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1 ***Pneumocystis* exhalation by infants developing *Pneumocystis* primary infection: putative**  
2 **infectious sources in hospitals and the community.**

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27 Abstract

28 *Pneumocystis jirovecii* DNA was detected using a-PCR assay in air samples collected using  
29 an air-liquid impaction device at one-meter distance from three out of fourteen infants who  
30 were developed *Pneumocystis* primary infection. *P. jirovecii* genotype identification was  
31 successful in one out of three pairs of air. Matching of *P. jirovecii* genotypes between the  
32 nasopharyngeal and air samples, suggested that *P. jirovecii* was effectively exhaled by the  
33 infected infant. These original results represent a proof of concept of the role of infants with  
34 primary *Pneumocystis* infection as infectious sources of *P. jirovecii* in hospitals and in the  
35 community.

36 Introduction

37 *Pneumocystis jirovecii* is a transmissible and uncultivable fungus, specific for humans, that  
38 causes severe pneumonia [*i.e.* *Pneumocystis* pneumonia (PCP)], in immunosuppressed adults  
39 and children [1]. *Pneumocystis* primary infection linked to first contact with the fungus occurs  
40 early in life in both immunosuppressed or immunocompetent infants, mostly those younger  
41 than 12 months [2–4]. *Pneumocystis* primary infection in immunocompetent infants appears  
42 to be a worldwide phenomenon [2–4], the infection being asymptomatic or symptomatic. In  
43 this latter case, it occurs contemporary to a self-limiting respiratory tract infection [3,4] in the  
44 course of which the fungus can be detected using PCR assays in nasopharyngeal aspirates  
45 (NPAs) [2–4].

46 *Pneumocystis* species are host specific and no environmental ecological niche for any of them  
47 has been identified so far. For these reasons, it is assumed that patients infected by *P. jirovecii*  
48 are the main part of the fungus reservoir. Indeed, the aerial interindividual transmission of  
49 *Pneumocystis murina* has been demonstrated in mouse models [5] and was strongly suggested  
50 for *P. jirovecii* in humans considering the occurrence of PCP case clusters in hospitals (see  
51 review in [6]). Moreover, *P. jirovecii* DNA has been detected in the air surrounding PCP  
52 patients or patients colonized by the fungus, consistent with *P. jirovecii* exhalation and spread  
53 from patients in their environment [7-9]. These patients represent potential infectious sources  
54 from which, susceptible other individuals may acquire *P. jirovecii*.

55 Considering the very high prevalence of *Pneumocystis* primary infection in infancy [2-4],  
56 infants in these circumstances may represent the main part of the human reservoir of the  
57 fungus. *P. jirovecii* detection in the surrounding environment of infants would strengthen their  
58 potential role as infectious sources. In this context, we performed the detection and  
59 characterization of *P. jirovecii* DNA in air samples from the surrounding environment of

60 infants diagnosed with *Pneumocystis* primary infection and admitted to our institution, the  
61 Brest University Hospital, Brest, France.

## 62 Patients and Methods

63 *Pneumocystis* primary infection was defined as detection of *P. jirovecii* in NPA samples from  
64 infant younger than 12 months, an infant who was a priori immune-naïve to *P. jirovecii* [4].  
65 The detection of *P. jirovecii* was routinely performed in our diagnostic laboratory in NPAs  
66 contemporary to that of viruses within the framework of investigation of pulmonary  
67 symptoms or fever. This detection was done using a real-time polymerase chain reaction  
68 (qPCR) assay targeting the gene of mitochondrial large subunit ribosomal RNA  
69 (*mtLSUrRNA*) on 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA)  
70 as described elsewhere [4].

71 Fourteen out of 87 infants younger than 12 months (sex ratio male/female 9/5, median age 2  
72 months 24 days [limits, 1 month 0 day – 9 months 19 days]), admitted at the Brest University  
73 Hospital, Brest, France between January 23<sup>th</sup>, 2018 and January 8<sup>th</sup>, 2020, and diagnosed with  
74 *Pneumocystis* primary infection, were enrolled in the study. Characteristics of the 14 infants  
75 are described in table I. All infants were hospitalized in the same pediatric ward, in a single or  
76 a two-bed room.

77 Fourteen air samples were collected in hospital rooms at one-meter distance from infants'  
78 head. One cubic meter of air (airflow, 100 L per min. during 10 min.) was collected using the  
79 Coriolis®  $\mu$  air sampler (Bertin Technologies, Montigny-le-Bretonneux, France) which  
80 collect and concentrates aerial biological particles into Phosphate-buffered Saline added with  
81 0.002% Tween80. DNA extraction of air samples was performed using the QIAamp DNA  
82 Mini Kit (Qiagen, Courtaboeuf, France) according to the recommendations by Choukri *et al.*  
83 [7]. DNA was eluted in 100  $\mu$ L of elution buffer and stored at  $-80$  °C until amplification  
84 using the same aforementioned PCR assay [4] but with 50 amplification cycles. All air  
85 samples and negative controls were run in triplicate whereas positive controls were run in  
86 duplicate. An internal positive control (TaqMan® Exogenous Internal Positive Control

87 Reagents; Life Technologies Corporation) was used to detect PCR inhibitors. Negative  
88 controls were used to managed putative cross contamination. Moreover, UDG was used to  
89 prevent putative cross contamination. The genotyping of *P. jirovecii* isolates from the  
90 nasopharyngeal aspirates and air samples was performed by examining the sequences of  
91 *mtLSUrRNA* gene. Genotypes are determined by single nucleotide polymorphisms at  
92 positions 85 and 248 [10].

93 The study was noninterventional, which did not require inform consents and ethical approval  
94 according to French laws and regulations (CSP Art L1121e1.1).

95 Statistical analyses were performed using Prism software (version 7.0, GraphPad Software,  
96 San Diego, CA, USA). Quantitative variables were compared using Mann-Whitney test. A  
97 two-sided P-value < 0.05 was considered significant.

98

100 The results of *P. jirovecii* DNA detection air samples collected in the surrounding aerial  
101 environment of infants' room are presented in table I. In the absence of inhibitors or  
102 contamination, *P. jirovecii* DNA was detected in three out of 14 air samples (21.4%). These  
103 three air samples were collected within one day of NPAs collection from infants I4, I9, and  
104 I10. *P. jirovecii* genotyping was successful in only one positive air sample that was collected  
105 near infant I9. The allele 2 (A<sub>85</sub>C<sub>248</sub>) was identified in both NPA and air samples,  
106 objectivizing a genotype match in this pair of samples. This match of genotype was consistent  
107 with the fact that *P. jirovecii* detected in the air sample was effectively exhaled by the infant.  
108 Infant I9 was hospitalized in a single bedroom.

109 Only 14 infants were enrolled the study, while 87 infants were diagnosed with *Pneumocystis*  
110 primary infection during the study period. The availability of the air sampling device, the  
111 disposal of the operator as well as that of the infants may have also hampered in air sampling.  
112 Although the detection of *P. jirovecii* in NPAs using PCR is a routine technique in our  
113 laboratory, it is performed only twice a week. *Pneumocystis* primary infection is a benign  
114 disease frequently contemporaneously to viral infection and with prompt improvement. Most  
115 infants infected with *P. jirovecii* in this context were already discharged from the hospital  
116 when the pediatricians were informed of the positive results of *P. jirovecii* detection in NPAs.  
117 Consequently, in this context the air sampling in infants' room was not be done. The time  
118 interval between the date of NPA sampling and that of *P. jirovecii* detection in NPAs also  
119 explains the time interval between the date of NPA sampling and that of air sampling in  
120 infants' room. The median of this latter was 3,5 days (range, 1 -10 days).

121 *P. jirovecii* DNA was detected using the real-time PCR assay in air samples in the rooms of  
122 infants I4, I9, and I10 with Ct values of 40.9, 40.6, and 42 respectively. Considering these  
123 values, the fungal burdens were probably low and below the quantification limit we



124 previously described elsewhere [9]. We did not perform the quantification in positive air  
125 samples for this reason. *P. jirovecii* DNA was not detected in air samples in the rooms of the  
126 remaining 11 infants (I1, I2, I3, I5, I6, I7, I8, I11, I12, I13, I14). Several uncontradictory  
127 hypotheses may explain these results: i) *P. jirovecii* exhalation by these 11 infants was faint or  
128 insignificant due to their low *P. jirovecii* pulmonary burdens. In favor of this hypothesis, it is  
129 noteworthy that the median of the Ct values of *P. jirovecii* DNA amplification in NPAs from  
130 these 11 infants was 39.6 whereas it was 36.2 in the three infants from whom *P. jirovecii*  
131 DNA was detected in air samples ( $P$ , 0.07, Mann-Whitney test); ii) the time interval between  
132 the date of NPA sampling and that of air sampling in infants' room may also be involved. It  
133 was one day for the three infants from whom *P. jirovecii* DNA was detected in air samples  
134 whereas it was 4 days (median) (range, 1-10 days) ( $P$ , 0.016, Mann-Whitney test) for the 11  
135 remaining infants from whom *P. jirovecii* DNA was not detected in air samples. This delay in  
136 air sampling was concomitant to infant improvement, *P. jirovecii* clearance from the lungs,  
137 and consequently, the decrease or disruption of *P. jirovecii* exhalation; iii) although, we used  
138 the same real time PCR assay that we previously described elsewhere [8], a 1-cubic-meter  
139 was collected per air sample (100 L per min. during 10 min.) instead of 1.5-cubic meter (300  
140 L per min. during 5 min.) in order to reduce the sound level of the device from 75 dB to 52  
141 dB close to the infants, and to respect the criteria of a non-interventional study. Thus, the air  
142 collection of *P. jirovecii* organisms may have been less efficient.

143 A plus/minus real-time PCR assay amplifying the mtLSUrRNA gene has been routinely used  
144 to perform diagnoses of *P. jirovecii* infection in our laboratory for many years. For this  
145 reason, the same gene was also chosen as the target of the qPCR assay for detection of *P.*  
146 *jirovecii* in air samples. This multicopy gene provides a highly sensitive amplification, which  
147 is required for detection of low fungal burden in NPAs from infants developing primary  
148 infection, and in the related air samples. For the same reasons, choosing this gene for *P.*

149 *jirovecii* genotyping based on DNA sequence analysis rendered it easy the comparison of  
150 putative genotypes in NPAs and air samples. The genotyping was successful in the air sample  
151 from infant I9 and unsuccessful in the two other air samples from infants I4 and I10. The  
152 vagaries between the real-time PCR-assay that was used for *P. jirovecii* detection in air  
153 samples and the conventional PCR assay that was used for the first step of the method of  
154 *mtLSUrRNA* gene sequencing in this context of low fungal burdens may explain these  
155 unsuccessful results. Additionally, some infants were more or less spreaders because they  
156 coughed or not, and they were awake or not. This is a hypothesis to explain why fungal loads  
157 in air samples were not strictly correlated with fungal loads in NPAs and why detection and  
158 genotyping of *P. jirovecii* failed.

159 In the course of the present study, none unoccupied rooms in the pediatrics department were  
160 available during the winter bronchiolitis epidemic to get negative controls of air samples.  
161 Nonetheless, it was noteworthy, that 11 out of 14 air samples were negative for *P. jirovecii*  
162 detection. Moreover, in our previous studies taken together [7, 8], 43 control air samples were  
163 negative providing data excluding putative environmental contaminations.

164 To sum up, despite the small number of infants enrolled in the study, we obtained the first  
165 data on *P. jirovecii* exhalation by infants with primary infection. This study provides  
166 additional data on *P. jirovecii* human reservoir. Infants may represent potential active  
167 infectious sources of the fungus in the hospital environment as previously shown for PCP  
168 patients. This observation may have repercussions in terms of infection control in pediatric  
169 units monitoring infants with respiratory infections. Infants with primary *Pneumocystis*  
170 infection may also represent potential active sources of infection in the community. Following  
171 these original but limited results, a new study considering the rapid turnover of infants  
172 hospitalized in pediatric units, and including the daily use of PCR for the detection of *P.*  
173 *jirovecii* in air samples is warranted.

174

175

176 Conflict of interest statement

177 None declared.

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181

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211

Table I: *Pneumocystis jirovecii* detection in the air surrounding infants developing *Pneumocystis* primary infection

Infant code (sex)	Age	Clinical presentation	Number of days between NPA sampling and air sampling	Results of <i>P. jirovecii</i> detection in NPA expressed in Ct	Results of <i>P. jirovecii</i> detection in air (Ct)	<i>mtLSUrRNA</i> genotype identification in air samples
I1 (M)	2 m, 12 days	Bronchiolitis* and pyelonephritis ( <i>E. coli</i> )	5	37.8	Negative	-
I2 (F)	8 m, 24 days	Rhinitis*	3	40.8	Negative	-
I3 (M)	3 m	Bronchiolitis (rhinovirus/enterovirus, <i>Haemophilus influenzae</i> concurrent infection)	3	34.9	Negative	-
I4 (F)	1 m, 23 days	Cow's milk protein allergy**	1	33.4	Positive (40.9)	Unsuccessful amplification
I5 (M)	7 m, 12 days	Acute obstructive respiratory deficiency due to rhinopharyngitis, Pierre Robin syndrome**	10	40	Negative	-
I6 (M)	8 m, 20 days	Bronchiolitis, asthma West Syndrome, brain malformation**	9	34.4	Negative	-
I7 (F)	1 m	Bronchiolitis (virus parainfluenzae type III)	4	39.2	Negative	-

and oral candidiasis						
I8 (M)	1 m, 5 days	Bronchiolitis (rhinovirus/enterovirus)	4	39.6	Negative	-
I9 (F)	9 m, 11 days	Bronchiolitis (RSV), acute otitis media	1	37.8	Positive (40.6)	Type 2 A <sub>85</sub> C <sub>248</sub> (identical to that identified in NPA)
I10 (M)	2 m, 28 days	Bronchiolitis, asthma. (coronavirus NL63, <i>Haemophilus influenzae</i> , <i>Staphylococcus aureus</i> )	1	36.2	Positive (42)	Unsuccessful amplification
I11 (M)	9 m, 19 days	Bronchiolitis, asthma (metapneumovirus), acute otitis media	1	41.4	Negative	-
I12 (M)	2 m, 14 days	Bronchiolitis (virus parainfluenzae type I)	3	37.9	Negative	-
I13 (M)	1 m, 19 days	Bronchiolitis. (rhinovirus/enterovirus)	6	40.8	Negative	-
I14 (F)	2 m, 21 days	Bronchiolitis (RSV)	6	40.7	Negative	-

\*: due to technical issue, virus detection was not performed. \*\*: negative for virus detection. M: male; F: female; m: month; RSV, respiratory syncytial virus, NPA: nasopharyngeal aspirate; Ct : threshold cycle of PCR amplification; -: not done