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Research Article

Carriage of Oropharyngeal Bacteria Among Children in a Rural Population Living in a Tropical Region in São Paulo, Brazil

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This study aimed to detect the carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in the oropharynx and a possible association with airway infections in children of a rural population living in a tropical settlement, São Paulo, Brazil. Demographic data were collected through standard questionnaires. Oropharyngeal samples were cultured and examined using the multiplex polymerase chain reaction. The molecular method had higher sensitivity and revealed a high rate of colonization by *S. pneumoniae* and *M. catarrhalis*. *H. influenzae* was not detected, highlighting the strength of Brazil's national immunization program. Low income was reported by 61.4% of participants. Carriage of *S. pneumoniae* was positively associated with being female (*P*=0.004) and being of brown color (*P*= 0.042). We identified risk factors for respiratory infections and vulnerabilities that may be widely applicable to other rural communities in Brazil.

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Introduction

Respiratory infections, both upper and lower, significantly contribute to global morbidity. Upper respiratory infections (URIs), caused mainly by *S. pneumoniae, H. influenzae,* and *M. catarrhalis*, are among the primary pathogens causing respiratory infections and the leading cause of acute disease incidence worldwide, while lower respiratory infections (LRIs) are a leading cause of morbidity and mortality. Globally, the incidence rate of URIs was 162 84·8 per 100 00 population in 2021, a decrease of 10·5% from 1990, when the incidence rate was 181 52·5 per 100 000 inhabitants.[1] Low- and middle-income tropical countries present the highest rates of colonization by these agents, especially in rural communities.[2][3][4][5] Socioeconomic and environmental conditions such as safe water, sanitation, and energy supplies, low family income, malnutrition, low levels of education, vaccination, and deficient prevention and treatment of infectious diseases contribute to these results. [6][7]

In Brazil, about 30 million people, or 16% of the population, live in rural areas. An increasing number of people live in rural settlements, presenting adverse conditions for health, such as inadequate waste disposal, deficiency in potable drinking water, and overcrowding in the home. It is well known that these conditions are directly linked to increased morbidity and mortality in children. [8][9] Rural settlements are found in different regions of São Paulo state, with the highest number in the western region, including the Dona Carmen settlement.[9] It has been hypothesized that the presence of socioeconomic and environmental risk factors in the settlement may favor the oropharyngeal carriage of microorganisms responsible for airway infections. Furthermore, the introduction of the *H. influenzaee* vaccine in the National Program of Immunization (NPI) may lead to a reduction in the carriage of these microorganisms in children.

Few studies have been conducted on infectious diseases in the vulnerable populations of rural settlements in Brazil.[10][11][12] This study aimed to detect the carriage of *Streptococcus pneumoniae*, *Haemophilus influenza*, and *Moraxella catarrhalis* in the oropharynx and to determine the association with airway infections in children of a population living in a tropical rural settlement, São Paulo, Brazil.

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Materials And Methods

Study design

Eligibility criteria

In the Dona Carmen settlement, located in the municipality of Mirante do Paranapanema, in the western region of São Paulo state, from February to May 2019, we conducted a cross-sectional and descriptive study; all specimens were collected from 44 of 522 (8.43%) individuals <18 years of age living in the settlement who agreed to take part in this study (Figure 1). Children and adolescents (people under 18) represented 19.8% of the Brazilian population. Of the 522 settlers, with a sampling error of 5% and a confidence level of 95%, the sample size under 18 years should be approximately 103 individuals. The study participants were randomly selected from healthy volunteers and ranged in age from 1 to 18 years. Informed consent was obtained from all of the participants before participation in the investigation. A short questionnaire interview was conducted to obtain information on demographics, clinical characteristics, and risk factors: age, gender, skin color, family income, use of antimicrobials, full vaccination, prematurity, breastfeeding, rhinitis, recurrent sinusitis, recurrent pneumonia, school attendance, and use of corticosteroids. The *H. influenzaee* (Hib) vaccine is part of the basic calendar of the National Program of Immunization (NPI), comprising the tetravalent vaccine (Diphtheria, tetanus, pertussis, and Hib); this was incorporated into the routine vaccination schedule in Brazil in 1999. The vaccine is applied in three doses at intervals of 60 days. Participants who received any antibiotic treatment within 72 hours of sample collection were excluded. By December 2022, the estimated population of São Paulo, the richest and most populous state of Brazil, was 46,024,937, 22.6% of the population of Brazil, estimated to be 203,062,512 according to the Brazilian Census of 2022[13][14] The state plays a crucial role in Brazil's public health, particularly through its Unified Health System (SUS), which provides access to healthcare services to all residents. The state also boasts a robust network of healthcare establishments, including numerous public and private hospitals, clinics, and health units, including very small municipalities.

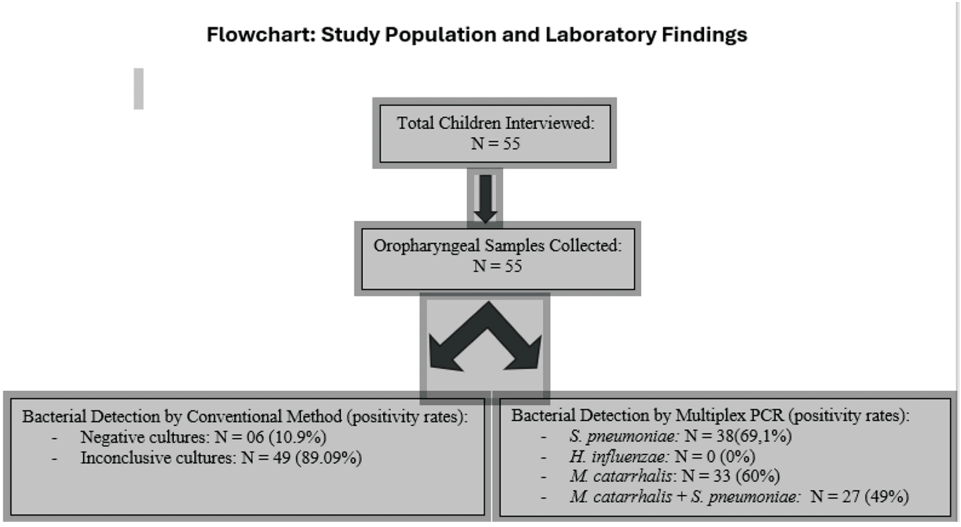

Figure 1.

Data collection

After collecting demographic data, two samples from the oropharynx were collected using a sterile saline-moistened swab (0.85%) with slight movements on the tonsils, always by the same examiner. The collected samples were stored in Stuart transport medium and transported to the laboratory immediately on ice. The analyses were conducted within 6 hours of collection.

Isolation and identification using conventional culture methods

In the microbiology laboratory, one swab was seeded on chocolate agar and blood agar and maintained at 37°C for 48 hours in an anaerobic jar. Five random colonies were selected based on their morphological characteristics. Bacterial identification of pathogens was performed using standard bacteriological procedures: *S. pneumoniae* was identified by optochin and bile testing; the identification of *H. influenzae*

was based on Gram staining, growth on chocolate agar, and lack of growth on trypticase agar with sheep blood supplemented with growth factor (Factor X and V). The identification of *M. catarrhalis* was based on Gram staining, a positive oxidase reaction, and a characteristic profile in biochemical tests. After the tests, bacterial colonies were stored in brain heart infusion broth enriched with 5% sheep blood and frozen at -70°C for further analysis by polymerase chain reaction (PCR).

Detection of S. pneumoniae, H. influenzae, and M. catarrhalis by polymerase chain reaction

The second swab, previously stored at -20˚C, was subjected to the phenol-chloroform technique to extract bacterial DNA. Briefly, the swab samples were suspended in 500 μL of lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, and 2.0% SDS), and 50 μL of 10% SDS. The samples were incubated for 1-3 h at a temperature of 56°C until dissolved. An equal volume of a phenol/chloroform/isoamyl alcohol (25:24:1) solution was added. Samples were then mixed by inverting the tubes for 3 min and centrifuged for 10 min at 10,000 × g (4°C). The aqueous layer in the supernatant was then transferred to a microcentrifuge tube with an equal volume of chilled isopropanol (Merck, Whitehouse Station, NJ, USA) added. The tubes were then cooled to -20°C for 1 h for precipitation. The samples were then centrifuged at 10,000 × g (4°C) for 10 min. After decanting the supernatant, 250 μL of 70% ethanol (Merck, Whitehouse Station, NJ, USA) was added, and the sediment was dissolved. The resulting mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was decanted. The resulting pellet was air-dried and suspended in 50 μL of nuclease-free water. Subsequently, the DNA was quantified, evaluated for purity and quality, and stored at -20°C.[15]

The genotypic analysis of the strains was based on genetic amplification using the multiplex PCR (polymerase chain reaction) technique, with previously extracted DNA samples. The multiplex PCR mix was composed of higher primers for *H. influenzaee* (1.4 mM), *M. catarrhalis* (0.2mM), *S. pneumoniae* (0.04 mM), and the lower common primer (0.4 mM), DNTPs (200 mM), buffer (10 mM Tris-HCl [pH 8.8)], MgCl2 (1.5 mM), KCl (50 mM), and 0.1% Triton X-100. For each reaction, 3U Taq polymerase was used. The reaction volume was 50 μl. The reaction amplification protocol consisted of: 3 min initial denaturation before enzyme addition, 38 cycles of 94°C/30s, 66°C/45s, and 72°C/1 min, followed by a 5-minute final extension at 72°C. The primers used in this study are described by Hendolin et al.[16] (Table 1).

Primers	Sequence	Amplicon (pb)
Lower primer 21-mer	5'-CTA CGC ATT TCA CCG CTA CAC-3'	
H. influenzae	5'-CGT ATT ATC GGA AGA TGA AAG TGC-3'	525
M. catarrhalis	5'-CCC ATA AGC CCT GAC GTT AC-3'	264
S. pneumoniae	5'-AAG GTG CAC TTG CAT CAC TAC C-3'	484

Table 1. Sequence of primers used in the Multiplex PCR for identification of *S. pneumoniae, H. influenzae,* and *M. catarrhalis* isolated from the oropharynx samples of residents of the rural settlement Dona Carmem, Mirante do Paranapanema-SP

Amplification products were evaluated by 1% agarose gel electrophoresis containing ethidium bromide (3 mg/mL) and visualized by UV light illumination. The DNA fragments were compared with a 1000 bp DNA marker. The presence (+) and absence (-) of the bands in the gel were indicative of the presence or absence of the bacterial species in the sample. The following strains were used as controls: *H. influenzae* INCQS 00434, *S. pneumoniae* INCQS 00752, and *M. catarrhalis* ATCC 25238. The previously stored bacterial samples were processed using the phenol-chloroform extraction technique to isolate genomic DNA. This method involves cell lysis followed by phase separation, allowing the removal of proteins and other contaminants through organic solvents. After extraction, the DNA concentration was measured using spectrophotometry, and its purity was assessed by calculating the A260/A280 and A260/A230 ratios. The integrity and quality of the extracted DNA were further evaluated by agarose gel electrophoresis. Finally, the DNA samples were aliquoted and stored at –20˚C until further use.

Human development index

The human development index (HDI) of the municipalities in the western region of São Paulo state was used to evaluate socio-economic development, especially those in the Pontal of Paranapanema (IBGE, 2021). The HDI value of the 45 municipalities in RHN11 was obtained from Fundação Sistema Estadual de Análises de Dados (SEADE) for 1999 to 2017, coded, and classified by the denominated quintile as a reference parameter (Figure 2C). Thematic maps were created for HDI using ArcGis 10.7.1 software. The HDI

was analyzed with support from a predictive method of interpolation surface generation: local polynomial interpolation (LPI). The Gaussian kernel method was used to generate the surface, classified according to the quintile. The gain in the LPI analysis in relation to the global polynomial interpolation presupposes it overlaps in several concentrations that a spatial representation may have, in our study, through a point in the centroid city of São Paulo state. The project was approved by the Research Ethics Committee of the University of Oeste Paulista, under registration CAAE 92660318.4.0000.5515.

Setting

São Paulo state is composed of 645 municipalities, and the western region has 45 municipalities, with an estimated population of 745,245 inhabitants in 2022 (Figure 2A and B). Among the 45 municipalities, 32 are in the Pontal of Paranapanema region (Figure 2C, blue map). Mirante of Paranapanema is located on the border of Paraná state (latitude 22º17′31″ S and longitude 51º54′23″W) at an altitude of 448 m above the Atlantic sea level and had an estimated population of 15,917 inhabitants in 2022. [13][14] (Figure 2C, violet map showing the urban area of Mirante of Paranapanema and Dona Carmen settlement, in the rural area). [13][14] In 2023, Mirante of Paranapanema had 32 rural settlements, the highest number in São Paulo state.

Statistical analysis

The results are shown as means ± standard error of the mean (for normally distributed variables). Dichotomous and nominal variables are expressed as frequencies and percentages. To compare the epidemiologic and clinical characteristics of the participants, the chi-squared test was used for categorical data and the t-test for continuous data after assessment of normality. Significant values were set at $P<0.05$. For the examination of potential risk factors for carriage, univariable odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression. Statistical analysis and graphics were performed using GraphPad Prism Software, Version 5.0 (San Diego, CA, USA) and the Sigma-Stat program, version 9.0 (Systat Software, Richmond, CA, USA).

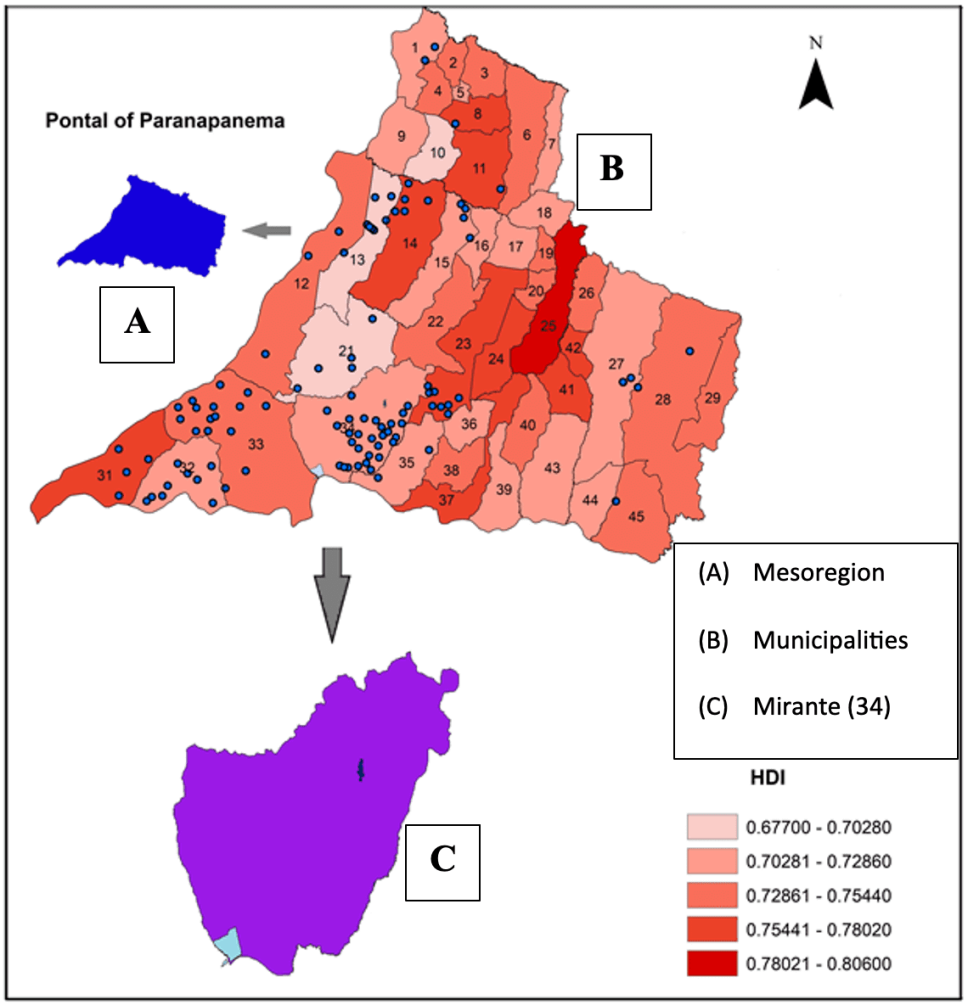

Figure 2. Spatial distribution of the HDI and localization of rural settlements in 45 municipalities of the RNHA11 mesoregion (A) and (B). Source: State System Data Analysis Foundation (SEADE, 2017). Base map: digital meshes of IBGE (2010). The municipalities are numbered as follows: 1, Paulicéia; 2, São João do Pau D'alho; 3, Monte Castelo; 4, Santa Mercedes; 5, Nova Guataporanga; 6, Junqueirópolis; 7, Irapuru; 8, Tupi Paulista; 9, Panorama; 10, Ouro Verde; 11, Dracena; 12, Presidente Epitácio; 13, Caiuá; 14, Presidente Venceslau; 15, Piquerobi; 16, Ribeirão dos Índios; 17, Emilianópolis;18, Flora Rica;19, Santo Expedito; 20, Alfredo Marcondes; 21, Marabá Paulista; 22, Santo Anastácio; 23, Presidente Bernardes; 24, Alvares Machado; 25, Presidente Prudente; 26, Caiabu; 27, Martinópolis; 28, Rancharia; 29, João Ramalho; 30, Quatá; 31, Rosana; 32, Euclides da Cunha Paulista; 33, Teodoro Sampaio; 34, Mirante do Paranapanema; 35, Sandovalina; 36, Tarabai; 37, Pirapozinho; 38, Estrela do Norte; 39, Narandiba; 40, Anhumas; 41, Regente Feijó; 42, Indiana; 43, Taciba; 44, Nantes; 45, Iepê.

Results

Epidemiological characteristics and spatial localization of the settlement

Figure 2C shows the 45 municipalities of the western region, highlighting Mirante do Paranapanema, the urban area, and the Dona Carmen settlement. Regarding the HDI, Mirante do Paranapanema (number 34) has one of the lowest HDI values, between 0.69701 and 0.71400, and is adjacent to Cuiabá Paulista (number 21), one of the poorest municipalities in São Paulo state. The mean age of the 44 individuals analyzed was 9.51 ± 4.28 years (interquartile range [IQR], 8.21-10.81 years; varying from 1 to 17 years). With regard to the epidemiologic and demographic characteristics, 9 (20.5%) were <6 years of age; 15 (34.0%) were 7-10 years; 15 (34.0%) were 11-14 years; and 5 (11.5%) were 15-18 years. The ratio of females to males was 1:1.45, and the ratio of brown to white color was 1:3.8. An income <US$173 per month was reported by 61.4% of the participants, use of antimicrobials more than twice a year by 40.9%, and attending schools regularly by 63.6%. The HDI was low in Mirante do Paranapanema and neighboring municipalities, varying from 0.67700 to 0.71400.

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Identification of pathogens by conventional and molecular methods

With the conventional culture method, 13.6% of the samples presented negative results; the percentage of inconclusive cultures was 79.5%. This high rate may be justified by the presence of microorganisms from other species and the fastidious characteristics of the agents: *S. pneumoniae, H. influenzae,* and *M. catarrhalis.* With the multiplex PCR identification method, *S. pneumoniae* was present in 68.2% (n=30) of the samples, followed by *M. Catarrhalis* in 68.2% (n=30). The molecular technique also allowed the simultaneous identification of the two species in 30.0% (n=13) of the samples. *H. influenzaee* was not detected. *M. catarrhalis* isolates were identified based on their characteristic morphology and biochemical properties. Colonies appeared as Gram-negative diplococci on Gram staining. The isolates were oxidase-positive and DNase-positive, consistent with *M. catarrhalis*. Additionally, they demonstrated growth at 37°C without fermenting carbohydrates. These combined features allowed for reliable differentiation of *M. catarrhalis* from other respiratory tract bacteria (Table 2).

Conventional culture method		**Multiplex PCR method**			
Negative	**Inconclusive**	***S. Pneumoniae***	***M. catarrhalis***	***H. Influenzae***	***S. pneumoniae + M. catarrhalis***
10.9% (N=6)	89.09 (N=49)	69.09% (N=38)	60% (N=33)	0% (N=0)*	49% (N=27)

Table 2. Pathogen identification by molecular and conventional techniques

**Statistically different value compared to that found for S. pneumoniae, M. catarrhalis, and both pathogens (p <0.05).*

Carriage of nasopharyngeal bacteria in individuals in different age groups

Regarding the carriage of bacteria (Figure 3), for *S. pneumoniae*, the highest colonization rate was found in children aged 7 to 10 years and those aged 11-14 years (33.3%), with a significant difference compared with those aged 15-18 years (*P*=0.03). For *M. catarrhalis*, the highest colonization rate was found in children <6 years of age (36.6%), with a significant difference compared with those aged 15-18 years (*P*=0.03).

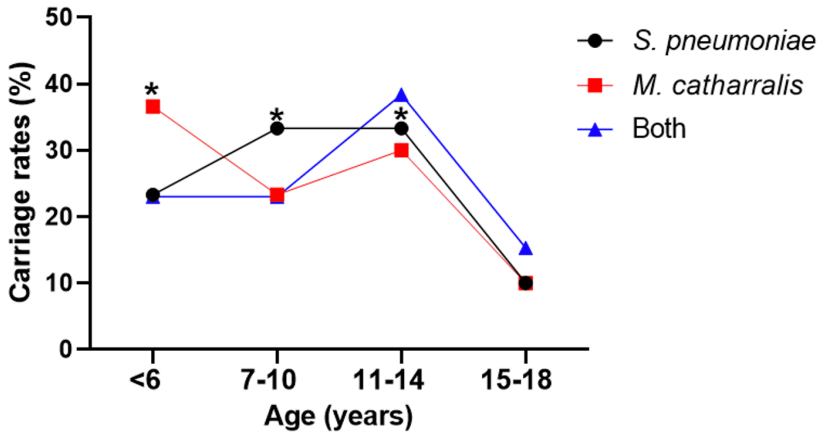

Figure 3. Carriage of nasopharyngeal bacteria in individuals of different ages. In children aged between 7 and 14 years, increased rates of *S. pneumoniae* were found compared with those aged 15-18 years (*P*= 0.03). In children <6 years, increased rates of *M. catarrhalis* were found compared with those aged 15-18 years (*P*= 0.03).

Risk factors for the carriage of S. pneumoniae and M. catarrhalis and both microorganisms

Table 3 shows the association between the participants' characteristics and the carriage of each species. The univariate analysis showed that being female (OR, 0.23; 95% CI, 0.08-0.61; *P*=0.004) and being brown color (OR, 0.35; 95% CI, 0.14-0.88; *P*= 0.042) were positively associated with carriage of pneumococci. No

associations were found for *M. catarrhalis*. Being brown color (OR, 0.30; CI, 0.12-0.79; *P*= 0.022) and using antimicrobials more than twice in a year (OR, 5.92; 95% CI, 1.96-17.91; *P*=0.001) were positively associated for both pathogens (Table 3).

Characteristics	*S. pneumoniae*, %; OR (95% CI); *P* value	*M. catarrhalis*, %; OR (95% CI); *P* value	Both microorganisms, %; OR (95% CI); *P* value
Gender			
Male (40.9%, *n*=18)	25.0	27.27	15.9
Female (59.1%, *n*=26)	45.45; 0.23 (0.08-0.61); 0.004	40.90; 0.54 (0.22-1.32); 0.265	27.7; 0.50 (0.17-1.43); 0.30
Color			
White (20.4%, *n*=9)	22.72	27.27	20.45
Brown (79.5%, *n*=35)	45.45; 0.35 (0.14-0.88); 0.042	45.45; 0.45 (0.18-1.0); 0.12	45.45; 0.30 (0.12-0.79); 0.022
Income			
<US$173 (61.4%, *n*=27)	43.18	38.63	27.27
>US$173 (38.6%, n=17)	25.00;2.28 (0.92-5.64); 0.11	29.54; 1.5 (0.61-3.64); 0.50	31.81; 0.80 (0.32-2.0); 0.81
Use of antimicrobials			
<2 times/y (59.1%, *n* =26)	40.9	29.5	43.1
≥2 times/y (40.9%, *n*=18)	27.2; 0.59 (0.24-15.47); 0.36	38.6; 1.21 (0.42-3.12); 0.81	11.36; 5.92 (1.96-17.91); 0.001
School attendance			
Yes (63.6%, *n*=28)	43.1	34.0	31.8
No (36.4%, *n*= 16)	27.2; 0.57 (0.24-1.34); 0.28	27.2; 1.0 (0.41-2.41); 1.11	27.2; 1.81 (0.68-4.78); 0.33

Table 3. Univariate analysis of risk factors for the carriage of *S. pneumoniae* and *M. catarrhalis* and both microorganisms in individuals living in Dona Carmen rural settlement, Mirante of Paranapanema, São Paulo State, Brazil

P values <0.05 are significant.

Discussion

In the current study, 79.5% of the samples did not provide conclusive results based on conventional culture techniques. Conversely, *S. pneumoniae* and *M. catarrhalis* were identified in 68.2% of the samples, respectively, using the multiplex PCR technique. *S. pneumoniae* has been cultured successfully in many low- and middle-income countries.[17] PCR offers significant advantages over traditional detection methods like culture or staining, including greater sensitivity, speed, and versatility. PCR can detect even minute amounts of genetic material, allowing for early infection detection, and is significantly faster, often yielding results in hours compared to days for traditional methods. Its versatility also allows for the detection of a wide range of pathogens, including viruses, bacteria, and fungi, making it a valuable tool in diagnosing various infectious diseases.[15][16]. In the identification of the bacteria responsible for chronic otitis media with effusion in children, a positive conventional culture was found in 13.6% of specimens, and using PCR, 73.5% of specimens were positive for at least one of the pathogens *H. influenzae*, *S. pneumonia*, and *M. catarrhalis*.[17][18][19] The superiority of PCR and the difficulties with growing pathogens by conventional culture were also demonstrated in children with acute otitis media in Finland. [20] However, in Brazil, due to the high costs, molecular techniques are not routinely available in laboratories, resulting in lower reliability of the results. We used the phenol-chloroform extraction technique, which is well known for yielding high-quality DNA. However, this method has limitations, such as the use of toxic reagents and greater operational complexity. Additionally, variations in yield may occur in samples with high levels of mucopolysaccharides or proteins. The risk of cross-contamination is also higher compared to commercial kits. These limitations should be considered in future studies.

The absence of bacterial growth in the cultures may be attributed to several factors. One possibility is the recent use of antibiotics by the children, even if not accurately reported, which could have inhibited the growth of the target bacteria. Additionally, despite the precautions taken during sample transport and storage, logistical limitations inherent to the rural setting may have affected microorganism viability. Another factor to consider is the specific composition of the oropharyngeal microbiota in this population, which may naturally exhibit a low prevalence of the investigated pathogens during the collection period. Finally, it is important to highlight that conventional culture techniques have limited sensitivity, and the use of molecular methods could enhance the accurate detection of these microorganisms.

Colonization of both nasopharyngeal and oropharyngeal airways by potential respiratory pathogens (*S. pneumoniae, H. influenzae*, and *M. catarrhalis*) is established early in childhood, although rates vary greatly according to locality, sampling frequency, and individual and social factors.[18][19][20] In this study, *S. pneumoniae* and *M. catarrhalis* were identified especially in children younger than 15 years of age (Figure 3). In line with our results, in Iran, *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* were the main bacteria isolated and the cause of otitis media with effusion in 45 children aged between 1 and 15 years.[21] The *H. influenzae* type b (Hib) vaccine was introduced into the Brazilian National Immunization Program (BNIP) in August 1999 and added to the routine immunization schedule for infants. Vaccination against *S. pneumoniae* with the 10-valent pneumococcal conjugate vaccine (PCV10) was introduced into the routine childhood immunization program in March 2010. In our study, all the participants were older than 20 years and had received *H. influenzae* and *S. pneumoniae* vaccines.

In our study, skin color and increased use of antimicrobials were also identified as risk factors for both pathogens. The role of the use of antibiotics as a risk factor for oropharyngeal carriage of *S. pneumoniae* is not well reported and is conflicting, depending on the study design.[22][23]. In Brazil and worldwide, the dangerous culture of self-medication in the population, including antibiotics, is well known and certainly contributed to our results.[24].

M. catarrhalis was significantly increased in children < 6 years of age compared to children aged 16–18 years. This is justified because, after the first year of life, children demonstrate the highest peak of colonization by this pathogen, followed by a decline with age due to the diversity of the microbiota and maturation of the immune system.[19][20]

We showed that *S. pneumoniae* carriage was associated with being female and of brown color. In a study in Brazil, there is an association between female gender and increased carriage of *S. pneumoniae* suggesting that females may be more prone to carrying the bacteria, although more research is needed to understand the reasons behind this association. Regarding the brown color, in 2022, 45.3% of Brazil's population identified as brown, making them the largest racial group. The brown population is also the most vulnerable in terms of social and economic inequalities. Despite the existence of the public healthcare system, SUS, these inequities manifest in various ways, including reduced access to healthcare services, poorer health outcomes, and racial discrimination within the healthcare system.[13][14] Taken together, we suggest that all these factors contributed to the significantly higher number of brown individuals carrying *S. pneumoniae* and both microorganisms compared to the White ones (0.042 and 0.022), respectively. It is well known that Brazil's rural areas, while housing 14.7% of the population, are disproportionately affected by poverty, accounting for 34.6% of all poverty cases in 2017–2018.[13][14] However, income was not significant in the studied population.

The negative results from both culture and PCR for *H. influenzae* can be traced back to the role of the *H. influenzaee* type b (Hib)-conjugate vaccine in Brazil and elsewhere.[25] The introduction of this vaccine markedly reduced the number of cases of meningitis caused by this microorganism.[26] We did not determine seroconversion of *S. pneumoniae* and *H. influenzae* vaccines. Serotype information is crucial for understanding vaccine coverage and its effectiveness. By identifying the different serotypes, we can better understand how well vaccines are protecting against various strains. Serotype information could have provided additional insights into vaccine coverage and bacterial pathogenicity.

Pontal of Paranapanema and Ribeira Valley are the poorest regions compared with other regions of São Paulo state.[27] Figure 2 highlights Mirante of Paranapanema, where the HDI is one of the lowest in the region. This is a reflection of the low income among residents in the Dona Carmen settlement; 61.4% of the families are living with less than US$160 per month. This value corresponds to living with less than one minimum salary per month, which is not enough to buy basic food. In these cases, health care, education, and other expenses must be secondary. Worldwide, in poor regions of developing countries, there is great inequality in health care, education, housing conditions, and occupational skills, directly affecting children and the elderly. In Brazil, the poorest regions are predominantly in rural areas and the outskirts of medium and large cities.[8]

Several shortcomings should be considered in the study. The low number of children interviewed and samples collected, difficulties with transport, low levels of education, and poor infrastructure directly influenced the adherence of the study participants, resulting in a smaller than expected sample size for the

settlement, with a low level of confidence for the study (12%). The interviewers visited the settlement on three different occasions, making house-to-house contact during the day; during that period, adults were working in agriculture or raising cattle, and only children and elderly people were available. In addition, settled families in Dona Carmen complained that many surveys are conducted with no reporting back to the community. The study focused on a highly vulnerable or specific population in one location, and the rest of the potentially susceptible population was not surveyed. We were also not able to determine the serotypes of *S. pneumoniae* or identify if the samples carried non-vaccine serotypes. DNA was not extracted from cultures, which is a better choice to minimize false negative rates by PCR. Antibiotic susceptibility data for the isolated bacterial species, especially given their origin from healthy individuals, would provide important insights into potential reservoirs of resistance in the community. However, due to logistical and resource limitations, antibiotic susceptibility testing was not included in the scope of the present study.

Conclusions

Our study may have regional and national importance because Brazil has a large number of rural settlements, mostly in tropical regions with socioeconomic and environmental similarities to the Dona Carmen settlement. Furthermore, there are few population-based studies in the context of respiratory diseases in children living in rural areas due to the difficulty in accessing these patients. Although the number of children assessed was small, as far as we know, this is the first study on oropharyngeal bacterial carriage of children from rural areas in Brazil. The findings from this study are of public health significance and an indication that the proper implementation and use of vaccines as an intervention can significantly reduce the burden of diseases locally and globally. They may better define public health policy actions in the promotion of regional and national health.

Statements and Declarations

Funding

This work was supported by the Sao Paulo Research Foundation - FAPESP (grant number 2018/08097-7).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical aspects

The project was submitted to the Research Ethics Committee of the University of Oeste Paulista, respecting Resolution 196/96 on research involving human subjects. All participants and/or guardians were asked to sign the informed consent form. The project is registered with the ethics committee under registration CAAE 92660318.4.0000.5515.

Data availability

The raw data supporting the conclusions of this article may be available by the authors upon reasonable request, pending ethical considerations regarding participant privacy.

Author contributions

Conceptualization: LKWE; Methodology: RNMM, GMS; Formal Analysis: LEPC, LKWE; Investigation: RNMM, GMS, EFF, VCP; Data Curation: LKWE; Writing – Original Draft Preparation: LEPC, LKWE; Writing – Review & Editing: RNMM, GMS, LE PC, E FF, VCP, LKWE.

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