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MAJOR ARTICLE

Early Acquisition of *Pneumocystis jirovecii* Colonization and Potential Association With Respiratory Distress Syndrome in Preterm Newborn Infants

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Background. *Pneumocystis* pneumonia is a well-recognized lung disease of premature and malnourished babies. Even though serologic studies have shown that children are exposed to *Pneumocystis jirovecii* early in life, the epidemiology of human *P. jirovecii* infection and the host–microorganism relationship in infancy remain poorly understood. The aim of the present study was to investigate the prevalence of *P. jirovecii* colonization in preterm infants and its possible association with medical complications.

Methods. A prospective observational study of preterm infants (birth weight <1500 g and/or gestational age <32 weeks) was carried out. Identification of *P. jirovecii* colonization was performed by means of molecular techniques in nasal aspirated samples at birth.

Results. A total of 128 preterm infants were included during the study period. *Pneumocystis* DNA was identified in 25.7% (95% confidence interval [CI], 17.8%–33.7%) of newborns studied. A significant increase of respiratory distress syndrome in colonized group, even after adjusting for confounding factors (odds ratio, 2.7 [95% CI, 1.0–7.5]; $P = .04$), was observed. No differences were observed in other medical conditions between the 2 groups.

Conclusions. *Pneumocystis jirovecii* colonization is frequent in preterm births and could be a risk factor to develop respiratory distress syndrome among preterm infants.

Keywords. pneumocystis; preterm infants; respiratory distress syndrome.

Pneumocystis jirovecii is an atypical opportunistic fungus with lung tropism and strong host species specificity, which causes pneumonia in immunosuppressed individuals worldwide [1]. Interstitial plasma cell pneumonia was the first human disease associated with *Pneumocystis* infection. This disease appeared endemically and in institutional epidemics in Central Europe during the period surrounding World War II, principally involving preterm or malnourished infants [2]. Since then, *Pneumocystis* pneumonia (PCP) has only been reported infrequently in patients with malignancies and solid organ transplants until the human immunodeficiency virus (HIV) pandemic turned PCP into a major medical and public health problem at the end of the 20th century [2].The AIDS epidemic stimulated research on PCP that can be considered just the tip of the iceberg of human *Pneumocystis* infection [3]. In fact, serological studies and use of molecular techniques to identified *P. jirovecii* colonization have shown that this microorganism probably is one of the more frequent infectious agents faced by humans in everyday life and that the first exposure to this pathogen happens in most children early in life [1, 4].

Newborns who have low birth weight, either because of early delivery or because of fetal growth restriction, are at increased risk for short- and long-term disabilities and death [5]. Preterm infants are at higher risk for infectious diseases such as invasive pneumococcal disease, with the highest risk in early infancy [6]. Fungal infections, other than those caused by *Candida* species, rarely are considered in the differential diagnosis for an acutely ill newborn infant because disorders of bacterial and viral etiology are vastly more common. Nevertheless, fungal infections do occur in neonates, especially in premature infants and those of very low birth weight (<1500 g), and can cause serious and frequently fatal disease [7]. As with any other infectious disease, the risk of fungal infection depends on the host and risk of exposure.

Recently, molecular evidence of *P. jirovecii* transplacental transmission in humans has been provided [8]. *Pneumocystis* DNA was identified in human placentas, and most importantly, in 35% of the lungs of fetuses dying in utero [8]. These data

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emphasize the need to study the possible role of this fungal organism in premature newborns. In this way, the aim of the present study was to investigate the prevalence of *P. jirovecii* colonization in preterm infants and its possible association with medical complications.

PATIENTS AND METHODS

A prospective observational study was carried out on all newborns <32 weeks of gestation and/or weighing <1500 g admitted to the Neonatal Intensive Care Unit of Virgen del Rocio University Hospital whose mothers did not have HIV infection or other causes of immunosuppression. In all cases, informed consent was obtained from the legal representatives of newborns before their inclusion in the study. The study protocol was approved by the hospital's ethics committee.

Every newborn underwent a clinical and biological examination using a standardized protocol, and biological samples were collected for analysis. Nasopharyngeal aspirate samples were obtained in all newborns within 10–15 minutes after birth, just prior to placement of a nasogastric tube. In brief, under sterile conditions, sterile saline was instilled inserting a tube attached to a syringe through the nose and directed toward the nasopharynx. After 1–2 seconds, the nasopharyngeal fluid was withdrawn using an aspiration system with a commercially available suction catheter connected to sterile recipient where secretions were collected. Samples were kept frozen at –20°C until DNA was extracted.

Identification of *P. jirovecii* colonization was done analyzing nasopharyngeal aspirate samples by means of a 2-step protocol for nested polymerase chain reaction (PCR) assay that amplifies a portion of the gene encoding the mitochondrial large-subunit ribosomal RNA [9]. Concisely, DNA from *P. jirovecii* was extracted using a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) with proteinase K digestion at 56°C, following the manufacturer's instructions. In the first round of amplification, the external primers pAZ102-E and pAZ102-H were used. This yielded a 346-base pair (bp) fragment. The second round of amplification utilized the internal primers pAZ102-X and pAZ102-Y and yielded a 260-bp product. Both rounds of PCR comprised 35 amplification cycles. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide and the bands were visualized by ultraviolet light. To prevent contamination, pipettes with filters were used in all manipulations. DNA extraction and preparation of the reaction mixture were performed in 2 different rooms using separate laminar-flow hoods. The PCR procedure and analysis of PCR products were performed in another room. Controls were run simultaneously with nasopharyngeal samples. Positive controls were bronchoalveolar lavage specimens from patients with PCP patients; negative controls were autoclaved water in the PCR mixture in the absence of the DNA template controls. All experiments were performed at least twice.

Additionally, all positive samples were retested by a real-time PCR assay, using a LightCycler 1.5 (Roche Molecular Biochemicals, Mannheim, Germany) according to a method previously described [10]. In brief, samples were simultaneously measured in duplicate PCR reactions performed in a final volume of 20 μL. The PCR mixture contained primers pAZ102-X and pAZ102-Y, with 20 pmol of each primer and 10–40 ng DNA, and was amplified using LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals) to a final 2.5 mM of magnesium ion. After an initial denaturation step at 95°C for 10 minutes, amplification was performed by 40 cycles of denaturation (95°C for 5 seconds), annealing (55°C for 15 seconds), and extension (72°C for 10 seconds). The amplification product was detected using SYBR Green I dye, which strongly increases its fluorescence after binding to double-stranded DNA. As *Pneumocystis* positive control, a 256-bp amplification product cloned into the pGEM T-EASY vector (Promega, Madison, Wisconsin) was run simultaneously with nasopharyngeal aspirate samples, and negative controls were autoclaved with water in the PCR mixture in the absence of the DNA template controls.

A newborn was considered colonized by *P. jirovecii* when his or her nasopharyngeal aspirate contained detectable *P. jirovecii* DNA by both nested PCR and reverse-transcription PCR in 2 independent analyses.

Infants were diagnosed with respiratory distress syndrome (RDS) when they had clinical signs of respiratory distress with requirements for supplemental oxygen and radiographic signs suggestive of RDS (diffuse, fine granular opacification in both lung fields). Bronchopulmonary dysplasia was diagnosed in accordance with the Shennan definition (need for supplemental oxygen at 36 weeks postmenstrual age) [11].

Statistical Analyses

Categorical variables were presented as number of cases with frequencies, and compared using the χ^2 or Fisher exact test. Continuous variables were presented as medians with ranges, and compared using the Mann-Whitney *U* test.

Univariate logistic regression was used to explore the effect of various factors on the development of RDS, while the combined effect of selected perinatal factors (predictors with clinical significance) and *Pneumocystis* colonization was assessed by multivariable logistic regression analysis. Statistics were performed using IBM SPSS software version 20.0 (IBM Corp, Armonk, New York). A *P* value of <.05 was considered significant in all instances.

RESULTS

A total of 128 preterm infants were included during the study period. *Pneumocystis* DNA was identified from nasopharyngeal aspirates in 33 of the 128 infants. Thus, *P. jirovecii* carriage was

detected in 25.7% of the patients. Table 1 shows gestational age and weight at birth of the infants and Table 2 summarizes the clinical characteristics of colonized and no colonized patients. No significant differences were observed in mean gestational age or birth weight between the groups. No difference was observed in maternal obstetrical history including use of prenatal corticosteroids.

The study observed a significant increase of RDS in the colonized group (72% vs 52%; $P = .04$). It was an increase in retinopathy of prematurity and bronchopulmonary dysplasia at a postmenstrual age of 36 weeks in the *P. jirovecii*–colonized group but without statistical significance. No differences were observed in other medical complications between the 2 groups (Table 3). During the study period, 17 infants died (8 cases due to respiratory failure; 5 cases due to shock; 2 cases due to intracranial hemorrhage; 1 case due to necrotizing enterocolitis; and 1 due to liver failure).

Multivariable regression analysis revealed that the negative effect of *P. jirovecii* colonization was preserved when adjusting for known risk factors, such as smaller gestational age, lower weight, and administration of antenatal corticosteroids, with the carriers of *P. jirovecii* having a 2.7-fold higher probability for RDS (Table 4).

Table 1. Gestational Age in Weeks and Weight At Birth

			Pneumocystis		
Weight Rank			Negative	Positive	Total
<500 g	GA	26	1	0	1
	Total		**1**	**0**	**1**
500–749 g	GA	23	1	1	2
		25	0	4	4
		27	0	1	1
	Total		**1**	**6**	**7**
750–949 g	GA	23	1	0	1
		25	3	0	3
		26	3	3	6
		27	5	0	5
		28	2	0	2
		29	2	0	2
		31	1	1	2
	Total		**17**	**4**	**21**
950–1249 g	GA	25	1	0	1
		26	1	0	1
		27	2	0	2
		28	5	3	8
		29	1	0	1
		30	1	0	1
		31	3	1	4
		32	6	1	7
		33	4	0	4
		34	2	0	2
	Total		**26**	**5**	**31**
1250–1500 g	GA	28	4	1	5
		29	7	3	10
		30	5	2	7
		31	5	1	6
		32	4	2	6
		33	1	0	1
		34	1	0	1
	Total		**27**	**9**	**36**
>1500 g	GA	29	3	1	4
		30	5	1	6
		31	6	5	11
		32	5	1	6
		33	3	0	3
		34	1	1	2
	Total		**23**	**9**	**32**
	TOTAL		95	33	128

Abbreviation: GA, gestational age.

DISCUSSION

This prospective observational study revealed a high prevalence of *P. jirovecii* colonization in preterm infants at birth, which has been associated, for the first time, with an increase in neonatal RDS risk.

Serologic studies have shown that most children are exposed to *P. jirovecii* early in life. Among healthy, immunocompetent infants in Chile, the seroconversion rate reached 85% by 20 months of age and *Pneumocystis* DNA was found in 32% of infants studied [12]. Similarly, in another study carried out among Spanish children, the overall seroprevalence of anti-*Pneumocystis* antibody was 73% [13].

Pneumocystis primary infection has been presumed to be an asymptomatic or mild nonspecific disease; however, recent reports indicate that it can present clinically as a self-limiting upper or lower acute respiratory tract infection [1]. One study carried out in Santiago, Chile, found that 32% of infants with mild respiratory symptoms harbored *P. jirovecii* [12]. Another study of infants with bronchiolitis found that 24% were colonized by *Pneumocystis* [14]. In another study of 422 children hospitalized with acute respiratory tract infection, *P. jirovecii* was detected by using a real-time PCR assay in nasopharyngeal aspirates in 16% of infants. However, a marked difference occurred in the age distribution, as the prevalence was 48% in infants aged 50–112 days, 13% in infants aged 113–265 days, and 2% in infants aged <49 days [15]. In a study carried out in Santiago, *Pneumocystis* DNA was detected by nested PCR in specimens from 51.7% of infants who died unexpectedly in the community, but only 15% had pneumonia [16]. The significance of these findings is unclear but shows the high prevalence of *P. jirovecii* infection in infants.

Even though the first human disease associated with *Pneumocystis* infection was interstitial plasma cell pneumonia, which typically affected preterm newborns, there are no data about prevalence of *Pneumocystis* colonization in premature infants. However, a recent study carried out in Seville found the presence of *P. jirovecii* DNA in 35% of lung tissues from fetuses

Table 2. Clinical Characteristics of *Pneumocystis*-Colonized and -Noncolonized Patients

Characteristic	*P. jirovecii* Positive (n = 33)	*P. jirovecii* Negative (n = 95)	*P* Value
Mothers			
Maternal age, y, median (IQR)	33 (28–36.5)	34 (30–38)	.066
Nulliparous	18 (54.5)	54 (56.8)	.819
Maternal blood pressure >140/90 mm Hg	4 (12.1)	13 (13.7)	1
Preterm labor	20 (60.6)	59 (62.1)	.879
Antenatal steroids	24 (72.7)	81 (85.3)	.106
Antibiotics in labor	12 (36.4)	42 (44.2)	.432
Chorioamnionitis	6 (30)	25 (36.8)	.578
Cesarean delivery	24 (72.7)	69 (72.6)	.992
Rupture of membranes >18 h before labor	10 (33.3)	22 (23.1)	.414
Infants			
Gestational age at birth, wk, median (IQR)	29 (26.5–31)	30 (28–32)	.165
Birth weight, g, mean (SD)	1251 (450)	1290 (352)	.615
Male sex	15 (45.5)	51 (53.7)	.415
Singleton birth	22 (66.7)	62 (65.3)	.884
Apgar score <6	5 (15.2)	12 (12.8)	.729
Intubated at birth	16 (48.5)	36 (37.9)	.286

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: IQR, interquartile range; *P. jirovecii*, *Pneumocystis jirovecii*; SD, standard deviation.

at 28 ± 8 weeks of gestation whose death occurred in utero, demonstrating vertical transmission of *Pneumocystis* via the transplacental route in humans [8]. The similar prevalence of *P. jirovecii* colonization found in preterm newborns in our study suggests the same route of infection.

RDS is an important cause of morbidity and mortality in premature infants. The incidence and severity of RDS is inversely related to gestational age. RDS is due to a deficiency of alveolar surfactant, along with the structural immaturity of the premature lung [17].

In our study, we found a significant increase of RDS among premature infants that harbor *P. jirovecii*. Interestingly, several studies have shown the ability of *Pneumocystis* infection to induce selective downregulation of surfactant protein B and C expression in animal models, with corresponding changes in surface tension [18, 19]. In this way, *Pneumocystis* infection could contribute to development of RDS in premature newborns. Moreover, a recent study has documented that *Pneumocystis* is associated with increased mucus (HCLCa1, a calcium-sensitive, chloride conductance family of proteins; and MU5AC, a mucus marker) in infant lungs [20]. These proteins are normally induced as part of the STAT6 pathway in the airways, and are part of the airway innate response.

Table 3. Outcomes of *Pneumocystis*-Colonized and -Noncolonized Patients

Outcome	*P. jirovecii* Positive (n = 33)	*P. jirovecii* Negative (n = 95)	*P* Value
Respiratory distress syndrome	24/33 (72.7)	50/95 (52.6)	.044
Mechanical ventilation	24/33 (72.7)	55/95 (57.9)	.131
High-frequency ventilation	8/33 (24.2)	13/95 (13.7)	.158
Interstitial emphysema	4/33 (12.1)	5/95 (5.3)	.235
Supplemental oxygen at 28 days	8/30 (26.7)	20/86 (23.3)	.707
Survival with bronchopulmonary dysplasia[a]	6/30 (20)	7/85 (8.2)	.080
Low blood pressure	7/33 (21.2)	20/95 (21.1)	.985
Hyperglycemia	8/33 (24.2)	28/95 (29.5)	.565
Retinopathy of prematurity (treatment administered)	8/30 (26.7)	10/85 (11.8)	.053
Brain injury by cranial ultrasonography examination	6/33 (18.2)	11/95 (11.6)	.336
Patent ductus arteriosus	7/31 (22.6)	14/92 (15.2)	.346
Late-onset bacterial infection	11/30 (36.7)	31/92 (33.7)	.766
Death	3/33 (9.1)	13/95 (13.7)	.492
Days of mechanical ventilation, median (IQR)	24 (0–180)	12 (0–120)	.156
Days of hospitalization, median (IQR)	40 (27-81)	37 (28–60)	.593

Data are presented as no./No. (%) unless otherwise indicated.

Abbreviations: IQR, interquartile range; *P. jirovecii*, *Pneumocystis jirovecii*.

[a]Bronchopulmonary dysplasia was assessed in 115 infants who were alive at a postmenstrual age of 36 weeks.

Table 4. Regression Analysis for Known Risk Factors of Respiratory Distress Syndrome

Risk Factor	*P. jirovecii* Positive (n = 33)	*P. jirovecii* Negative (n = 95)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
RDS	24/33 (72.7)	50/95 (52.6)	2.4 (1.010–5.703)	2.757 (1.005–7.564)
Antenatal steroids	24/33 (72.7)	81/95 (85.3)	0.461 (.178–1.196)	0.422 (.153–1.163)
GA at birth <29 wk	13/33 (39.4)	29/95 (30.5)	1.479 (.649–3.371)	0.725 (.221–2.378)
Birth weight <1000 g	13/33 (39.4)	25/95 (26.3)	1.820 (.790–4.192)	1.494 (.489–4.560)

Data are presented as no./No. (%) unless otherwise indicated.

Abbreviations: CI, confidence interval; GA, gestational age; OR, odds ratio; *P. jirovecii, Pneumocystis jirovecii*; RDS, respiratory distress syndrome.

Bronchopulmonary dysplasia of prematurity (BPD), the most common chronic lung disease in infants, has been associated with multifactorial etiologies including hyperoxia, barotrauma, surfactant deficiency, nutritional deficiencies, fluid overload, patent ductus arteriosus, inflammation of the lungs, and infection [21, 22]. Acute RDS in premature newborns is often the first step to the subsequent development of BPD. In our study, we observed an increase in bronchopulmonary dysplasia at a postmenstrual age of 36 weeks in *P. jirovecii*–colonized infants but without statistical significance, probably due to the small sample size. In fact, several microorganisms have been implicated in the development of BPD such as *Chlamydia trachomatis*, cytomegalovirus, adenovirus, and *Ureaplasma urealyticum* [23–26]. Interestingly, a recent study of the airway microbiome at birth has shown that consistent temporal dysbiotic changes in the airway microbiome were seen from birth to the development of BPD in extremely preterm infants, with a decrease in the phyla Firmicutes and Fusobacteria and increase in abundance of Proteobacteria over time [27]. In this way, a metagenomic study of the lung microbiome of patients with idiopathic interstitial pneumonia has shown a clearly negative relation among the presence of *P. jirovecii* and bacteria, suggesting an antagonistic relationship [28]. In this way, *Pneumocystis* colonization could also play a role in BPD.

In conclusion, in this study a high rate of *Pneumocystis* colonization was shown in preterm infants at birth, which suggests vertical transmission of *P. jirovecii*. Furthermore, *Pneumocystis* colonization seems to be a risk factor until now unknown for acute RDS in premature infants and probably for BPD.

Our findings could be of potential clinical importance and could open a new field of research that deserves exploration. Further research should assess the role of *Pneumocystis* colonization in the pathophysiology of neonatal respiratory pathology as well as to study how this microorganism interacts with bacterial microbiota as well as with lung cells of newborns.

Notes

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