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Pneumocystis primary infection in non-immunosuppressed infants in Lima, Peru.

Short title. *Pneumocystis* primary infection in Peru

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Key words: *Pneumocystis jirovecii;* pulmonary colonization, primary infection; infants, Peru,

Objectives. To provide original data on *Pneumocystis* primary infection in non-immunosuppressed infants from Peru.

Methods. A cross sectional study was performed. Infants less than seven months old, without any underlying medical conditions attending the "well baby" outpatient clinic at one hospital in Lima, Peru were prospectively enrolled during a 15-month period from November 2016 to February 2018. All had a nasopharyngeal aspirate (NPA) for detection of *P. jirovecii* DNA using a PCR assay, regardless of respiratory symptoms. *P. jirovecii* DNA detection was considered to represent pulmonary colonization contemporaneous with *Pneumocystis* primary infection. Associations between infants' clinical and demographic characteristics and results of *P. jirovecii* DNA detection were analyzed.

Results. *P. jirovecii* DNA was detected in 45 of 146 infants (30.8%) and detection was not associated with concurrent respiratory symptoms in 40 of 45 infants. Infants with *P. jirovecii* had a lower mean age when compared to infants not colonized ($p < 0.05$). The highest frequency of *P. jirovecii* was observed in 2-3-month-old infants ($p < 0.01$) and in the cooler winter and spring seasons ($p < 0.01$). Multivariable analysis showed that infants living in a home with ≤ 1 bedroom were more likely to be colonized; Odds Ratio =3.03 (95%CI 1.31-7.00; $p = 0.01$).

Conclusion. *Pneumocystis* primary infection in this single site in Lima, Peru, was most frequently observed in 2-3-month-old infants, in winter and spring seasons, and with higher detection rates being associated with household conditions favoring close inter-individual contacts and potential transmission of *P. jirovecii*.

Introduction

The primary infection with *Pneumocystis jirovecii* appears to be highly common during the first year of life in non-immunosuppressed infants. Indeed, in the early 1970s sero-epidemiological surveys suggested that first contacts with the fungus contemporaneously occurred with primary infection in 90% of humans during infancy [1, 2]. Initially, primary infection occurring in this context, was thought to be essentially asymptomatic [1, 2]. In the early 2000s, a study conducted in Chile showed that primary infection in non-immunosuppressed infants can be asymptomatic but also symptomatic [3]. *P. jirovecii* was detectable using a PCR assay in nasopharyngeal aspirate (NPA) samples from symptomatic infants developing primary infection with a frequency of 32% [3]. These low burdens of *P. jirovecii* detected using PCR were considered to reflect pulmonary colonization rather than overt *Pneumocystis* Pneumonia (PCP) since the infants recovered in the absence of specific anti-*Pneumocystis* treatment. Another study conducted in France showed that primary infection in non-immunosuppressed infants can be symptomatic and may or may not be associated with viral or bacterial infections, *P. jirovecii* being also detectable in NPAs using a PCR assay [4]. More recently, in Denmark, it was reported that primary infection in non-immunosuppressed infants frequently occurred in the context of a self-limiting upper respiratory tract infection (URTI) [5]. Data on primary *Pneumocystis* infection in non-immunosuppressed infants living in emerging countries from South America are scarce. In this context, the general objective of the present study was to provide original data on primary *Pneumocystis* infection in Peru, a upper-middle-income country of South America characterized by a Gross Domestic Product per capita of 6,710 US $ in 2017. The specific objectives were to detect *P. jirovecii* in a cohort of *a priori* healthy infants, i.e. non-immunosuppressed infants at risk for primary infection considering their age, and to correlate *P. jirovecii* presence with putative specific clinical and demographic characteristics.

Methods

Hospital settings and infant population.

The free National Health System hospital network in Lima, Peru is divided into areas with outpatient clinics that serve populations living in these geographical locations. The outpatient clinics at Cayetano Heredia Hospital provide care to an infant population that lives in a densely populated neighborhood.

Criteria for enrollment of infants

Infants less than seven months of age, attending outpatient clinics for well-baby checks were prospectively enrolled in this cross-sectional study during a 15 month-period from November 2016 to February 2018. They had an NPA for *P. jirovecii* detection regardless of respiratory symptoms. Infants were placed into one of six groups based on age: one month, two months, three months, four months, five months, and six months. Infants' clinical and demographic data were collected using a case report form. Demographic data consisted of age, sex, type of birth and birth weight. At enrollment, information about respiratory symptoms that had occurred during the previous two weeks, including the day of enrollment, were collected. Parents were subsequently contacted by telephone 15 days later to ask about occurrence of any respiratory symptoms following the clinic visit.

Infants with significant chronic disease (e.g. HIV infection, primary immunodeficiency, congenital heart disease, Down syndrome), previous hospitalization (except that related to the birth period and who were discharged $\leq$3 days after birth), mothers <18 years of age, or infants whose parents could not be contacted for the 15 days follow up, were not enrolled.

Sampling and *P. jirovecii* detection.

One NPA specimen was collected per infant. Specimens were transported to the clinical mycology laboratory at Universidad Peruana Cayetano Heredia in Lima in a cooler with ice packs. DNA was extracted using a commercial kit following manufacturer's instructions (QIAamp® DNA Mini Kit, Qiagen). *P. jirovecii* DNA detection was performed using a nested-PCR assay that amplifies the mitochondrial large subunit rRNA gene. The primers and methods were described elsewhere [3, 6, 7]. Each specimen was also examined with a PCR assay targeting the β-globin gene to estimate cellular quality of specimens and the presence of potential inhibitors [8]. To avoid contamination, each step of the PCR assay was performed in different areas of the laboratory with different sets of micropipettes. To monitor for possible contamination, negative controls were included in each experiment and PCR round. A positive control (*P. jirovecii* DNA positive) was added in each PCR experiment. Each positive NPA specimen was controlled in a second experiment.

Possible influence of climatic conditions.

Weather data for Lima, Peru between November 2016 and February 2018 were obtained from www.worldweatheronline.com

Data analyses.

Data analyses were performed with Stata version 15 (StataCorp, College Station, USA). A descriptive analysis was carried out where frequency measurements of variables were estimated according to the main dependent variable, i.e. *P. jirovecii* DNA detected, or not, using the χ^2 test or Fisher exact test when appropriate. Student T test was used to compare quantitative variables.

Association between variables and *P. jirovecii* DNA detection results was estimated by Odds Ratios (OR) with 95% confidence intervals (95% CI), in bivariable and multivariable logistic regression models. A two-sided p value <0.05 was considered significant.

The study was approved by the Institutional Ethics Committees of Universidad Peruana Cayetano Heredia and Hospital Cayetano Heredia (May 31 2016, registration code: 66639). One or both parents signed the Informed Consent to allow their infant to participate.

Results

A hundred and forty-six infants were enrolled. Demographic and clinical data of infants examined are listed in Table 1. Infants' mean (SD) age was 3.7 months (± 1.7 months), mean (SD) birth weight was 3.3 (± 0.4 Kg), and 60.3% were exclusively breast-fed. *P. jirovecii* DNA was detected in 45 out of 146 infants, i.e. an overall prevalence of 30.8%. These 45 infants were considered to be colonized by *P. jirovecii* contemporaneous with *Pneumocystis* primary infection because they were mostly asymptomatic and they improved despite the absence of specific treatment against *P. jirovecii*. The characteristics of infants colonized by *P. jirovecii* or not, did not differ apart from those related to infants co-sleeping with their parents [42/45 (93%) vs. 70/101 (69%), p <0.01], the number of bedrooms at home ≤ 1 [32/45 (71%) vs. 50/101 (49.5 %), p <0.05], and the lower mean age of the infants [3.1 months vs. 4 months, p <0.05]. Infants colonized by *P. jirovecii* had a lower rate of URTI, mainly a common cold, within the 15 days prior to and including the day of enrollment, compared to infants who were not colonized [5/45 (11.1%) vs. 27/101 (26.7%); p <0.05] however this difference was not observed at 15 days post clinic visit (Table 2). No infant had clinical signs compatible with lower respiratory tract infection (LRTI), such as wheezing or overt pneumonia (Table 2).

A significant difference in the proportion of colonized infants was observed by age group (p <0.01). The highest frequency of colonization was observed in 2-month-old and 3-month old infants 61% and 47%, respectively (Figure 1). A significant difference in the proportion of colonized infants by season of the year was also observed. Higher rates of colonization were

observed in winter and spring, 50% and 36%, respectively (when average daily temperature was 21.3°C and 21°C, respectively) whereas lower rates of colonization were observed in summer and fall, 29 % and 8 %, respectively (when average daily average temperature was 25.3°C and 26.3°C, respectively); p <0.01. (Figure 2).

Using bivariable analysis, the ORs of colonization in infants living in a home with ≤ 1 bedroom or co-sleeping with parents were 2.51 [(95% CI 1.18-5.33, p =0.02)] and 3.4 [95% CI 0.94-12.30, p =0.06], respectively (Table 3). Using multivariable analysis, the only remaining significant factor associated with *P. jirovecii* colonization was having ≤1 bedroom at home; OR =3.03 [(95% CI 1.31-7.00, p =0.01)] (Table 3).

Discussion

This study provides original data on *Pneumocystis* primary infection among apparently healthy infants in Lima, Peru. *P. jirovecii* presence and its detection in infants less than 7 months-old was considered to be related to first contacts with the fungus and consequently, to be contemporary with primary infection. This definition of primary infection could be discussed because putative *P. jirovecii* reinfection could not be strictly ruled out in some cases. Nonetheless, it is noteworthy that *P. jirovecii* was mainly detected in young infants [2-month-old (61%) and 3-month-old (47%)], and less frequently in older infants. *P. jirovecii* appears highly prevalent in infants mostly asymptomatic, its overall rate being 30.8%. This rate is similar to that previously observed in Chile (32%) [3] but differs significantly to those previously observed in France (from 18.2 % to 25.3%) [4, 9] and Denmark (16%) [5]. There is a higher prevalence in South America than in Europe (P < 0.001, $\chi 2$ test) [9]. Several hypotheses could be considered to explain these results, including differences in study design (prospective vs. retrospective), in infant populations (asymptomatic vs. symptomatic), in socioeconomical characteristics (middle income countries vs. high income countries), in methods of *P. jirovecii* detection (nested-PCR assay vs. real-time PCR assay), and in

geographic and climatic characteristics (tropical/hot temperate climate vs. cool/temperate climate).

The highest rate of *P. jirovecii* detection in infants in Peru was at 3 months (IQR, 2-4 months) whereas it was 5 months (IQR, 4-6 months) and 3 months 24 days (IQR, 75-113 days) in France [4, 9], 5 months (IQR, 3.7–9.3 months) in Chile [3] and 3 months (IQR, 73–112 days) in Denmark [5]. Comparing the results of these different studies must be done cautiously, nonetheless the values of median and IQR ages of positive infants are similar. These common characteristics suggest that first contacts with *P. jirovecii*, and consequently primary infection in infants, is a common process occurring at the same time in life, regardless the geographical location.

This study was not designed to establish correlations between *P. jirovecii* detection and putative specific symptoms. Indeed, it was conducted in an outpatient clinic for well-baby checks and consequently most of enrolled infants were asymptomatic (114 out of 146 infants: 78%). *P. jirovecii* was detected in 45 infants of whom 40 did not have symptoms. Moreover, it is noteworthy that at enrolment, infants with URTI were less likely colonized than asymptomatic infants (table 2). The survey performed 15 days after the date of infants' enrollment confirmed absence of symptoms after *P. jirovecii* detection. These original results on primary infection represent an important advance in knowledge of *Pneumocystis* epidemiology. Indeed, in the study of Vargas and colleagues [3] it was suggested that primary infection could be asymptomatic but this observation was based on seroconversion against *Pneumocystis* antigens whereas only NPAs from symptomatic infants were examined for *P. jirovecii* detection. In Larsen and colleagues' study and Nevez and colleagues' study, no asymptomatic infants were enrolled and *P. jirovecii* detection was associated with URTI and LRTI respectively [5, 9]. In the early nineteen eighties, the association of *P. jirovecii* and pneumonitis in immunocompetent infants was suggested by Stagno and colleagues [10].

However, they used *Pneumocystis* antigen detection in serum whereas *P. jirovecii* detection was positive in only one pulmonary sample [10].

Recently, it was shown that *P. jirovecii* was frequent in preterm infants in Europe [11]. Preterm infants with *P. jirovecii* had a 2.7-fold higher probability of developing respiratory distress syndrome than infants without *P. jirovecii* [11]. Nonetheless, premature infants cannot be considered as healthy and strictly immunocompetent. This population differs from that we examined, likewise do the infant populations in who an unexplained association between *P. jirovecii* and sudden infant death syndrome (SIDS) that occurs at the first months of life worldwide, has been observed. Indeed, *P. jirovecii* was detected in infants with SIDS in Santiago, Chile, Oxford, UK [12], Rochester, New York, New Haven, Connecticut [13], and San Diego, CA, USA [14]. Although the role of *P. jirovecii* as the causative infectious agent of SIDS could not be established, it was noteworthy that its presence in the lungs was associated with increased mucus and activity of chloride channel accessory1 (hCLCA1), the mucus precursor proteins [8, 15]. Whether these observations in deceased infants with putative primary infection may be applicable to primary infection in alive infants remains an open question.

The fact that *P. jirovecii* may have a pathogenic effect in infants with asymptomatic primary infection is prompted by recent data showing that subclinical *Pneumocystis* primary infection in rats was associated with a progression of Th2 cell–type inflammation and airway remodeling [16]. To sum up, primary infection in non-immunosuppressed or apparently healthy infants can be symptomatic contemporaneous with self-limited URTI or LRTI, or asymptomatic but in this later case *P. jirovecii* could not be entirely nonpathogenic. For obvious ethical reasons, this hypothesis could not be tested in our infant population which could not undergo invasive lung tissue sampling.

The sources from which infants acquired *P. jirovecii* are still unclear. Although an environmental reservoir cannot be strictly ruled out considering the report of *P. jirovecii* DNA detection in the air from the countryside [7], no environmental niche has clearly been identified. By contrast, there are several populations of patients who may contribute to the human reservoir of *P. jirovecii*. Interindividual transmission of *Pneumocystis* sp. has clearly been established in rodent models [17, 18] and is highly probable in humans (review in [19]). It is known that pregnancy which is associated with physiological changes in the immune system [20] may predispose to pulmonary colonization with *P. jirovecii* [21]. The mothers may have transmitted the fungus by airborne route to their newborns or infants, this mode of transmission being suggested in humans [22] and demonstrated for *P. oryctolagi* in rabbits [23]. Likewise, the transplacental transmission of *P. oryctolagi* in rabbits has been demonstrated [23] and suspected for *P. jirovecii* in humans. Montes-Cano and colleagues detected *P. jirovecii* DNA in 11 lungs of miscarriage fetuses and 8 placenta samples [24]. Szydlowicz and colleagues recently discussed transplacental transmission, specifically in newborns [25]. It the present study, no newborns, i.e. infants younger than one month, were enrolled. However, the mothers may not necessarily be the source of *P. jirovecii*. Indeed, in Spencer and colleagues' study, 5 out of 41 women and 5 out of 60 infants were colonized by *P. jirovecii* but none of the colonized women or children were members of the same family [26]. In another recent investigation of *P. jirovecii* detection and genotyping in sample pairs from mothers and newborns, the newborns were more frequently colonized than their mothers (74.4% vs. 46.5%, $p < 0.01$) and no perfect matches of *P. jirovecii* genotypes in mothers/newborns sample pairs were observed [27]. Moreover, in a previous study, we detected *P. jirovecii* in only 5 out of 92 pregnant women near term (5.43%) while it was not detected in their newborns nor in their placentas [28]. Thus, other human sources of *P. jirovecii* including acquisition in households may be possible. For example, putative *P.*

jirovecii transmission between a grand-father and his grand-son has been described by Rivero and colleagues [29]. The higher rate of *P. jirovecii* that we observed in infants living in a dwelling with only one or no bedroom suggests that *P. jirovecii* may have been acquired under overcrowded conditions within the family. Unfortunately, in this study, we could not investigate *P. jirovecii* acquisition by infants from their family members.

A significant difference in the proportion of infants colonized or not colonized by *P. jirovecii* by season was observed ($\chi 2$ test $p < 0.01$). Higher rates of *P. jirovecii* in infants were observed in winter and spring whereas the lower rates were observed in summer and fall. These results suggesting seasonal variation could be related to a bias. The highest frequency of *P. jirovecii* detection was observed in 2-month-old and 3-month-old infants and these two infant groups may have been over represented. The numbers of infants in the six groups of age (from one to six months) and in each month over the 15 months of the study were not strictly identical on the one hand and insufficient for a highly powerful analysis on the other hand.

Be that as it may, the higher rate of *P. jirovecii* detection in infants was effectively observed in the cooler months of the spring and the winter in Lima when the average temperature was around 21°C. This may be consistent with reports described elsewhere showing an association of PCP occurrence in immunosuppressed patients with average temperatures from 10°C to 20°C rather than with the seasons themselves [30]. In Chile, the highest rate of *P. jirovecii* detection in autopsied infants was in winter, when the average temperatures were 16°C-18°C whereas the lowest rate was in the fall when the average temperatures were 20°C-28°C [31]. However, the seasons and/or temperatures may not represent strictly independent factors.

Finally, we provide original data on *Pneumocystis* primary infection in Lima, Peru. We demonstrated using microbiological data, i.e. *P. jirovecii* detection in NPAs, that *Pneumocystis* primary infection could be asymptomatic, as it was previously established on

serological data [3]. The higher detection rate was associated with household conditions favoring close inter-individual contacts and therefore potential transmission of *P. jirovecii*. Moreover, *Pneumocystis* primary infection was most frequently observed in infants 2-3 months of age and during the cooler months of the winter and spring. Our overall results combined with the analysis of the literature show that *Pneumocystis* primary infection occurs in the first months of life worldwide in both high income and middle-income countries.

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Table 1. Demographic and clinical characteristics of infants with or without *Pneumocystis* primary infection, Lima, Peru.

	All infants (n = 146)	Colonized infants (n = 45)	Non-colonized infants (n = 101)	p value
Gender, female	76 (52.1%)	23 (51.1%)	53 (52.5%)	p = 0.88 [a]
Mean age, months (SD)	3.7 (± 1.7)	3.1 (± 1.6)	4 (± 1.7)	p < 0.05 [b]
Type of birth, C-section*	78 (53.4%)	26 (57.8%)	52 (51.5%)	p = 0.48 [a]
Birth weight (Kg)				
2.5 - <3.0	30 (20.6%)	10 (22.2%)	20 (19.8%)	p = 0.33 [c]
3.0 - <3.5	72 (49.3%)	26 (57.8%)	46 (45.5%)	
3.5 - <4.0	40 (27.4%)	8 (17.8%)	32 (31.7%)	
4.0 - 4.5	4 (2.7%)	1 (2.2%)	3 (3.0%)	

Exclusively breastfed	88 (60.3%)	30 (66.7%)	58 (57.4%)	p = 0.29 [a]
Parents smoking tobacco at home	10 (6.9%)	1 (2.2%)	9 (8.9%)	p = 0.17 [c]
Siblings at home	70 (48.0%)	18 (40.0%)	52 (51.5%)	p = 0.20 [a]
Co-sleeping with parents	112 (76.7%)	42 (93.3%)	70 (69.3%)	p < 0.01 [a]
Number of bedrooms ≤ 1	82 (56.2%)	32 (71.1%)	50 (49.5%)	p = 0.015 [a]
Waste disposal, indoor public network	120 (82.2%)	40 (88.9%)	80 (79.2%)	p = 0.15 [a]
Antibiotics since birth [d]	12 (8.2%)	2 (4.4%)	10 (9.9%)	p = 0. 34 [c]
Antibiotics given in the last 15 days [d]	3 (2.1%)	0 (0%)	3 (3.0%)	p = 0.55 [c]
Symptoms in the last 15 days [e]				
Rhinorrhea	21(14.4%)	4 (8.9%)	17 (16.8%)	p = 0.20 [a]
Cough	10 (6.8%)	2 (4.4%)	8 (7.9%)	p = 0.72 [c]
Fever	6 (4.1%)	1 (2.2%)	5 (5.0%)	p = 0.66 [c]
Wheezing	3 (2.1%)	1 (2.2%)	2 (2.0%)	p = 1 [c]
Diarrhea	2 (1.4%)	0 (0%)	2 (2.0%)	p = 1 [c]
Subcostal retractions	1 (0.7%)	0 (0%)	1 (1.0%)	p = 1 [c]
Vomiting	1 (0.7%)	0 (0%)	1 (1.0%)	p = 1 [c]
Sibling and/or parents with symptoms	4 (2.7%)	0 (0%)	4 (4.0%)	p = 0. 31 [c]

Key: *C-section = Caesarean section; [a] $\chi 2$ test, [b] Student T test, [c] Fisher exact test
[d] mostly beta-lactams or aminoglycosides; [e] including the day of enrolment

Table 2. Symptoms and putative respiratory diseases in infants attending "well baby" clinics with or without *Pneumocystis* primary infection, Lima, Peru.

	At enrollment		On day15 of follow-up	
	Colonized infants (n = 45)	Non-colonized infants (n=101)	Colonized infants (n=45)	Non-colonized infants (n=101)
Asymptomatic	40 (88.9%) [a]	74 (73.3%) [a]	37 (82.2%)	77 (76.2%)
Putative URTI	5 (11.1%)[a]	27 (26.7%) [a]	8 (17.7%)	21 (20.8%)
Common cold	5 (11.1%)	26 (25.7%)	4 (8.9%)	7 (6.9%)
Pharyngitis	0	1 (1.0%)	4 (8.9%)	14 (13.9%)
Acute otitis media	0	0	0	0
Laryngotracheitis	0	0	0	0
Putative LRTI	0	0	0	3 (3.0%)
Bronchitis	0	0	0	2 (2.0%)
Bronchiolitis	0	0	0	1 (1.0%)
Asthma/bronchospasm	0	0	0	0
Pneumonia	0	0	0	0

Key : [a] $p<0.05$ (0.03) (χ^2 test) ; URTI =Upper respiratory tract infection ; LRTI =Lower respiratory tract infection

Table 3. Bivariable and multivariable analyses of factors associated with diagnoses of *Pneumocystis* primary infection in infants, Lima, Peru.

Variables	Bivariable analysis				Multivariable analysis			
	OR	95% CI		p value	OR	95% CI		p value
Gender								
Female	1				1			
Male	1.06	0.52	2.13	0.88	1.30	0.58	2.91	0.52
Type of birth								
Vaginal	1				1			
C-section	1.29	0.63	2.62	0.48	1.67	0.72	3.86	0.23
Birthweight (Kg)								
2.5 - <3	1				1			
3.0 - < 3.5	1.13	0.46	2.78	0.79	1.07	0.40	2.87	0.89
3.5 - < 4	0.50	0.17	1.48	0.21	0.47	0.14	1.55	0.21
4.0 - 4.5	0.67	0.06	7.25	0.74	1.25	0.07	22.26	0.88
Parents smoking tobacco at home								
No	1				1			
Yes	0.23	0.03	1.89	0.17	0.19	0.02	2.34	0.19
Siblings at home								
No	1				1			
Yes	0.63	0.31	1.28	0.20	1.02	0.45	2.32	0.96
Antibiotics used since birth								
No	1				1			
Yes	0.42	0.09	1.99	0.27	0.50	0.07	3.82	0.51
Wheezing								
No	1				1			
Yes	1.13	0.10	12.74	0.92	3.34	0.09	122.01	0.51
Cough								
No	1				1			
Yes	0.54	0.11	2.65	0.45	0.58	0.06	5.52	0.63
Fever								
No	1				1			
Yes	0.44	0.05	3.85	0.46	1.50	0.03	65.45	0.83
Rhinorrhea								
No	1				1			
Yes	0.48	0.15	1.52	0.21	0.70	0.16	3.13	0.64
Number of bedrooms								
≤1	2.51	1.18	5.33	0.02[a]	3.03	1.31	7.00	0.01[a]
>1	1				1			
Co-sleeping with parents								
No	1				1			
Yes	3.40	0.94	12.30	0.06	1.77	0.39	8.07	0.46

Key: C-section = Caesarean section; [a] Using bivariable and multivariable analysis, the number of bedrooms at home ≤ 1 remained the only significant factor associated with *Pneumocystis* primary infection.

Figure 1. Proportion of infants with or without *Pneumocystis* primary infection by age groups.

Foot notes. There was a significant difference in the proportion of infants with or without *Pneumocystis* primary infection by age groups (χ^2 test $p < 0.01$).

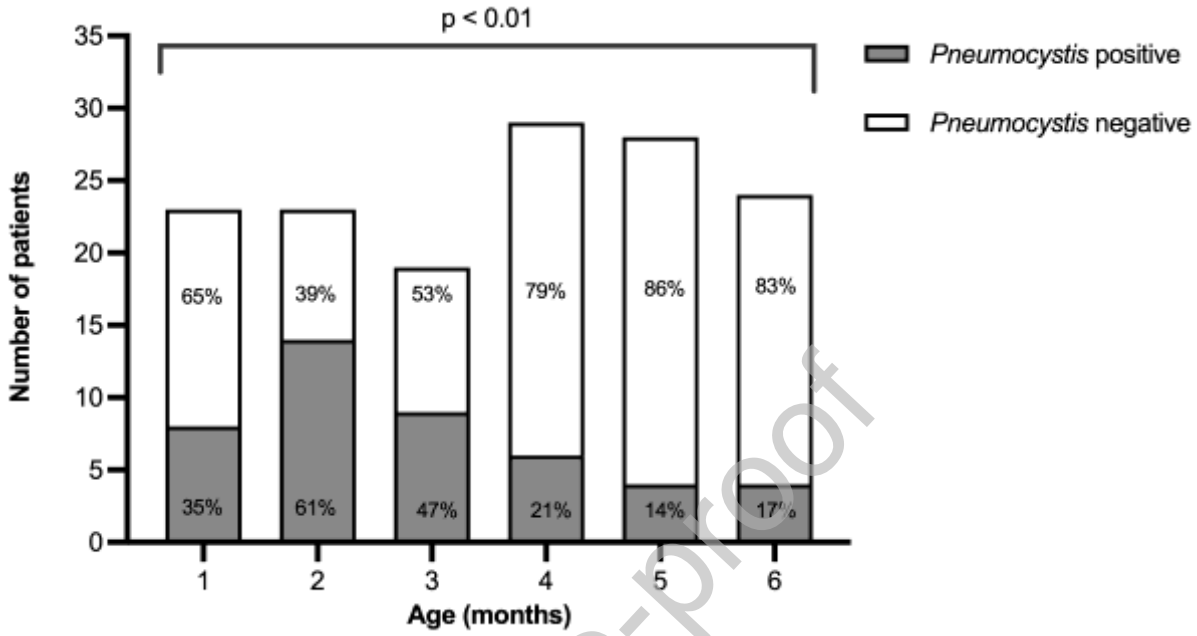

Figure 2. Proportion of infants with or without *Pneumocystis* primary infection by season of the year.

Foot notes. A significant difference in the proportion of infants with or without primary infection by season was observed (χ^2 test $p < 0.01$).