Degree and frequency of inhibition in a routine real-time PCR detecting *Pneumocystis jirovecii* for the diagnosis of *Pneumocystis* pneumonia in Turkey

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Routine laboratory diagnosis of *Pneumocystis jirovecii* is currently achieved by PCR in almost all laboratories with sufficient equipment due to its high sensitivity and specificity compared to staining methods. A current issue that limits the reliability and sensitivity of PCR is the degree of inhibition caused by inhibitory substances in respiratory samples. The present study aimed to analyse the degree and frequency of inhibition in real-time PCR detecting *P. jirovecii* in respiratory specimens submitted to a *Pneumocystis* pneumonia (PcP) diagnosis laboratory in Ege University Medical School, Turkey. Between July 2009 and December 2010, 76 respiratory specimens [63 bronchoalveolar lavage (BAL) fluid, 10 sputum samples, two tracheal aspiration fluid and one thoracentesis fluid] obtained from 69 PcP-suspected patients were investigated for the presence of *P. jirovecii* using real-time PCR targeting the *cdc2* gene. Of these samples, 42 of the specimens were stained and examined by microscopy according to the request of the clinicians. PCR was positive in 15 specimens in the initial run. Of the remaining 61 samples, 41 of them were negative with positive internal inhibition controls (i.e. true-negative group). The frequency of inhibition in the initial run was 26.31 % (20/76) as determined by spiked negative controls. All of the inhibited samples were resolved after 1 : 2, 1 : 5, 1 : 10 and 1 : 20 dilutions. *P. jirovecii* was detected by PCR in two inhibited specimens after retesting with diluted samples which were also positive by microscopy. The incidence of *P. jirovecii* in respiratory specimens was 22.36 % (17/76) as determined by real-time PCR and 7.14 % (3/42) by microscopy. Overall, the incidence of *P. jirovecii* in respiratory samples was 23.68 % (18/76) as detected by both methods. In conclusion, inclusion of spiked positive controls in each sample and retesting with diluted samples to resolve inhibition increased the reliability of the real-time PCR assay in terms of determining false-negative results and influencing the treatment of the patient. Furthermore, results of the present study determined for the first time the frequency and degree of inhibition in a real-time PCR detecting *P. jirovecii* in respiratory specimens during routine diagnosis of PcP.

INTRODUCTION

*Pneumocystis jirovecii* most commonly causes *Pneumocystis* pneumonia (PcP) in patients with AIDS and patients receiving intensive or prolonged immune suppressive treatment for malignancy, transplantation and immune disorders (Lu et al., 1995; Wakefield, 2002; Gianella et al., 2010; Louie et al., 2010). Currently, PcP most commonly occurs as an AIDS-associated illness in patients who are not aware that they are HIV-positive or those who are not receiving combined anti-retroviral therapy (Lu et al., 1995; Wakefield, 2002).

Since *P. jirovecii* cannot be cultured, definitive diagnosis of pneumocystosis is usually achieved by the demonstration of *Pneumocystis* in respiratory samples using staining methods, immunofluorescence techniques using anti-*Pneumocystis* antibodies and PCR (Lu et al., 1995; Kaiser et al., 1999; Wakefield, 2002). Staining methods, such as Gram Weigert, Wright–Giemsa, modified Papanicolaou, calcofluor white, cresyl echt violet, Giemsa, Gomori methenamine silver or toluidine blue O and more sensitive monoclonal antibodies, have been developed for...
identifying *P. jirovecii* in respiratory specimens (Carmona & Limper, 2011).

In the 1990s, the introduction of PCR facilitated the diagnosis of *P. jirovecii* by offering increased sensitivity and specificity compared to methods using staining and monoclonal antibodies and by decreasing the requirement for experienced personnel to differentiate *P. jirovecii* from artefacts and other non-specific staining (Arcenas et al., 2006; Rohner et al., 2009). To diagnose PcP, various *P. jirovecii* genes were targeted by PCR, such as major surface glycoprotein (MSG) (Huang et al., 1999; Fischer et al., 2001; Helweg-Larsen et al., 2002; Larsen et al., 2002b, 2004; Flori et al., 2004; Linssen et al., 2006; Fillaux et al., 2008), dihydropteroylate synthase (Demanche et al., 2001; Alvarez-Martinez et al., 2006; Linssen et al., 2006; Jiancheng et al., 2009), dihydrofolate reductase (Schluger et al., 1991; Lu et al., 1995; Larsen et al., 2002a; Bandt & Monecke, 2007), internal transcribed spacer regions of the rRNA (Lu et al., 1995; Torres et al., 2000), mitochondrial large subunit rRNA (mtLSU rRNA) (Wakefield et al., 1990; Leibovitz et al., 1995; Lu et al., 1995; Wakefield, 1996; Huang et al., 1999; Kaiser et al., 2001; Olsson et al., 2001; Helweg-Larsen et al., 2002; Flori et al., 2004; Gupta et al., 2009; Jiancheng et al., 2009; Choukri et al., 2010), mitochondrial small-subunit rRNA (Hunter & Wakefield, 1996; Helweg-Larsen et al., 2002), 5S rRNA (Kitada et al., 1991; Wakefield et al., 1991; Lu et al., 1995; Ribes et al., 1997; Sandhu et al., 1999; Pinlaor et al., 2004), 18S rRNA (Lipschik et al., 1992; Lu et al., 1995), 28S rRNA (Sandhu et al., 1999), HSP70 (Huggett et al., 2008), β-tubulin (Brancart et al., 2005; Rohner et al., 2009), thymidylate synthase (Olsson et al., 1993; Lu et al., 1995), Kox-1 (Rohner et al., 2009) and the cyclin-dependent kinase gene (*cdc2*; Kaiser et al., 2001; Arcenas et al., 2006).

Recently, development of real-time PCR platforms has increased specificity by decreasing cross-contamination, enabling quantification of target gene copies and rapid turnover time per sample (Kaiser et al., 2001; Larsen et al., 2002a, b, 2004; Flori et al., 2004; Brancart et al., 2005; Alvarez-Martinez et al., 2006; Arcenas et al., 2006; Linssen et al., 2006; Bandt & Monecke, 2007; Fillaux et al., 2008; Huggett et al., 2008; Jiancheng et al., 2009; Choukri et al., 2010).

A current issue that limits the reliability and sensitivity of diagnostic PCR systems is the inhibition of PCR. Inhibitors in blood, faeces, respiratory specimens and other complex biological samples have been shown to inhibit PCR and cause false-negative results (Wilson, 1997; Kern et al., 2009). To reduce the likelihood of false-negative results due to inhibition, internal inhibition controls targeting human genes or plasmids containing target sequences were included in the majority of diagnostic *P. jirovecii* PCR development studies. However, the degree and frequency of inhibition in any PCR assay detecting *P. jirovecii* during routine diagnosis of PcP has, to our knowledge, not yet been investigated. Thus, the present study aimed to analyse the frequency and degree of inhibition in real-time PCR detecting *P. jirovecii* in respiratory specimens from patients admitted to Ege University Medical School in Turkey.

**METHODS**

**Patients and respiratory specimens.** Between July 2009 and December 2010, 76 respiratory specimens [63 bronchoalveolar lavage (BAL) fluid, 10 sputum samples, two tracheal aspiration fluid and one thoracentesis fluid] obtained from 69 patients (18 female and 51 male) with clinically suspected PcP admitted to Ege University Medical School were investigated for the presence of *P. jirovecii* using microscopy and real-time PCR. Among these patients, four were infected with HIV and the remainder were patients receiving intensive or prolonged immunosuppressive treatment. As requested by clinicians, real-time PCR was performed on all specimens, and 42 of the specimens were stained and examined by microscopy. An institutional review board of Ege University approved the use of all specimens included in this study.

**Giemsia and Gram Weigert staining.** Initially, one volume of mucus-containing specimens was treated with two volumes of 0.1 % (w/v) diethiothreitol. Then, each specimen was centrifuged at 1500 g for 5 min and the supernatant was discarded. Specimens containing red blood cells were additionally treated with an equal volume of 0.1 % (w/v) saponin and centrifuged at 1500 g for 5 min. After discarding the supernatant, resuspended specimens were cytocentrifuged at 1500 g for 5 min, air-dried and stained by the Giemsa and Gram Weigert methods as described previously for examination of the presence of *Pneumocystis carinii* cysts and trophozoites (Rosen et al., 1975; Gill et al., 1988; Walker et al., 1989).

During Giemsa staining, methanol-fixed slides were covered with 10 % (v/v) Giemsa solution and incubated for 30 min at room temperature. Then, slides were washed with distilled water to remove excess dye and examined under light microscopy with immersion oil.

During Gram Weigert staining, air-dried slides were stained with 1 % Eosin-Y solution for 5 min. Then, slides were rinsed with distilled water for 2 min to remove excess dye and stained with crystal violet solution [5 % (w/v) crystal violet; 10 % (v/v) ethanol; 2 % (v/v) aniline oil] for 5 min. Excess crystal violet was rinsed off with Gram’s iodine solution (3.61 mM potassium iodide; 1.18 mM iodine). Rinsed and blot-dried slides were washed with aniline oil/xylene solution [50 % (v/v) aniline; 50 % (v/v) xylene] for decolorization. Further decolorization was stopped by xylene washing. Slides were examined by two qualified parasitologists under light microscopy with immersion oil.

**Specimen processing and DNA extraction.** Initially, specimens were processed as described for DNA extraction (Flori et al., 2004; Binnicker et al., 2007; Khot et al., 2008). Briefly, 8–10 ml raw respiratory fluid (BAL, tracheal aspiration and thoracentesis fluids) was centrifuged at 3000 g for 10 min. The supernatant was carefully discarded using a pipette and the pellet was resuspended in 500 μl supernatant. Sputum samples were used directly without any centrifugation. Five hundred microlitres of resuspended pellet or sputum was added to a 1.5 ml tube containing 100 μl proteinase K (Qiagen), 0.1 mm glass beads and 2.0 mm zirconia beads (BioSpec Products). Samples were then incubated at 55 °C for 15 min at 1400 r.p.m. on a thermomixer (Lab4You) and subsequently incubated at 95 °C for 5 min. After incubation, samples were vortexed for 2 min on a Disruptor Genie (Scientific Industries) to facilitate lysis and release of nucleic acid. Next, samples were centrifuged briefly at 3000 g, and processed specimens, collected from the bottom of the tube, were used for DNA extraction. Isolation of DNA was performed with the QIAamp DNA mini kit according to the manufacturer’s
LightCycler PCR. Real-time PCR targeting the cdc2 gene of P. jirovecii (GenBank accession no. AF026546) was performed as described previously (Arcenas et al., 2006). Briefly, the primers used for amplifying the 166 bp cdc2 gene fragment were 5'-AGTGAGCTGGATTACAAAA-3' (20 nt, forward primer) and 5'-CCTGTTGCT-TGGAAACC-3' (16 nt, reverse primer). The FRET hybridization probes were 5'-GATCCAAAGGGACCAATGATG-fluorescein-3' (25 nt) and 5'-Red-640-TTAAAAATCCTGGCTAGAACGA-AG-phosphate-3' (27 nt) (TIB Molbiol). Each 20 μl PCR mix included 5 μl purified patient DNA template or controls, 1× FastStart mix (Roche), 4 mmol MgCl₂ l⁻¹, 0.4 μmol Red-640 labelled probe I⁻¹ and 0.5 μmol each primer I⁻¹, PCR amplification reactions were performed using the following calculated control protocol: 10 min preincubation step at 95 °C, followed by 45 cycles of 10 s at 95 °C, 15 s at 55 °C and 15 s at 72 °C.

A positive control plasmid containing a P. jirovecii cdc2 gene fragment was prepared as described previously (Arcenas et al., 2006). Positive controls contained 10-fold dilutions of positive control plasmid ranging from 6 x 10⁶ to 6 copies of cdc2 μl⁻¹. P. jirovecii-negative and -positive respiratory samples determined by previous PCR and microscopy methods were used as external controls. One negative control, prepared by replacing template DNA with distilled water, was used in each run. Quantification analysis for each sample was performed with a 1.2 LightCycler Real-time instrument using LightCycler software version 4.0 (Roche).

Assessment of the degree of PCR inhibition in respiratory samples. To analyse the PCR inhibition attributable to the specimen matrix, PCRs were prepared in duplicate for each patient. One of the reactions contained only purified patient DNA sample and the other reaction contained 10 copies of cdc2 μl⁻¹ spiked into the purified patient sample. To generate a crossing point threshold (CPₚ) mean value and SD, three reactions containing 10 copies of cdc2 μl⁻¹ in distilled water were run. Observed CPₚ values for the spiked respiratory sample containing positive control plasmid differing by more than 2.5 SD from the mean CPₚ value were considered to be indicative of PCR inhibition. Subsequently, additional PCR runs were performed in duplicate with 1:2, 1:5, 1:10 and 1:20 dilutions of patient DNA samples to assess the degree of inhibition, and analysed as described above.

RESULTS

Results of staining and initial real-time PCR
According to the results of the Giemsa- and Gram Weigert-stained respiratory specimens, P. jirovecii was identified in three BAL fluids by microscopy (7.14%; 3/42).

The mean CPₚ value and SD of real-time PCR detecting P. jirovecii was 35.41 ± 0.19 as determined by spiked runs containing internal control plasmid. According to the CPₚ values of 76 respiratory samples and their spiked controls, among the 17 P. jirovecii real-time PCR-positive samples, 15 samples (12 of them were BAL fluids and three were sputum samples) were positive during the initial run, wherein internal inhibition controls were also positive (i.e. true-positive group) (Table 1). Among the remaining 61 samples, 41 were negative in PCRs which internal inhibition controls were positive (i.e. true-negative group).

The frequency of inhibition in the initial run was 26.31% (20/76) as determined by negative spiked controls.

Among the 15 microscopy-negative and PCR-positive samples, real-time PCR detected P. jirovecii in 11 specimens in the initial run. One of the microscopy-positive samples was real-time PCR-negative in the initial run and the internal inhibition control was also positive (named as a discordant sample). This sample was from an HIV-positive patient. The remaining three HIV-positive patients were positive by PCR and negative by microscopy.

Degree of inhibition assessed by diluted samples
After 1:2 dilution of inhibited samples, inhibition resolved in five samples and inhibition frequency decreased to 19.73% (15/76). Among the 15 inhibited samples, inhibition resolved in six samples after dilution to 1:5 and the inhibition rate reduced to 11.84% (9/76). Further dilution of the nine inhibited samples by 1:10 resolved five more samples. Dilution of the remaining four samples by 1:20 resolved inhibition and two samples belonging to the same patient were PCR-positive (Table 2). Overall, the incidence of P. jirovecii as detected by real-time PCR was 22.36% (17/76).

The inhibition frequency of BAL samples in the initial PCR run was 23.80% (15/63), whereas in sputum the rate of inhibition was 50% (5/10). Inhibition of sputum samples decreased to 40% (4/10) and 20% (2/10) in 1:2 and 1:5 dilutions, respectively, and in the run using 1:10 diluted samples, none of the sputum samples were completely inhibited. Inhibition frequency of BAL samples decreased to 17.46% (11/63), 11.11% (7/63) and 6.34% (4/63) as the samples were diluted 1:2, 1:5 and 1:10, respectively. Inhibition in all of the BAL samples was resolved at 1:20 dilution. Inhibition was not detected in tracheal aspiration or thoracentesis fluids (Table 2).

DISCUSSION

Routine diagnosis of P. jirovecii is currently achieved by PCR in almost all laboratories with sufficient equipment due to its high sensitivity and specificity compared to common staining methods. Numerous conventional, nested or real-time PCR platforms have been developed to detect almost 15 gene targets of P. jirovecii and to increase the diagnostic utility of PCR. However, the degree and frequency of inhibition during routine P. jirovecii PCR still remains an issue to be resolved.

Due to the probability of inhibition, internal controls have been included in diagnostic P. jirovecii PCR protocols. Since a negative PCR result does not necessarily indicate the absence of PcP and may influence the treatment of the patient, a cellular gene sequence which is accepted to be present in all specimens or a plasmid DNA with primer binding regions identical to a target sequence can be used as an internal control to increase the reliability of PCR results (Rosenstraus et al., 1998).
To reduce the likelihood of false-negative results due to inhibition, PCR runs targeting the human β-globin gene (Ribes et al., 1997; Sandhu et al., 1999; Olsson et al., 2001; Pinlaor et al., 2004; Fillaux et al., 2008; Gupta et al., 2009) or plasmids containing the target gene (Fischer et al., 2001; Larsen et al., 2002b, 2004; Helweg-Larsen et al., 2002; Arcenas et al., 2006; Jiancheng et al., 2009), the mouse galactose-1-phosphate uridyltransferase gene (Flori et al., 2004) or a commercial Taqman internal control (Brancart et al., 2005; Choukri et al., 2010) were included in the majority of diagnostic *P. jirovecii* PCR protocols.

Sensitivity and reliability of diagnostic assays can be limited by inhibitory substances in blood, faeces, respiratory and other complex biological samples (Wilson, 1997; Tong et al., 1999; Al-Soud et al., 2000; Levidiotou et al., 2003; Wilson et al., 2004; Maaroufi et al., 2006; Kern et al., 2009). Although the mechanisms of inhibition are often unclear, most of the physical and chemical inhibitory substances either bind to polymerase or interact with sample DNA or/and polymerase during primer extension (Wilson, 1997; Opel et al., 2010). Several types of respiratory specimens, such as sputum, BAL fluid, tracheal aspiration fluid or thoracentesis fluid, can be used for the detection of *P. jirovecii* by PCR. BAL fluids are less likely to contain inhibitors compared to sputum specimens (Raggam et al., 2002; Apfalter et al., 2003; Heginbothom et al., 2003; Iinuma et al., 2003; Levidiotou et al., 2003; Wilson et al.,

### Table 1. Results of PCR performed on 76 respiratory specimens in comparison with 42 samples examined by microscopy

At the request of the clinicians, real-time PCR was performed on all specimens and 42 of the specimens were stained and examined by microscopy. NA, Not available.

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Total</th>
<th>Microscopy-positive (n=42)</th>
<th>PCR-positive (n=76)</th>
<th>Microscopy- or PCR-positive</th>
<th>Microscopy- and PCR-positive</th>
<th>Microscopy-positive, PCR-negative</th>
<th>Microscopy-negative, PCR-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td>63</td>
<td>3</td>
<td>14</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Sputum</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Tracheal aspiration fluid</td>
<td>2</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thoracentesis fluid</td>
<td>1</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>3</td>
<td>17</td>
<td>18</td>
<td>2</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

### Table 2. Degree and frequency of inhibition of real-time PCR detecting *P. jirovecii* in 76 respiratory specimens

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Initial run (no dilution)</th>
<th>Second run (1:2 dilution)</th>
<th>Third run (1:5 dilution)</th>
<th>Fourth run (1:10 dilution)</th>
<th>Fifth run (1:20 dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninhibited samples</td>
<td>48</td>
<td>52</td>
<td>56</td>
<td>59</td>
<td>63</td>
</tr>
<tr>
<td>Inhibited samples</td>
<td>15</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Rate of inhibition (%)</td>
<td>23.80</td>
<td>17.46</td>
<td>11.11</td>
<td>6.34</td>
<td>0.0</td>
</tr>
<tr>
<td>Sputum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninhibited samples</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Inhibited samples</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rate of inhibition (%)</td>
<td>50</td>
<td>40</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tracheal aspiration</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Uninhibited samples</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Inhibited samples</td>
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<td>0</td>
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<td>Rate of inhibition (%)</td>
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<td>Uninhibited samples</td>
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<td>1</td>
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<td>1</td>
</tr>
<tr>
<td>Inhibited samples</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Rate of inhibition (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninhibited samples</td>
<td>56</td>
<td>61</td>
<td>67</td>
<td>72</td>
<td>76</td>
</tr>
<tr>
<td>Inhibited samples</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Rate of inhibition (%)</td>
<td>26.31</td>
<td>19.73</td>
<td>11.84</td>
<td>5.26</td>
<td>0.0</td>
</tr>
</tbody>
</table>
2004; Kais et al., 2006; Halse et al., 2010). Previously, mucolytic agents, haemoglobin and other undefined substances in sputum samples have been reported to inhibit PCR (Wilson, 1997; Amicosante et al., 1995, Deneer & Knight, 1994). Pretreatment to reduce viscosity in respiratory samples prevents inhibition of PCR (Bencini et al., 2007). Overall, the specimen type and use of an internal control for the detection of inhibition are the two major aspects that influence the reliability of diagnostic P. jirovecii PCR methods.

PCR inhibition frequency by respiratory specimens has often been reported in PCR studies targeting microorganisms other than P. jirovecii. In a study on Mycobacterium tuberculosis, 4% inhibition in the initial run and 2.2% inhibition in retesting of a PCR system using respiratory samples was reported (Levidiotou et al., 2003). Relatively low levels of inhibition such as 0.2% and 0.7% have also been reported in PCR studies with M. tuberculosis using respiratory specimens (Inuma et al., 2003; Halse et al., 2010). In these studies, DNA samples were not further diluted to resolve inhibition. The absence of PCR inhibition using BAL fluid and/or sputum specimens has also been reported previously (Raggam et al., 2002; Apfalter et al., 2003; Heginbothom et al., 2003).

In some studies, to resolve inhibition detected in initial PCR runs, DNA of respiratory specimens was diluted in subsequent runs (Tong et al., 1999; Halse et al., 2010). In a multiplex PCR study detecting micro-organisms other than P. jirovecii, among the 53 known respiratory samples, inhibition was determined in four samples using a human internal control (7.5%). Inhibition resolved in three samples after retesting with 1:20 diluted DNA samples and levels of target DNA were detectable. Subsequently, 279 sputum samples were analysed and nine (3.2%) of them inhibited PCR. After resolution of inhibition by dilution, all samples remained negative (Tong et al., 1999). In a real-time PCR study, three respiratory specimens were reported to inhibit PCR, as determined by a plasmid internal control, and inhibition was not resolved even after using 1:5 dilutions of DNA samples (Halse et al., 2010).

In the present study, the inhibition rates of BAL and sputum samples in the initial PCR run were 23.80% and 50%, respectively. Inhibition decreased as the samples were diluted and none of the samples was completely inhibited. Interestingly, inhibition in the four remaining 1:10 diluted BAL samples was resolved by further dilution to 1:20 and two samples belonging to the same patient had detectable levels of P. jirovecii DNA (Table 2). The other two samples remained PCR-negative. Further dilutions of inhibited samples reduce the cost-effectiveness of the real-time PCR; however, it was beneficial for this specific patient.

Although BAL fluids are less likely to contain inhibitors compared to sputum specimens, inhibition in two sputum samples resolved at 1:10 dilution compared to four BAL samples that resolved at 1:20 dilution (Raggam et al., 2002; Apfalter et al., 2003; Inuma et al., 2003; Levidiotou et al., 2003; Wilson et al., 2004; Kais et al., 2006; Halse et al., 2010). In a real-time PCR optimization study, three out of 15 sputum samples (20%) partially inhibited PCR (Kais et al., 2006). In another study, none of the sputum samples inhibited PCR (Heginbothom et al., 2003).

During the development of real-time PCR targeting the cdc2 gene of P. jirovecii by Arcenas et al. (2006), spiked controls prepared from 18 pooled P. jirovecii-negative BAL samples (500 µl BAL was used per sample) did not cause inhibition in PCR. The incidence of P. jirovecii was 12.8% during analysis of 214 BAL fluids obtained from PcP-suspected patients using real-time PCR without performing spiked control runs for each sample (Arcenas et al., 2006). According to the results of the present study, the incidences of P. jirovecii in BAL fluids and sputum of PcP-suspected patients were 20.63% (13/63) and 30% (3/10), respectively. The DNA extraction protocol used in the present study was similar to that of Arcenas et al. (2006) with the exception that the BAL sample size was increased from 500 µl to 8–10 ml BAL in the present study. Inclusion of spiked controls and an additional concentrating step used during the processing of 8–10 ml BAL fluids may be the cause of the increase in the observed incidence of P. jirovecii and higher inhibition frequency compared to lower inhibition rates reported in previous PCR studies using respiratory specimens (Raggam et al., 2002; Apfalter et al., 2003; Inuma et al., 2003; Levidiotou et al., 2003; Arcenas et al., 2006; Halse et al., 2010).

In the present study, the incidence of P. jirovecii in respiratory samples was 22.36% (17/76) as determined by real-time PCR targeting of the cdc2 gene of P. jirovecii and 7.14% (3/42) by microscopy (Table 1). Only one sample differed in the microscopy and PCR results. Two microscopy-positive samples, which were PCR-positive after dilution, increased the sensitivity of PCR by 11.76%. Overall, the incidence of P. jirovecii in respiratory samples was 23.68% (18/76) as detected by both methods. Leibovitz et al. (1995) compared PCR targeting mtLSU rRNA with Giemsa and modified methenamine silver stains in 284 respiratory samples and reported P. jirovecii incidences of 28.1% and 24.2%, as determined by PCR and staining techniques, respectively. Of the positive samples, 13 were microscopy-positive and PCR-negative, whereas 24 samples were microscopy-negative and PCR-positive. Pinlaor et al. (2004) compared Wright–Giemsa and Gomori’s methenamine silver staining methods with PCR targeting 5S rRNA in 160 respiratory specimens. The incidence of P. jirovecii was 10% by both microscopy methods and 15.6% by PCR. Gomori’s methenamine silver staining was positive for the two PCR-negative samples (Pinlaor et al., 2004).

In some studies, PCR was able to detect P. jirovecii DNA in all of the microscopy-positive samples. In a study comparing direct examination methods (Gomori’s methenamine silver and Calcofluor white staining) with PCR targeting 5S rRNA in 129 respiratory specimens, the
incidence of *P. jirovecii* was 28.6% for direct examination and 46.5% for PCR. All of the positive samples as detected by direct examination methods were also positive with PCR (Ribes et al., 1997). Similarly, Flori et al. (2004) compared Giemsa and Gomori–Grocott staining with conventional and real-time PCR targeting mtLSU rRNA and MSG genes of *P. jirovecii* in 173 BAL fluids obtained from 150 clinically PcP-suspected patients. The reported incidences as determined by microscopy, conventional PCR and real-time PCR were 3.4%, 6.3% and 6.3%, respectively. All microscopy-positive samples were also positive with PCR (Flori et al., 2004). During a comparison of toluidine blue staining and calcofluor white staining with PCR targeting of the *Kex*-1 gene in 186 samples collected from 143 PcP-suspected patients, the incidences of *P. jirovecii* as determined by microscopy using both staining methods and *Kex*-1 PCR were 11.3% and 17.7%, respectively. Similarly, all microscopy-positive samples were positive with PCR (Rohner et al., 2009).

In conclusion, the results of the present study showed that inclusion of spiked positive controls in each sample during real-time PCR detection of *P. jirovecii* provided enhanced sensitivity compared to PCR without spiked controls. Also, retesting with diluted samples to resolve inhibition can increase the sensitivity of the PCR protocol used in the present study. Overall, these approaches increased the reliability of the *P. jirovecii* real-time PCR and influenced the treatment of the patients in terms of determining false-negative results. Moreover, the present study assessed for the first time the degree and frequency of inhibition of a real-time PCR detecting *P. jirovecii* in respiratory specimens in Turkey.

REFERENCES


infections by PCR directed to genes encoding for mitochondrial 5S and 28S ribosomal RNA. *Diagn Microbiol Infect Dis* 33, 157–162.


