Prevalence of *Pneumocystis jirovecii* colonization in autopsy cases in Turkey

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Detection of *Pneumocystis jirovecii* and its DNA in clinically asymptomatic people is defined as colonization. The aim of this study was to reveal the colonization prevalence of *P. jirovecii* and affecting factors in an immunocompetent population. The study included 200 cases undergoing forensic autopsy between February 2015 and April 2015. The cause of death was non-medical conditions (group 1) in 111 cases (55.5 %), medical conditions (group 2) in 73 cases (36.5 %) and undetermined (group 3) in 16 cases (group 3). Tissue specimens about 1 g in weight were taken from the right upper pulmonary lobe. After DNA extraction, nested PCR targeting mitochondrial large subunit rRNA was used to detect *P. jirovecii*. Of 200 cases, 37 (18.5 %) had *P. jirovecii* DNA. There was not a significant difference in place of living, gender, smoking status and medication use between the cases with *P. jirovecii* and those without *P. jirovecii*. A significantly high rate of *P. jirovecii* colonization was detected in group 2 ($\chi^2=7.674; P=0.022$). *P. jirovecii*-colonized cases also had a chronic disease in 2 of 13 (group 1), 12 of 20 (group 2) and 1 of 4 (group 3) cases ($\chi^2=5.571; P=0.062$). A significantly high rate of the cases aged 0–1 year had *P. jirovecii* (5/11; 45.5 %) ($\chi^2=5.639; P=0.018$). The results of the study suggest that infants and patients with chronic diseases like cardiac or pulmonary diseases can be at risk for *P. jirovecii* colonization.

Since the number of parasites is low, conventional staining techniques fail to show the colonization (Morris & Norris, 2012). With the introduction of PCR-based molecular methods, there has been an increase in studies on *P. jirovecii* colonization. Sensitivity of molecular tests has been increased by using multi-copied mitochondrial large subunit rRNA (mt-LSUrRNA) gene (Morris et al., 2008; Calderon & Dei-Cas, 2010; Morris & Norris, 2012).

People with *P. jirovecii* colonization play an important role in the spread of the agent and can be at risk for PCP development when immunosuppression appears (Morris et al., 2008; Mekinian et al., 2011; Gutiérrez et al., 2011; Tasaka & Tokuda, 2012). It is argued that the colonization may trigger chronic pulmonary diseases, exacerbate diseases already present and cause drug resistance (Morris et al., 2008; Mekinian et al., 2011; Gutiérrez et al., 2011; Calderon et al., 2007). It has been shown in the literature that *P. jirovecii* colonization can be detected in completely healthy people in addition to immunocompromised patients (Peters et al., 1992; Medrano et al., 2005; Nevez et al., 1997, 1999; Maskell et al., 2003).

**INTRODUCTION**

*Pneumocystis jirovecii* is a human-specific *Pneumocystis* and causes opportunistic pulmonary infections called *Pneumocystis jirovecii* pneumonia (PCP) in immunocompromised people (Krajiceka et al., 2008). Detection of *P. jirovecii* in people without clinical or radiological findings of PCP is defined as *P. jirovecii* colonization (Calderon & Dei-Cas, 2010; Morris & Norris, 2012).

**Abbreviations:** mt-LSUrRNA, mitochondrial large subunit ribosomal RNA; nPCR, nested PCR; PCP, *Pneumocystis jirovecii* pneumonia.
METHODS

**Autopsy cases.** Pulmonary tissue specimens taken consecutively from 200 cases autopsied at the İzmir Group Chairman Morgue Department, Council of Forensic Medicine, İzmir, between February 2015 and April 2015 were used. Exclusion criteria were disruption of tissue structure in specimens (severe burns, advanced stages of decomposition and remaining in water for a long time) and/or death after a long hospitalization. As for permanent place of living, 198 cases lived in the Aegean region, and two cases lived outside the Aegean region (in Ankara). Of cases who lived in the Aegean region, 126 lived in İzmir, 35 lived in Manisa, 19 lived in Aydın, 7 lived in Usak, 6 lived in Balıkesir, 4 lived Denizli and 1 case lived in Kutahya (Fig. 1). Data about known diseases, immunosuppressant drugs used, causes of death and descriptive characteristics like age, gender and smoking status were recorded. Ethical approval was obtained from the Non-Invasive Research Ethical Committee of Dokuz Eylül University.

**Collection of pulmonary tissue specimens.** When the chest was opened during the autopsy, approximately 1 g tissue specimen was removed from deep lung tissue through a 2 cm incision in the decorticated surface. The obtained specimens were put in sterile Falcon tubes with screwed caps, and the tubes were kept at −20 °C until they were examined.

**Processing pulmonary tissue specimens.** Prior to processing, tissue specimens were allowed to return to room temperature, and they were sliced into small pieces in the biosafety cabinet. Afterwards, they were homogenized mechanically for 30 min in 20 ml sterile PBS (0.04 M, pH 7.2). The obtained suspension was filtered through sterile ribbon gauze and centrifuged at 690 g for 10 min. Following centrifugation, the pellet was added into 700 µl sterile PBS. Two hundred microlitres of this suspension was used for DNA extraction (Ponce et al., 2010).

**DNA extraction.** DNA extraction was performed with an extraction kit (Macherey-Nagel) in accordance with the manufacturer’s guidelines. Extracted DNA samples were stored at −20 °C until they were used for amplification. Ultrapure distilled water was used as negative control, and *P. jirovecii* isolate obtained from a previously diagnosed PCP patient was used as positive control (Özkoç et al., 2010).

**nPCR assay for *P. jirovecii*.** In nPCR, *P. jirovecii* mt-LSU rRNA gene region was targeted. In the first PCR round, the primers pAZ102-E (5′-GATGCGCTTTCTAAGCCCA-3′) and pAZ102-H (5′-GTGTAATGTTGCAAGTACCTC-3′) were used, and in the second PCR round, pAZ102-X (5′-GTGAAATACAAATCGGACTAGG-3′) and pAZ102-Y (5′-TCACCTTAATATATTGGAAGGC-3′) were used (Tia et al., 2012). For each reaction, 2.5 µl 10× reaction buffer, 2.5 µl MgCl2 (25 mM stock solution), 2.5 µl dNTP (2 mM stock solution), 1 µl Taq DNA polymerase (1 U µl −1 stock solution), 0.75 µl primer (10 µM stock solution) and 5 µl DNA sample were added, and the final volume was completed to 25 µl with distilled water. Amplification was performed under the following conditions: after an initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, at 56 °C for 1 min, at 72 °C for 1.5 min and final extension at 72 °C for 5 min (Tia et al., 2012). For the second round, a 2 µl specimen taken from the first amplification product was amplified under the same conditions as in the first reaction. To evaluate amplification of target DNA, 10 µl amplification products were analysed by electrophoresis on 1.5% agarose gel under 100 V for 1 h. Then the gel was stained with 1 µg ml −1 ethidium bromide and visualized under UV light. The detection of an amplicon 267 bp in length showed that a specimen was positive for *P. jirovecii* (Tia et al., 2012). PCR was repeated three times for each specimen, for confirmation of results.

Statistical analysis. Data were analysed with Statistical Package for Social Sciences 15.0 program. Chi-squared test and t-test were used to compare characteristics of patients positive and negative for *P. jirovecii*. P<0.05 was considered significant.

RESULTS

**Demographic features of patients**

Of 200 cases, 143 (71.5%) were male, and 57 (28.5%) were female. The median age of the cases was 49 years (range, 1–86 years). Eleven cases were infants (aged 0–1 year). Of the remaining cases, 6 were aged 1–9 years, 10 were aged 10–19 years, 55 were aged 20–39 years, 50 were aged 40–59 years and 68 were older than 60 years.

At autopsy, the possible cause of death was non-medical conditions in 111 cases (55.5%; group 1), medical conditions in 73 cases (36.5%; group 2) and undetermined in 16 cases (8%; group 3) (Table 1).

All the infant cases were found dead at home or were dead when they were brought to hospital. None of the cases had a history of hospitalization for more than 1 day at birth or later. Two infants died 1–2 days after birth. In autopsy reports, the cause of death was not stated in seven infants and was suspected pneumonia and/or there were signs of pneumonia in the other four.

None of the cases received immunosuppressive therapy. Only one infant was thought to have primary immunosuppression due to suspected Griscelli syndrome. Ninety-seven cases were found to be active smokers (based on patient records and history taken from family members). Thirty-seven patients were found to take medications regularly for chronic diseases including diabetes, hypertension, coronary artery disease, chronic obstructive lung disease, asthma, thyreoiditis and epilepsy.

**nPCR results of *P. jirovecii***

nPCR revealed that 37 of 200 cases (18.5%) were positive for *P. jirovecii* DNA (Fig. 2). The distributions of *P. jirovecii* according to possible cause of death are shown in Table 1.

There was not a significant difference in the distribution of cases positive and negative for *P. jirovecii* DNA in terms of place of living, age, gender, smoking status, chronic diseases and medication use (Table 2). However, a significantly high rate of the cases in group 2 was positive for *P. jirovecii* DNA ($\chi^2=7.674; P=0.022$). *P. jirovecii*-colonized cases also had a chronic disease in 2 of 13 (group 1), 12 of 20 (group 2) and 1 of 4 (group 3) cases ($\chi^2=5.571; P=0.062$).

The difference between infants and older people with respect to *P. jirovecii* positivity was significant ($\chi^2=5.639; P=0.018$) (Table 2). Of seven infants with unknown causes of death, two had *P. jirovecii* colonization, and of four infants with suspected pneumonia, three were positive for *P. jirovecii* DNA.
DISCUSSION

In Turkey, studies on *P. jirovecii* have mostly been directed towards evaluation of efficacy of diagnostic methods and give indirect information about its colonization. They were performed on different groups of patients, and various regions like mt-LSUrRNA, internal transcribed spacer and major surface glycoprotein gene were amplified with PCR in those studies. They showed *P. jirovecii* positivity in 8–24% of the cases (Günes et al., 2004; Döskaya et al., 2011; Tekinsen et al., 2013; Tosun et al., 2013; Özmen et al., 2013). In prior studies, we also evaluated different groups of patients with the method we used in the present study. In one study, we showed that 70% of 30 patients with underlying pulmonary diseases had *P. jirovecii* colonization. In another study, we found that 33.7% of 92 patients, most of whom were immunocompromised, were positive for *P. jirovecii* (Özkoç et al., 2014; Özkoç & Delibaş, 2015). To our knowledge, the present study is the first to examine pulmonary specimens of a group of patients outside the hospital and showed *P. jirovecii* positivity in 18.5% of the cases.

*P. jirovecii* colonization may appear in varying rates of immunocompetent individuals using PCR analysis (Nevez et al., 1997, 1999; Maskel et al., 2003). Ponce et al. (2010), in their study in Chile, revealed that 55 of 77 autopsy cases were positive for *P. jirovecii*. Another study revealed that bronchoalveolar lavage fluid specimens from 20% of 169 cases with respiratory symptoms but without PCP were positive for *P. jirovecii* (Nevez et al., 1997). Medrano et al. (2005) showed *P. jirovecii* colonization in oral lavage fluid from 20% of 50 healthy cases. They also underlined that these cases did not have a known contact with patients with PCP (Medrano et al., 2005). However, there have been
studies reporting no *P. jirovecii* positivity in nasal swabs, sputum specimens, induced sputum specimens and pulmonary tissue specimens (Peters et al., 1992; Leigh et al., 1993; Nevez et al., 2006; Morris et al., 2008). The conflicting results of the studies show that different patient groups, different methods used and different geographical regions may affect *P. jirovecii* colonization. None of the cases in the present study, except an infant with suspected Griscelli syndrome (primary immunosuppression), had immunosuppression. Therefore, the results of this study can be considered as reflection of *P. jirovecii* colonization in an immunocompetent sample of the Turkish population.

There has been conflicting results that several features like gender and smoking affect *P. jirovecii* colonization. Fritzsche et al. (2012) detected no relation between smoking and *P. jirovecii* colonization, while Vidal et al. (2006) reported that a significantly high rate of the patients with interstitial disease and *P. jirovecii* colonization were smokers. Calderon et al. (2007) showed similar results in their study on chronic obstructive lung disease patients with *P. jirovecii* colonization. However, Mekinian et al. (2011) found that a significantly high rate of the non-smoker patients had *P. jirovecii* colonization. They also indicated that a significantly high rate of the male patients had *P. jirovecii* colonization (Mekinian et al., 2011). The present study showed no relation between *P. jirovecii* positivity and

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<th>Table 1. The distribution of <em>P. jirovecii</em> according to possible causes of death at autopsy</th>
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<tr>
<td><strong>No. of cases, n (%)</strong></td>
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<tr>
<td>Deaths due to non-medical conditions (group 1)</td>
</tr>
<tr>
<td>1. Traffic accidents</td>
</tr>
<tr>
<td>2. Firearm injuries</td>
</tr>
<tr>
<td>3. Hanging</td>
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<tr>
<td>4. Poisoning</td>
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<tr>
<td>(carbon monoxide, medications, alcohol, drugs)</td>
</tr>
<tr>
<td>5. Other</td>
</tr>
<tr>
<td>Drowning</td>
</tr>
<tr>
<td>Fall from height</td>
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<tr>
<td>Electric shock</td>
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<tr>
<td>Sharp force injuries</td>
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<tr>
<td>Burns</td>
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<tr>
<td>Deaths due to medical conditions (group 2)</td>
</tr>
<tr>
<td>1. Cardiovascular conditions, myocardial infarction</td>
</tr>
<tr>
<td>2. Pulmonary infection, oedema</td>
</tr>
<tr>
<td>3. Subarachnoid haemorrhage</td>
</tr>
<tr>
<td>4. Other</td>
</tr>
<tr>
<td>Pericardial tamponade</td>
</tr>
<tr>
<td>Epileptic seizures</td>
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<td>Intestinal obstruction</td>
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<td>Undetermined (group 3)</td>
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Figs. 2. nPCR results of a group of cases. M, marker; NC, negative control; PC, positive control (267 bp).

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<th>Table 2. A comparison of demographic features between cases positive for <em>P. jirovecii</em> colonization and those negative for <em>P. jirovecii</em> colonization</th>
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<tr>
<td><strong>P. jirovecii nPCR (+) (n=37)</strong></td>
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<tr>
<td>Mean age (range, years)</td>
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<tr>
<td>Gender, male, n (%)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
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<tr>
<td>Medication use, n (%)</td>
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<tr>
<td>Infants, n (%)</td>
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gender and smoking. Also, none of the cases had a history of corticosteroid or other immunosuppressive agent use, which is considered as a risk for \textit{P. jirovecii} colonization.

Serological and molecular studies have pointed out that \textit{P. jirovecii} colonization more frequently appears in children and especially in those with lower pulmonary tract infections (Morris \textit{et al.}, 2008; Morris & Norris, 2012; Vargas \textit{et al.}, 2001; Nevez \textit{et al.}, 2001). Autopsy studies in infants showed \textit{P. jirovecii} positivity in pulmonary specimens from 10 to 100% of the cases (Morris \textit{et al.}, 2008; Beard \textit{et al.}, 2005; Vargas \textit{et al.}, 1999, 2005). One of these studies showed a significant relation between \textit{P. jirovecii} positivity and sudden infant death syndrome (Vargas \textit{et al.}, 1999). In the present study, the difference in \textit{P. jirovecii} positivity between the infants (45.5%) and the general population (18.5%) was statistically significant. However, because of the small number of infant cases, the relation between \textit{P. jirovecii} colonization and sudden infant death syndrome and pneumonia could not be evaluated. In addition, 33.3% of the children younger than 10 years (1–9 years) had \textit{P. jirovecii} positivity. Consistent with the literature, the current study showed that \textit{P. jirovecii} is encountered in the early years of life.

A significantly high rate of the cases in group 2 were positive for \textit{P. jirovecii}. In addition, the relation between \textit{P. jirovecii} colonization and presence of chronic diseases was statistically significant in this group. This suggests that underlying diseases can pose a risk of \textit{P. jirovecii} colonization. Another striking feature of group 2 was that the cases were older than the other groups (median age, 60 years). Both old age and high possibility of presentation to hospital might have increased the risk of contact with \textit{P. jirovecii}. Age is also known to be an important risk factor for the distribution of hospital-associated \textit{P. jirovecii}, which can be transmitted from person to person (Medrano \textit{et al.}, 2005; Tasaka & Tokuda, 2012).

In the present study, of 73 cases in group 2, 59 (81%) died of cardiovascular or other conditions, and 14 (19%) died of pulmonary infections (pneumonia and its complications). On PCR, of the 14 cases who died of pneumonia, 5 (35.7%) were positive for \textit{P. jirovecii} DNA, and of 59 cases who died of other conditions, 15 (25.4%) were positive for \textit{P. jirovecii} DNA. Although the rate of \textit{P. jirovecii} positivity was higher in the cases who died of pulmonary conditions, the difference between the two groups was not statistically significant ($P=0.061$). Since the microbiological identification could not be done in those pulmonary infections, we do not know whether the causative agents of pneumonia were \textit{P. jirovecii} and/or other agents.

To the best of our knowledge, this is the first study to reveal the prevalence of \textit{P. jirovecii} colonization in immunocompetent people in our country. We could say that a considerable rate of the Turkish population have \textit{P. jirovecii} colonization. Although the results of this study showed that infants and patients with chronic diseases were at risk for \textit{P. jirovecii} colonization, further studies with larger sample sizes are needed to obtain more reliable data.

**ACKNOWLEDGEMENTS**

This work was supported by the Scientific and Technological Research Council of Turkey (TÜB TAK; project no. SBAG-214S052). We also thank the Izmir Group Chairman, Council of Forensic Medicine (Republic of Turkey Ministry of Justice), for the support and permission given during the preparation of the present scientific study.

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Pneumocystis jirovecii in autopsy cases


