UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

Streptococcus pneumoniae nasopharyngeal carriage in indigenous Kichwa communities of Otavalo, Ecuador: serotypes, risk factors, and associated respiratory pathogens

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DEDICATORIA

A mi esposo y mi hijo, mi apoyo incondicional, mi fuerza y mi mayor motivación A mis padres y mi hermana, mi sangre y mi alegría en la vida

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A Ismar, por su vocación de enseñanza y generosidad de conocimiento.
Al Dr. Enrique Terán por su guía y apoyo durante la realización de este proyecto.
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A mi hermana, por su dedicación amorosa y guía en cada parte de esta experiencia.

RESUMEN

Antecedentes: *Streptococcus pneumoniae* ha sido reconocido a nivel mundial como uno de los agentes etiológicos de otitis media, neumonía, septicemia y meningitis; con altas tasas de mortalidad. No existen estudios que evalúan la colonización por patógenos respiratorios en países de América Latina como Ecuador, especialmente en grupos de alto riesgo, como la población indígena.

Métodos: Se realizó un estudio transversal en 5 comunidades Kichwa, en el que se evaluaron factores socioeconómicos y de riesgo para la portación de *S.pneumoniae* y otros patógenos respiratorios en 63 familias, 100 niños entre 0-12 años de edad y sus padres.

Se tomaron hisopados nasofaríngeos para identificar la colonización por *S. pneumoniae, H. influenzae, M*. *catarrhalis* o *S. aureus* a través de métodos microbiológicos estándar. La identificación de serotipos de neumococo se realizó a través de PCR multiplex secuencial. Los análisis estadísticos se realizaron con IBM SPSS Statistics V24 para Windows.

Resultados: Se identificaron condiciones de pobreza en el 90.5% de la población evaluada. La colonización por neumococo se observó en el 50% de los niños y 6.3% de los padres. La colonización incrementó con la edad y presentó un pico entre los 61-138 meses. Los serotipos más prevalentes identificados fueron No tipables, 6A/D/C y 19A.

Los patrones de co-colonización identificados fueron *S. pneumoniae-M.catarrhalis* en niños < 60 meses y *S. pneumoniae-H. influenzae* en niños entre 61 a 138 meses de edad. *S. aureus* fue el patógeno identificado con mayor prevalencia en los padres estudiados. El único predictor de colonización de neumococo identificado fue la exposición a cocina de leña (*P*=0.047), con mayor riesgo de colonización en niños mayores a 60 meses (*P*=0.022 OR=3.2 IC95% 1.2-8.8) y mujeres (*P*=0.034 OR=3.5 IC95% 1.1-11.4).

Conclusiones: Este es el primer estudio que identifica la colonización por neumococo en grupos étnicos indígenas del Ecuador. Se pudieron identificar serotipos vacunales colonizando niños indígenas, por lo tanto se considera necesario extender el estudio de colonización por neumococo y otros patógenos respiratorios a nivel nacional en población en general.

Palabras clave: Neumococo, colonización nasofaríngea, población indígena, serotipos, niños.

ABSTRACT

Background: *Streptococcus pneumoniae* has been recognized worldwide as one of the main causative agents of otitis media, pneumonia, septicemia and meningitis; with high rates of mortality. Studies that explore respiratory pathogens colonization in Latin American Countries like Ecuador are absent for higher-risk groups, such as the indigenous population.

Methods: A cross sectional survey was carried out in five Kichwa villages, assessing 63 families, 100 children between 0 to 12 years old and their parents regarding socio-economic conditions and risk factors related to *Streptococcus pneumoniae* carriage. Nasopharyngeal swabs were collected in order to identify *S. pneumoniae, H. influenzae, M*. *catarrhalis* or *S. aureus* colonization by means of microbiological standard methods. Sequential multiplex PCR approach was used for serotyping and identification. Statistical analyses were done using IBM SPSS Statistics V24 for Windows.

Results: Poverty conditions were identified in 90.5% of the assessed population. Pneumococcus colonization was identified in 50% of the children and 6.3% of the caregivers. Colonization increased with age and peaked at 61-138 months. Non Tippable, 6A/D/C and 19A were the most prevalent serotypes identified.

S. pneumoniae-M.catarrhalis was the more prevalent co-colonization pattern in children < 60 months and *S. pneumoniae-H. influenzae* in children from 61 to 138 months old. *S. aureus* was the most prevalent identified pathogen colonizing caregivers. The presence of wooden stove was identified as the only predictor for pneumococcus colonization (*P*=0.047), with a higher risk for colonization in children older than 60 months (*P*=0.022 OR=3.2 CI95% 1.2-8.8) and females (*P*=0.034 OR=3.5 CI95% 1.1-11.4).

Conclusions: This is the first study that identifies pneumococcal colonization in indigenous ethnical groups in Ecuador.Vaccine type serotypes were identified colonizing indigenous children making it necessary to extend the assessment of pneumococcal colonization nationwide to general population.

***Key words*:** Pneumococcus, nasopharyngeal colonization, indigenous population, serotypes, children.

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PART I

GENERAL INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a facultative anaerobic Gram positive coccus that colonizes the mucosal surfaces of the upper respiratory tract of healthy children and adults.(Murray Patrick, Rosenthal Ken, 2013) This opportunistic pathogen was first described by Pasteur and Steinberg over 100 years ago, and has an oval shape and arranges in pairs, named diplococcus, or short chains. (Murray Patrick, Rosenthal Ken, 2013) This bacterium grows in enriched media complemented with blood supplements, is soluble in bile and its colonies are recognizable by their characteristic umbilicated shape, due to autolysis, and alpha hemolysis on blood agar. (Murray Patrick, Rosenthal Ken, 2013; Skovsted, 2017) The colonies morphology usually depend on the presence or absence of bacterial polysaccharide capsule, which is also related to virulence. Generally, the encapsulated strains colonies are bigger, round and mucoid, while the non-encapsulated ones are smaller and flattened.(Murray Patrick, Rosenthal Ken, 2013)

Clinical Disease and Epidemiology

Pneumococcus has been recognized worldwide as one of the main causative agents of bacterial diseases such as otitis media, pneumonia, empyema, septic arthritis, septicemia and meningitis (Abdullahi et al., n.d.; Tuomanen, 2001)

Due to a vast extent of virulence factors, pneumococcus has the ability to evade or take advantage of the host immune and inflammatory responses, determining its further transmission, colonization and invasion.(Weiser, Ferreira, & Paton, 2018)

Even though colonization of the upper respiratory tract presents asymptomatic; if pneumococcus gains access to normally sterile parts of the airway, either by aspiration, local spread or bacteremia, an inflammatory response triggers pathogenicity and invasive disease. Therefore, carriage is necessary in order to enable the development of pneumococcal disease and transmission to other individuals. (Kadioglu, Weiser, Paton, & Andrew, 2008; Weiser et al., 2018)

Approximately 27-65% of children are carriers of this opportunistic pathogen worldwide (Weiser et al., 2018), and it´s been described that the carriage prevalence reaches 95% in healthy children under 3 years old in developing countries, thus constituting the main reservoir of this pathogen (Obaro & Adegbola, 2002).

Pneumococcus disease represents a threat for global health since mortality due to this pathogen infection is high. Pneumococcal pneumonia was estimated to be the leading cause of death due to lower respiratory infections (LRI) for children younger than 5 years old in 2017 with 808,000 deaths worldwide, while 43,900 deaths were also registered at age 5 to 14.(Dicker et al., 2018) LRI were ranked fourth in terms of total years of life lost (1515 per 100000 habitants) and first in low income countries, and even tough mortality rate has decreased in the last 27 years, the high burden of disease, difference in age patterns and rising antimicrobial resistance have led the World Health Organization (WHO) to include pneumococcus as one of 12 priority pathogens in 2017(Dicker et al., 2018; Weiser et al., 2018). In Latin America and the Caribbean, pneumonia is responsible for 14% of deaths in children under 5 years old, and in Ecuador it was the second cause of childhood death with 5,8% in 2016.(Instituto Nacional de Estadísticas Y Censos, 2016)

Pneumococcus is also responsible for 70000 deaths from meningitis and sepsis in developing countries each year and is the leading pathogen associated to acute and recurrent otitis media, which is 1000 times more common than invasive disease, while pneumonia has 10 times more impact worldwide(Bardach et al., 2017; Obaro & Adegbola, 2002; Verhagen et al., 2017)

Risk factors and ethnical influence

Identification of risk factors associated with pneumococcal colonization plays a relevant role in prevention of invasive disease development. Age, day-care centers attendance, presence of siblings, recent antibiotic use, respiratory tract infections and crowded living conditions have been recognized as the principal ones (Jacoby et al., 2011; Ueno et al., 2013). Exposure to tobacco or firewood smoke, low socio-economic status and weather, have also been described as risk factors (Sun et al., 2012).

Seasonality also contributes to the development of pneumococcal disease in Ecuador, where it coincides with viral pneumonia due to Respiratory Syncytial Virus and Parainfluenza virus during late winter (Jonnalagadda et al., 2017). This phenomenon could be due also to the presence of respiratory virus neuraminidase activity that allows an increased pneumococcal adhesion to the respiratory tract epithelium during the rainy season of the year.(Rivera-Olivero et al., 2007)

Ethnic and regional influence on pneumococcal colonization have been noted as relevant risk factors worldwide, where economic conditions are important in explaining racial health disparities. Indigenous population living in poor conditions present an increased susceptibility towards infectious diseases, with a higher prevalence and mortality rate.(Gracey & King, 2009) Evidence shows that indigenous populations have poor social and health outcomes in relation to their non-indigenous counterparts due to

restricted access to clean water supplies, fresh food and health services. Children's health status is reflected by the nutritional and health status of the mother and it has been described that indigenous populations worldwide have generally higher infant and maternal mortality rates, child malnutrition and lower life expectancy at birth in relation to the non-indigenous population. (Anderson et al., 2016; Gracey & King, 2009) Thus an increased prevalence of pneumococcal infections has been described in indigenous people in Alaska, Canadian artic, Navajo Native American, White Mountain Apache, New Zealand Maoris and Venezuelan Waraos. (Lynch & Zhanel, 2010; Rivera-Olivero et al., 2007) It has also been described that Australian aborigines under 14 months old present higher prevalence of pneumococcal colonization with a 40% versus a 20% for non - Aboriginal children. (Jacoby et al., 2011)

In Ecuador, regional and ethnic differences regarding the general health status of the population are historically reflected by higher prevalence of growth retardation and much lower weight in indigenous groups; who are more affected by problems such as child mortality, poverty, indigence and illiteracy, especially in indigenous populations of the Andean region of the country. (Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2010) During the period 2011 to 2013, growth retardation prevalence in Indigenous Ecuadorian population between 0-60 months of age reached 42.3%, and 36.5% in the group between 5 to 11 years, while in the mestizo population it reached 24% and 13.7% respectively. (Instituto Nacional de Estadísticas y Censos, 2013) By year 2017, poverty status due to unsatisfied basic needs, considered as education, clothing, housing, health, nutrition and employment, reached 65.2% for indigenous population while the national prevalence reached 32.1%, also 35% lived in extreme poverty conditions; 60% had access to water supply by public network and 22%

lived in crowding conditions, while the national prevalence reached 7.9%, 82.6% and 11% respectively. (Secretaría Nacional de Planificación y Desarrollo, 2017) In Ecuador statistical information regarding infectious diseases is limited, and there are no previous epidemiological studies regarding pneumococcus colonization prevalence in the entire country from indigenous populations.

Colonization and transmission mechanisms

Pneumococcus is mainly located in the mucus layer covering the epithelial surface of the upper respiratory tract (URT) in carriers, who transmit the pathogen trough nasal secretions. The main means of transmission are by direct contact with other people and by airborne transmission with further spreading through contaminated surfaces. Activities such as coughing and sneezing or even talking produce respiratory droplets that carry bacteria that then deposits on dry surfaces and forms desiccation resistant biofilms, where pneumococcus can survive at room temperature for a month. (Brown, Hammerschmidt, & Orihuela, 2015; Verhagen et al., 2014; Weiser et al., 2018)

Levels of shedding depend on the amount of inflammation of the URT in the actual host since the latter is associated with a greater mucus production and mucin glycoproteins expression in order to facilitate entrapment and clearance of bacteria present in the URT trough ciliary beating towards the pharynx for a posterior elimination via fecal- oral route. (Brown et al., 2015) Influenza A virus co-infection and pneumococcus virulence factors such as the capsule and the pro-inflammatory pneumococcal toxin pneumolysin (PLY) are associated to URT inflammation process. Pneumococcus expresses one of more than 90 polysaccharide capsules that attach covalently to the cell wall, and due to the presence of pyruvate, uronic acid and

phosphate it has a negative charge, which repels the negatively charged N-acetylneuraminic acid present in mucin that binds to positively charged particles, preventing entrapment and clearance, while PLY forms pores within the phagosome, releasing its content in the cytosol of the phagocytic cell and facilitating the inflammatory response with the production of cytokines and chemokines. Therefore the higher the nasal secretion, the higher the density of pneumococcus; facilitating the detachment of the URT of the colonized host and consequently transmission. (Brown et al., 2015; Weiser et al., 2018).

Colonization of the upper respiratory tract is most common during early childhood and is apparently asymptomatic, but if the pathogen reaches sterile areas of the airway, an intense inflammatory response occurs, ending in disease development such as otitis media, sinusitis, pneumonia, arthritis, pericarditis, meningitis and septicemia. (Kadioglu et al., 2008; Obaro & Adegbola, 2002) Therefore, within the life cycle of this pathogen, carriage is a vital element for transmission to other individuals and subsequent disease development. (Weiser et al., 2018) It has been proved that colonization of the upper respiratory tract with pneumococcus can occur in multiple episodes from the first years of life, but even though colonization precedes disease, most children tolerate several events of colonization without developing invasive or local pneumococcal infection, constituting an ideal source of transmission. (Wootton, Aston, & Gordon, 2014) Different serotypes of pneumococcus can be found colonizing the respiratory tract at the same time or subsequently for several months, but most infections occur after acquiring a new serotype. (Obaro & Adegbola, 2002)

In order to achieve a successful colonization of the URT, pneumococcus displays a series of virulence factors that allow adherence to several cells and tissues,

overcoming innate and adaptive immune system and avoiding mucociliary clearance. (Abdullahi et al., 2012) Pneumococcus needs to persist in the URT, and initial adherence to mucin provides a first step in colonization that provides nutrients and an ideal niche, so that the pathogen uses extracellular glycosidases that help reveal host glycan receptors; from these, Neuraminidase A (NanA), β-galactosidase (Bga) and β- N-acetylglucosaminidase (StrH) have not only enzymatic but also adherence activities, acting sequentially to remove terminal sugars on human glucoconjugates, unmasking adherence receptors and degrading mucus, therefore blocking mucociliary clearance. (Kadioglu et al., 2008; Weiser et al., 2018) Mucus has a series of antimicrobial compounds, during colonization pneumococcus uses the peptidoglycan N-acetylglucosamine deacetylase (PgdA) and O-acetyltransferase to modify pneumococcal peptidoglycan, making it less recognizable by human lysozyme, a muramidase that binds to the bond between N-actetyl glucosamine and N-acetyl muramic acid residues of the peptidoglycan, favoring cell wall degradation. (Brown et al., 2015) Subsequently, during transport across the mucus layer, pneumococcus uses an autolysin (LytA), which allows pneumolysin (Ply) release in order to generate epithelial damage and prevent ciliary beating. This will allow further access to the epithelium, where pneumococcus adheres using the surface pneumococcal adherence and virulence proteins (Pav) A, B and Enolase, which bind to fibronectin and plasminogen, Choline binding protein A (CbpA) which binds to vitronectin, secretory forms of immunoglobulin and H factor in the host, this surface protein is non-covalently anchored to phosphorylcoline (ChoP) which mimics platelet- activating factor (PAF), therefore granting the ability to adhere to rPAF; a receptor that is present within the host tissues, activating host signaling. Pneumococcal surface protein A (PspA) and pneumococcal histidine triad protein (Pht)

avoid complement recognition of the capsule and the first also binds to lactoferrin to acquire iron, inhibiting the antimicrobial effect of apolactoferrin. (Kadioglu et al., 2008; Weiser et al., 2018; Zangari, Wang, & Weiser, 2017) Pneumococcus must face the presence of antibodies on the mucosal surface of the URT and compete with the existing microbiota during URT colonization process. The immunoglobulin IgA1 is the most abundant on mucosal surfaces of the upper respiratory tract and its protective activity is based on its agglutination function, which favors the elimination of pathogens through mucociliary flow. To evade this host defense system, pneumococcus uses a zinc-dependent metalloprotease called ZmpA, which cuts the hinge region of this immunoglobulin, eliminating its agglutination capacity. (Kadioglu et al., 2008; Weiser et al., 2018) Polysaccharide conjugate vaccines (PCVs) and natural colonization induce high levels of serotype specific IgG in mucus, and even though pneumococcus lacks enzymes to cleave this antigen, high levels of antibody could overcame by a large inoculum. (Brown et al., 2015)

S. pneumoniae is associated to a less stable microbiome profile during the first two years of life and co-colonization of different strains of pneumococcus is associated to an increased production of peptides with antimicrobial activity, providing new DNA sources for this natural competent bacteria. (Weiser et al., 2018) Although pneumococcus has developed efficient mechanisms to avoid co-colonization by organisms with which it competes for the same niche, such as hydrogen peroxide production, it has been described that pathogens as *Haemophilus influenzae* stimulate complement dependent phagocytic killing of pneumococcus. (Brown et al., 2015)

Pneumococcal invasive disease

Once colonization is achieved, epithelial adherence and local microbiota interaction allow either biofilm formation with further lung access through aspiration or translocation across the epithelium with further access to the bloodstream. (Tuomanen, 2001) During invasion processes ChoP and CbpA proteins bind to their respective receptors PAFr and polymeric immunoglobulin receptor (PIGR) in the epitelium, allowing pneumococcus endocytosis and further exocytosis in the interstitium, the same mechanism allows pneumococcus to cross the endothelium and reach the bloodstream. Paracellular invasion is an alternative for invasion of this pathogen when Ply and hydrogen peroxide harm the epithelium. (Weiser et al., 2018) Pneumococcus requires several adaptations in order to survive after the environmental change induced by bacterial translocation, with a greater expression of genes codifying ion transporters for Manganese, Zinc and Iron, fundamental cofactors for bacterial metabolism and enzyme function. (Kadioglu et al., 2008; Weiser et al., 2018) A phenomenon called phase variation gives pneumococcus versatility in order to adapt to different niches, it has been described that during phase variation key virulence factors vary their expression, among them capsular polysaccharide is fundamental; the thickness of the polysaccharide capsule gives it a specific phenotype known as opaque or transparent, where the opaque phenotype, characterized by a thicker CPS can better survive in the niche of the nasopharynx, while the transparent can do better in the blood. (Kadioglu et al., 2008)

Pneumococcal vaccines and surveillance

Pneumococcus has had a great impact worldwide due to the effects of invasive disease on health and economy, constituting a major public health problem. Before the antibiotic era, more than 70% of pneumococcal infected patients died (Butler, Shapiro,

& Carlone, 1999) and by the year 2016 pneumococcal pneumonia remained as the leading cause of pneumonia mortality globally. (Troeger et al., 2018) Since colonization precedes invasion, controlling the first one is a key element to prevent invasive pneumococcal disease development, and when a humoral immunological response against pneumococcus was confirmed in 1886 by Albert Fränkel and 1891 by Klemperer brothers, control mechanisms such as vaccination became a fundamental resource for this purpose. The first whole–cell heat treated bacteria vaccine was commercialized in 1909 as "Pneumo- Bacterin" in USA (Grabenstein & Klugman, 2012); during this decade realization of serotype specificity of pneumococcus raised with Neufeld and Händel´s description of the Quellung reaction in order to distinguish serotypes of pneumococci, and after Lister´s description of predominant serotypes of pneumococci, a new approach to controlled bivalent, trivalent and four-valent vaccine trials and the herd effect theory developed (Grabenstein & Klugman, 2012); allowing knowledge acquisition regarding administration, dosage and methods of vaccine preparation.

The realization of pneumococci's capsule polysaccharide presence and its association to bacterial serotypes during 1920´s defined it as a critical virulence factor and led, during the 1940´s, to the development of new polyvalent vaccines, containing purified capsular polysaccharide of pneumococcal serotypes, reaching a vaccine efficacy of 84% in preventing pneumococcal pneumonia and reduction of vaccine type carriage. (Siber, Klugman, & Mä, 2008)

By 1977 after the Food and Drug Administration and Canada´s Health Products approval, the first 14 valent vaccine was commercialized as "Pneumovax" and contained 50ug of purified polysaccharide of pneumococcal serotypes 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F,

14, 18C, 19F, 23F and 25F based on the most prevalent serotypes in USA, Europe and South Africa. (Butler et al., 1999; Grabenstein & Klugman, 2012)

Even though pneumococcal vaccines proved effective against invasive disease, during the 1980´s a low immunological response in children under 2 years old was evidenced, leaving a potential reservoir and susceptible risk group unprotected. This led to the development of a conjugated vaccine which contained pneumococcal polysaccharide serotype 6A conjugated with type b *Haemophilus influenzae* (Hib) capsular polysaccharide and serum albumin, thus inducing a cellular immune response, increased antibody production in children and by default protection against Hib also. (Butler et al., 1999; Grabenstein & Klugman, 2012) In the year 2000 a 7 valent, Hib conjugated vaccine, was released providing coverage for serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, but serotype invasive disease due to serotype 19A prevalence increased, a phenomenon known as serotype replacement, therefore new 10 and 13 valent vaccines were developed. This last two are known as “Synflorix” and “Prevenar 13” which are commercialized until today by GlaxoSmithKline and Wyeth laboratories respectively, and provide coverage for the following serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F for 10 valent and additional serotypes 3, 6A and 19A for 13 valent vaccine. (Butler et al., 1999; Grabenstein & Klugman, 2012; Siber et al., 2008)

Nowadays, vaccine development targets the most prevalent serotypes in certain areas of the world, but these vaccines may not be useful for others, where different serotypes prevail. Pneumococcus possess a great versatility for capsule switching, and besides the limited vaccines serotype coverage, serotype replacement has become the main reason why new vaccines aiming different pneumococcal antigens that allow a universal serotype independent vaccine development is essential, in order to prevent

and control IPD. Active and passive immunizations with recombinant pilus proteins associated to adherence and host inflammatory response have shown immunogenicity. (Seib, Zhao, & Rappuoli, 2012) PspA and C and also pneumolysin are under study for protein based vaccines development. (Daniels, Rogers, & Shelton, 2016)

In order to carry out IPD control strategies, it is necessary to gather epidemiological information of great relevance, such as the geographic distribution and temporal variation of IPD. This is achieved through epidemiological surveillance in which sentinel institutions are assigned due to a key geographic location and availability of scientific knowledge to diagnose the disease and produce high quality data. (Organización Panamericana de la Salud, 2018a)

Due to the public health impact of IPD in Latin America, in 1993 the Pan American Health Organization (PAHO) implemented a regional surveillance program based on a network of hospitals and sentinel laboratories with delegates from 20 countries known as SIREVA (System of Surveillance Networks of the Responsible Agents of Pneumonia and Bacterial Meningitis) and in 2004 SIREVA II, responsible for providing information regarding the distribution of serotypes and antibiotic susceptibility of S*. pneumoniae, H. influenzae* and *N. meningitidis*. In addition, the information compiled by this program allows estimating the burden of these diseases and the development of more efficient and specific vaccines for the requirements of this region. (Organización Panamericana de la Salud, 2018a)

In 1995 the Emerging Infections Program Network from the Centers for Disease Control (CDC) stablished a core component named Active Bacterial Core surveillance, (ABCs) which consists of a collaboration between CDC state health department and several universities in order to establish a surveillance system in 10 States, providing

demographic and laboratory information and infrastructure for further public health research regarding group A Streptococcus, group B Streptococcus (GBS), *Haemophilus influenzae, Neisseria meningitides* and *Streptococcus pneumoniae.* (CDC, 2018a)

In 2005 the European Centre for Disease Prevention and Control (ECDC) was established aiming to identify current and emerging infectious diseases within 28 countries from the European Union; this program collects information and performs indicator and event based surveillance analyzing and disseminating information on 56 pathogens and associated diseases including IPD. (European Center for Disease Prevention and Control, 2018)

PART II

SCIENTIFIC ARTICLE

Streptococcus pneumoniae nasopharyngeal carriage in indigenous Kichwa communities of Otavalo, Ecuador: serotypes, risk factors, and associated respiratory pathogens

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ABSTRACT

Background: *Streptococcus pneumoniae* has been recognized worldwide as one of the main causative agents of otitis media, pneumonia, septicemia and meningitis; with high rates of mortality. Studies that explore respiratory pathogens colonization in Latin American Countries like Ecuador are absent for higher-risk groups, such as the indigenous population.

Methods: A cross sectional survey was carried out in five Kichwa villages, assessing 63 families, 100 children between 0 to 12 years old and their parents, regarding socio-economic conditions and risk factors related to *Streptococcus pneumoniae* carriage. Nasopharyngeal swabs were collected in order to identify *S. pneumoniae, H. influenzae, M*. *catarrhalis* or *S. aureus* colonization by means of microbiological standard methods. Sequential multiplex PCR approach was used for serotyping and identification. Statistical analyses were done using IBM SPSS Statistics V24 for Windows.

Results: Poverty conditions were identified in 90.5% of the assessed population. Pneumococcus colonization was identified in 50% of the children and 6.3% of the caregivers. Colonization increased with age and peaked at 61-138 months. Non Tippable, 6A/D/C and 19A were the most prevalent serotypes identified.

S. pneumoniae-M.catarrhalis was the more prevalent co-colonization pattern in children < 60 months and *S. pneumoniae-H. influenzae* in children from 61 to 138 months old. *S. aureus* was the most prevalent identified pathogen colonizing caregivers. The presence of wooden stove was identified as the only predictor for pneumococcus colonization (*P*=0.047), with a higher risk for colonization in children older than 60 months (*P*=0.022 OR=3.2 CI95% 1.2-8.8) and females (*P*=0.034 OR=3.5 CI95% 1.1-11.4).

Conclusions: This is the first study that identifies pneumococcal colonization in indigenous ethnical groups in Ecuador. Despite the sample size, vaccine type serotypes were identified colonizing indigenous children making it necessary to extend the assessment of pneumococcal colonization nationwide to general population.

***Key words*:** Pneumococcus, nasopharyngeal colonization, indigenous population, serotypes, children.

INTRODUCTION

Known as one of the main causative agents of otitis media, pneumonia, meningitis and septicemia worldwide. (Abdullahi et al., 2012; Tuomanen, 2001) *Streptococcus pneumoniae* (pneumococcus) is a facultative anaerobic Gram positive coccus that colonizes the mucosal surfaces of the upper respiratory tract of healthy children and adults. (Murray Patrick, Rosenthal Ken, 2013) Carriage prevalence reaches 95% in healthy children under 3 years old in developing countries and 65% worldwide. (Kadioglu et al., 2008; Obaro & Adegbola, 2002) Colonization of the upper respiratory tract (URT) is most common during early childhood, and is apparently asymptomatic, but if the pathogen reaches sterile areas of the airway, an intense inflammatory response occurs, ending in disease development. (Kadioglu et al., 2008; Obaro & Adegbola, 2002) Therefore, within the life cycle of this pathogen, carriage is a vital element for transmission to other individuals and subsequent disease development. (Weiser et al., 2018)

The mortality associated to the invasive disease and rising antimicrobial resistance patterns of this opportunistic pathogen represent a global health threat. Pneumococcal pneumonia mortality was the leading cause of death due to lower respiratory infections in children under 5 years in 2017, (Dicker et al., 2018) which is the reason why the World Health Organization included pneumococcus as one of the 12 priority pathogens the same year. (Dicker et al., 2018; Weiser et al., 2018)

Studies that explore the etiology of pneumonia in Latin America are limited. In Ecuador mortality during childhood due to pneumonia reached 5,8% in 2016 (Instituto Nacional de Estadísticas Y Censos, 2016) but information regarding the disease etiology is absent. According to Jonnalagadda et al.,(Jonnalagadda et al., 2017) by 2017

respiratory syncytial virus (RSV) was the leading viral cause of pneumonia, followed by *S. pneumoniae* as the leading bacterial pathogen in hospitalized children in Quito, Ecuador.

Information regarding pneumococcal colonization rates and risk factors in this country is absent. Considering that factors such as age, day-care centers attendance, presence of siblings, recent antibiotic use, respiratory tract infections, cooking method and crowded living style are the main ones, the lack of identification of these relevant factors related to colonization opens a gap to the implementation of public health policies that could help avoid the development of invasive disease by pneumococcus, and thus high health expenditure associated with invasive pneumococcal disease. Another relevant factor to consider is that due to associated poor living conditions, indigenous people present an increased susceptibility towards infectious diseases, with a higher prevalence and mortality rate. (Gracey & King, 2009) Ethnic and regional influence on pneumococcal colonization have been noted as relevant risk factors worldwide, where economic conditions are important in explaining racial health disparities. Evidence shows that indigenous populations have poor social and health outcomes in relation to their non-indigenous counterparts due to restricted access to clean water supplies, fresh food and health services. (Anderson et al., 2016; Gracey & King, 2009) In Ecuador 7% of the general population belongs to the indigenous ethnic group, which is divided in 13 different nationalities. (Sistema de Indicadores Sociales del Ecuador, 2018) For decades, regional and ethnic differences regarding the general health status of this population are historically reflected by higher prevalence of growth retardation and much lower weight. They are also more affected by problems such as child mortality, poverty, indigence and illiteracy, especially in indigenous populations of

the Andean region of the country. (Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2010)

Controlling pneumococcal colonization is a key element to prevent invasive pneumococcal disease development and therefore, first approach control mechanisms such as vaccination and sentinel surveillance have become a fundamental resource for this purpose. Ecuador is part of the regional surveillance program based on a network of hospitals and sentinel laboratories with delegates from 20 countries known as SIREVA (System of Surveillance Networks of the Responsible Agents of Pneumonia and Bacterial Meningitis) which is responsible for providing information regarding the distribution of serotypes and antibiotic susceptibility of S*. pneumoniae, H. influenzae* and *N. meningitidis*. In addition, the information compiled by this program allows estimating the burden of diseases caused by these pathogens and the development of more efficient and specific vaccines for the requirements of this region. (Organización Panamericana de la Salud, 2018a)

In 2008 conjugated vaccines with Hib capsular polysaccharides for the prevention of pneumococcal disease were introduced in Ecuador as PCV7 for risk groups. (Organización Panamericana de la Salud, 2017) Later in 2011, the decavalent pneumococcal conjugated vaccine, PCV10, was implemented into the national immunization schedule, offering protection against the most invasive serotypes of pneumococcus at that time, 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. By 2017 the coverage of the third dose of this vaccine corresponded to 84% of the target population. (Organización Panamericana de la Salud, 2018b) and the most prevalent serotypes circulating around the country, causing invasive disease, where 3, 6B, 19A, 19F, 15A and 15B. (Brandileone, 2015)

Despite the fact that Ecuador belongs to this regional surveillance system and has records of circulating serotypes that generate invasive disease in patients under 5 years of age, there is currently a lack of up-to-date information regarding the prevalence of colonization by this pathogen in the population in general and even less in higher-risk populations such as the indigenous population. Therefore, in this study we evaluated the prevalence of pneumococcus nasopharyngeal colonization and its serotypes, risk factors for infection and concomitant airway pathogens in children under 12 years old and their caregivers in different Kichwa communities of Otavalo, Ecuador.

PARTICIPANTS AND METHODS

Study site and population.

A cross sectional survey was carried out between February and April 2018 in five kichwa villages, assessing 63 family groups belonging to seventeen different communities of Otavalo and Cotacachi; located in the Imbabura province at the northern Highlands of Ecuador.

Otavalo has a population of 110.461 habitants, where the rural inhabitants occupy most of the territory, with 65.925 habitants. (Gobierno Autonomo Descentralizado Municipal del Cantón Otavalo, 2018) This area is characterized by having a large indigenous population, represented by 101 indigenous communities, 85 of them are identified as kichwas otavalos and 14 as kichwas kayambis; and 77 of this indigenous communities are located in rural parishes. (Cevallos, 2017; Otavalo, 2012) Cotacachi as a smaller population, 40.036 habitants, with 77% of its inhabitants distributed in rural areas, and 40.55 % recognized as indigenous, located in 4 parishes. (Otavalo, 2012)

Family groups considered in the study included children between 0 to 12 years old and their parents, who were interviewed regarding socio-economic conditions of the family group and risk factors related to *Streptococcus pneumoniae* (pneumococcus) carriage such as age, gender, vaccination status, daycare assistance, breastfeeding history, exposure to indoor cooking or tobacco smoke, upper respiratory tract infection (URTI), prematurity, prior pneumonia or otitis events, antibiotic use in the prior 30 days and living conditions. General health status was evaluated by physical examination, and nutritional status was assessed by anthropometric measurements.

Sampling and laboratory procedures.

Nasopharyngeal swabs were collected from all the study participants following the World Health Organization (WHO) standard methods for measuring pneumococcal nasopharyngeal carriage. (Katherine L O'Brien & Nohynek, 2003; Satzke et al., 2013) Briefly, a rayon tipped swab (Transystem™ Copan Italia) was inserted into the posterior nasopharynx of the patient and rotated 180 degrees, the tip was cut out and stored in 1ml skim milk-tryptone-glucose-glycerol (STGG) medium (K. L. O'Brien et al., 2001) at 4°C for a maximum of 24 hours before final storage at -80°C at the microbiology laboratory in School of Medicine at Universidad San Francisco de Quito until analysis.

Swabs were plated on sheep blood, sheep blood plus gentamicine, chocolate and mannitol salt agar plates, the first three where incubated overnight at 37°C in 5%CO2 and the last at 37°C in aerobiosis. S. *pneumoniae*, H*. influenzae*, M. *catarrhalis* or S. *aureus* isolates were identified using microbiological standard methods. (Murray & Microbiology, 1995)

DNA extraction of the pneumococcal isolates was carried out by means of boiling methods (Le Polain De Waroux, Flasche, Prieto-Merino, & Edmunds, 2014) and

sequential multiplex PCR approach was used for serotyping and identification (Pai Rekha , Gertz Robert E., 2006) based on the list of oligonucleotide primers recommended by the Centers for Disease Control and Prevention (CDC) for pneumococcal capsular polysaccharide (cps) identification, allowing serotype deduction of 38 pneumococcal serotypes in 7 different multiplex reactions. (CDC, 2018)

Anthropometric measurements.

Data regarding size (cm) and weight (kg) were taken as previously described (Ministerio de Salud Pública del Ecuador, 2012) in children under 10 years old and their caregivers. The records were evaluated according Z scores for weight for height and height for age based on WHO standard reference populations and Public Health Ministry of Ecuador. (Ministerio de Salud Pública del Ecuador, 2012; World Health Organization, 2008)

Statistical analysis.

Statistical analyses were done using IBM SPSS Statistics V24 for Windows (SPSS Inc, Chicago, IL, USA). Descriptive and regression analysis were performed to determine association between risk factors for carriage and S*. pneumoniae*. M. *catarrhalis*, S. *aureus* and H. *influenzae* rates of colonization were also identified. Univariate regression analysis was adjusted for sex and age. A p-value of <0.05 was considered statistically significant.

Ethics.

The study protocol was evaluated and approved (2016-183M) by the bioethics committee of Universidad San Francisco de Quito, Ecuador. A written informed consent was signed by the primary caregiver in order to enroll the family group in the study.

RESULTS

Sociodemographic characteristics of the population.

In general, poverty conditions were identified. The studied population comprised families with several members, 52.4% of them were integrated by 5 or more individuals, with an average of 3 children per family. Caregivers, who were mostly of female gender and young adults, had a basic or middle school level of education. Caregiver's general information is resumed in Table 1.

Characteristics		Number (%)
Sex		
	Female	60(95,2)
Mean age		33 years
	min	17 years
	max	52 years
Education level		
	Analphabet	9(14,3)
	Elementary School	41(65,1)
	Highschool	11(17,5)
	College	2(3,2)

Table 1 General characteristics of caregivers' population

A total of 63 families were assessed, from them 92.1% lived in rural housing and 90.5% in poverty conditions, of which 30.2% belonged to a state of critical poverty. The sociodemographic characteristics identified are resumed in Table 2.

Characteristics	Number (%)
Type of housing	
Rural	58(92,1)
Household inhabitants	
More than 5	33(52,4)
Up to 5	30(47,6)
Mean number of people in the family group	5
min	2
max	13
Mean number of children per family	3
min	1
max	11
Economic Stratum	
Critical poverty	19(30,2)
Relative poverty	38(60,3)
Middle class	6(9,5)

Table 2 Sociodemographic characteristics of the population

A total of 163 nasopharyngeal samples were obtained, 63 from caregivers and 100 from children; from them, 33% belonged to children under 5 years and 67% to 5-11.5 years. The child population distribution was homogeneous, 51% were female and 49% male. Child sex by age group distribution is resumed on Table 3.

Age in months	Sex		Total
	F	M	
1-59	13	20	33
60-138	38	29	67
Total	**51**	**49**	**100**

Table 3 Child sex population, distribution by age group

Prevalence of pneumococcal colonization.

Pneumococcus colonization was identified in 92% (58/63) of the studied families. From these, in only 3.2% (n=2) both, caregiver and at least one child, were colonized. Colonization reached 50% of the children and 6.3% of the caregivers, among adolescents (120-138 months) only, colonization rates reached 35%. In children, colonization

increased with age and peaked at 61-138 months. Pneumococcal colonization in children by age group is resumed in Figure 1.

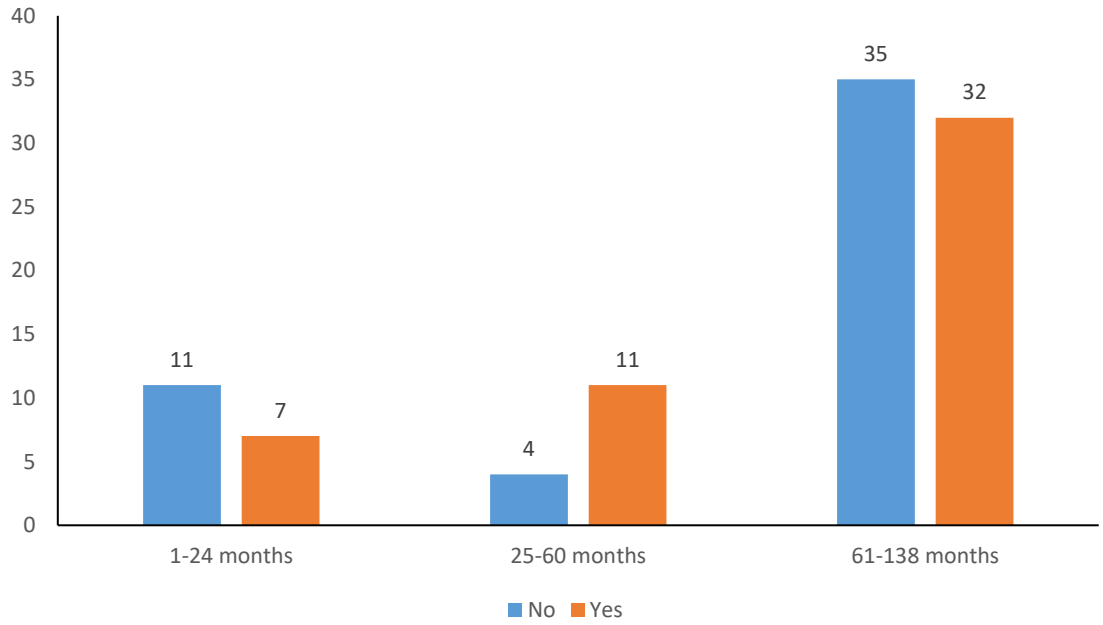

Figure 1 Prevalence of S. pneumoniae colonization in children by age group

Seven Multiplex PCR reactions were used in order to identify pneumococcal serotypes in the microbiological samples obtained. Only one of the 63 families was colonized by the same serotype (11A/D). The most frequent serotypes are resumed in Table 4.

Serotype	Prevalence (%)
3	1(1.85)
6A/D/C	6(11.11)
7A/F	1(1.85)
10A	3(5.55)
11A/D	3(5.55)
15 B/C	3(5.55)
19A	5(9.25)
22AF	2(3.73)
23A	3(5.55)
23B	1(1.85)
23F	1(1.85)
31	3(5.55)
34	2(3.73)
35B	3(5.55)
35F/47F	3(5.55)
37	1(1.85)
38	2(3.73)
NT	11(20.37)
TOTAL	54(100)

Table 4 S. Pneumoniae Serotype Prevalence

Co-colonization patterns.

Standard microbiological methods were used in order to determine the presence of *S. pneumoniae*, *M. catarrhalis, H. influenzae*, *S. aureus* and co-colonization patterns. All the 4 pathogenic agents previously described were found colonizing children. In younger children (< 60 months) the most frequently identified co-colonizing pattern was *S. pneumoniae-M. catarrhalis,* while in older children (60 to 138 months) *S. pneumoniae-H. influenzae* was the most frequent co-colonization pattern. The data is resumed in Figure 2, Figure 3 and Figure 4.

Pneumococcal serotypes 6A/D/C, 7AF, 10A, 11A/D, 15 B/C, 23A, 31, 34 and 38 were the most prevalent in *S. pneumoniae- M. catarrhalis* co-colonization pattern, while 6A/D/C, 11A/D, 15B/C, 19A, 22AF, 23A, 23F, 35B and 38 were the most prevalent in *S. pneumoniae-H. influenzae co-colonization pattern.*

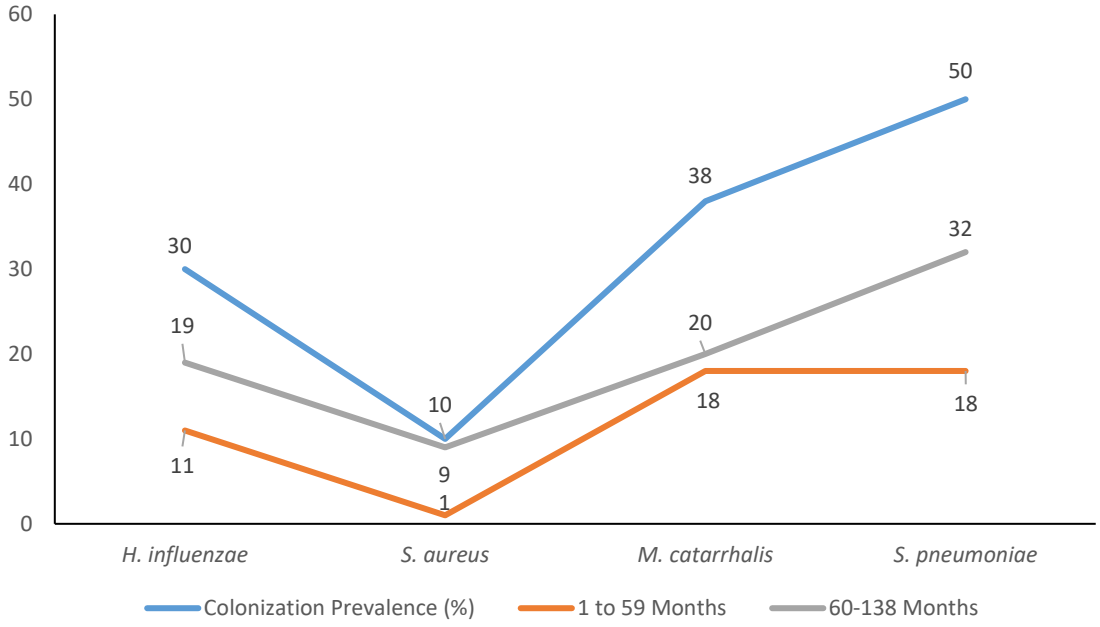

Figure 2 Prevalence of respiratory pathogens colonization in children

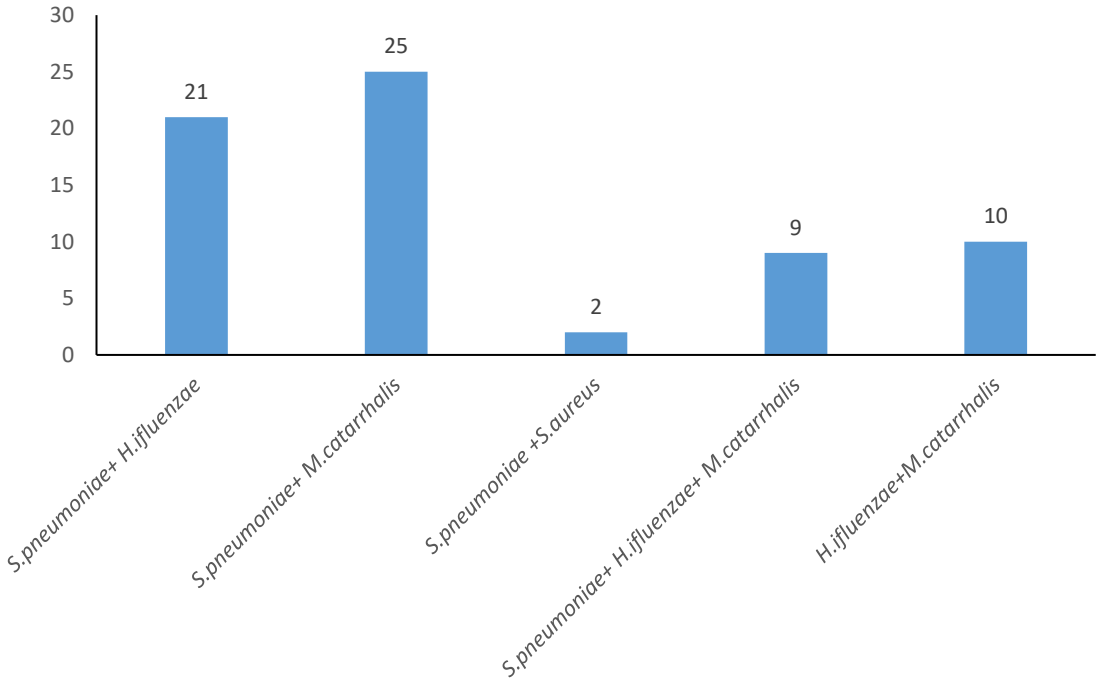

Figure 3 Co-colonization patterns in children

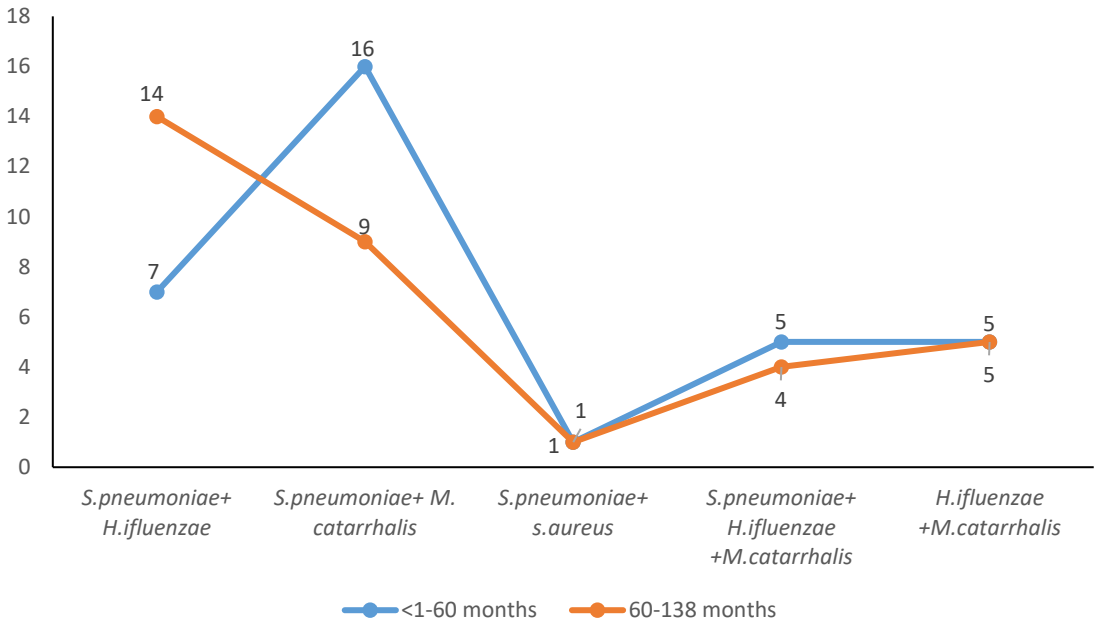

Figure 4 Co-colonization patterns by age group in children

Caregivers presented different colonization patterns than children, with *S. aureus* as the most prevalent pathogen. Co-colonization cases such as *S. pneumoniae-M. catarrhalis* were identified in 1.6% of caregivers, and *S. pneumoniae-H. influenzae* in 3.2%. Caregivers´ colonization patterns are resumed in Figure 5.

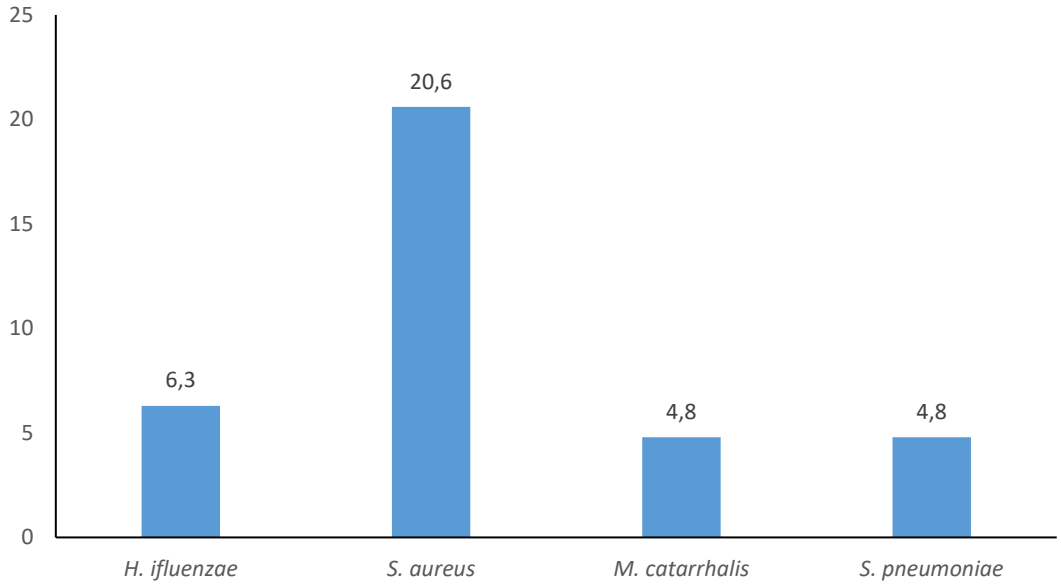

Figure 5 Prevalence of respiratory pathogens colonization in caregivers

Risk factors for nasopharyngeal colonization.

It was observed that 55.6% of the studied population cooked using a wooden stove. From them, 65.7% had at least one member of the family colonized by pneumococcus. From the families that had at least one member colonized, 89.7% lived in rural housing, 64% had a caregiver with a basic instruction level and 23% had smokers at home.

After the logistic regression, the presence of wooden stove was identified as the only predictor for pneumococcus colonization (*P*=0.047), with a higher risk for colonization in children older than 60 months (*P*=0.022 OR=3.2 CI95% 1.2-8.8) and females (*P*=0.034 OR=3.5 CI95% 1.1-11.4). No statistical significance could be demonstrated for the rest of the assessed risk factors for pneumococcal colonization, as summarized in Table 5.

Characteristic		***S. pneumoniae* colonization**			
		Prevalence	**p value**	**OR**	**(95%IC)**
Housing	**urban**	7%	0.255	2.667	0.492-14.44
	rural	97%			
Sex	**Male**	49%	0.549	1.272	0.580-2,790
	Female	51%			
Socioeconomic stratum	**Medium**	9%	0.100	3.907	0.770-19.831
	Poverty	91%			
More than 5 habitants		57%	0.840	1.085	0.491-2.395
URTI		8%	0.159	3.273	0.627-17.071
Antibiotics use		5.1%	0.665	1.500	0.240-9.391
Small for the age		25%	0.817	1.113	0.450-2.753
Low weight for age		5%	0.648	1.532	0.245-9.587
Day care assistance		27%	0.887	1.077	0.386-3.005
Breastfeeding		95.9%	0.518	0.446	0.039-5.147
History of pneumonia		15.5%	0.124	3.048	0.736-12.615
History of prematurity		8.5%	0.971	1.031	0.194-5.492
Wooden Stove	**Inside**	55.6% 34%	0.047	**2.253**	**1.011-5.019**
	Outside	65.7%			
Wooden stove by age (≥60 months)	**<60 Months**	16%	0.022	**3.217**	**1.181-8.761**
	≥60 Months	36%			

Wooden stove by sex (female)	**Male**	24%	0.034	**3.532**	**1.099-11.358**
	Female	28%			
Smoke (tobacco or stove)		58%	0.107	1.941	0.867-4.346

Table 5 Risk factors for S. pneumoniae colonization in children, Binary logistic regression

Regarding nutritional status, most of the children population had normal weight (89.5%) and size (71.3%) for the age; and despite what was reported in other studies, it was found that the majority of pneumococcal colonized children had also normal weight (88.9%) and size (70.2%) for the age.

DISCUSSION

The present study is the first approach to nasopharyngeal carriage prevalence and risk factors for *Streptococcus pneumoniae* and other potential respiratory pathogens colonization in children and their caregivers among indigenous communities in Ecuador. Pneumococcal carriage data in general population of South America and the Caribbean is scarce and even more so for indigenous population with limited access to health services.

It was possible to identify that the majority of the assessed population in this study had a lifestyle that has previously been associated in the literature with the presence of risk factors for pneumococcal colonization, which was identified in 39.8% of the studied population. Carriage prevalence reached 15.8% for caregivers and 55% for children. Unlike what has been established in other studies, the children population presented a particular pattern of colonization, since pneumococcus carriage increased with age and reached a peak among older children (5 to 11.5 years). This colonization pattern has been previously described by Ueno et al., (Ueno et al., 2013) in a group of unvaccinated younger children in Japan, where the mean carriage rate was 22% and reached a peak among children between 3 to 6 years old (28%), with a higher prevalence

of colonization in children who attended day care and had older siblings. Despite having a similar tendency, in our study no association or statistical significance could be demonstrated regarding the presence of siblings or day care attending.

As it was demonstrated, pneumococcal colonization affects mostly vulnerable groups, such as senior population and children under two years of age, (Bogaert et al., 2004) colonization patterns investigation in other groups of age has been relegated to few studies. Adolescent's colonization study, for example, has been incipient and although it was not the main objective of this study, the colonization patterns during the first two years of adolescence in indigenous children were identified. Among people between 10 and 12 years old, colonization rates reached 35% (7/20), and despite no statistical significance could be demonstrated for sex, higher rates of colonization were identified in women, reaching 85%. Pneumococcal colonization in adolescents from 10 to 19 years old has been previously described by other authors, with carriage prevalence that vary from 8 to 43%, (Bokaeian, Khazaei, & Javadimehr, 2011; Cardozo et al., 2008) Cardozo et al.,(Cardozo et al., 2008) demonstrated that pneumococcal colonization was related to sex and younger age during adolescence. The study, carried out among people between 10 to 19 years in Brazil, presented pneumococcal colonization rates that reached 8.2%, with higher colonization rates for adolescents among 10, 11 and 12 years, with 11.6%, 11.3% and 11.7% respectively. Taking into account that the observed colonization prevalence among adolescents in this study could be explained by the age of the assessed group, and the fact that the colonization pattern demonstrated a tendency to increase with age; risk factors for carriage and colonization patterns identification among a more representative group of adolescents between 10-19 years

old in indigenous populations in Ecuador becomes a necessity, in order to determine the role of this age group in pneumococcal transmission and niche.

In general, carriage rates data correlate with those described by other authors who have studied communities with similar characteristics to those of the population of this study in other parts of the world. Bello et al. and Rivera-Olivero et al., (Bello et al., 2010; Rivera-Olivero et al., 2007) identified pneumococcal carriage rates in children under 5 years old and their caregivers in indigenous Panare and Warao rural communities in Venezuela, reaching colonization rates of 69% and 49% respectively for children, with higher prevalence in children under 2 years old, and rates of 11% for Panare caregivers. It´s relevant to consider that these two authors studied indigenous populations that had not been previously vaccinated, since access to health services and their contact with society was much more limited than the population of our study. On the other hand, Neves et al.,(Neves et al., 2008) developed a study in slum communities in Brazil, in which despite the fact that the studied population was not indigenous, socioeconomic conditions were similar. In the study, general carriage rate in slum communities reached 36%, with 66% carriage rates in children under 5 years old and 16.2% in young adults. It was identified that URTI increased the risk for pneumococcal colonization, while breastfeeding was related to a decreased risk for carriage. Despite the fact that there were no significant results regarding these variables in our study, a similar tendency was observed, and a more heterogeneous sample of the population may allow us to compare the obtained results in order to determine the presence of significant association between these variables and colonization in other groups of the Ecuadorian population, such as mestizos and other vulnerable ethnic groups. The largest number of individuals belonging to the indigenous ethnic group was captured in the

region of Cotacachi and Otavalo areas, and given the results obtained, a larger and more diverse sample will be assessed in a future study, considering different ethnical groups around the country.

The presence of wooden stove fumes was significantly associated with pneumococcus carriage as a relevant identification of our study. In this particular case, sex and age were relevant factors determining the predisposition for colonization. Exposure to wooden stove fumes in older children (5 to 12 years old) increased 3.2 times the possibilities for colonization and 3.5 times in females. According to the WHO, about 3 billion people around the world still cook using solid fuels such as wood, crop wastes, charcoal, coal and dung in open fires. This activity is more common in developing countries due to its low cost and easy access, and it's responsible for the presence of high levels of household air pollution and the premature death of 3.8 million people around the world due to illnesses such as pneumonia, stroke and ischemic heart disease. (World Health Organization, 2018) It´s been demonstrated that exposure to household air pollution doubles the risk for childhood pneumonia, is responsible for 45% of all pneumonia deaths in children less than 5 years old and is 28% of all adult pneumonia deaths. (World Health Organization, 2018) The health effect of indoor air pollution is determined by the level of pollution and time of exposure. People in developing countries are exposed to high levels of pollution for 3 to 7 hours a day for several years, and due to a more related role in cooking, women are more exposed, and therefore have higher risk to develop nasopharyngeal colonization and further disease. (Patel, Okocha, Narayan, & Sheth, 2013) Indigenous cultural aspects in Ecuador involve women in activities related to home and children care with more responsibility than men, especially the ones related to feeding and family health. (Méndez Torres, 2009;

Radcliffe, 2014) Due to this characteristic, it´s plausible that infants and female children spend more time around their mother during cooking and home care activities; this could explain the increased risk for colonization observed in young female patients in this study. Given this background, it would be logical to expect similar pneumococcal colonization rates both in children and their mothers; in this study, mother- child pneumococcal colonization was identified in only two out of sixty three families, with higher pneumococcal colonization rates in children and S. *aureus* colonization in mothers. This pattern of colonization in adult females can be explained by a cumulative development of immunological memory due to repeated pneumococcal colonization events during life, generating fast bacteria clearance of the nasopharynx before colonization stablishes during adult life or lower colonization density in the nasopharynx, (Brown et al., 2015) allowing colonization of different bacteria, such as S. *aureus*. Lower rates of identification via microbiological methods due to low bacterial density could also explain this pattern, and molecular methods such as qPCR could be useful to determine the presence or absence of this bacteria in adult patients due to its high sensitivity.

Despite SIREVA II sentinel vigilance reports that Ecuador presents an 84% coverage for pneumococcal vaccine, (Pan American Health Organization, 2018) no information regarding ethnical groups access to the vaccine is available, and governmental programs for vaccine control report vaccination coverage by ethnical groups only for five different vaccine types for children under two years old: BCG, DPT+HB+Hib, DPT, OPV and SRP. (Sistema de Indicadores Sociales del Ecuador, 2018) Even though all the interviewed caregivers reported verbally that their children

vaccination status was updated, no vaccination card or registry was available at the moment of the assessment, and pneumococcal vaccination could not be proved.

In 2011 PCV10 vaccine was implemented into the national immunization schedule in Ecuador, since pneumonia became the first morbidity cause for general population with 18153 hospitalizations for children under 5 years old, and the second mortality cause for children after birth related causes. (Ortiz-Prado, Iturralde, Hernández, & Galarza, 2014) Only after 2014, three complete doses of pneumococcal vaccine were finally implemented for children under 2 years in the country. (Ministerio de Salud Pública del Ecuador; Secretaría Nacional de Planificación y Desarrollo; OPS/OMS, 2017) According to SIREVA II regional reports, before the vaccine was implemented in the national immunization schedule, by 2009, a total of 48 cases of invasive pneumococcal disease were reported, and the responsible serotypes were PCV10 vaccine types 14, 9V, 23F, 19F and 6B and PCV13 vaccine types 3, 6A and 19A. (Organización Panamericana de la Salud, 2011) It´s important to consider that the burden estimation of pneumococcal disease by standard diagnostic methods has low sensitivity, only 0,5 to 16% from blood samples for microbiological isolation, leading to information bias and underestimating the real burden of disease, since only the most severe cases are identified. (Bardach et al., 2017) According to vaccine probe trial studies in Latin American countries, cases of prevented clinical pneumonia were 10 times greater than cases of culture- confirmed pneumonia. (Bardach et al., 2017) This is the reason why colonization studies are so relevant regarding pneumococcal disease prevention and niche identification. Two years after the implementation of the PCV10 vaccine in Ecuador, the number of pneumococcal disease cases decreased to 25, and kept a similar pattern the next year; by 2015 only 9 cases were identified. Despite the

number of reported cases decreased, according to this last 3 SIREVA II regional reports for Ecuador, from 2013 to 2015, the most prevalent serotypes identified as causative agents of invasive pneumococcal disease, mostly diagnosed from blood cultures from pediatric patients with pneumonia, were 6A/C, 15A, 19A, 19F and 23F, with higher prevalence in children under 12 months and 2 to 5 years old. (Brandileone, 2015) From them, serotypes 6A/6C and 19A were the most predominant. Worldwide, PCV7 implementation reduced pneumococcal disease associated to this vaccine´s serotypes, while non vaccine types increased, including serotype 19A, which emerged in different countries. This serotype has an increased potential to cause invasive disease due to penicillin resistance and capsular switching (Isturiz et al., 2017) reason why controlling and preventing colonization of this serotype is fundamental in Ecuador.

Pneumococcal serotype diversity allows this bacteria to respond effectively to the selective pressure generated by immunization, allowing serotype replacement but not carriage variation, as was demonstrated by Gladstone et al.,(Gladstone et al., 2015) in a longitudinal trial that evaluated pneumococcal carriage in children population of the United Kingdom during five winters in a row. In this study, conventional microbiology and whole genome sequencing technologies were utilized to characterize pneumococcal isolates. After PCV implementation in this country, vaccine serotypes decreased 98% while non vaccine types increased 68%; carriage rates maintained but serotypes 7F, 19A and 22F increased their prevalence with the years. This phenomenon was accompanied by parallel changes in genotype prevalence for associated sequence types with clonal expansion contributing to replacement. By the end of this study serotype coverage of PCV7 and PCV13 was1% and 11% respectively.

The Ecuadorian public health system lacks a supervising protocol of pneumococcal carriage, so there is no background data to compare the results obtained in our study, in which the most prevalent serotypes identified were 19A with 9.25% and 6A/D/C with 11.11%. This results suggest that this two serotypes are circulating in the country and probably generating pneumococcal disease in pediatric patients who carry this pathogen. It has been described that immunological cross-reactivity between serotypes 19F and 19A could theoretically result in cross-protection against pneumococcal disease caused by serotype 19A, (Isturiz et al., 2017) but considering that serotypes 19A and 6A have been the most prevalent causing pneumococcal disease in Ecuador for the last 3 years, and that colonization has been proved in a vulnerable ethnical group; identification of pneumococcal colonization in general population including different ethnical groups around the country becomes a necessity in order to validate the effectiveness of the actual vaccination protocol. Given the fact that pneumococcal vaccination nationwide reaches 84%, immunological cross-protection developed by vaccinated population should be assessed, and it would be important to define if switching to PCV13 vaccination could help to prevent further pneumococcal colonization in vulnerable groups, decreasing the risk of further pneumococcal transmission and disease, and also the expense generated by pneumococcal disease and patient´s medical care, hospitalization and treatment. According to the only economic analysis performed in Ecuador regarding PCV10 and PCV13 affordability in 2014, disease costs prevented by PCV13 implementation in the country would be close to $3.4 million each year, avoiding medical expenses related to 323 potential cases of pneumococcal invasive disease, 3.480 pneumonia cases and 8136 acute otitis media cases, just by

covering the three additional serotypes included in the PCV13 vaccine. (Ortiz-Prado et al., 2014)

Regarding other nasopharyngeal pathogens assessed in our study, co-colonization showed an age related pattern, as higher rates of S.pneumoniae-H.influenzae co-colonization were identified in older children (>60 months), while higher rates of S.pneumoniae-M.catarrhalis co-colonization were the most prevalent in younger ones (<60 months). Despite no specific pneumococcal serotypes presented this patterns of co-colonization, serotypes 6A/D/C, 11A/D, 15 B/C, 23A and 38 were identified in both co-colonization groups. From these, 15 B serotype was reported responsible of invasive disease in our country by SIREVA II report in 2015, (Brandileone, 2015) which also reported 8 invasive disease cases by H. influenzae the same year in this country.

According to this report, type B H. influenzae invasive disease cases, such as pneumonia and meningitis, are still being recorded in our country despite that the reported H. influenzae vaccine coverage for the same year reached 83% for general population and 85% for indigenous population. (Secretaría Nacional de Planificación y Desarrollo, 2017)

In the present study, only Non Typeable serotypes of *H. influenzae* were identified among colonized children and their caregivers, but given the facts that: invasive disease by type B *H. influenzae* has been previously reported in the country and pneumococcal vaccine PCV10 has been previously associated with indirect immunological protection against *H. influenzae* invasive disease(Sáez-Llorens et al., 2017), investigation regarding *H. influenzae* colonization and serotype identification

among a more diverse group of population around the country becomes a necessity in order to contrast the identified results.

Systematic reviews and meta-analysis performed in Latin American countries in order to assess the impact of PCV10 and 13 on the most relevant clinical syndromes of pneumococcal disease have demonstrated pneumococcal vaccine effectiveness with a decrease in pneumococcal disease hospitalization, especially in X-ray confirmed pneumonia. (Alicino et al., 2017; De Oliveira et al., 2016) Due to several variations within the studies assessed in this reviews, particularly at the population level, the impact or superiority of either PCV10 or PCV13 effectiveness can´t be determined. Factors such as baseline trends in pneumonia, pneumococcal serotype distribution and the prevalence of nasopharyngeal carriage of vaccine-type serotypes, the prevalence of factors that may affect immunogenicity (such as HIV or malnutrition), vaccine coverage, implementation of catch-up campaigns, and organizational aspects such as cold chain capacity determine heterogeneity in the results obtained. (Alicino et al., 2017; De Oliveira et al., 2016)

CONCLUSIONS

- This is the first study that identifies pneumococcal colonization in indigenous ethnical groups in Ecuador. This country belongs to the sentinel surveillance group SIREVA II, which reports pneumococcal disease, prevalent serotypes and antibiotic sensibility yearly. This has led to the development of pneumococcal disease prevalence studies in which hospitalized children with etiological agents responsible for pneumonia have been studied. There are no reference or background studies in the country that could be used as a base line for

comparison; therefore, the results presented in this study reflect a pilot sampling of a vulnerable group of interest.

- This study demonstrated that the use of wooden stoves is directly associated with pneumococcal colonization, while the colonization risk is associated with age and sex. A relevant result is that, unlike other studies, colonization prevalence rates were higher in children between 5 to 12 years old. Since colonization rates reached 35% in the first two years of adolescence, it becomes clear that new studies approaching adolescent population are essential to understand the role they play in pneumococcal behavior transmission and niche in our country.
- It is important to point that since the introduction of PCV10 in Ecuador, serotype replacement has taken place with a gradual reduction of vaccine types through the years and an increase of serotypes 19A and 6A. This could be prevented by the use of PCV13, which is not actually included in the national health vaccination protocol.
- In addition, co-colonizing patterns with other pathogen agents were also identified as part of the study. The most prevalent ones were *S. pneumoniae+ M. catarrhalis* in children under 60 months old and *S. pneumoniae+ H. influenzae* in children between 5 to 12 years old.
- Despite the sample size of this study, vaccine type serotypes were identified colonizing indigenous children, making it necessary to extend the assessment of pneumococcal colonization nationwide to general population. A larger sample may provide enough statistical power in order to determine the association of

other risk factors that have been previously identified in other studies around the world and that had similar tendencies in our study.

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