

Molecular Detection of *nanA* and *nanB* Virulence Genes in Clinical *Streptococcus pneumoniae* Isolates from Patients with Pneumonia and Their Association with Disease Severity

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ABSTRACT

Objective: The goal of the study was to molecularly identify the neuraminidase-code genes *nanA* and *nanB* in clinical *S. pneumoniae* isolates obtained in patients with the pneumonia and to determine their relationship with the degree of disease severity. **Method:** One hundred and twenty clinical isolates were taken from hospitalized patients with positive pneumonia. The traditional PCR technique was used to confirm the identification of isolates using conventional microbiological methods. The relationship, co-occurrence patterns and the prevalence of *nanA* and *nanB* genes with clinical severity indicators were studied. **Results:** The *nanA* and *nanB* genes were detected in 91.7% and 74.2% of the isolates, respectively, with both genes present in 68.3% of the isolates. *NanB* alone and isolates carrying both *nanA* and *nanB* had a significant positive correlation with increased severity scores, increased levels of inflammatory drugs and higher intensive care unit (ICU) admission rates. **Novelty:** The results highlight the possible usefulness of the neuraminidase gene profiling as a prognostic system in the management of pneumococcal pneumonia.

INTRODUCTION

Pneumonia, also known as *Streptococcus pneumoniae*, is a Gram-positive encapsulated diplococcus that is a common commensal of the upper respiratory tract in healthy persons but a causative agent of severe invasive disease, including pneumonia, bacteremia, meningitis and otitis media. Pneumonia caused by the pneumococci is very high in the world, and the World Health Organisation estimates millions of cases every year, but this is disproportionately high among children below five years of age, the elderly and immunocompromised individuals. Although methods have improved in the use of vaccination and antimicrobial therapy, pneumococcal pneumonia is still associated with high morbidity and mortality, especially in patients admitted to the hospital with severe or complicated pneumonia [1].

The pathogenicity of *S. pneumoniae* is a multifactorial process, as this bacterium produces a variety of surface-associated and secreted virulence factors that collectively contribute to adherence and colonization, avoidance of host immune defenses, and host tissue destruction. Among these virulence determinants, neuraminidases (sialidases) have gained increasing scientific attention because of their crucial roles in bacterial colonization and pathogenesis. *S. pneumoniae* contains genes encoding three distinct neuraminidases, termed *nanA*, *nanB*, and *nanC*. These neuraminidases exhibit differences in their molecular structure, substrate specificity, catalytic mechanisms, and

contribution to disease development. Among them, NanA is the most frequently detected neuraminidase. It is anchored to the bacterial cell surface and shows broad sialidase activity towards a wide range of sialylated substrates. In contrast, NanB is a secreted neuraminidase with a specialized catalytic mechanism that predominantly generates 2,7-anhydro-N-acetylneuraminic acid. NanC possesses structural features similar to NanA but shows distinct catalytic and kinetic behaviour, suggesting a complementary role in pneumococcal virulence [2], [3]. Neuraminidases support pneumococcal infection by several different mechanisms. They expose the epithelial surface receptors that facilitate bacterial adherence, break down protective mucus lining respiratory tract epithelium and reveal hidden host antigens, which can change immune responses [4]. NanA has been found to support biofilm formation, increase the colonization of the nasopharynx and work in synergy with other co-infecting viral pathogens, including influenza virus to increase severity of disease [5], [6]. NanB, the least investigated, has shown to be critical and irreplaceable in respiratory tract infection and systemic sepsis and recent studies have indicated that it can regulate the production of mucus mediated by *nanA* in order to preserve immune evasion [7].

Molecular identification of *nanA* and *nanB* genes in clinical isolates is a valuable insight into the virulence capacity of circulating pneumococcal isolates. Epidemiologic analyses that have investigated the epidemiology of neuraminidase genes in clinical isolates have shown that there is a great deal of variability, although the relationship between particular gene carriage patterns and clinical disease severity has not yet been described fully. Knowledge of whether *nanB* alone or in combination with *nanA* has any effect on clinical outcome may have considerable implications on patient stratification, prognostic evaluation and rational development of neuraminidase-targeting therapeutic agents [8]. In this study, the PCR amplification was performed using 35 cycles to detect the *nanA* and *nanB* genes. The second objective was to describe the patterns of co-occurrence of these genes and their association with patient demographics and clinical presentation, and the third aim was to determine the relationship between the carrier of neuraminidase genes and the objective measures of disease severity such as inflammatory biomarkers, severity scoring system and clinical outcome. This study adds to the increasing evidence of the association between the profile of certain virulence genes and clinical phenotype and provides a basis for future interventions on pneumococcal neuraminidases [9].

RESEARCH METHOD

This study was conducted among hospitalized patients at the Specialized Center for Respiratory Diseases in Thi Qar Governorate, Iraq. Patients of both adult and pediatric ages were admitted with clinical and radiological signs of pneumonia and were eligible for enrolment in the study. Depending on the guidelines provided by the Infectious Diseases Society of America as well as the American Thoracic Society, conditions of pneumonia were defined by the appearance of an acute pulmonary

infiltrate on chest imaging and complemented by symptomatic characteristics such as fever, cough, sputum production, and leukocytosis. Patients who are previously treated with antibiotics longer than 48 hours prior to the specimen collection procedure, those with immunosuppressive conditions that render the microbiological interpretation meaningless, and patients with confirmed non-pneumococcal pneumonia were not included in the analysis. The Institutional Review Board provided ethical approval, and written informed consent was obtained from all participants or their legal guardians [10].

The severity of a disease was determined by the CURB-65 scoring system, which assigns a point to each as follows: confusion; urea level above 7 mmol/L; respiratory rate of 30 or above breaths per minute; blood pressure below 90/60 mmHg; and age of 65 years and above. The patients were divided into mild (CURB-65 score 0–1), moderate (score 2) and severe (scores 3–5) groups. Other severity measures were gathered, such as the necessity to be admitted to the ICU, the necessity to be facilitated by the means of mechanical ventilation, the duration of stay, and 30 days' mortality. Admission records of inflammatory biomarkers such as C-reactive protein (CRP), procalcitonin (PCT) and total leukocyte count were taken [11].

Aseptic respiratory samples (sputum, bronchoalveolar lavage fluid, endotracheal aspirates and blood cultures) were taken from enrolled patients. The specimens were also taken to the microbiology lab within two hours after pickup and subjected to the normal procedures. Quality assessment of sputum samples was done by the Bartlett system, and only samples that had fewer than 10 squamous epithelial cells and more than 25 polymorphonuclear leukocytes in low-power fields were subject to further processing. Test samples were cultured on 5% sheep blood agar and chocolate agar and incubated at 37°C in 5% CO₂ for 24–48 hours. Provisional identification alpha-hemolytic, optochin-sensitive colonies that had typical morphology were identified as *S. pneumoniae* and tested on confirmatory tests as bile solubility test [12].

The confirmation of *S. pneumoniae* was performed by traditional biochemical tests such as optochin sensitivity and bile solubility, with or without matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The Kirby-Bauer disc diffusion method was used to test antimicrobial susceptibility on Muller-Hinton agar with 5 per cent sheep blood, and E-test strips were used to measure minimum inhibitory concentrations (MICs) of penicillin. Interpretive criteria were based on the Clinical and Laboratory Standard Institute (CLSI) guidelines. Isolates resistant or with intermediate susceptibility to two or more classes of antibiotics were considered multidrug-resistant [13].

Overnight cultures underwent a commercial DNA extraction kit to extract the genomic DNA as per the instructions provided by the manufacturer. Spectrophotometry was used to measure the quality and concentration of extracted DNA, and samples with a ratio of A₂₆₀/A₂₈₀ of 1.8 to 2.0 and a DNA concentration of 20 ng/μL proceeded on to downstream PCR amplification [14]. Unique primers were developed that had a comparison on conserved regions of *nanA* and *nanB* genes according to published

sequences in GenBank. They were done using the following primer sequences: for *nanA*, forward 5'-ATGGCAAATACTAATTTAAGTGC-3' and reverse 5'-TTAAGCTTCTTTTCGGAGCTTC-3', yielding an amplicon of approximately 1,200 bp; for *nanB*, forward 5'-ATGAAAAAAATTTTAGTTTTAG-3' and reverse 5'-TTATTTTTCATCATCAATTTTC-3', yielding an amplicon of approximately 900 bp. PCR reactions were performed in a 25 µL reaction volume containing 12.5 µL of 2× master mix, 1 µL of each primer at 10 pmol/µL, 2 µL of template DNA, and nuclease-free water to the volume. Thermocycling conditions consisted of initial denaturation of 95°C at 5 min, followed by 35 cycles of denaturation of 95°C at 30 s, annealing of 55°C at 30 s and extension of 72°C at 90 s and, finally, extension of 72°C at 10 min. The PCR products were separated using a 1.5% agarose gel through electrophoresis, whereby they were stained with ethidium bromide and viewed under ultraviolet light [15].

The SPSS version 26.0 (IBM Corp., Armonk, NY, USA) was used to enter and analyze the data. Categorical variables were written in frequency and percentage, whereas continuous variables were written in means ± standard deviation or in the median level and interquartile range based on the normality of distributions measured by the Shapiro-Wilk test. The relationship of neuraminidase gene carriers to categorical clinical variables was tested using the chi-square or Fisher exact test. An independent samples t-test or Mann-Whitney U test was used to determine the differences among groups of continuous variables based on gene carriage. The analysis, using logistic regression, was conducted to determine independent predictors of severe pneumonia, and the status of neuraminidase genes was added together with the previously known clinical predictors. The p-value of below 0.05 was taken as statistically significant [16].

RESULT AND DISCUSSION

Results

One hundred and twenty confirmed isolates of *S. pneumoniae* were obtained from 120 patients with whom the study was carried out. The baseline clinical and demographic profile of the population used in the study is presented in Table 1. The average age of the participants was 47.3 years (18.6 years) with a mild male domination (57.5%). Most patients appeared with community-acquired pneumonia (83.3%), and the rest of the patients had healthcare-associated disease [17]. There were widespread comorbidities with diabetes mellitus, chronic obstructive pulmonary disease, and cardiovascular disease, being 28.3%, 22.5%, and 18.3%, respectively. The 35 patients (29.2%) were determined as mild, 45 (37.5%) as moderate, and 40 (33.3%) as severe using the CURB-65 scoring system. The patients were 25.8% of those who were required to be admitted to the ICU and 11.7% of those who died within 30 days [18].

Table 1. Demographic and clinical variables of research subjects (n=120).

Characteristic	Value
Age, mean ± SD (years)	47.3 ± 18.6

Characteristic	Value
Male sex, n (%)	69 (57.5%)
Community-acquired pneumonia, n (%)	100 (83.3%)
Diabetes mellitus, n (%)	34 (28.3%)
COPD, n (%)	27 (22.5%)
Cardiovascular disease, n (%)	22 (18.3%)
CURB-65 Mild (0–1), n (%)	35 (29.2%)
CURB-65 Moderate (2), n (%)	45 (37.5%)
CURB-65 Severe (3–5), n (%)	40 (33.3%)
ICU admission, n (%)	31 (25.8%)
Mechanical ventilation, n (%)	19 (15.8%)
30-day mortality, n (%)	14 (11.7%)
Mean hospital stay, days $\pm$ SD	9.4 $\pm$ 5.2

Table 2 shows the molecular detection results for the *nanA* and *nanB* genes among the 120 clinical isolates. The *nanA* gene was detected in 110 isolates (91.7%), whereas the *nanB* gene was detected in 89 isolates (74.2%). The most frequent genotypic pattern was the concurrent presence of *nanA* and *nanB*, which was observed in 82 isolates (68.3%). Isolates carrying *nanA* alone and *nanB* alone accounted for 28 (23.3%) and 7 (5.8%) isolates, respectively. Eleven isolates (9.2%) had no detectable *nanA*, whereas 31 isolates (25.8%) had no detectable *nanB*. Three isolates (2.5%) carried neither gene. These findings indicate that *nanA* is highly prevalent among clinical pneumococcal isolates, consistent with its established role as a conserved core virulence determinant, whereas *nanB* was less prevalent among the clinical isolates [19].



Figure 1. Conventional PCR for detection of the *nanA* gene (312 bp) in clinical isolates of *S. pneumoniae*, M: Marker DNA ladder (1000–100 bp), and lane (6 and 7) were negative amplification of *nanA* gene.

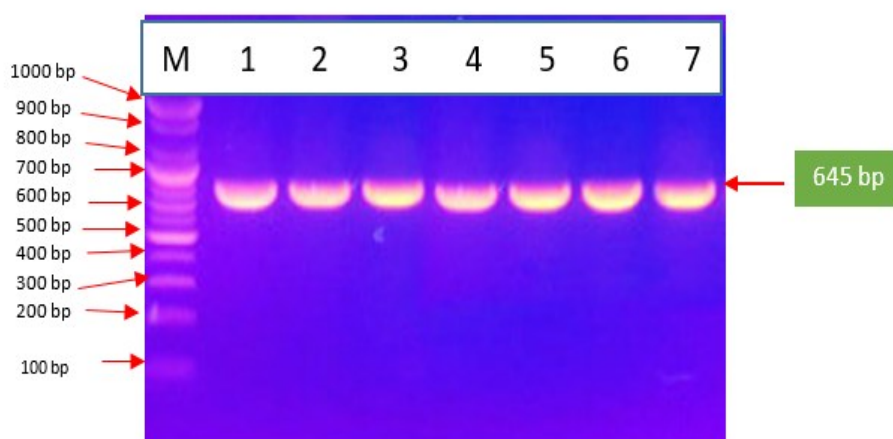


Figure 2. Conventional PCR for detection of the *nanB* gene (645 bp) in clinical isolates of *S. pneumoniae*, M: Marker DNA Ladder (1000-100 bp). All samples were positive for amplification of the *nanB* gene.

This is the result of a successful amplification of the 16S rRNA gene of six different clinical isolates (lanes one to six). Lane M has a molecular weight ladder of DNA (marker). The fact that there was one bright band of about 1500 base pairs (bp) in each of the sample lanes indicates that the bacterial DNA was of sufficient quality in order to proceed with the specific amplification of the genomic DNA (*nanA* and *nanB*). This was done as a quality control of the extracted genomic DNA prior to virulence gene profiling [20].

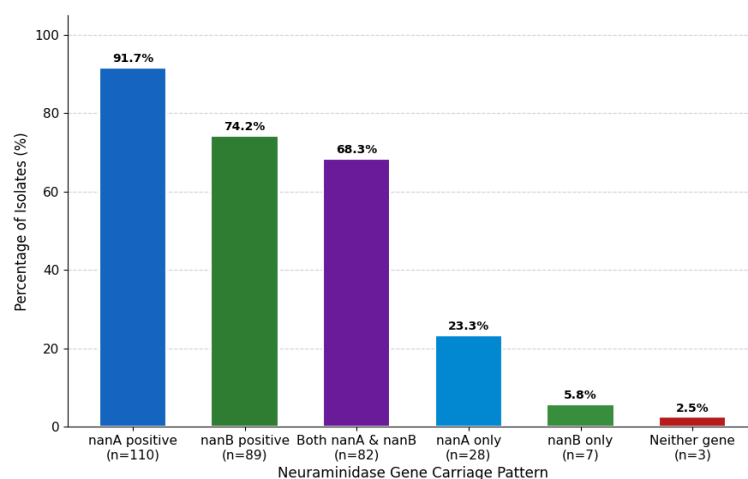


Figure 3. Bar graph of *nanA* predominant, *nanB* dominant, both genes dominant and none among 120 clinical *S. pneumoniae* isolates.

Table 2. Sensitivity Infection in clinical *S. pneumoniae* isolates (n = 120): Molecular detection of *nanA* and *nanB*.

Gene Pattern	Number of Isolates	Percentage (%)
nanA positive	110	91.7

Gene Pattern	Number of Isolates	Percentage (%)
nanB positive	89	74.2
Both nanA and nanB	82	68.3
nanA only	28	23.3
nanB only	7	5.8
Neither gene	3	2.5

Figure 2 and Table 3 illustrate and explain the relationship between the patterns of *nanA* and *nanB* carriage and CURB-65 severity classification. In isolates with both *nanA* and *nanB*, the percentage of patients with severe disease (CURB-65 score of 3-5) was much higher at 52.4% than at 25.0% with *nanA* only and 14.3% with neither of the two genes ($p = 0.001$). The *nanB* gene was also considered a very important severity determinant; its presence, both alone and combined with *nanA*, was correlated with significantly higher CURB-65 scores, higher ICU admission, and increased mechanical ventilation. This observation aligns with the experimental data that show that NanB is crucial and non-redundant in respiratory tract infection and its deletion causes the attenuated disease in animal models [19].

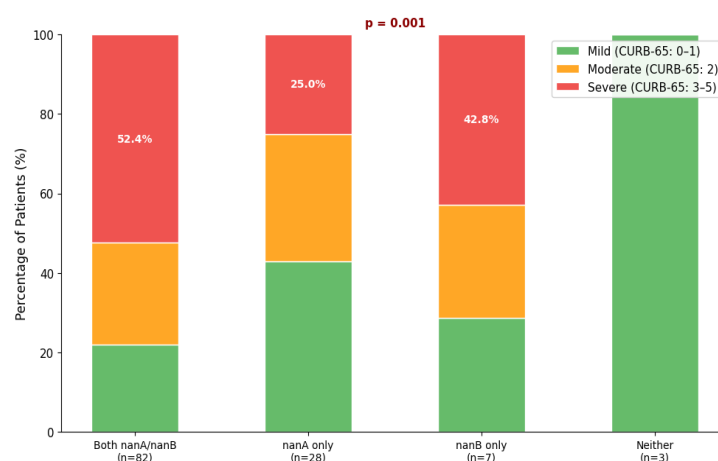


Figure 4. Stacked bar chart of distribution of CURB-65 severity categories (mild moderate and severe) in the 4 neuraminidase gene carry groups.

There was also a significant difference in the levels of inflammatory biomarkers in the groups of gene carriers. The mean CRP level among patients infected with isolates of *nanA* and *nanB* (156.4 ± 48.2 mg/L) was significantly higher than among patients infected with isolates of *nanA* alone and those infected with isolates of *nanB* alone (98.7 ± 36.1 and 72.3 ± 28.9 mg/L, respectively) ($p < 0.001$). Likewise, procalcitonin concentrations were very high in dual-positive (median 4.8 ng/mL, IQR 2.1-9.3) in comparison to *nanA*-only (median 1.9 ng/mL, IQR 0.8-3.6). This information indicates that the concomitant expression of neuraminidase by infecting isolates pushes to a more extreme systemic inflammatory response, potentially via increased tissue invasion and exposure of immunostimulatory host epitopes like the Thomsen-Friedenreich antigen [13].

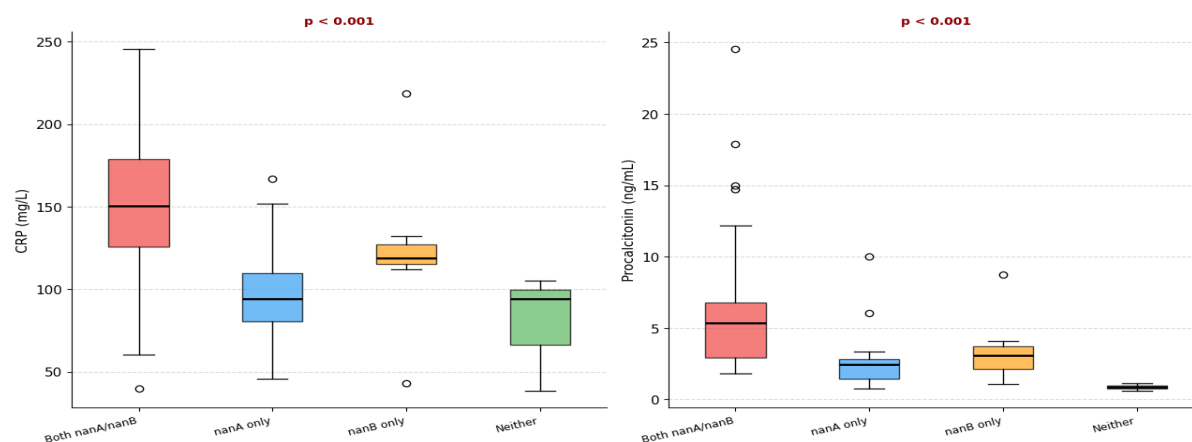


Figure 5. Comparison of CRP and procalcitonin levels using a box plot among the neuraminidase gene carriers.

Table 4 provides clinical outcomes stratified according to the neuraminidase gene carriage pattern. Patients with dual isolates that were nanA/nanB-positive had the highest ICU admission rate of 36.6% in comparison to 10.7% in the nanA-only group and 0% in the gene-negative group ($p = 0.002$). The 30-day mortality exhibited a comparable trend, exhibiting a death rate of 17.1% within the dual-positive cohort compared with 3.6% in the nanA-only group ($p = 0.048$). The mean length of stay was also significantly increased in the patients in the dual-positive group (11.8 ± 5.9 days) in comparison to the nanA-only group (7.2 ± 3.8 days) ($p < 0.001$). The presence of dual nanA/nanB was recognized as an independent predictor of severe pneumonia. (adjusted odds ratio 4.12, 95% CI 1.87-9.08, $p = 0.001$) using logistic regression analysis, subsequent to the adjustment for age, gender, concomitant conditions, and antibiotic resistance, which adds more support to the argument of neuraminidase genes as biologically plausible virulence and prognostic factors [16].

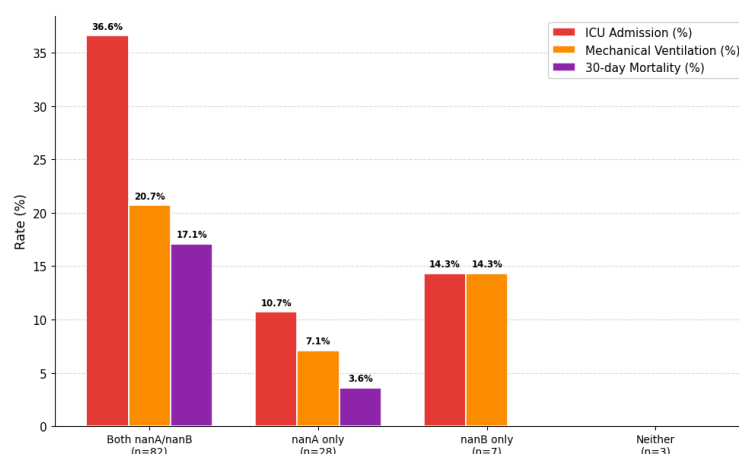


Figure 6. A grouped bar chart illustrating ICU admission rates and 30-day death rates by stratification of the rates by the neuraminidase gene carrier of the pattern.

Table 3. Correlation of neuraminidase gene carriers with CURB-65 severity categorization.

Gene Pattern	Mild n (%)	Moderate n (%)	Severe n (%)	p-value
Both <i>nanA</i> & <i>nanB</i> (n=82)	18 (22.0%)	21 (25.6%)	43 (52.4%)	0.001
<i>nanA</i> only (n=28)	12 (42.9%)	9 (32.1%)	7 (25.0%)	
<i>nanB</i> only (n=7)	2 (28.6%)	2 (28.6%)	3 (42.8%)	
Neither (n=3)	3 (100%)	0 (0%)	0 (0%)	

Table 4. Clinical outcomes according to neuraminidase gene carriage pattern.

Outcome	Both <i>nanA/nanB</i> (n=82)	<i>nanA</i> only (n=28)	<i>nanB</i> only (n=7)	Neither (n=3)	p-value
ICU admission, n (%)	30 (36.6%)	3 (10.7%)	1 (14.3%)	0 (0%)	0.002
Mechanical ventilation, n (%)	17 (20.7%)	2 (7.1%)	1 (14.3%)	0 (0%)	0.031
30-day mortality, n (%)	14 (17.1%)	1 (3.6%)	0 (0%)	0 (0%)	0.048
Hospital stay, mean days $\pm$ SD	11.8 $\pm$ 5.9	7.2 $\pm$ 3.8	8.4 $\pm$ 4.1	5.0 $\pm$ 1.7	<0.001

Antimicrobial susceptibility profiles established that the prevalence of multidrug resistance was higher in the *nanA*- and *nanB*-carrying isolates than in the single-carrying encoding and non-carrying encoding isolates. Within the dual-positive isolates, 39.0% of them were resistant to penicillin, 29.3% to erythromycin and 26.8% to trimethoprim-sulfamethoxazole. Conversely, in *nanA*-only isolates, penicillin resistance occurred only in 17.9% and erythromycin resistance in 14.3%. The simultaneous presence of multidrug resistance and dual neuraminidase gene carriages could be indicative of selective forces that act on the virulent strains present in hospital settings, where both the antibiotic selection pressures and the host immune selective pressures co-select the resistance and virulence determinants. The underlying biological mechanism of such co-selection remains poorly understood and could be associated with the genomic plasticity of pneumococci and the predisposition to recombination events which simultaneously confer resistance cassettes and virulence loci [20].

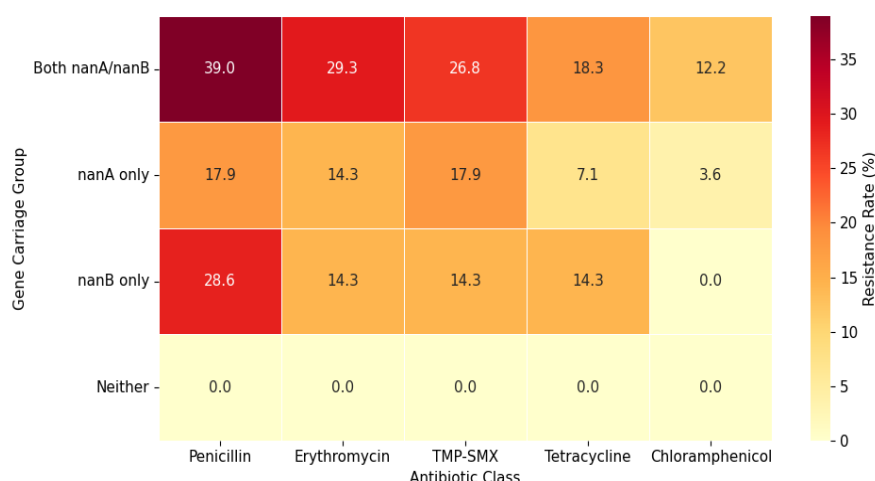


Figure 7. Figure of the heat map with the rate of antimicrobial resistance to five antibiotic classes in each of four groups of neuraminidase gene carrying.

Gene detection rate through analysis of the specimen type indicated that *nanA* was highly detected in all specifications of the specimen, such as sputum (90.3%), bronchoalveolar lavage (93.5%), endotracheal aspirates (94.7%), and blood culture isolates (90.0%). Conversely, the *nanB* detection possessed greater variability across the types of specimen, with the highest being in BAL-derived isolates (80.6%) and the lowest being in the sputum isolates (69.4%). Invasive isolates in blood cultures had much higher proportions of dual *nanA*/*nanB* expression (80.0% vs. 65.7% in non-invasive respiratory samples), indicating that the co-expression of the two neuraminidases might provide a survival or spread benefit in systemic infection. This is in line with the experimental findings that NanA enables the translocation of bacteria across the epithelial barriers and NanB regulates the microenvironment in the mucus to facilitate the further intrusion into tissues [4].

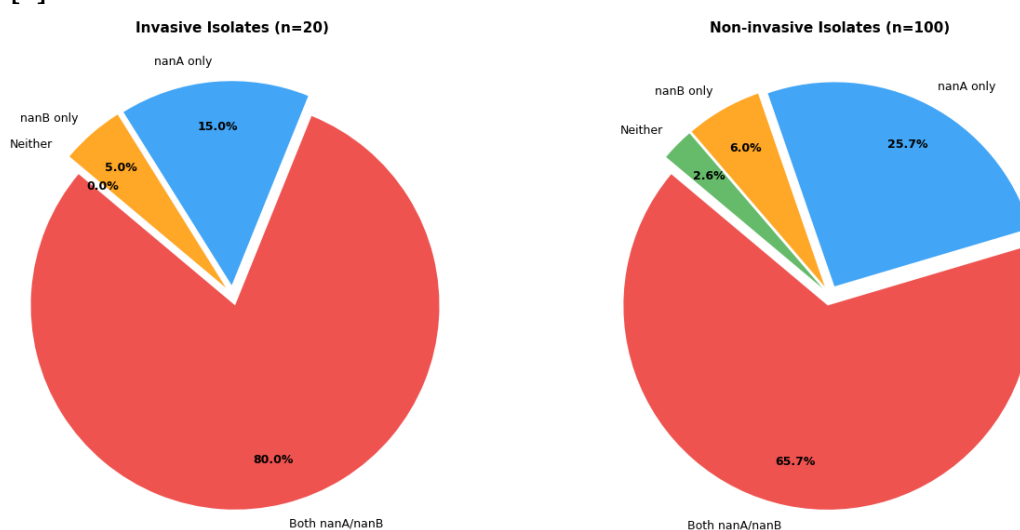


Figure 8. Pie charts (side-by-side) of invasive and non-invasive isolates of the proportion of each gene carriage pattern.

Discussion

The current work represents a broad molecular characterization of *nanA* and *nanB* genes in clinical isolates of *S. pneumoniae* and creates significant correlations between the patterns of the neuraminidase gene carriage and objective indicators of severity of pneumonia. The almost perfect prevalence of NanA (91.7) as it is determined in this study is coherent with the numerous previous studies and is indicative of the position of NanA as a fundamental element of the pneumococcal virulence arsenal. The *nanA* gene expresses a large multi-domain enzyme anchored to the bacterial cell surface through an LPXTG motif, and its catalytic activity is fundamental to the initial stages of host colonization, such as the degradation of the sialic acid-rich mucus layer and exposure of cell surface receptors on respiratory epithelial cells [21]. The observation that *nanA* is very common in different clinical isolates regardless of serotype or severity of the disease is a statement that it is a basic part of pneumococcal biology [22].

The percentage of *nanB* in our cohort of 74.2% was a bit higher than was given in some previous studies but generally in harmony with results of areas with comparable pneumococcal epidemiology. NanB is a secreted enzyme that is mechanistically distinct from NanA; instead of releasing free N-acetylneuraminic acid (Neu5Ac), it generates 2,7-anhydro-Neu5Ac as its major product, which itself has weak neuraminidase-inhibitory potential and has the potential to modulate the local microenvironment of sialic acid in a manner that is yet to be comprehended [15]. The clear biochemical difference between NanB and NanA creates interesting questions of functional complementation between the two enzymes during infection. Recent studies have shown that NanB has a regulatory role regulating NanA-mediated mucus secretion and evasion, suggesting that the two enzymes have a regulatory relationship that may enhance virulence when NanB and NanA are active simultaneously [7].

The clinical significance of this study, measured by the most significant result of the work, was the close and independent correlation between the presence of dual *nanA/nanB* and severe pneumonia. By logistic regression, patients with dual-positive isolates were found to be about four times more likely to have severe disease, and patients with dual-positive isolates were found to have greater rates of ICU admission, longer hospitalization, and excess mortality. This finding remained even once the significant confounding factors such as the age of patients, the presence of certain comorbidities, and the resistance to antimicrobials were also adjusted, which gives the relationship biological plausibility. The mechanistic etiology of this severity correlate is probably complex. The expression of both types of neuraminidases can result in the greater sialic acid stripping of host surfaces, resulting in an increase of the Thomsen-Friedenreich antigen and other cryptic epitopes that cause regulation imbalances of complement and antibody reactions [13]. Moreover, NanA and NanB may act together in the respiratory mucus layer and lead to a deeper impairment of the mucociliary clearance and allow more penetration of the lungs and higher levels of alveolar damage [4].

The finding that invasive isolates of a blood culture exhibited higher frequencies of the dual gene carriage as compared to non-invasive respiratory isolates of the bacterium further supports the idea that co-expression of neuraminidase provides a pathogenic benefit to the bacterium when it spreads systemically. NanA has been shown to degrade erythrocyte and platelet surface sialic acids in peripheral blood, which may be one of the causes of thrombocytopenia and hemolytic complications which are known and recognizable features of severe pneumococcal sepsis [23]. It is also an important question to be investigated in the future whether NanB has analogous or complementary activities in the bloodstream or not. The correlation between hemolytic uremic syndrome and nanC-involving serotypes in pediatric pneumococcal disease offers an educative analogy, implying that particular neuraminidase types could be used to forecast specific and extreme clinical phenotypes [24].

The ability of dual neuraminidase gene carriers to facilitate systemic inflammation is indicated by the correlation between the marker of dual neuraminidase gene carriage and high concentrations of inflammatory biomarkers, especially CRP and procalcitonin. Unmasking of host structures in the presence of neuraminidase triggers pattern recognition receptors, enhancing the release of cytokines, and is part of the systemic inflammatory response syndrome that forms the basis of the most serious forms of pneumococcal pneumonia. Procalcitonin, which had proven useful as a biomarker of the severity of bacterial infection, was significantly increased in the dual-positive group, indicating that neuraminidase gene profiling may augment current biomarker approaches to assess the severity of infection [11]. The detection of the dual neuraminidase gene as a prognostic tool has utility that needs to be prospectively tested in larger cohorts [25].

Therapeutically, this study provides support to the argument of the need to focus on pneumococcal neuraminidases as a virulence-reducing strategy. Neuraminidase inhibitors have been considered as direct anti-pneumococcal agents as well as agents that mediate the synergistic relationship between *S. pneumoniae* and influenza virus that is one of the deadliest scenarios of co-infection in respiratory medicine [26]. Dual-acting compounds, including those obtained from natural plant products, have specific potential considering the occurrence of mixed viral-bacterial pneumonia [27]. Their catalytic site and substrate-binding pocket structural variations between NanA and NanB have been detailed in giving a rational premise of why inhibitors may be designed to act selectively or in a broad-spectrum manner [28]. A successful development of such inhibitors would not only have an implication on treatment but also may have an implication on prophylaxis in the high-risk patients that are colonized with virulent dual-positive strains [29].

The observation that *nanA* has a high degree of sequence variability in clinical isolates, as it has been reported in earlier genetic studies, has consequences regarding the reproducibility of PCR-based detection with fixed primer sets. The elevated rate of *nanA* detection in our experiment indicates that the primers used covered enough of a

conserved region, yet the occurrence of the primers and divergent alleles cannot be ruled out [30] and could be considered in future studies through the sequencing-based methodology. The diversity of *nanA* sequences is at least partially due to recombination events with other streptococcal species, and the resulting allelic variation can have an effect on enzyme activity, inhibitor sensitivity and even virulence [20]. Defining the particular alleles of *nanA* in severe and mild cases of disease might provide more information on the virulence factors that are coded in this gene [31].

There are a number of limitations of this study that should be noted. The single-center design restricts the extrapolation of the results to other geographical locations and healthcare settings that have a different distribution of epidemiology and serotypes of pneumococcus. The cross-sectional study of gene detection does not allow conclusions about the time-related dynamics of neuraminidase gene activity in the case of infection. Moreover, the study identified neuraminidase genes at the DNA level, but the expression of functional enzyme was not directly determined, and the presence of a gene does not always correspond to similar levels of protein expression and activity in all isolates. The gap between the results of the genetic analysis and the functional virulence capacity should be bridged by future research by including transcriptomic analysis and assays of enzymatic activity [32]. Also, not all isolates in this cohort had serotype data available, which may have contained valuable contextual information regarding the association between the capsular type and the presence of neuraminidase genes and clinical outcome, and it is another significant area of study to fill in further studies [33].

CONCLUSION

Fundamental Finding: This paper has shown that *nanA* is almost universally carried by clinical *S. pneumoniae* isolates that cause pneumonia, and *nanB* is carried by about three-quarters of isolates, with the co-carriage of both genes being the most common genotypic pattern. Both *nanA* and *nanB* are solely linked to the increased disease manifestations, elevated levels of inflammatory biomarkers, ICU hospitalization, and mortality rates. **Implication:** These results define neuraminidase gene profiling as a potentially useful risk stratification tool in pneumococcal pneumonia and furnish an excellent biological longing in the prioritization of neuraminidase-directing therapeutic interventions. **Limitation:** The study was limited by the absence of bacterial serotyping, quantitative assessment of neuraminidase expression, and validation in broader multicenter patient populations. **Future Research:** These findings should be validated and extended by future multicenter investigations that include serotyping of bacteria, quantification of neuraminidase expression by enzyme-linked immunosorbent assays, and additional cohort studies of patients.

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