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ORIGINAL ARTICLE

Prevalence of colonization and mitochondrial large subunit rRNA mutation of *Pneumocystis jiroveci* among Iranian children

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ABSTRACT

Background and Objectives: *Pneumocystis jiroveci* is an important causative agent of *Pneumocystis* pneumonia. During childhood, exposure to *Pneumocystis* occurs and antibody was built in early childhood. The aim of this study was to describe the molecular epidemiology of *P. jiroveci* in children without any respiratory syndrome and survey the distribution of different mitochondrial large subunit, ribosomal ribonucleic acid (mtLSU- rRNA) genotypes.

Materials and Methods: Mini-bronchoalveolar lavage (mini-BAL) fluids from pediatric patients with no history of lung disorders were obtained during a 14-month period. *P. jiroveci* colonization was confirmed by immunofluorescence staining, nested PCR and sequencing. Genotypic characterization at the mitochondrial large subunit rRNA gene was performed by direct sequencing.

Results: Of 172 BAL specimens from patients, with mean age of 4.9 years, the prevalence of *P. jiroveci* colonization was 3.5% (6 samples). The results of sequencing revealed the two polymorphisms; 85/A; 248/C in 3 cases, and 85/T; 248/C in other cases. One sample also showed a mutation replacement at position 258 (T-to-C change), which was not reported previously.

Conclusion: Colonized person as an environmental reservoir might play an important role in the progression of infection in immunocompromised patients. Diagnosis of the reservoir and genotyping can be essential for the prevention of nosocomial infections.

Keywords: *Pneumocystis jiroveci;* PCR; Genotypic; Pediatrics

INTRODUCTION

Pneumocystis jiroveci is an important causative agent of *Pneumocystis* pneumonia (PCP) in immunocompromised hosts (1). During childhood, exposure to *Pneumocystis* occurs and antibody was built in early childhood; so by 2–3 years of age, 70–90% of the healthy children have serum antibodies (2, 3). The seroprevalence of antibody in 233 Spanish children was 73% generally, with an age-related increase from 52% to 80% (4). This organism colonizes in healthy persons who may act as *P. jiroveci* carriers (5). Diagnosis of PCP is challenging task due to lack of ability to an invitro culture system, so staining

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method and immunofluorescence assay in respiratory specimens are used to diagnose the infection (6, 7). PCR assays have been used for the diagnosis of this infection and several genes have been used for this purpose. MtLSU- rRNA, is one of the highly sensitive and specific genetic regions because there are multiple copies of genomes in it (8-10). The objectives of the present study were to describe the molecular epidemiology of *P. jiroveci* in children without any respiratory syndrome and survey the sequencing using mtLSU-rRNA and genotypic characterization of them in a tertiary health care center in Shiraz, southern Iran.

MATERIALS AND METHODS

Clinical specimens. In this cross sectional study, 184 mini-BAL fluid specimens were collected from pediatric patients with no history of lung disorders, according to the recorded clinical and radiologic symptoms and with normal CD4 count during a 14-month period from January 2012 to March 2013. The patients were admitted in wards and underwent general surgery due to non-infected diseases. Mini-BAL was obtained in anesthetic stage in the operating room.

Ethical consideration. The study protocol was approved by the ethics committee at Prof. Alborzi Clinical Microbiology Research center, Shiraz University of Medical Sciences. Informed written consents were obtained from the parents. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration.

Staining and DNA extraction. All specimens were centrifuged at 1500 rpm for 20 minutes. 100 µl of sediment was used for extracting by a commercial extraction kit (QIAamp ® DNA Mini Kit), as the manufacturer described for body fluid, and two smears were stained by indirect immunofluorescence assay (MonofluoKit ® *P. carinii,* Diagnostics Pasteur, Marnes-La-Coquette, France). Based on manufacturer's instructions, one to five fluorescent oocysts per slide is considered as equivocal.

Semi nested PCR. The primers were described previously (6, 11), able to amplify a 346-bp segment from a specific region at the mitochondrial 5S rRNA gene of *P. jiroveci*. Amplification was performed by adding 5 µl extracted material into a reaction mixture (50 µl) containing 1x PCR buffer (10 mM-Tris-HCl [pH 8.3]), 50 mMKCl; 2.5 mM $MgCl_2$, 0.25 mMdNTPs, 0.25 µM primers, and 2.5 U of *Taq* polymerase (Cinna Gen co.). In the first round of amplification, the primers were: pAZ102-E (5′-GATGGCTGTTTCCAAGCCCA-3′) and pAZ102-H (5′-GTGTACGTTGCAAAGTACTC-3′). Amplification steps included: initial denaturation step at 94°C for 5-min, followed by DNA amplification for 40 cycles. Each cycle included: 94°C for 20 s, 56°C for 20 s, 72°C for 20 s, and final extension step at 72°C for 5 min (Thermal cycler; applied biosystem, Singapore).

The second round was carried out with adding 1 µl of the first round PCR product as DNA template into a reaction mixture using; 1x PCR buffer (10mM Tris-HCl [pH 8.3], 50 mM KCl; 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 µM primers, and 2.5 U of *Taq* polymerase. The primers pAZ102-E and pAZ102-L2 (5′-ATAAGGTAGATAGTCGAAAG-3′) were used as the internal primers. Amplification protocol included; initial denaturation step at 94°C for 5-min followed by DNA amplification for 40 cycles. Each cycle consisted of; 94°C for 20 s, 52°C for 20 s, 72°C for 20 s, and final extension step at 72°C for 5 min. Both rounds of PCR comprised 40 amplification cycles. The expected fragment at second round was 120-bp. To avoid contamination, aerosol-barrier pipette tips were used for transfer of all samples and reagents, and DNA extraction and amplification was performed in a separate room. Negative amplification controls were used in all rounds of tests.

Sequencing and genotyping. The PCR products were purified using ethanol precipitation. Sequencing was performed in three steps. At first step, Big Dye terminator cycle sequencing ABI kit was used. Then, the products of cycle sequencing were purified by ethanol precipitation method and finally applying an ABI 3130 sequencer (Hitachi, Japan), following the manufacturer's protocols for DNA sequencing. Sequence data analysis was carried out by using Chromas lite version 2.1.1 software (Technelysium Pty, Ltd.). The sequence submitted by Sinclair et al. 1991 (12) was used as prototype sequence. The sequences were aligned with the prototype, by BLAST software.

RESULTS

In this study, 184 children were enrolled but due to clinical evidence and probable lung disorders, 12 were excluded due to received previous chemotherapy, corticotherapy, radiotherapy, transplantation and HIV infection. Cases with abnormal chest X-ray and primary or secondary immunodeficiency were also excluded from the study. The most admission reasons of these patients were hypospadias and abdominal surgery. The mean age was 4.9 years (1 month to 15 years) and including 59 girls and 113 boys (Table 1).

From 172 samples collected in this study, four were equivocal by staining (1-4 oocyst per whole slide) and PCR and two were positive only by nested PCR. By sequencing procedure at the mt LSU-rRNA locus, 6 samples of children (3.5%) were confirmed as positive *P. jiroveci* colonization, we can consider these children as carriers. In the remaining 166, diagnosis of PCP DNAs was not confirmed. The results of sequencing, carried out on these PCR products showed two genotypes at the positions 85 and 248. At position 85, either adenine (A) or thymine (T) was seen, but at position 248 only cytosine (C) was observed (Table2). Sequence of one sample also showed a replacement at position 258 (T-to-C change), which was not reported previously. The sequences of PCR products were deposited in GeneBank under accession numbers KJ733279, seq 2 KJ733280, seq 3 KJ733281, seq 4 KJ733282 and Bankit1588391.

DISCUSSION

In this study, mini-BAL samples were used for the detection of colonization in children. Among a variety of specimens such as lung biopsy (invasive method), BAL, induced and expectorated sputum, nasopharyngeal aspirates and oral washings, BAL has been the sample of choice for the diagnosis of *P. carinii (P. jiroveci)* pneumonia (13). The interpretation of the

Table 1. Baseline demographic characteristic of children participant in evaluation of *P. jiroveci* colonization study, South Iran, 2013

Variables		Number (%)
Sex	Male	113 (66)
	Female	59 (34)
Age (years)	≤1	35 (20.34)
	1-5	73 (42.44)
	5-10	42 (24.4)
	10-15	22 (12.79)
Reason for admission	Abdominal Surgery	45 (26.1)
	Hypospadias	32 (18.6)
	Laparactomy	23 (13.4)
	Undescended Testis	23 (13.4)
	Foreign Body Aspiration	23 (13.4)
	Hernia	10 (5.8)
	Urinary Fistula, Appendicitis	13 (7.6)
	Others	3 (1.7)

Table 2. Concordance of the PCR assay with direct immunoflouresent staining and sequencing

	IFA staining			Genotype
		Positive	Negative	Nucleotide position identity
PCR result (6 positive and 166 negative)	Positive (6)	4	2	85/A; 248/C (3 cases) 85/T; 248/C (3 cases)

(A)= adenine, (T)= thymine, (C)= cytosine

results for colonization is difficult in the studies on children with underlying lung diseases. However, in children with *P. jiroveci* and with no underlying diseases, *P. jiroveci* can be indicative of real colonization. The route of transmission of *P. jiroveci* infection is unknown but person-to-person transmission or transmission by aerosol is controversial (8, 14). Colonized persons as environmental reservoir might play a role in the development and progression of infection in immunocompromised patients.

Although there are reports of the presence of antibody in pediatrics, reports on the colonization of *P. jiroveci* in them is limited. Using molecular semi nested PCR, 3.5% of children (6 samples) were found to be colonized with *P. jiroveci* in this study. The rates of colonization with *P. jiroveci* in pediatrics with respiratory syndromes vary in the literature, in infants with mild respiratory symptoms 32% (3), in infants with bronchiolitis 24% (15), in children of HIV-infected mothers with upper respiratory symptoms 3.5% (16), and in children with leukemia 15-20% (17).

In this study, six children were colonized with *P. jiroveci* and only four of them were positive by staining and all the PCR positive patients were confirmed by sequencing. Therefore, the sensitivity of staining in this study for the diagnosis of colonized patients was %66.6 and use of molecular methods are useful for diagnosis of colonized patients.

P. jiroveci genotyping serves exclusively as a diagnostic method to reveal association between genotypes, epidemiology, and virulence of organism (18). In this study, six sequences were deposited in GenBank. The results indicate a significant homology with previously reported fungal sequences. Polymorphisms were detected at nucleotide positions 85 and 248 [85/A; 248/C (%50); 85/T; 248/C (%50)]. Results of genotypic characterization of the mtLSU-rRNA locus of *P. jiroveci,* isolated from patients with cystic fibrosis in Spain, reveal the identified polymorphisms were 85C/248C (45.4%), 85T/248C (27.2%) and 85A/248C (18.1%) (19) and in Brazil were 85C/248C (genotype 1) and 85T/248C (genotype 3) (20). A mutation was observed in one sample. Typing studies have demonstrated subclinical colonization and carriage, clinical manifestations.

CONCLUSION

There have been limited studies reporting on the colonization of *P. jiroveci* in children with healthy lungs. The diagnosis of reservoir can be essential to the prevention of person to person transmission in critical wards in hospitals and nosocomial infections in high risk patients.

Our genotyping results represent original data of *P. jiroveci* genomic characteristics in children with no past history of travel outside the region, southern Iran. As revealed, *P. jiroveci* mtLSU-rRNA mutants can occur. Our findings indicate that such genotyping studies should be conducted in different geographical areas in order to manage more efficiently the high risk patients.

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