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Coronavirus Pandemic

Nasopharyngeal pneumococcal carriage among unvaccinated healthy Sri Lankan children during the COVID-19 pandemic

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Abstract

Introduction: *Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide, especially in children. This study focused on the prevalence, serotypes, antibiotic resistance, and biofilm formation of pneumococci colonising an unvaccinated Sri Lankan children cohort aged ≤ 2 years during the COVID-19 pandemic.

Methodology: This descriptive, cross-sectional study was carried out between April to August 2021 among healthy children visiting routine immunisation clinics in the Colombo district, Sri Lanka. Nasopharyngeal swabs (NPS) from healthy children were collected and cultured on sheep blood agar to isolate *S. pneumoniae* and confirmed by lytA gene-specific PCR. All confirmed *S. pneumoniae* isolates underwent capsular sequence typing to detect serotypes. Antibiotic susceptibility was determined. In-vitro biofilm-forming ability was assessed using the crystal violet assay, tetrazolium reduction assay, and scanning electron microscopy.

Results: The *S. pneumoniae* colonization rate of healthy children was 5.7% (20/350). Serotype 19F was the commonest, and 80% (16/20) of isolates were covered by the 13-valent pneumococcal conjugate vaccine (PCV13). All isolates were sensitive to levofloxacin, vancomycin, and linezolid but showed significant non-susceptibility to penicillin (70%, 14/20) and cefotaxime (15%, 3/20) at non-meningitis break points. All isolates formed biofilms.

Conclusions: A comparatively lower rate of pneumococcal colonisation was observed among this cohort compared to the current literature. The most prevalent serotype identified was 19F. Serotype pattern was similar to the pre-vaccine era pattern reported globally. Antibiotic non-susceptibility rates were high for penicillin and erythromycin. Almost all isolates showed evidence of in vitro biofilm formation.

Key words: *Streptococcus pneumoniae;* pneumococcal vaccine; colonization; biofilms.

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Introduction

Streptococcus pneumoniae is a Gram-positive, lancet-shaped diplococcus that commonly colonizes the human nasopharynx. It causes fatal infections globally, particularly in developing countries. *S. pneumoniae* colonization is generally a prerequisite for the development of pneumococcal infection, including invasive disease [1–3].

Previous systematic reviews indicate that pneumococcal colonization rates are higher in low and middle-income countries than in high-income countries [4,5]. Three previous Sri Lankan studies conducted before the COVID-19 pandemic reported varying nasopharyngeal colonization rates (21% to 32%) among children [6–8]. The prevalence of pneumococcal colonization is higher in the first 24 months of life and varies depending on socioeconomic and geographic factors [9]. Pneumococci are transmitted through direct contact and indirectly via contaminated surfaces [1–3,10]. Infants and young children serve as reservoirs, with frequent transmission occurring in day care centers [11].

Pneumococcal conjugate vaccines (PCVs) have significantly reduced the burden of pneumococcal infections in children, which target up to 13 serotypes (serotype 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) responsible for a majority of the infections. It is also shown to be effective in preventing carriage of vaccine serotypes [12]. Yet, after the introduction of PCVs, there has been a global rise in pneumococcal disease caused by non-vaccine serotypes (6C, 12A/F, 23B, and 34) [13].

While the pneumococcal vaccine is included in the National Immunization Programs (NIP) of many developing countries, it has not yet been introduced into the NIP in Sri Lanka. However, some children who attend fee-levying immunization clinics do receive the pneumococcal vaccine. In Sri Lanka, pneumonia is a leading cause of death in children under five [14]. In the absence of PCVs in the NIP, *S. pneumoniae* is likely the leading cause of most pneumonia cases.

Pneumococcal vaccination helps combat the growing threat of antimicrobial resistance by reducing disease incidence, minimizing antibiotic use, and lowering the risk of resistant infections [15]. Furthermore, Antibiotic resistance has risen among isolates from nasopharyngeal and clinical specimens worldwide [16]. Biofilm formation plays a role in colonization dynamics as well as antibiotic resistance. The protective matrix formed in biofilms enables bacteria to evade immune responses, promoting persistence and spread [17]. Few studies have investigated biofilm formation by *S. pneumoniae* in low-income countries [17,18], and none have addressed this issue in the Sri Lankan context.

During the COVID-19 pandemic, the incidence of pneumococcal disease reported was decreased, likely due to social distancing and enhanced respiratory precautions and cleaning practices [19]. However, studies investigating the prevalence of pneumococcal colonization during this period have yielded conflicting results [20–22]. Therefore, this study aimed to investigate pneumococcal colonization prevalence, serotype distribution, antibiotic susceptibility, and biofilm formation in children aged ≤ 2 years in an urban, community-based sample in Sri Lanka during the COVID-19 pandemic.

Methodology

Study design and setting

This descriptive, cross-sectional study was carried out between April to August 2021 among healthy children visiting routine immunization clinics. Healthy children visit immunization clinics in Sri Lanka to receive scheduled vaccines, monitor growth, and obtain essential health guidance as part of the national child health programme. Ethical approval for the study was granted by the Ethics Review Committees of the Faculty of Medical Sciences, University of Sri Jayewardenepura (ERC/FMS 11/20). Written informed consent was obtained from each parent /guardian before enrolment.

Study population and sampling

The study was carried out with a statistically analyzed sample size to describe the prevalence of *Streptococcus pneumoniae* colonization among healthy children aged ≤ 2 years in the Colombo District. Sampling was carried out as a multistage sampling method. Two medical officers of health (MOH) areas (Homagama and Kesbawa) were selected randomly from the Colombo district. Eight public health midwife (PHM) areas were also selected randomly from the selected MOH areas. Among the children who attended the clinics during the time of sample collection, all eligible children from each PHM were recruited for the study until the total sample size of 350 was obtained.

The sample number was calculated based on the formula:

$$n = \frac{Z^2 . P(1 - P)}{d^2}$$

where, (n) = sample size, (Z) = 1.96 for 95% confidence level, (d) = 5% and the expected prevalence to detect, (P) is set to 31.8% according to the available data [6]. The minimum sample size was calculated for 333. After 5% correction for the non-responsive rate, the final sample size was 349 from the Colombo District.

Healthy children aged 2 years or below [a child was considered healthy if the child attended the immunization clinic without any recent (within the past 15 days) or persisting respiratory infections and carried overall good general health with no history of a long-term illness] were considered as the inclusion criteria. Children diagnosed with COVID-19 within the previous month, who received the pneumococcal vaccine, who had current or recent (within the last 15 days) respiratory infection, who had chronic long-term illnesses (renal, cardiac, etc.), and who received at least one dose of any antibiotic treatment during the previous 15 days were excluded.

Data collection

A pre-piloted interviewer-administered questionnaire was used to collect data. Clinical, demographic, and general health information were obtained from a parent/guardian. Nasopharyngeal samples were obtained using an age-appropriate nylon-tipped sterile flocked swab. Swabs were dipped immediately into a screw-capped vial with skim milk, tryptone, glucose, and glycerine (STGG) medium. The swabs were transported on ice and stored at -80°C until further processing.

Laboratory testing

Nasopharyngeal swabs were thawed and vortexed, and 10 µL were inoculated on sheep blood agar and incubated up to 48 hours. Suspected colonies of pneumococci were identified by colony morphology and standard microbiological tests, including optochin susceptibility and bile solubility. A PCR assay was performed to confirm *S. pneumoniae* with the lytA gene. Capsular Sequence Typing (CST) was based on the previously published protocol by Marmara et al [23]. Sanger sequencing was carried out at Macrogen Inc. (Seoul, Korea) using BigDye chemistry with capillary electrophoresis in an ABI 3730 XL genetic analyzer. Forward and reverse sequences were obtained using M13F and M13R primers for each DNA fragment [23]. The sequencing results were viewed using Chromas version 2.6.6 (Technelysium Pvt Ltd). Sharp, evenly spaced peaks were considered in each forward and reverse DNA sequence, and upstream sequencing areas without sharp, evenly spaced peaks were removed from the sequence analysis. The trimmed nucleotide sequences were saved as a new corrected file and exported as a FASTA file for the CST Typing tool insertion. The edited nucleotide sequences were imported into the *S. pneumoniae* CST Typing Tool (https://www.rivm.nl/mpf/typingtool/spn, version 0.0, National Institute for Public Health and the Environment, Ministry of Health, Netherlands (accessed on 10 December 2022) (free online database of the National Institute for Public Health and the Environment, RIVM, Netherlands), and the *S. pneumoniae* serotype was automatically assigned.

Antibacterial susceptibility testing for all PCR-confirmed S. *pneumoniae* isolates was conducted using the disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. The minimum inhibitory concentration (MIC) for penicillin and cefotaxime was determined using the Epsilometer (E-Strip, Oxoid, UK) test method.

S. pneumoniae isolates were tested for biofilm-forming ability using crystal violet and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction assays (MTT) to quantify the bacterial biomass and cell viability, respectively. Biofilms were grown on sterile cover slips.

Crystal violet assay: The wells were fixed with 200 µL of 2% sodium acetate to adhere the biofilms to the wells. Staining was done with 100 µL of 0.1% crystal violet. The excess stain was removed by thorough washing with distilled water, and the plates were air-dried for 2 hours. Thirty percent acetic acid was added to each well (200 µL), and the absorbance of the final solution was measured at 595nm [24,25]. MTT assay: Fifty microliters of 1mg/mL MTT was added to each well and incubated at 37°C for 3 hours after covering the plates with aluminum paper. Following incubation, the remaining MTT reagent was removed by gently tapping, and 100 µL dimethyl sulfoxide (DMSO) was added to each well and mixed on a shaker for 10 minutes. The absorbance of the dissolved formazan product was obtained at 570 nm with a 630 nm reference filter [26]. The assays were carried out in triplicate to ensure reproducibility. Each isolate was classified as shown below [27]. Non-biofilm producer = OD (isolate) < ODc, Weak biofilm producer = ODc < OD (isolate) ≤ 2 x ODc, Moderate biofilm producer = 2 ODc < OD (isolate) ≤ 4 x ODc, Strong biofilm producer = 4 ODc < OD (isolate).

ODc - Optical density of cut-off value, OD (isolate) - Optical density of isolate. Cut off (ODc) = Average OD of negative control + (3 x Standard Deviation of negative control).

Ten biofilms grown on sterile coverslips were randomly selected, processed, and examined using a scanning electron microscope (SEM) for the architecture of biofilms. Limiting of SEM examination to ten selected coverslips was due to financial restrictions.

Table 1. Socio-demographic data of healthy children (n = 350).

Parameter	Total (n = 350)
Age in months	
Mean (SD)	8.9 (5.3)
Median	9.0
Sex	
Male	180 (51.4%)
Female	170 (48.6%)
Mother's age in years	
Mean (SD)	31.17 (5.8)
Median	31.0
Mother employed	98 (28.0%)
Mother's education	
Up to ordinary level	139 (39.7%)
Advanced level or above	211 (60.3%)
Father's education	
Up to ordinary level	154 (44.0%)
Advanced level or above	196 (56.0%)
Household income (SRs)	
Not informed	114 (32.6%)
≤ 40,000	146 (41.7%)
> 40,000	90 (25.7%)

Statistical analysis

Statistical analysis was performed using SPSS version 21. Categorical variables were expressed as proportions and continuous variables as mean (SD) or median (IQR).

Total household income was categorized by taking the estimated median value (approx. 40,000LKR) of total household income of a family in Sri Lanka [28]. Education level was categorized as primary or less (No education or grade 1-5), secondary/collegiate (Grade 6-12), and tertiary [29].

Results

Baseline characteristics

Three hundred and fifty healthy children were enrolled in the study. The participants were recruited from two out of thirteen MOH areas in an urban district. The median age was 9 months (IQR_{25-75}: 4-12). The percentage of males and females was 51.4% (180/350) and 48.6% (170/350), respectively. There were only five day-care attendees due to social restrictions during

Table 2. Antibiotic susceptibility patterns of the *S. pneumoniae* isolates (n = 20).

Antibiotic name	MIC (µg/mL)	Antibiotic susceptibility		
		Sensitive	Intermediate	Resistant
*Penicillin	0.002-32	-	-	20 (100)
**Penicillin	-	6 (30)	14 (70.0%)	-
*Cefotaxime	0.002-32	17 (85)	3 (15.0%)	-
**Cefotaxime	-	20 (100	-	-
Levofloxacin	-	20 (100)	-	-
Erythromycin	-	6 (30)	-	14 (70)
Clindamycin	-	14 (70)	-	6 (30)
Vancomycin	-	20 (100)	-	-
Chloramphenicol	-	16 (80)	-	4 (20)
Tetracycline	-	14 (60)	-	6 (40)
Linezolid	-	20 (100)	-	-

*Meningitis break point, ** Non-meningitis break point.

the COVID-19 pandemic, and parents were working from home. The main caretaker was the mother. Most of the mothers (60.3%, n = 211) had received secondary education or above. A substantial percentage (42%) had less than the median household income (Table 1).

Laboratory results

Of 350 screened children, only 20 were *S. pneumoniae* carriers (5.7%, 20/350). Serotype 19F was the most abundant (45%, n = 9). None of them were day-care attendees. Serotypes 3 and 9N/L were each detected in 15% (n = 3), and serotype 6B was identified in 10% (n = 2) of the pneumococcal isolates. For three isolates, serotype identification was inconclusive, in which 2 (10%) isolates were serotype 6A/B and one (5%) was 17A/F, 35B/C, 33C. The majority (16/20, 80%) of the serotypes were covered by the PCV13 vaccine (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) while PCV10 vaccine (serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F) covered 55% (11/20) of serotypes.

The antibiotic susceptibility patterns of S. pneumoniae

The antibiotic sensitivity test (ABST) was performed for all 20 isolates of PCR-confirmed *S. pneumoniae* (Table 2). All isolates were sensitive to levofloxacin, vancomycin, and linezolid. Resistance to penicillin and erythromycin was high. There were no fully resistant

Table 3. Antimicrobial non-susceptibility of different serotypes of *S. pneumoniae* (n = 20).

Antibiotic	Non-susceptibility							
	19F, n = 9	3, n = 3	6B, n = 2	6A/B, n = 2	PCV-13 n = 16 (%)	9N/L, n = 3	17A/F,35B/C, 33C, n = 1	Non-PCV13 n = 4, (%)
Penicillin* (non-meningitis)	5	3	2	2	**12 (75)**	2	0	**2 (50)**
Cefotaxime* (meningitis)	1	1	0	1	**3 (18.8)**	0	0	**0**
Erythromycin	6	3	1	2	**12 (75)**	1	1	**2 (50)**
Clindamycin	2	2	1	0	**5 (31.3)**	1	0	**1 (25)**
Chloramphenicol	3	1	0	0	**4 (25)**	0	0	**0**
Tetracycline	5	2	0	0	**7 (43.8)**	1	0	**1 (25)**

* Intermediate resistance.

Figure 1. Biofilms of *S. pneumoniae* on coverslips by scanning electron microscopy.

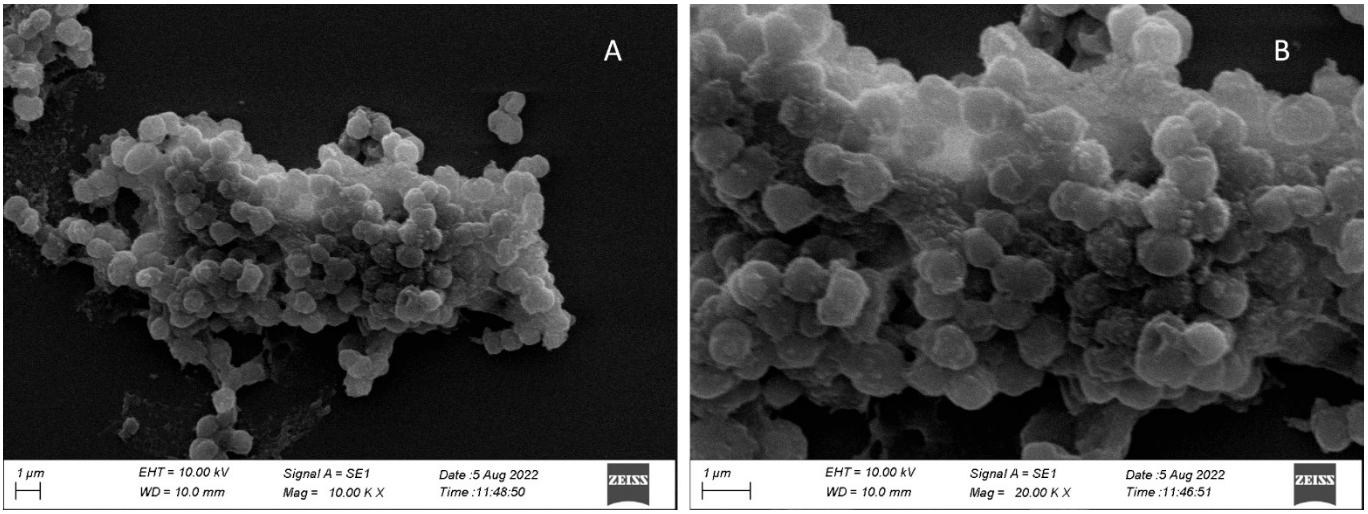
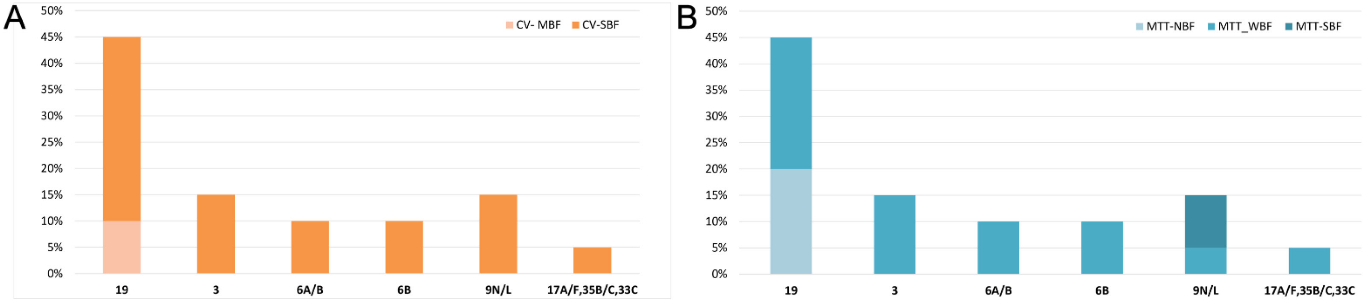


Figure 2. Biofilm formation with serotypes of *S. pneumoniae* by crystal violet (CV) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction assays (MTT).



isolates to cefotaxime at both meningitic and non-meningitic breakpoints.

The non-susceptibility profiles (resistant and intermediate-sensitivity) of different serotypes to antibiotics were analyzed. Overall, serotypes 19F and 3 exhibited a higher non-susceptibility rate to most of the antibiotics tested, while an uncertain serotype (17A/F, 35B/C, 33C) showed a relatively lower resistance. The serotypes were grouped according to their coverage by the 13-valent pneumococcal conjugate vaccine and compared with the corresponding antibiotic non-susceptibility (Table 3).

Biofilm formation ability of S. pneumoniae

Ninety percent (n = 18) of the isolates of pneumococci were strong biofilm formers, and only two isolates were moderate biofilm formers by biomass assessment through CV assay. By MTT assay assessing the viability of cells, the majority (70%, n = 14) were weak biofilm formers, while two isolates demonstrated strong biofilm formation. The remainder (n = 4) did not demonstrate viable biofilm formation through the MTT assay. On coverslips, one architecturally mature biofilm was detected by SEM (Figure 1)

The majority of 19F showed high biomass by CV assay (77.8%, n = 7), and in the MTT assay, 55.6% (n = 9) showed low cell viability (Figure 2).

The biofilm formation ability of the isolated pneumococci was compared with their antibacterial susceptibility patterns (Table 4). Strong biofilm formers in the CV assay had higher non-susceptibility rates (66.7%-100%) for all the antibiotics tested compared to moderate biofilm formers. Biofilm formers on MTT assay revealed 64.3%-100% non-susceptibility rates to most of the antibiotics, excluding chloramphenicol, compared to non-biofilm formers.

Discussion

Our study represents the first community-based study investigating pneumococcal colonization among healthy Sri Lankan children in an urban locality, and we report a very low rate of colonization (5.7%, n = 20) among this study population during the COVID-19 pandemic. Notably, most (80%, n = 16) of the isolates were vaccine serotypes (PCV-13). Antibiotic non-susceptibility was common, particularly to penicillin and erythromycin. Almost all isolates showed at least some evidence of biofilm formation.

In Sri Lanka, previous studies showed colonization rates of 26.4% in healthy children aged ≤5 years in Galle (2019), 31.8% in healthy children aged between 2 months and 2 years in Kandy (2017-2018), and 21% in children aged 2 months to 5 years admitted with signs and symptoms of meningitis, pneumonia, or very severe disease in Colombo (2005-2007) [6–8]. The low rate of pneumococcal colonization observed in the current study may be attributed to COVID-19 pandemic control measures, which were in place during the study period, such as social distancing, mask-wearing, minimal social gatherings, and lockdowns, which reduced children's exposure to potential carriers. However, the impact of COVID-19 preventive measures on pneumococcal

Table 4. Antibiotic non-susceptibility patterns of pneumococci against the biofilm forming ability.

Antibiotic (non-susceptible)	MTT (%)			CV (%)	
	NBF (n = 4)	WBF (n = 14)	SBF (n = 2)	MBF (n = 2)	SBF (n = 18)
Penicillin (non-meningitis) (14/20)	21.4	64.3	14.3	14.3	85.7
Cefotaxime (meningitis) (3/20)	-	100	-	33.3	66.7
Erythromycin (14/20)	21.4	71.4	7.1	7.1	92.9
Clindamycin (6/20)	33.3	50.0	16.7	-	100.0
Chloramphenicol (4/20)	50.0	50.0	-	25.0	75.0
Tetracycline (8/20)	12.5	75.0	12.5	25.0	75.0

NBF: non-biofilm former; WBF: weak-biofilm former; MBF: moderate-biofilm former; SBF: strong-biofilm former.

carriage is debated. Studies from Belgium and France found no significant reduction in pneumococcal carriage, while research from Vietnam and Israel reported lower rates during certain periods of the pandemic [22,30–32]. Especially, the reduction in Vietnam was due to decreased capsulated pneumococcal carriage, which is crucial for invasive disease [30]. Further, the low colonization rate observed with non-pharmaceutical interventions could be due to less number of viral respiratory infections. Since respiratory viruses are known to increase pneumococcal carriage density, transmission, and disease, interventions targeting these viruses may help reduce invasive pneumococcal disease.

Epidemiological surveillance of *S. pneumoniae* serotype carriage is essential to set up effective immunization programs and evaluate their impact. Before the introduction of pneumococcal conjugate vaccines (PCVs), serotypes 6A, 6B, 19A, 19F, and 23F were the most prevalent causes of pneumococcal illness in low-income countries [4]. In our study, serotype 19F was the most common among healthy children, accounting for 45% of cases. The next most prevalent serotypes were 3 (15%), 9A/F (15%), 6B (10%), and 6A/B (10%). Similarly, Vidanapathirana et al. (2020) and the ANSORP study [32] also identified serotype 19F as the most common colonizing serotype in Sri Lanka. Post-PCV introduction studies have shown that while the overall *S. pneumoniae* carriage rates remain unchanged, the carriage rates of vaccine serotypes decline and are replaced by non-vaccine serotypes [34]. Therefore, our study findings support the inclusion of PCVs in the Sri Lanka national immunization programme.

Various studies have investigated the antibiotic susceptibility of pneumococci isolated from healthy children, revealing differing resistance rates. In the present study, at the meningitis breakpoint, all isolates were non-susceptible to penicillin, while 15% showed intermediate resistance to cefotaxime. Similarly, high levels of penicillin non-susceptibility have been shown in other studies conducted in Sri Lanka [6,7] and other countries [16,35]. Vidanapathirana et al. also reported high non-susceptibility rates to cefotaxime at meningitis breakpoints (62.9%). These findings are consistent with studies on invasive pneumococcal disease, which reported over 90% penicillin resistance and approximately 47% cefotaxime non-susceptibility [8].

All 20 *S. pneumoniae* isolates in this study were sensitive to levofloxacin, vancomycin, and linezolid. Other studies from Sri Lanka [6,7] found 100% levofloxacin susceptibility but higher resistance rates for erythromycin, similar to our findings (70%). Kim et al. (2012) noted 78.9% erythromycin resistance in Asia. Lee et al. (2001) reported lower resistance rates, while studies from South Africa [35,36] showed varying resistance profiles.

The ability of pneumococci to form biofilms, particularly in healthy children, has been extensively studied. Biofilm formation is crucial for nasopharyngeal colonization [17,37]. In our study, a significant portion of pneumococci were strong biofilm formers using the CV assay, while the MTT assay, which focuses on metabolic activity, revealed the majority as weak biofilm formers. This difference arises because the MTT assay detects the viable cells but does not stain various other biofilm components. Thus, the CV assay quantifies the biomass of the biofilm, while the MTT assay estimates bacterial viability within the biofilm [38]. SEM images confirmed in vitro biofilm formation in only a small fraction of isolates. The reasons could be that biofilms grown on abiotic surfaces have delayed growth, low biomass, and lack characteristic structures, emphasizing the importance of an in vivo environment to study biofilms [17]. Especially, as *S. pneumoniae* is a nutritionally fastidious organism, its growth on abiotic surfaces could be affected to a larger extent.

The above findings should be interpreted with the following considerations. Firstly, the results cannot be generalized to rural and estate sectors since the study sample was drawn from an urban population. Comparability among studies is limited due to heterogeneity, particularly related to study sample, laboratory methods, and vaccination status. Another limitation of the study is the relatively small sample size, which may affect the generalizability of the findings and was considered when interpreting the results.

The sample collection for the study of nasopharyngeal carriage of pneumococci was carried out during the COVID-19 pandemic period. This could have affected the rate of PC colonization detected in this study, as this particular time period does not exactly show the true picture of community behaviour and environmental patterns (strict rules of wearing face masks at all social gatherings, no school or day-care activities, limitations on social gatherings, less air pollution, etc.). Also, because of the challenges posed by the COVID-19 pandemic, we encountered limitations in selecting the MOH areas in the Colombo district for our study and in obtaining nasopharyngeal swabs from healthy children. The biofilm formation of *S. pneumoniae* could hardly be observed by SEM when they were formed in vitro on glass cover slips. Many studies have been performed the biofilm in vivo using animal models. Continuous-culture biofilm system is suitable for characterizing biofilm formation by *S. pneumoniae* strains because the biofilm developmental process in *S. pneumoniae* is complex compared to other bacteria. The biofilm development process of *S. pneumoniae* is accompanied by an increased production of several proteins involved in attachment, resistance, and virulence. However, due to financial and technical constraints and ethical considerations in vivo or biofilm model could not be applied to the present study. In future studies, it will be interesting to test the capacity of *S. pneumoniae* to form biofilms, using more than one model system. Also, it is worth trying to develop and apply ex vivo models in pneumococcal biofilm studies, which may be an important technical step forward.

Conclusions

In conclusion, the current study found a comparatively lower rate of pneumococcal colonization among healthy children aged two years or below during the COVID-19 pandemic. The most prevalent serotype identified was 19F. The serotype pattern was similar to the pre-vaccine pattern reported globally, and the majority is covered by the available pneumococcal vaccines. Antibiotic non-susceptibility rates were high for penicillin and erythromycin. Almost all isolates showed at least some evidence of biofilm formation, indicating its significance in colonization dynamics.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Authors Contributions

MG: conceptualization, methodology, formal analysis, writing the original draft, reviewing and editing; GL, MW: methodology, writing, reviewing and editing the manuscript and supervision; RP: methodology, formal analysis, writing, reviewing and editing the manuscript; TD: conceptualization, funding acquisition, methodology, writing, reviewing and editing the manuscript and supervision. All authors approved the final manuscript.

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Conflict of interest

No conflict of interest is declared.

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