Prevalence and Genotype Distribution of *Pneumocystis jirovecii* in Cuban Infants and Toddlers with Whooping Cough

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Running Head: Epidemiology of *P. jirovecii* in young Cuban children

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Abstract:
This study describes the prevalence and genotype distribution of *Pneumocystis jirovecii* obtained from nasopharyngeal (NP) swabs of immunocompetent Cuban infants and toddlers with whooping cough (WC). One hundred sixty three NP swabs from 163 Cuban young children with WC and admitted to the respiratory care units at two pediatric centers, were studied. The prevalence was determined by a quantitative PCR (qPCR) assay targeting the *P. jirovecii* mitochondrial large subunit (mtLSU) rRNA gene. Genotypes were identified by direct sequencing of mtLSU rDNA and restriction fragment–length polymorphism analysis of the dihydropteroate synthase (DHPS) gene amplicons. qPCR detected *P. jirovecii* DNA in 48/163 (29.4%) samples. mtLSU rDNA sequence analysis revealed the presence of three different genotypes in the population. Genotype 2 was most common (48%) followed in prevalence by genotype 1 (23%) and 3 (19%); mixed genotype infection was seen in 10% of the cases. RFLP analysis of DHPS PCR products revealed four genotypes, 18% of which were associated to sulfa drugs resistance. Only contact to coughers (prevalence ratio (PR) 3.51, 95% CI: 1.79–6.87, *P*=0.000) and exposure to tobacco smoke (PR 1.82, 95% CI: 1.14–2.92, *P*=0.009) were statistically associated with being colonized by *P. jirovecii*. The prevalence of *P. jirovecii* in infants and toddlers with WC and the genotyping results provide evidence that this population represents a potential reservoir and transmission source of *P. jirovecii*.

Keywords: *Pneumocystis*, colonization, genotypes, epidemiology, infants, toddlers, Cuba.
INTRODUCTION

*Pneumocystis jirovecii* is a well-recognized major opportunistic fungus with worldwide distribution that causes *Pneumocystis* pneumonia (PaP) in immunosuppressed individuals (1). Non-immunocompromised patients may occasionally develop PaP (2). Low levels of *Pneumocystis* DNA can be detected in respiratory samples from subjects without signs and symptoms of PaP; this is known as “colonization”, “carriage”, “subclinical infection” or “asymptomatic infection” (3, 4). Because of the low burden of organisms associated with colonization status, PCR-based techniques are often required to determine the presence of *P. jirovecii*, especially in cases where histochemical staining methods are negative (3). The most common respiratory specimens used to detect colonization by PCR include sputum, bronchoalveolar lavage (BAL), oral wash, and lung tissue (5). However, acquisition of these samples is difficult in infants and toddlers. Therefore, non-invasive sampling such as collection of nasopharyngeal (NP) swabs is highly useful for this population (6).

The mitochondrial large subunit (mtLSU) rRNA gene is a commonly used target for *P. jirovecii* diagnosis and quantification (7). This gene is also useful for molecular epidemiology studies and determination of population genetic structure (7, 8). Dihydropteroate synthase (DHPS) is the target of sulfonamides, the first-line drugs for PaP prophylaxis or treatment. Mutations in the DHPS locus may be a consequence of exposure to sulfonamide drugs and may be related to the emergence of resistant strains of *P. jirovecii* to these agents (9). Both loci, mtLSU rRNA and DHPS, have been used to identify human reservoirs of this microorganism (7, 9).

Colonization with *P. jirovecii* increases the risk of developing acute PaP and has been associated with sudden infant death syndrome (SIDS) and bronchiolitis in susceptible hosts (3, 10, 11). *Pneumocystis* is the most prevalent microorganism identified in the lungs of...
infants with sudden unexpected death, and infection due to *P. jirovecii* is most common between the ages of 3-4 months (11). Upper respiratory infections appear to be a major risk factor for *Pneumocystis* colonization in small children (3).

Whooping cough (WC) is a vaccine-preventable disease and is a major cause of childhood morbidity and mortality. In infants, case-fatality rates in developing countries are estimated to 4% (12).

To the knowledge of the authors there is no data available about the prevalence of *P. jirovecii* associated to WC in infants and toddlers. This study describes the prevalence and genotype distribution of *P. jirovecii* obtained from NP swabs of an immunocompetent Cuban pediatric population with WC.
MATERIALS AND METHODS

Samples. From April 2010 to March 2013, all available NP swabs sent to The Institute of Tropical Medicine ‘Pedro Kouri’ for bacterial and viral analyses from young children with clinical diagnosis of WC (according to WHO criteria: individual with a cough lasting at least 2 weeks with at least one of the following symptoms: paroxysms of coughing, inspiratory ‘whooping’ or post-tussive vomiting without other apparent cause), were included in the study (12). Infants and toddlers were hospitalized in the pediatric hospitals Juan Manuel Marquez and William Soler, Havana, Cuba. Patients included had experienced no effect of antibiotic treatment. An informed consent was obtained from all parents or tutors of the study participants, and ethical clearance to conduct the study was acquired from our Institutional Ethical Committee.

DNA extraction from nasopharyngeal swabs. DNA extraction was performed with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Purified DNA was eluted in 150 µL of elution buffer and stored at −20°C until use.

P. jirovecii qPCR assay. The quantitative PCR developed by Dini et al. (13) targeting the mtLSU rRNA gene was used with minor modifications. A 77 bp amplicon was amplified by the primers LSU1 (5’-AAA TAA ATA ATC AGA CTA TGT GCG ATA AGG-3’) and LSU2 (5’-GGG AGC TTT AAT TAC TGT TCT GGG-3’) and detected by the hydrolysis probe LSUPN1, labeled with 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (TAMRA) 5’-AGA TAG TCS AAA GGG AAA C-3’ (TAMRA Copenhagen A/S, Copenhagen, Denmark). The amplification was carried out in a Applied Biosystems 7500 Fast Real-Time PCR System, in a final volume of 25 µL, using the
TaqMan® Universal PCR Master Mix (Applied Biosystems, California, USA), with 0.4 µM each primer, 0.2 µM probe, and 5 µL of the extracted DNA. Thermal cycling was 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 55°C. Absolute quantification was based on extrapolation to standard curves generated by amplification of five serial dilutions tested in triplicate of a linearized pCR™2.1-TOPO® (Invitrogen, California, USA) plasmid containing a *P. jirovecii* DNA fragment amplified by primers LSU1 and LSU2 diluted into negative control sample DNA. The results were expressed in mtLSU gene copies x µL⁻¹ (14). PCR efficiency (Pe) was calculated as follows: 

\[ Pe = 10^{-1/slope} - 1 \]

For qPCR experiments “The Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE) guidelines were followed (15).

**External controls and contamination prevention.** During each PCR, a *P. jirovecii* DNA positive control (BAL sample from a patient with PcP and *P. jirovecii* positive by Giemsa stain and immunofluorescence assay) and a DNA elution buffer negative control were used. From each extract, amplification of the human β-actin gene was performed to confirm successful DNA extraction and the absence of PCR inhibitors. This PCR assay was carried out separately using the SYBR® Green PCR Core Reagents (Applied Biosystems, California, USA). The primers were βac1 (5’-CCT TCC TGG GCA TGG AGT CCT G-3’) and βac2 (5’-GGA GCA ATG ATC TTG ATC TTC-3’) described by Ghossein et al. (16). To avoid contamination, each step (master mix preparation, DNA extraction, amplification and addition of the PCR product to nested PCR reaction mixture) was performed in separate areas with different micropipettes and barrier tips in laminar-flow cabinets.
Analysis of mtLSU genotypes. Clinical samples with \( \geq 3 \) mtLSU gene copies x \( \mu L^{-1} \) of extracted DNA were selected for mtLSU genotype analysis. For this assay a nested PCR (260 bp product length) with the primers pAZ102-H and pAZ102-E in the first amplification round and the primers pAZ102-X and pAZ102-Y in the second round was used as previously described (17). Amplifications were carried out in a 30 \( \mu L \) volume containing 15 \( \mu L \) 2X HotStarTaq Plus Master mix (QIAGEN, Hilden, Germany), 0.4 \( \mu M \) of each primer and 5 \( \mu L \) of extracted DNA (2 \( \mu L \) of the first PCR products for the second round of PCR). The same conditions were used for the first and second amplification: one step at 95°C for 5 min, 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 10 min at 72°C. PCR amplifications were performed in a Mastercycler® personal (Eppendorf, Germany). PCR products were visualized by agarose gel electrophoresis under ultraviolet light and purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced on both strands (Big-Dye Terminator Method, Capillary electrophoresis CEQ Sequencer 8800, Beckman Coulter, USA). Consensus sequences were obtained with ChromasPro (version 1.7.4, Technelysium Pty Ltd., Australia) and aligned using CLUSTAL X (version 2.1) with the reference sequence (accession number M58605.1) deposited by Sinclair et al. (18). Genotypes were called on the basis of polymorphism at nucleotide positions 85 and 248 described by Beard et al. (19).

Analysis of DHPS genotypes. Clinical samples with \( \geq 20 \) mtLSU gene copies x \( \mu L^{-1} \) of extracted DNA were selected for analysis of genotypes of the DHPS gene. For amplification of DHPS region a nested PCR was used. In the first round the primers DHPS F1 (5’-CCT GGT ATT AAA CCA GTT TTG CC-3’) and DHPS B45 (5’-CAA TTT AAT 7

\[ 7 \]
AAA TTT CTT TCC AAA TAG CAT C-3’) which yield a 895 bp fragment were used. For
the second round, the primers DHPS AHum (5’-GCG CCT ACA CAT ATT ATG GCC
ATT TTA AAT C-3’) and DHPS BN (5’-GGA ACT TTC AAC TTG GCA ACC AC-3’)
generating a 370 bp fragment (20) were used. Amplifications were carried out in a 30 µL
volume containing 15 µL 2X HotStarTaq Plus Master mix (QIAGEN, Hilden, Germany),
0.4 µM of each primer and 5 µL of extracted DNA (2 µL of the first PCR products for the
second round of PCR). The same conditions were used for the first and second
amplification: one step at 95°C for 5 min, 40 cycles of 1 min at 94°C, 1 min at 57°C, and 1
min at 72°C, followed by 10 min at 72°C. DHPS PCR products were subject to restriction
fragment length polymorphism (RFLP) analysis using AccI and HaeIII endonucleases
(Sigma-Aldrich, Missouri, USA) as described by Helweg-Larsen et al. (21). *P. jirovecii*
DHPS genotypes were determined as described elsewhere (19) based on polymorphisms at
codons 55 and 57. PCR products and restriction fragments were visualized under the same
conditions defined previously.

**Clinical data collection.** Demographic and clinical data were obtained from patient
medical records using uniform data forms including information on age, sex, ethnicity,
history of previous PcP episodes, immunosuppressive therapy, asthma status, tobacco
smoke exposure, documented contact to coughers, hematological malignancy, solid tumors,
solid organ transplants, HIV-infection, other organisms identified in the specimen, anti-PcP
treatment/prophylaxis, and season of sampling.

**Statistical analysis.** SPSS v.18.0 software (SPSS Inc., Chicago, IL, USA) for Windows
was used for statistical analysis. Categorical variables were compared using χ² or Fisher’s
exact tests. Results were considered statistically significant at P values <0.05.
RESULTS

Children and demographics characteristics. A total of 163 samples from 163 young children (aged 27 days to 72 months; median, 8 [inter-quartile range, IQR 4.0 – 15.0] months) were considered for this study (Table 1). Eighty eight (54%) were female.

qPCR assay validation. The limit of detection of the qPCR assay was 3 copies of plasmid per PCR. The standard curve was generated automatically ($R^2=0.997$, intercept=35.986595 and slope=-3.282599). The mean overall standard deviation (SD) and coefficient of variation (CV) of the Cq values was 0.14 (range, 0.10 to 0.19) and 1.93% (range, 1.11 to 3.63), respectively. The efficiency of qPCR amplification was 101.67%.

DNA amplification. qPCR detected *P. jirovecii* DNA in 48 (29.4%) of the 163 samples: 28 (31.8%) of 88 female and 20 (26.7%) of 75 male patients. Sixteen (33.3%) positive specimens contained ≥ 20 mtLSU gene copies x µL$^{-1}$. In all 163 cases human β-actin genes could be amplified.

Genotypes of mtLSU rRNA. Genotypes were determined in 48 specimens detected by qPCR with ≥ 3 mtLSU gene copies x µL$^{-1}$. The observed predominant genotype among infants and toddlers was the genotype 2 (48%), followed by genotype 1 (23%), and genotype 3 (19%). Mixed genotypes were detected in five samples (10%) (Table 2).

Other nucleotide mutations were detected in several strains. Mutations in positions 123 (C to T), 252 (A to T) and 258 (A to T) were observed (Table 3). Mutations at position 252 and 258 were found in 14 (29.1%) and 1 (2.1%) of 48 analyzed sequences, respectively. A mutation in position 123 (C to T) was detected in 2/48 samples (4.2%); this mutation has
not been described before and these novel nucleotide sequences were deposited in GenBank under accession number KC937058 and KC937059.

Genotypes of the DHPS gene. The DHPS gene amplifications were successful in all of the 16 specimens detected by qPCR with \( \geq 20 \) mtLSU gene copies x µL\(^{-1}\). After enzymatic digestion it was observed that 13/16 specimens (82%) presented a wild type (Wt, genotype 1), two samples (12%) were classified as mutant types (genotypes 2 and 3) and one specimen (6%) contained mixed genotypes (genotypes 1 and 4) (Table 2). This last specimen was obtained from the same patient who showed multiple mtLSU rRNA genotypes.

Statistical analysis. Patients colonized with \( P. jirovecii \) were similar to non-colonized patients in terms of age, gender, ethnicity, and asthma status (Table 1). The season of the sample collection and the identification of other organisms were not related with \( P. jirovecii \) colonization. Contact to coughers (Prevalence ratio (PR) 3.51, 95% CI: 1.79–6.87, \( P=0.000 \)) and exposure to tobacco smoke (PR 1.82, 95% CI: 1.14–2.92, \( P=0.009 \)) were statistically associated with \( P. jirovecii \) colonization (Table 1).
DISCUSSION

In this study, *P. jirovecii* DNA was detected and quantified in NP swabs in 29.4% (48/163) of infants and toddlers with WC admitted to two pediatrics hospitals. Similar prevalence figures for *P. jirovecii* colonization have been found in infants with mild respiratory infection (33%) and with bronchiolitis (24.4%) (2, 10). The prevalence of colonization in infants dying of SIDS and other causes ranges from 9.4% to 100% (22-25). These results are higher than the prevalence reported in a similar study in Poland in young children with respiratory tract infections, where only 2.9% of NP swabs contained *P. jirovecii* (26).

Molecular tools have proved to be reliable for the detection of *P. jirovecii* colonization due to high sensitivity and since these provide the possibility of strain identification, quantification and genotyping using non-invasive samples as described in this report.

During *Pneumocystis* colonization there are low levels of fungal DNA in the host. Nevertheless, *Pneumocystis* may play a role in the development of lung damage which could favor other respiratory infections and SIDS (3, 11). In this study, 29.4% of patients (48/163) had between 3 and 50 mtLSU gene copies x µL\(^{-1}\) with no previous history of sulfa drug prophylaxis/treatment. This group was defined as “colonized”. Patients with higher concentrations of *P. jirovecii* should be actively followed since they could be in an early PcP stage.

All 48 specimens with ≥ 3 mtLSU gene copies x µL\(^{-1}\) quantified by qPCR were amplified by nested-PCR of the *P. jirovecii* mtLSU rRNA locus (a multicopy gene), and for all 16 (100%) of the specimens with ≥ 20 mtLSU gene copies x µL\(^{-1}\) detected by qPCR the DHPS locus (a single-copy gene and, consequently, a less sensitive target) could be successfully
amplified. These cutoffs for gene amplification of *P. jirovecii* have proved to be useful in samples with low fungal load and colonization status.

The mtLSU rRNA gene is involved in basic metabolic functions and has been widely used for PCR-based detection of *P. jirovecii* and for discerning associations between specific genotypes and geographical locations (6, 17, 19, 27).

In the present study, genotype 2 (85A/248C) was the most frequent genotype (48%) followed by genotype 1 (85C/248C, 23%), and only 19% of the samples belonged to genotype 3 (85T/248C). In contrast, in a study conducted in Cuba by de Armas et al. (28), mtLSU rRNA genotype 3 was the most frequently detected genotype (93.8%) in paraffin embedded post mortem lung specimens obtained at autopsy of HIV-positive patients. The difference in genotype distribution between these two studies may rely on the type of cohorts studied (infants/toddlers with WC vs. patients with HIV/AIDS), *Pneumocystis* presentation in the host (colonization vs. PcP), different underlying disease and independent transmission cycles, as previously suggested (25, 29).

Other studies of HIV-positive and HIV-negative patients with PcP revealed that genotype 2 was the most frequent, while genotype 4 (85C/248T) was identified in relatively low numbers (19, 30-32). Our results agree with these studies in that genotype 2 was the most common. However, genotype 4 has not yet been found in Cuba. On the other hand, the frequency distribution of mtLSU rRNA genotypes showed to be similar to previous studies of Italian, Indian, and Britain *P. jirovecii* strains (31-33). In contrast, in Spain, a different frequency distribution was observed with genotype 3 being the second most prevalent and genotype 2 being the third most common. Moreover, in Australia genotype 2 was absent and genotype 1 was the most frequent (29, 34-36). These differences imply that genetic variation in *P. jirovecii* has a geographical component. It has been suggested that the
epidemiological factors inherent to geographic location may influence the transmission and
circulation of different *P. jirovecii* genotypes (8, 28, 37).

Based on mtLSU rDNA sequence analysis, mixed genotypes (two or more genotypes in the
same sample) in colonized patients have been reported (19, 32, 38, 39). Here, mixed
genotypes were identified in 10% of the cases. A somewhat similar prevalence was
reported in Spanish children (3.7% and <10%) (29, 34) but it is lower than the prevalence
described in the USA (45.6%) (25). High *P. jirovecii* burden in 3/5 samples with mixed
genotypes was detected, suggesting that mixed genotypes may be related to parasite burden
in colonized patients. The finding of multiple mtLSU rRNA genotypes in infants shows
that infection with *P. jirovecii* is not necessarily clonal and multiple different strains can be
found simultaneously in the same host, which in turn may influence the severity of
infection. However, this interpretation requires confirmation from other studies.

The demonstration of nucleotide changes (without polymorphic positions 85 and 248) in
35.4% (17/48, including a novel mutations) of the analyzed genes, suggests a high genetic
variability among Cuban *P. jirovecii* strains. Additionally, the mutations observed at the
positions 252 (A to T) and 258 (A to T) in several strains were previously described in a
nosocomial cluster of PcP infection in kidney transplant recipients in Australia (40).

Because *P. jirovecii* cannot be cultivated in vitro, determination of resistance by traditional
assays is not possible. The DHPS gene, is involved in the essential biosynthesis of folic
acid and has been used as marker for changes in susceptibility levels against sulfa and
sulfone drugs, as well as for *P. jirovecii* characterization (9). Variations in this gene have
been associated with previous sulfa drug exposition (9, 41). In the present study, 16/48
specimens (33.3%) were genotyped by PCR-RFLP analysis of the DHPS locus. However,
other studies have reported a reduced success of DHPS gene amplification (19, 41) while
Dimonte et al. (31) showed a similar amplification rate (33.3%, 23/67). Most of the 16 studied patients (13, 81.2%) exhibited a Wt pattern (genotype 1), two were classified as mutant type (change in codon 55 and 57, genotype 2 and 3) and one had a mixture of Wt and double-mutant type (genotype 1 and 4). In this study, the patients with DHPS mutant types (single or double) had not suffered any sulfonamide selection pressure (i.e. no previous sulfa prophylaxis or treatment). In Cuba, cotrimoxazole is used as standard chemoprophylaxis against PcP in HIV-infected patients and as second-line drug in WC unresponsive to antibacterial therapy. In this sense, it is not surprising to find circulating *P. jirovecii* strains with DHPS mutations. These results suggest that such *P. jirovecii* DHPS mutant strains are circulating in the infant/toddler population in Cuba. Continuous monitoring of the evolution of mutant *P. jirovecii* strains in susceptible populations is important; this in turn could help to clarify the mechanisms involved in sulfa drugs resistance and clinical implications.

The Cuban samples showed a high prevalence of the DHPS Wt and these data are in concordance with studies from Portugal (93%) and Spain (78%) (35) but in contrast to studies performed in the US where the prevalence is lower and the frequency of DHPS mutations ranges between 43% – 68% (19, 42-45). These data support the hypothesis about the existence of geographical variation in the prevalence of *P. jirovecii* DHPS gene mutations because of intrinsic epidemiological factors of each location which influence the circulation and transmission of different genotypes, and which also may reflect and contribute to the differences in the use and effect of sulfonamides for PcP prophylaxis and treatment of bacterial diseases (9, 35, 45, 46).

However, the effect of mutant strains on clinical illness and outcome of PcP has not been clearly defined. Some studies have found no significant association between symptoms and
patients with Wt and mutant strains, respectively, while others have reported that patients infected with mutant strains have a poorer prognosis and high rate of mortality (21, 36, 45, 47). In a recent study of 301 HIV-infected patients with PcP, DHPS mutants, independently of definition (single or double mutant), were not associated with increased mortality ($P > 0.05$) (48).

In the infants/toddlers population studied, no cases of current or previous PcP prophylaxis/treatment, hematological malignancy, solid tumors, immunosuppressive therapy, solid organ transplant recipients or HIV-infection were found. It is essential to develop a similar approach to study healthy children and other populations with the disorders described above, since these conditions have been previously described as predisposing factors to PcP or being asymptomatic carrier (2, 3). No significant differences have been found between colonized patients with regard to age, gender, ethnicity, or asthma status.

Exposure to tobacco smoke increases the risk of developing pneumonia and severe pneumonia in childhood (49). In HIV-positive patients, tobacco use increases the risk of PcP (50). In this study, certain risk factors, such as tobacco smoke exposure and contact to coughers appeared to influence the risk for *P. jirovecii* colonization. None of the genotypes found for the mtLSU rRNA and DHPS loci were associated with demographic and clinical data in univariate analysis. In the present study, no correlation among mtLSU rRNA and DHPS genotypes were detected, and no single genotype predominated or had a specific distribution by group.
Based on the results of this study, efforts to reduce the incidence of *P. jirovecii* colonization in infants and toddlers should include minimizing exposure to coughers and tobacco smoke combined with the provision of good ventilation in residential houses.

We have reported for first time the prevalence of *Pneumocystis* in patients with WC, from two Pediatrics Hospitals in Havana, Cuba. We demonstrated a high diversity of the mtLSU rRNA gene and a low prevalence of DHPS mutations in colonized Cuban infants and toddlers. These results provide evidence that this infant population is exposed to *P. jirovecii* and represents a potential reservoir and transmission source of this fungus to susceptible individuals. This study supplies the first data in the scientific literature on the distribution of *P. jirovecii* genotypes in infants/toddlers in Cuba and their association with whooping cough.
Acknowledgements

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Declaration of interest

None of the authors of this article have a conflict of interest with any product or company listed in this article.
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12. WHO–recommended standards for surveillance of selected vaccine-preventable diseases. WHO/V&B/03.01


associated with duration of sulfa or sulfone prophylaxis exposure in AIDS patients.


Table 1. Demographic and clinical characteristics of enrolled infants and toddlers with whooping cough.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>P. jirovecii-positive patients (n = 48)</th>
<th>P. jirovecii-negative patients (n = 115)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>28 (58.3%)</td>
<td>60 (52.2%)</td>
<td>0.292</td>
</tr>
<tr>
<td>Age (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 months</td>
<td>24 (50%)</td>
<td>41 (35.6%)</td>
<td>0.063</td>
</tr>
<tr>
<td>6-11 months</td>
<td>10 (20.8%)</td>
<td>32 (27.8%)</td>
<td>0.233</td>
</tr>
<tr>
<td>12-24 months</td>
<td>8 (16.6%)</td>
<td>28 (24.3%)</td>
<td>0.193</td>
</tr>
<tr>
<td>&gt;24 months</td>
<td>6 (12.5%)</td>
<td>14 (12.2%)</td>
<td>0.340</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>35 (72.9%)</td>
<td>79 (68.7%)</td>
<td>0.367</td>
</tr>
<tr>
<td>Mestizo</td>
<td>11 (22.9%)</td>
<td>31 (26.9%)</td>
<td>0.480</td>
</tr>
<tr>
<td>Black</td>
<td>2 (4.2%)</td>
<td>5 (4.3%)</td>
<td>0.625</td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer (May to October)</td>
<td>22 (45.8%)</td>
<td>45 (39.2%)</td>
<td>0.340</td>
</tr>
<tr>
<td>Winter (November to April)</td>
<td>26 (54.2%)</td>
<td>70 (60.9%)</td>
<td>0.267</td>
</tr>
<tr>
<td>Contact to coughers</td>
<td>40 (83.3%)</td>
<td>54 (46.9%)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Asthma status</td>
<td>7 (14.6%)</td>
<td>17 (14.8%)</td>
<td>0.599</td>
</tr>
<tr>
<td>Tobacco smoke exposure</td>
<td>27 (56.2%)</td>
<td>40 (34.8%)</td>
<td>0.009*</td>
</tr>
<tr>
<td>Other organisms identified\b</td>
<td>38 (79.2%)</td>
<td>90 (70.3%)</td>
<td>0.539</td>
</tr>
</tbody>
</table>

\*In Cuba the climate is tropical wet with only two seasons, summer and winter (51).
\bIncluding Haemophilus influenzae, Moraxella catarrhalis, Bordetella pertussis, Streptococcus pneumoniae, Mycoplasma pneumoniae, Ureaplasma urealyticum, Candida albicans, Aspergillus fumigatus, Influenza A, Influenza B, Respiratory Syncytial virus, Adenovirus, Parainfluenza virus (type 1, 2 and 3), Rhinovirus/Enterovirus.
*Statistically significant.
Table 2. Frequency of (*P. jirovecii*) genotypes observed in the study.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nucleotide (codon) identity</th>
<th>Genotype</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtLSU rRNA</td>
<td>85 C/248 C</td>
<td>1</td>
<td>11 (23)</td>
</tr>
<tr>
<td></td>
<td>85 A/248 C</td>
<td>2</td>
<td>23 (48)</td>
</tr>
<tr>
<td></td>
<td>85 T/248 C</td>
<td>3</td>
<td>9 (19)</td>
</tr>
<tr>
<td></td>
<td>85 C/248 C and 85 A/248 C</td>
<td>mixed (1 and 2)</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>85 C/248 C and 85 T/248 C</td>
<td>mixed (1 and 3)</td>
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mtLSU rRNA = mitochondrial large subunit rRNA; DHPS = dihydropteroate synthase.
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* Based on the sequences deposited in the GenBank (last access 29/05/13), used primers pAZ102X and pAZ102Y (17).

* Arbitrary numbers.

* Without the polymorphic positions 85 and 248.

* Countries refer to accession nos. in last column.

* Reference sequence (18).