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Research Article

Carriage of oropharyngeal bacteria among children in a vulnerable 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 influenza*, and *Moraxella catarrhalis* in the oropharynx and a possible association for airway infections in children of a vulnerable population living in a tropical rural 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. influenza* 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 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 and other settings of developing countries.

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Introduction

Respiratory infections are the main cause of morbidity around the world, mainly due to pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenza*, and *Moraxella catarrhalis*. Low-and middle-income tropical countries present the highest rates of colonization of these agents, especially in rural communities.[1][2][3][4] 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. [5][6]

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

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conditions are directly linked to increased morbidity and mortality in children.[7][8] Rural settlements are found in different regions of São Paulo state, with the highest number in the western region, including Dona Carmen settlement. [8] 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 *H. influenzae* 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.[9][10][11] This study aimed to detect the carriage of *Streptococcus pneumoniae*, *Haemophilus influenza*, and *Moraxella catarrhalis* in the oropharynx and to determine the association for airway infections in children of a vulnerable population living in a tropical rural settlement, São Paulo, Brazil.

Materials and Methods

Study design and settings

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 (Instituto Brasileiro de Geografia e Estatística (IBGE, 2022) (Figure 1A).[12][13] 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 1A and B). Among the 45 municipalities, 32 are in the Pontal of Paranapanema region (Figure 1C, blue map). Mirante of Paranapanema is located in the border of Parana state (latitude 22°17′31″ S and longitude 51°54′23″W) at an altitude of 448 m above the Atlantic sea level had an estimated population of 15,917 inhabitants in 2022 (IBGE, 2022)) (Figure 1C, violet map showing the urban area of Mirante of Paranapanema and Dona Carmen settlement, in the rural area). [12][13][14] In 2023, Mirante of Paranapanema had 32 rural settlements, the highest number in São Paulo state. We conducted a transverse and descriptive study and all specimens were collected from February to May 2022 from 44 of 522 (8.43%) individuals <18 years of age living in the settlement who agreed to take part in this study. 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. influenzae* (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 h of sample collection were excluded.

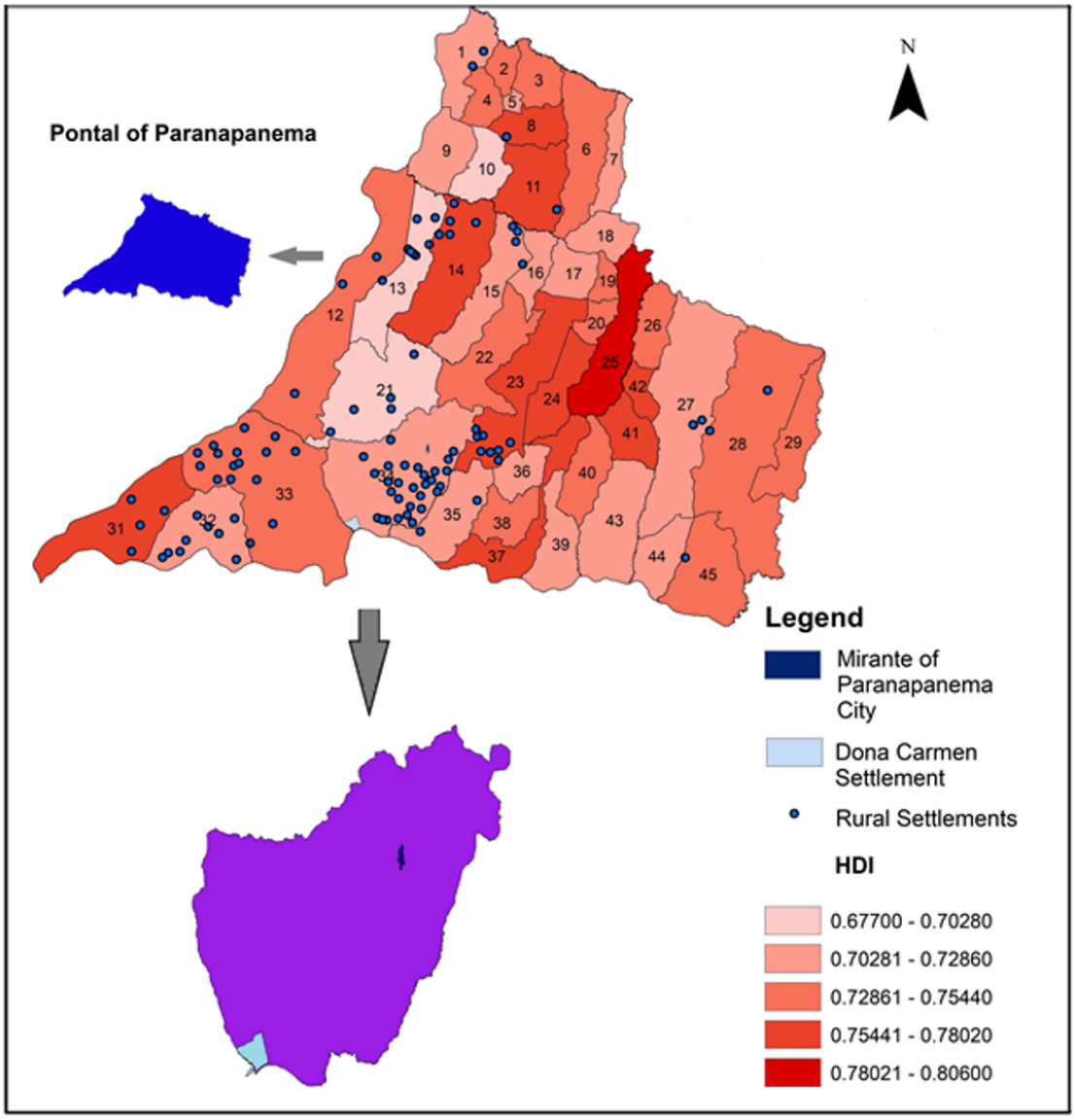

Figure 1. Spatial distribution of the HDI and localization of rural settlements in 45 municipalities of RNHA11 mesoregion. Source: State System Data Analysis Foundation (SEADE, 2017). Base map: digital meshes of IBGE (2010). The municipalities are numbered as follows: 1, Paulicéia; 2, São João do Pau 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.

Collection of samples

After collecting demographic data, two samples of the oropharynx were collected using a sterile saline moistened swab (0.85%) with slight movements on the tonsils. The collected samples were stored in Stuart transport medium and transported to the

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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 h in an anaerobic jar. Five random colonies were selected based on the 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, positive oxidase reaction, and 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 centrifuging 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. influenzae* (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 minutes final extension at 72°C. The primers used in this study are described by Hendolin et al. (1997)

Amplification products were evaluated by 1% agarose gel electrophoresis containing ethidium bromide (3 mg/mL) 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: *Haemophilus influenzae* INCQS 00434, *Streptococcus pneumoniae* INCQS 00752 e *Moraxella catarrhalis* ATCC 25238.

Human development index

The human development index (HDI) of the municipalities in the western region of São Paulo state was used to evaluate the 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 1C). 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.

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 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 Software (San Diego, CA, USA) and the Sigma-Stat program (Systat Software, Richmond, CA, USA).

Results

Epidemiological characteristics and spatial localization of the settlement

Figure 1C shows the 45 municipalities of the western region, highlighting Mirante of Paranapanema, the urban area, and Dona Carmen settlement. Regarding the HDI, Mirante of Paranapanema (number 34) has one of the lowest HDI values at between 0.69701 and 0.71400, and is adjacent to Cuiabá Paulista (number 21), one of the poorest municipalities of 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 of Paranapanema and neighboring municipalities, varying from 0.67700 to 0.71400.

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. influenzae* was not detected.

Carriage of nasopharyngeal bacteria in individuals in different age groups

Regarding the carriage of bacteria (Figure 2), 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 ($P=0.03$).

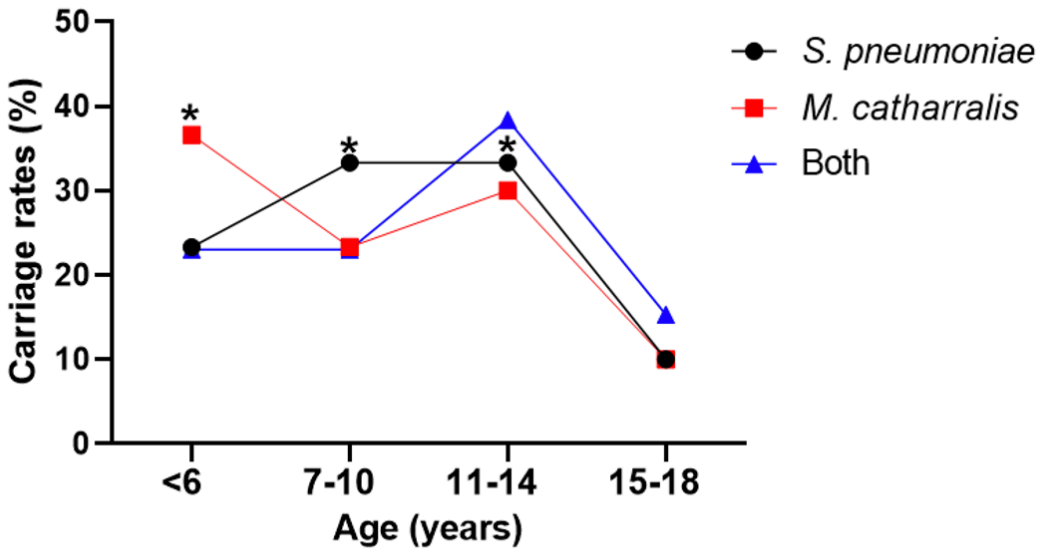

Figure 2. Carriage of nasopharyngeal bacteria in individuals of different ages. In children aged between 7 and 14 years, increased rates of *S. pneumoniae* were found compared with those aged 15-18 years (*P*= 0.03). In children <6 years, increased rates of *M. catarrhalis* were found compared with those aged 15-18 years (*P*= 0.03).

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

Table 1 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 1).

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 1. 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.[16] PCR offers greater sensitivity in the detection of bacterial species, allowing the identification of more than one pathogen quickly and effectively.[15][17] 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*.[16][18][19] Superiority of PCR and the difficulties with growing pathogens by conventional culture was 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.

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 2). In line with our results, in Iran, *S. pneumoniae*, *M. catarrhalis*, and *H. influenza* were the main bacteria isolated and cause of otitis media with effusion in 45 children aged between 1 and 15 years.[21]

In our study. female gender and increased use of antimicrobials were also identified as risk factors for both pathogens. The role of 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 the auto-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]

The negative results from both culture and PCR for *H. influenzae* can be traced back to the role of *H. influenzae* 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]

Pontal of Paranapanema and Ribeira Valley are the poorest regions compared with other regions of São Paulo state.[27] Figure 1 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 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.[7]

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. The interviewers visited the settlement on three different occasions making house-to-house contact during the day; in that period, adults were working in agriculture or raising cattle, and only children and old people were available. In addition, settled families in Dona Carmen complained that many surveys are conducted with no reporting back to the community. We were also not able to determine the serotypes of *S. pneumoniae* and identify if the samples carried non-vaccine serotypes.

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. As far as we know, this is the first study in the field in Brazil, although the number of children assessed was small.

In our studied population we identified risk factors associated to the carriage of *S. pneumoniae* and *M. catarralis* and vulnerabilities such as low income and low HDI that may be widely applicable to other rural communities in Brazil and other settings with similar characteristics worldwide. They may better define public health policy actions in the promotion of regional and national health.

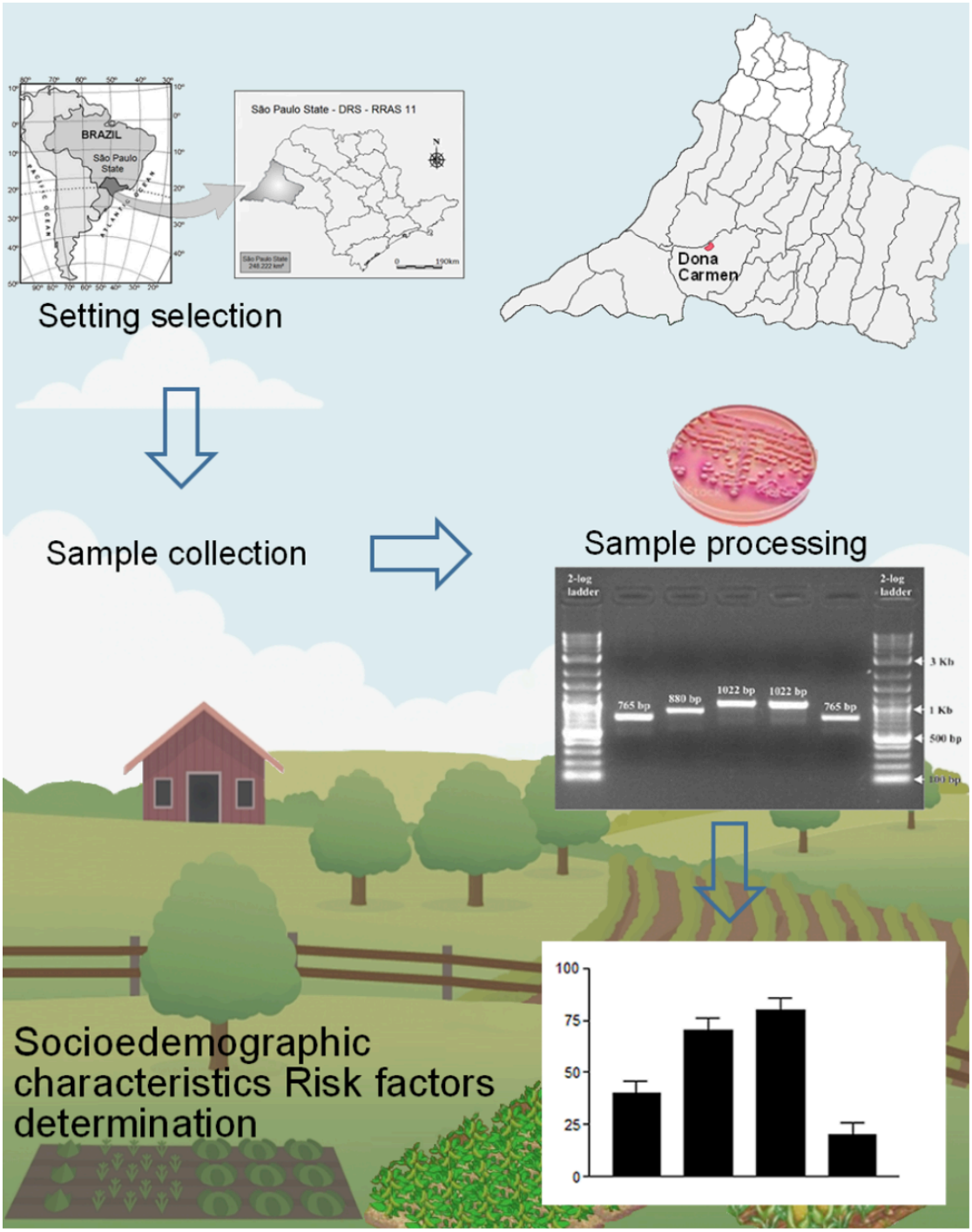

Figure 3. Infographic designed for the study. Selection of the setting, municipalities of the western region of São Paulo state, location of the rural settlements in each municipality, and selection of Dona Carmen rural settlement in Mirante of Paranapanema; sample collection and processing, culture, and DNA analysis from an oropharynx swab; sociodemographic characteristics and risk factors.

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Statements and Declarations

Acknowledgments

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Conflict of interest statement

The authors declare that they have no conflict of interests.

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.

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