Typing of *Pneumocystis jirovecii* isolates from Iranian immunosuppressed patients based on the Internal Transcribed Spacer (ITS) region of the rRNA gene

MARYAM-FATEMEH SHEIKHOLESLAMI*†, JAVID SADRAEI†, PARISSA FARNIA*, MEHDI FOROZANDEH MOGHADAM‡ & HAMID EMADI KOCHAK§

*Mycobacteriology Research Center, NRITLD, Masih Daneshvary Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran, Departments of †Parasitology and ‡Biotechnology, Tarbiat Modares University Tehran, Iran and §Iranian HIV/AIDS Research Center, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran

Since there have been no published molecular studies of *Pneumocystis jirovecii* isolates from Iranian patients, we investigated the genotypes of such isolates recovered from HIV-infected patients, those undergoing cancer chemotherapy and patients with chronic obstructive pulmonary disease (COPD). *P. jirovecii* typing, based on ITS1 and ITS2 sequence analysis, was performed on 34 isolates from Iranian immunosuppressed patients. In total, 44 genotypes were detected of which relative to ITS1, eight known genotypes (A, B, C, E, G, H, N and O) and one novel sequence were noted. Eight known genotypes (b, c, e, g, h, i, j and n) were also found with ITS2. The most frequent ITS1 and ITS2 genotypes were E (21/44, 47.7%) and g (22/44, 50%), respectively. From determined haplotypes, the four most frequent ones were Eg (11/44, 25%), Gg (5/44, 11.3%), Gi (4/44, 9.1%), Ei (3/44, 6.8%), and Hg (3/44, 6.8%). Two novel haplotypes (Hb and Hi) were also identified, along with mixed infections as seven (20.5%) patients were found to have more than one haplotype. It is suggested that novel haplotypes in Iranian patients may be generated through sexual recombination within the host.

**Keywords** *Pneumocystis jirovecii*, immunosuppressed patients, genotyping, Internal Transcribed Spacer 1 (ITS1), Internal Transcribed Spacer 2 (ITS2), ribosomal DNA, DNA sequencing

Introduction

One of the major causes of morbidity and mortality in immunosuppressed patients, especially those positive for Human Immunodeficiency Virus (HIV), is *Pneumocystis* pneumonia (PcP) [1]. Previous molecular epidemiological studies have revealed that, worldwide, there are many strain types of this microorganism [2–14]. *Pneumocystis* biodiversity [14], environmental reservoirs [13,15], person-to-person transmission [16,17], recurrent infection [18], subclinical colonization and carriage [19,20], clinical manifestations [21], and sulfa exposure and suspected treatment or prophylaxis failures [22–24] have all been demonstrated by typing studies. A variety of typing methods have been used for *Pneumocystis jirovecii* genetic analysis and a large number of gene loci have been examined [25]. To detect biodiversity and to type *P. jirovecii* isolates [25], direct DNA sequencing of the Internal Transcribed Spacer 1 (ITS1), located between the 18S and 5.8S ribosomal RNA (rRNA) genes, and Internal Transcribed Spacer 2 (ITS2), located between the 5.8S and 26S rRNA genes, have been performed [26]. These two regions are useful for investigating variation among *P. jirovecii* isolates [25].

Although *P. jirovecii* was reported in Iranian orphans in 1951 [27], there have been no recent studies addressing *P. jirovecii* in Iran. For this reason, we used the direct DNA sequencing of the ITS1 and ITS2 regions to
investigate the molecular epidemiology of *P. jirovecii* isolates from Iranian immunosuppressed patients.

**Materials and methods**

**Patients and *P. jirovecii* isolates**

A total of 449 respiratory specimens were collected during 2010–2011 from 372 Iranian immunosuppressed patients (196 samples from 130 HIV positive patients, 153 samples from 153 cancer patients undergoing chemotherapy and 100 samples from 89 Acute Exacerbation Chronic Obstructive Pulmonary Disease (AECOPD) patients) at two referral centers for HIV/AIDS and tuberculosis and lung disease in Iran during 2010–2011. Respiratory specimens included sputum (119 samples), induced sputum (105 samples), bronchoalveolar lavage fluid (183 samples), tracheal aspiration (three samples) and nasopharyngeal washes (39 samples).

The mean age of the HIV infected patients was 35 ± 10 years, who all had CD4 T cell counts below 200 cells per microliter (mean = 93 cells per microliter) and had been admitted due to pneumonia or other pulmonary symptoms. All HIV positive patients received Highly Active Anti Retrovirus Therapy (HAART) and co-trimoxazole as prophylaxis against PcP.

The non-HIV positive immunosuppressed patients were divided into two groups, i.e., AECOPD and cancer patients undergoing chemotherapy. The mean age of AECOPD patients was 66 ± 11 years, all were male and 35% were categorized in stage III or IV of infection. The mean age of the cancer patients was 43 ± 11 years, 51.6% were male, and pneumonia was diagnosed in 67/153 of them (44%).

*P. jirovecii* infection was diagnosed by molecular assay of samples collected from cancer and AECOPD patients, but HIV positive patients were also diagnosed through the use of Giemsa staining. For molecular diagnosis, DNA was extracted from respiratory samples using the QIAamp DNA MiniKit (Qiagen), as per the manufacturer’s instructions. Subsequently, a 260 bp fragment of the mitochondrial large subunit of rRNA gene (mt LSU rRNA) was amplified by nested PCR [28, 29]. In total, *P. jirovecii* was detected in 37 samples (8.2%). PcP was diagnosed in 12.3% of HIV positive patients. *P. jirovecii* DNA was also detected in 7/89 (7.9%) of AECOPD patients and in 11/153 (7.2%) of cancer patients. *P. jirovecii* was potentially the cause of active disease in 7/67 (10.5%) of cancer patients suffering from pneumonia. All positive samples were used to determine the genetic pattern of *P. jirovecii* isolates.

**P. jirovecii** typing

*P. jirovecii* isolate genotypes were identified based on analysis of the ITS1 and ITS2 sequences. To amplify the ITS1 and ITS2 sequences, a nested PCR assay was performed with two pairs of primers specific for *P. jirovecii*: 1724F (5′-AAG TGG ATC ATA AAA TTT GTGTC-3′) with ITS2R (5′-CTC GGA CGA GGA TCC TCG CC-3′) for the first round, as previously described [26], and ITS1F (5′-CGT AGG TGA ACC TGC GGA AGG ATC-3′) and ITS2R1 (5′-GTT CCG AGG ATC CCT CTT G-3′) [30] for the second round. Similar PCR conditions were used for both rounds of PCR. Reactions contained 1 μM of each primer, 3 mM MgCl2, 10 mM Tris HCl, 200 μM of each deoxynucleoside triphosphate (dNTP set; Fermentas), and 1 U HotstarTaq plus DNA polymerase (Qiagen) in a 50 μl reaction mixture. The first round contained 50–100 ng of extracted DNA and the second round of PCR used 5 μl of the first-round PCR product. PCR thermal conditions were slight modifications of those previously described [26,30]; the first PCR reactions was done with denaturation at 94°C for 1.5 min, annealing at 62°C for 1.5 min, and extension at 72°C for 2 min for five cycles, followed by 35 cycles of 94, 60 and 72°C, each for 1 min. The second PCR reaction had five cycles at 94, 58, 72°C, each carried out for 1.5 min, followed by 35 cycles of 94, 56, 72°C, each for 1 min. PCR products were analyzed by 1.5% agarose gel electrophoresis. PCR products were purified and sequenced, using primers ITS1F and ITS2R1 and an ABS 3100 DNA sequencer, by Genefnavaran (Iran).

Sequence alignment was performed with Clustal W Software version 1.81 and a phylogenetic tree drawn by maximum likelihood software with bootstrap set at 5000. To identify the ancestral haplotype in these isolates, TCS analysis was performed.

ITS1 and ITS2 alleles were characterized using the score previously described by Lee et al. [26]. *P. jirovecii* ITS types were defined by the combination of the alleles of the two loci. Any new insertion or deletion mutations were confirmed by repeating the analysis. Cases with confused sequence patterns (observation of two or three peaks instead of one peak in the chromatogram) were considered as a mixed infection and PCR studies were repeated to confirm the result. GenBank accession numbers of sequences are JQ365707–JQ365749.

The study was approved by the Ethical Committees of Tarbiat Modares University, Iranian HIV/AIDS Research Center and Mycobacteriology Research Center. Written informed consent was obtained from all subjects.

**Results**

In this study we recovered 37 isolates of *P. jirovecii* from three different kinds of immunosuppressed patients and used nested PCR and direct sequencing to type them based on ITS1 and ITS2 regions of rRNA operon genes. In total,
44 genotypes were detected, due to mixed infection in seven cases.

Direct sequencing identified a total of nine ITS1 genotypes, eight known (A, B, C, E, G, H, N and O) and one novel that had a deletion of a T in region 62–70 bp of ITS1. This genotype had been found in isolates recovered from two HIV positive patients and named it EdelT because its sequence was similar to genotype E. The deletion was confirmed by repeating the entire procedure. Eight known ITS2 genotypes were detected (b, c, e, g, h, i, j and n). Type E was the most frequent ITS1 genotype 21/44, 47.7%) and type g for ITS2 (22/44, 50%).

The combination of ITS1 and ITS2 described nineteen P. jirovecii ITS haplotypes, the most frequent were Eg (11/44, 25%), Gg (5/44, 11.4%), Gi (4/44, 9.1%), Ei (3/44, 6.8%) and Hg (3/44, 6.8%). The remaining haplotypes (Ai, Bi, Cg, Eb, Ec, Ee, Eg, EdelTg, Eh, Ej, Gh, Hb, Hg, Ni and On) occurred at frequencies of less than 5% (Table 1). Two new haplotypes (Hb and Hi) were identified in Iranian immunosuppressed patients and have not been reported in any other region of the world.

The phylogenetic analysis revealed that the Iranian haplotypes formed six branches (Fig. 1). The first and biggest branch showed the greatest similarity and highest frequency among the isolates. Type Cg formed an independent branch. The third branch showed the greatest diversity. The fourth branch comprised two sub-branches. The fifth branch did not show any diversity. In the sixth branch two Eg haplotypes were seen which were different from the other Eg haplotypes in the first branch. Type Eg was the most common haplotype and was considered as the ancestral haplotype of Iranian isolates. This network of haplotypes showed two unresolved loops: the first consisted of types Eg, Hg, Gg, Gi and Hi and the other complex loop consisted of two types of Eg with type EdelTg (Fig. 2).

The data demonstrate that seven patients (19%) were infected with more than one haplotype, i.e., one with three haplotypes and the others with two (Table 2). The mixed infection in these patients was confirmed by repeating the entire procedure.

**Discussion**

ITS-based typing of P. jirovecii isolates is a good and reliable method for molecular epidemiologic studies [13]. To the best of our knowledge, this is the first such investigation conducted with Iranian patients, and the first study to compare Iranian P. jirovecii haplotypes with those from other regions of the world [31,32].

Our study demonstrated that most of Iranian P. jirovecii isolates harbored similar ITS diversity as other from other regions of the world [20,33–35]. Although genotype E (47.7%) is the most frequent ITS1 genotype as found elsewhere, two P. jirovecii isolates from two HIV positive patients possessed a novel ITS1 genotype, EdelT. Recently, a similar genotype was reported for the first time in Australia [36] and India [31,36].

Movement of P. jirovecii appears to reflect the classical migration of humans, where the microorganism moved from Africa to south of Iran, reached India, and then made its way to China and Europe, before returning to Africa. It appears that the frequency of ITS1 genotype EdelT increased during its movement to India, where it reached its highest level. The frequency of the allele then decreased during movement to eastern countries, such as Australia and results clearly suggest that it has a low frequency in European countries. We believe this deletion occurred randomly in the ITS1 region, persisted and gradually increased in frequency. It can be distributed to the other regions of the world by movement of the population [37]. We suggest that the EdelT genotype increased from near 0% in Europe and Africa to 4.5% in Iran and reached 16.3% in India [31] and 8% in Australia [36]. It may be presumed that the high frequency of this genotype in India is due to increased ability to infect the human population in this part of the world.

Among ITS2 alleles, allele g was the most common (50%) followed by allele i (25%), h (9%), b (6.8%), with all of the alleles previously reported in other parts of the world [20,26].

**Table 1** Iranian Pneumocystis jirovecii Internal Transcribed Spacer regions haplotypes detected in immunosuppressed patients.

<table>
<thead>
<tr>
<th>ITS haplotype</th>
<th>HIV+ patients No. (%)</th>
<th>Malignant patients No. (%)</th>
<th>AECOPD patients No. (%)</th>
<th>Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ai</td>
<td>1(4.2)</td>
<td>1(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bi</td>
<td>1(12.5)</td>
<td>1(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cg</td>
<td>2(8.3)</td>
<td>1(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eb</td>
<td>1(4.2)</td>
<td>1(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ec</td>
<td>1(8.3)</td>
<td>1(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eg</td>
<td>4(16.6)</td>
<td>3(25)</td>
<td>4(50)</td>
<td>11(25)</td>
</tr>
<tr>
<td>EdelTg</td>
<td>2(8.3)</td>
<td>2(4.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eh</td>
<td>2(8.3)</td>
<td>2(4.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ei</td>
<td>1(4.2)</td>
<td>2(16.7)</td>
<td>3(6.8)</td>
<td></td>
</tr>
<tr>
<td>Ej</td>
<td>1(12.5)</td>
<td>1(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gg</td>
<td>3(12.5)</td>
<td>2(16.7)</td>
<td>5(11.3)</td>
<td></td>
</tr>
<tr>
<td>Gh</td>
<td>1(4.2)</td>
<td>1(8.3)</td>
<td>2(4.5)</td>
<td></td>
</tr>
<tr>
<td>Gi</td>
<td>3(12.5)</td>
<td>1(8.3)</td>
<td>4(9.1)</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>3(12.5)</td>
<td>1(8.3)</td>
<td>1(2.3)</td>
<td></td>
</tr>
<tr>
<td>Hg</td>
<td>3(12.5)</td>
<td>1(8.3)</td>
<td>1(2.3)</td>
<td></td>
</tr>
<tr>
<td>Hi</td>
<td>1(4.2)</td>
<td>1(12.5)</td>
<td>1(2.3)</td>
<td></td>
</tr>
<tr>
<td>On</td>
<td>1(4.2)</td>
<td>1(0.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24(54.5)</td>
<td>12(27.3)</td>
<td>8(18.2)</td>
<td>44(100)</td>
</tr>
</tbody>
</table>

*ITS haplotypes are formed by the combination of ITS1 and ITS2 regions. AECOPD: Acute Exacerbate Chronic Obstructive Pulmonary Disease.*
Fig. 1  Phylogenetic analysis of Iranian haplotypes isolated from immunosuppressed patients based on likelihood analysis. Bootstrap = 5000.
Studies have confirmed that a high diversity of *P. jirovecii* ITS types exist in different populations in different geographical areas [14,20,26,35,36,38,39]. In most studies, a relatively small number of samples were analyzed, therefore determining the distribution of types in different geographical areas is difficult. To date, Eg has been the most common type found worldwide [26,36,40], similar to our results from Iran. The second most common haplotype isolated from Iranian immunosuppressed patients was Gg (11.3%), similar to South African isolates [40]. Two unique types, Hb and Hi, were detected in Iranian isolates. Unique types of *P. jirovecii* can be found in a specific group of populations [32], allowing these types to be used as genetic markers to study evolution. Our study demonstrated that these unique combination types were found in two patients suffering from cancer who were undergoing chemotherapy, and appear to be restricted to the Mediterranean region. Analysis suggests that type Hi is not a new strain type since four strain types appear to be derived from it. However, type Hb is located in the end of a branch and has no sub-branches, suggesting that this is a new strain type (Fig. 2). The probable cause for appearance of this strain type is recombination due to sexual reproduction or occurrence of mutation in the existing strains.

Fig. 2  ITS haplotype network of Iranian strains. Each line represents a single mutational step connecting two haplotypes. Empty nodes indicate haplotype states that were absent in the samples.
Most Iranian types of \textit{P. jirovecii} were similar to those of European countries, such as Denmark, The Netherlands, France and Portugal, and of African countries, such as the Ivory Coast and South Africa [26]. However, specific haplotypes, such as Ne (the predominant haplotype from England and France) or Eu (reported in South Africa) were not found in Iran. On the other hand, some of our haplotypes, such as Ai, Bi, Eb, Ec, Gg, Gh, were also reported from the USA and Thailand, and others like Ee and Eh have also been described as occurring in the USA [26,33]. Types Eb, Eh, Gg have also been reported in South Africa [40] and it appears that they have worldwide distribution. In Iran, types specific to the USA [26], e.g., Be, Hh, Nb, Nc, or specific to South Africa [40], e.g., Eo, Je, Ge, were not found. In a Thai study a new Ir haplotype was reported as the most frequent type; however, our results were not in accordance with this study [33].

The most common haplotype isolated from AECOPD patients, as was the case with HIV positive patients was Eg (50%), but at significantly higher levels (50% vs. 16.6%; Table 1). It appears that these patients may play a role as reservoirs of infection in the population as suggested by the fact that some less common haplotypes, such as Bi, Cg, Ej and Ni, were found only in this group of patients. This may be due to sexual recombination within the host. The absence of these haplotypes in HIV-positive patients may be due to the time required to adapt to these individuals and given that the age of the HIV-positive patient has been so low it did not allow the genotypes to appear and be detected in the phylogenetic study. We suggest that if this study was continued for a longer period of time, and more strains were isolated from HIV-positive patients, the above-mentioned haplotypes might be found.

Most studies show that mixed infections, with more than one type of \textit{P. jirovecii}, occur in 25–82% of cases [20,32,33,40,41]. In our study, mixed infection was detected in 20.5% of Iranian immunosuppressed patients. It should be noted that mixed infection occurred at much higher frequencies in HIV-positive patients relative to the other types of immunodeficiency disorders. It could be proposed that the type of immunodeficiency and the affected cells are involved in this status. The reason for mixed infection is not clear to us and the question still remains as to whether mixed infection is due to recurrence of an old \textit{P. jirovecii} infection. A single copy of ribosomal RNA (rRNA) has been reported in \textit{P. jirovecii} [42], therefore a single copy is transcribed occurs, with multiple copies suggesting mixed infection. Mixed infection, with more than one type, could be due to recombination within the host. Sexual reproduction has been proven in the life cycle of \textit{P. jirovecii} [3] and could be the origin of the haplotype diversity and occurrence of mixed infections.

As we mentioned above, haplotype network analysis showed that the ancestral haplotype in Iranian isolates was Eg, which is similar to the findings from other studies [8,26,40,43,44]. This suggests that the novel Iranian genotype (EdelT) has evolved from this haplotype, and again suggests that the ITS regions are prone to frequent homoplastic and/or in vivo recombination events [36]. Despite low numbers of positive samples in Iranian immunosuppressed patients, the variation in \textit{P. jirovecii} haplotypes was high. Distribution of \textit{P. jirovecii} types demonstrated that \textit{P. jirovecii} has moved from Europe to Africa to reach Iran, following the Silk Road [45].

In conclusion, in this study two novel Iranian \textit{P. jirovecii} ITS haplotypes and a new genotype were identified. The predominant haplotype was Eg and, like studies from other regions of the world, this haplotype was the ancestral haplotype of Iranian isolates. The two new haplotypes may have arisen in Iranian isolates due to recombination or due to mutation of an old and frequent haplotype. Mixed infections with more than one type of \textit{P. jirovecii} were not rare in the patients investigated.

\textbf{Acknowledgments}

The authors thank Dr Payam Tabarsi (Mycobacteriology Research Center) for presenting HIV-positive patients, and also Dr AliReza Nadji (Virology Research Center) for providing DNA from AECOPD patients and Dr Majid Pirestani (Tarbiat Modares University) for identifying cancer patients, Mrs Leila Moazami (Head nurse of HIV/AIDS Research Center) for collecting samples from HIV-positive patients, and also Mr Kasra Vahidi and Akbar Nikzad Farokhi for editing the text. This work was supported in part by the Iranian HIV/AIDS Research Center, Mycobacteriology Research Center/ National Research Institute of Tuberculosis and Lung Disease, and Tarbiat Modares University.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
ITS haplotype & HIV $^*$ patients & Malignant patients & AECOPD patients \\
& No. (%) & No. (%) & No. (%) \\
\hline
Ec, Eg, Hg & 1/16 (6.25) & 1/34 (2.9) \\
Eb, Eh & 1/16 (6.25) & 1/34 (2.9) \\
Gg, Ai & 1/16 (6.25) & 1/34 (2.9) \\
Eg, Hg & 1/16 (6.25) & 1/34 (2.9) \\
EdelT, Eg & 1/16 (6.25) & 1/34 (2.9) \\
Eg, Gi & 1/11 (9.1) & 1/34 (2.9) \\
Eg, Cg & 1/7 (14.3) & 1/34 (2.9) \\
\hline
Total & 5/16 (31.5) & 1/11 (9.1) & 1/7 (14.3) & 7/34 (20.5) \\
\hline
\end{tabular}
\caption{Frequency of mixed infection with more than one haplotype in Iranian immunosuppressed patients.}
\end{table}
Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

Typing of *Pneumocystis carinii* f. *sp. hominis* isolates from nasopharyngeal aspirates of immunocompetent infants with bronchiolitis by dihydropteroate synthase gene analysis. *J Eukaryot Microbiol* 2001; (Suppl.): 121S.

Genetic diversity at the internal transcribed spacer regions of the rRNA operon among isolates of *Pneumocystis carinii* from AIDS patients with recurrent pneumonia. *J Infect Dis* 1996; 174: 141–156.


This paper was first published online on Early Online on 17 July 2013.