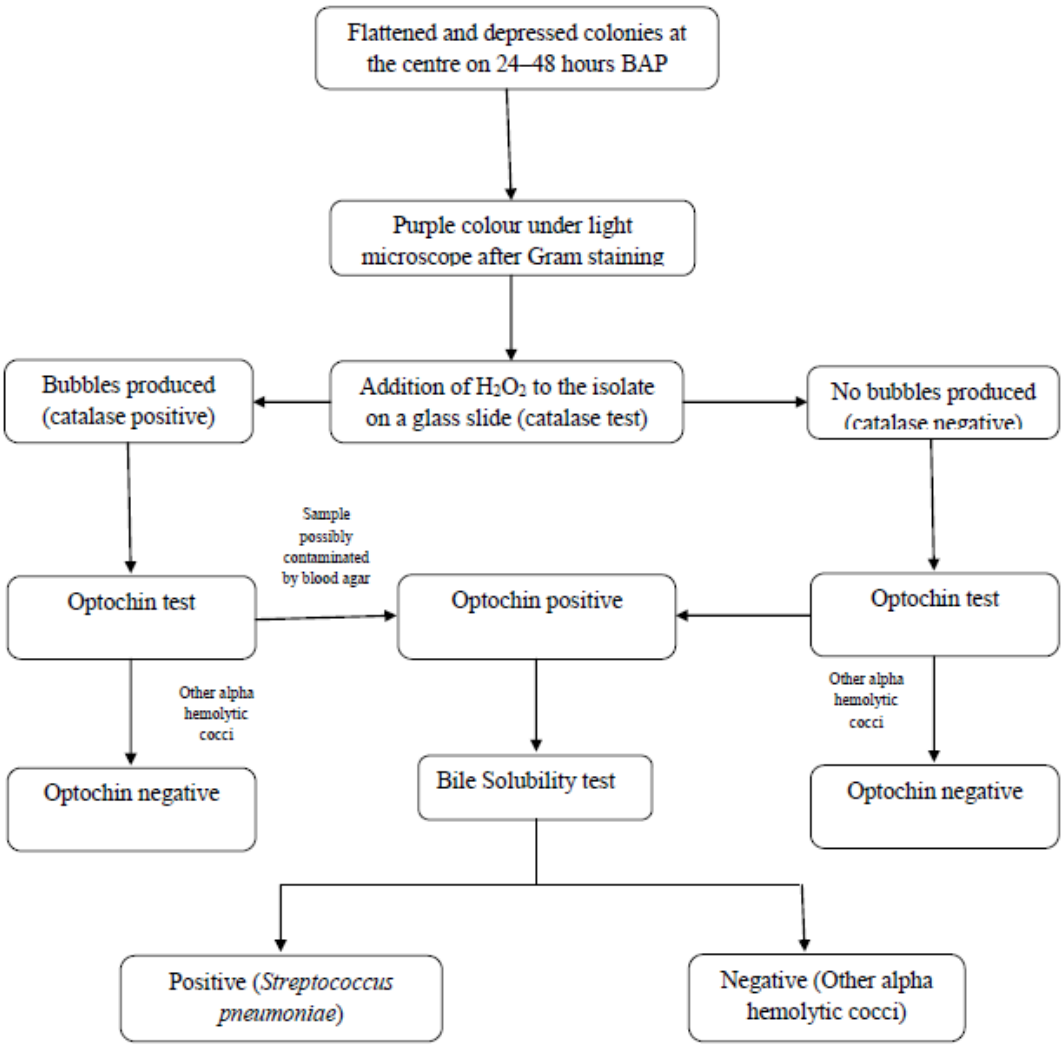

Figure 1: Flowchart for biochemical identification of *Streptococcus pneumonia.*

Molecular Detection and Characterization of *Streptococcus pneumoniae*

Pneumolysin is a virulent toxin produced by all invasive *S. pneumoniae*. Its major functions are to promote adherence to the host and lyses of immune cells.[36] Polymerase chain reaction (PCR) can be used for the detection of pneumolysin in the blood, sputum, urine, or cerebrospinal fluid of an infected patient. Pneumolysin as a diagnostic tool is potentially more reliable than capsular polysaccharides, which are prone to error of cross-reaction with structurally related bacterial antigens.[37,38]

Several techniques abound for molecular characterizations of bacterial cells. These include DNA-DNA hybridization which is employed for novel isolates assignment. However, the DNA-DNA hybridization procedure is labour-intensive, time-consuming, and quite expensive.[37] The two suitable and commonly employed housekeeping genes for molecular identification of *S. pneumoniae* are 16S rRNA and *rpoB*.[22,38,39] 16S rRNA gene is a nuclear fragment present in almost all bacterial cells. It has three properties that make it suitable for the identification, classification, and quantification of most bacterial cells. Its nucleotide sequence is fairly predictable and has not been swept aside by cellular evolution.[40] The gene is fairly sizeable. At 1500 bp in size, it is a broad repertoire of vital scientific information. However, *S. pneumoniae* shares, to a high degree, 16S rRNA sequence homology with *S. mitis* and *S. oralis.* This makes it difficult to discriminate these organisms using 16S rRNA genetic information.[41] For improved discrimination between these genetically related Streptococci, *rpoB*-based phylogeny serves as a better genomic marker because of its lower similarities among these species.[39]

Clinical Diagnosis of *Streptococcus pneumoniae*

For prompt treatment and recovery of patients with pneumococcal infections, a sensitive, specific, and rapid diagnostic procedure is a necessity in standard clinical practices. Isolation and identification of *S. pneumoniae* using blood culture take 2 to 3 days. Adding to the low sensitivity of this diagnosis, culture is not ideal for clinical practice, especially when the patient's health is critical and requires urgent medical attention.[42] S. *pneumoniae* cell wall contains an antigenic molecule called C-polysaccharide, which can be detected in the urine of a carrier. The first pitfall of this procedure is that the C-polysaccharide antigen is also present in the urine of healthy children with opportunistic *S. pneumoniae* colonizing their nasopharyngeal region.[43] Other pitfalls of this *S. pneumoniae* urinary antigen test are the possibility of a false positive test caused by pneumococcal vaccine in immunized patients and

the presence of pneumococcal C-polysaccharide-like compound secreted by *S. mitis.* The antigenic C-polysaccharide has also been detected in the urine of patients several days after treatment and recovery from the infection. This assay may not be suitable for detecting *S. pneumoniae* infection in populations with high colonization of the nasopharynx.[44]

There are some commercially available kits that utilize the principle of agglutination in the identification of *S. pneumoniae* from BAP. The kits are designed with beads coated with rabbit antibody which agglutinates with pneumococcal capsular antigen. The only limitation to this procedure is the possibility of cross-reaction with other streptococci in an aged culture.[45]

For efficient and timely discrimination between *S. pneumoniae* infection and nasopharynx colonization, the putative proteinase maturation protein A (PpmA) should be exploited. This is because PpmA of *S. pneumoniae* is one of the three classes of peptidyl-prolyl cis–trans isomerases (Pplases) which contribute to their virulence and offer immunogenic protection to the bacteria.[44,46] The lipoprotein is relatively the same across several *S. pneumoniae* serotypes, making it suitable for drug and vaccine targets.[47] Since PpmA is also one of the bacterial extracellular molecules present in a urine sample, infection due to *S. pneumoniae* can be detected using rabbit antibody and western blot techniques. This molecule can be detected in the urine using western blot assay.[45] It is quite significant to note that PpmA-deficient serotypes of *S. pneumoniae* have shown reduced virulence.

DISCUSSION AND CONCLUSION

Streptococcus pneumoniae shares major biochemical characteristics with other organisms of its genetic lineage, making it difficult for prompt clinical identification. This has adversely affected the treatment of pneumococcal infection and delayed the time of the patient's recovery.[39,48] Culture characteristics and biochemical tests such as colony morphology, optochin, and bile solubility tests can be time-consuming and less sensitive.

A molecular technique called DNA-DNA hybridization used in measuring genetic similarities among DNA sequences has been deployed extensively in microbial taxonomy. However, identification of *S. pneumoniae* using DNA-DNA hybridization is quite tedious and expensive. This is why the procedure is not suitable for clinical adaptation.[40]

16S rRNA and *rpoB* housekeeping genes have been developed as efficient methods for bacterial identification and novel strain assignment. 16S rRNA is relatively preserved in all bacterial cells. It is sizeable enough to hold important genetic information, making it a suitable tool for cell identification. However, *S. pneumoniae* shares up to 98% 16S rRNA gene sequence similarity with some organisms of its phylogenetic lineage and, hence, has poor resolution in strain substructure. The *rpoB* housekeeping gene, which has higher genetic resolution would be required as a complement for high throughput pneumococcal discrimination.[40,42]

The C-polysaccharide present in the *S. pneumoniae* cell wall can be promptly detected in the urine. It is a prompt and easy immunological procedure but enamored with drawbacks. C-polysaccharide antigen has been detected in the urine of healthy children with opportunistic *S. pneumoniae* colonizing their nasopharyngeal region.[43] It had also been detected in the urine of patients who had recovered from the infection. These indicate the possibility of false positive tests caused by pneumococcal vaccine and nasal colonization.[44]

The putative proteinase maturation protein A (PpmA) of *S. pneumoniae* is a class of Pplases that play significant roles in pneumococcal virulence and immunogenicity.[44,46] The lipoprotein is relatively the same across several *S. pneumoniae* serotypes and is present in urine samples of infection due to *S. pneumoniae.* This molecule can be detected in the urine using western blot assay.[45] This serves as an efficient tool for discrimination between infections due to *S. pneumoniae* and nasopharyngeal colonization.

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