



Original article

Pneumocystis Jirovecii detection and comparison of multiple diagnostic methods with quantitative real-time PCR in patients with respiratory symptoms

Mohammad Y. Alshahrani^{a,*}, Mohammed Alfaifi^a, Irfan Ahmad^a, Ali Gaithan Alkhatami^a, Abdulrahim Refdan Hakami^a, Hafiz Ahmad^b, Osama M. Alshehri^c, Megh Singh Dhakad^d

^a Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia

^b Department of Medical Microbiology and Immunology, RAK Medical & Health Sciences University, Ras Al Khaimah, United Arab Emirates

^c Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Najran University, Najran, Saudi Arabia

^d Department of Microbiology, Lady Hardinge Medical College, New Delhi, India



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ABSTRACT

Pneumocystis jirovecii (PCP) remains a significant cause of mortality and morbidity in patients with respiratory infections. Accurate diagnosis of PCP is still a diagnostic challenge. Hence, the main objectives were to study the incidence of *Pneumocystis Jirovecii* pneumonia infection among respiratory problems patients and to compare the real-time quantitative PCR technique with various diagnostic methodologies. Patients who have respiratory symptoms of PCP like breathlessness, cough, and fever were enrolled. Bronchoalveolar lavage (BAL) samples were collected and homogenized, and then smears were prepared for examination by Gomorimethanamine silver staining (GMSS), Immunofluorescent staining (IFAT), Toluidine blue O (TBO), and Giemsa staining. Further, RT-PCR was also performed for the detection of PCP. The mean patients' age was 52 (SD ± 16) years. 41% were female, and 59% of the patients were male. Weight loss (80%), fever (92%), cough (100%), and dyspnea (76%) were the most common complaints. Twenty-eight patients have been diagnosed with pulmonary infiltrates using chest X-ray. Out of 100 patients, 35% were positive for PCP. The organism was detected using IFAT in all the 35 specimens, 15 of 35 (42.86%) by GMSS, 8 of 35 (17.6%) by Giemsa stain, and 1 of 35 (2.8%) was detected by TBO stains. RT-PCR showed that 39 patients was found to be positive for PCP. Thirty-five of these 39 patients had a positive IFAT (89.74%); the IFAT was negative or undefined in 4 samples. All 39 patients (100%) had signs and symptoms for PCP. Our results suggest that RT-PCR is still the most highly sensitive method for *Pneumocystis Jirovecii* detection. In poor resource settings where RT-PCR and IFAT is not available, diagnosis of *Pneumocystis jirovecii* pneumonia remains a complicated issue. In settings where RT-PCR & IFAT are not available, GMSS staining may be the next best choice to detect PCP.

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* Corresponding author at: Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, P.O. Box 61413, Abha 9088, Saudi Arabia.

E-mail addresses: moyahya@kku.edu.sa (M.Y. Alshahrani), mhalfaifi@kku.edu.sa (M. Alfaifi), agaithan@kku.edu.sa (A.G. Alkhatami), ahakami@kku.edu.sa (A.R. Hakami), omalshehri@nu.edu.sa (O.M. Alshehri).

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1. Introduction

Respiratory tract infection, especially *Pneumocystis pneumonia* (PCP), is a significant causative agent of fatal diseases (Hirama, 2016). The organism *Pneumocystis Jirovecii* previously known as *Pneumocystis carinii*, is the causative agent of *Pneumocystis jirovecii* pneumonia (Muhlethaler et al., 2012). In developed countries, the rate of *Pneumocystis jirovecii* pneumonia co-infection with HIV has reduced while controlling the prevalence of co-infection in developing countries remains challenging (Ravinder et al., 2015). Many factors can make PCP diagnosis tricky such as nonspecific symptoms, coexistence of other infections, and difficulty in establishing a

reliable culturing system of this pathogen (Kelly et al., 2018). Rapid and accurate identification of infections and suitable recommended treatment, which is based on accurate microbiological results, is still required; however, the current standard clinical tests lack high sensitivity and flexibility (Hirama, 2016).

The standard direct microscopic characterization of *Pneumocystis jirovecii* from different samples such as bronchoalveolar lavage (BAL), lung biopsy, or induced sputum is the hallmark method for diagnosis (Kelly et al., 2018). Gomorimethanamine silver staining (GMSS) is considered the more sensitive immunofluorescence assay for microscopic detection of the lower respiratory system samples (Kovacs et al., 1988). However, molecular methods such as PCR have much higher sensitivity than microscopic identification (Kelly et al., 2018; Arcenas et al., 2006). Different PCR platforms, patient populations, and different specimen types have been extensively clarified (Kelly et al., 2018; Arcenas et al., 2006). Real-time (RT-PCR) is ideal for standard microscopic techniques as PCR utilizes a closed system which minimizes contamination and offers the cycle thresholds function, which helps to detect the real infection instead of airway colonization (Arcenas et al., 2006; Wilson et al., 2011).

BAL and sputum are ideal samples for detecting the nucleic acids of *Pneumocystis* using RT-PCR, and the results are more sensitive than microscopic identification (Kelly et al., 2018). However, RT-PCR allows accurate and specific quantification of DNA. Moreover, RT-PCR has the potential ability to discriminate between asymptomatic carriage of *Pneumocystis jirovecii* and clinical disease based on the load of pathogen copies (Huggett et al., 2008). There is a need for an alternate method for rapid and accurate laboratory techniques in order to diagnose the disease in limited resources countries and prescribe appropriate medication. Hence, our main objectives were to study the incidence of PCP among patients with respiratory problems, and to compare QRT-PCR with various diagnostic methodologies.

2. Methodology

2.1. Study population and design

The sample size was calculated by using $n = Z\alpha^2 (p \times q)/d^2$ formula based on prevalence 30.3% (Abubakar et al., 2016). A total of 100 suspected cases for PCP, which met the essential medical manifestations, radiological criteria, and microbiological findings, from tertiary care hospitals in the period between January 2019 and August 2019, were included into this study. Patients with common respiratory symptoms such as shortness of breath, cough, chest pain, and typical interstitial pulmonary infiltrates in X-ray, or computed tomography scan were enrolled. The clinical data and medical history of the patients were documented. BAL samples were collected using 0.9% sodium chloride solution and instantly sent to the laboratory for the required analysis.

2.2. Ethics approval

The study obtained ethical approval from the Research Ethics Committee, Deanship of Scientific Research, King Khalid University, Abha, Kingdom of Saudi Arabia with wide approval number is (ECM-2019-73) - (HAPO-06-B-001).

2.3. *Pneumocystis* RT-PCR

Isolation and purification of *Pneumocystis jirovecii* DNA from BAL sample was done by using QIAamp DNA Mini Kit manufactured by Qiagen (Germany). *Pneumocystis* RT-PCR assay was done by using MycAssay *Pneumocystis* kit, manufactured by Myconos-

tica (UK). The assay was designed to detect the mitochondrial ribosomal large subunit (mLSU). MycAssay kit is a commercial qualitative real-time PCR technique that utilizes molecular beacons for *Pneumocystis jirovecii* pneumonia detection. The assay kit includes an internal amplification control (IAC) sequence and DNA fragment leading to confirmed amplification as *Pneumocystis jirovecii* lack both IAC and DNA fragment.

The target sequence of *Pneumocystis jirovecii* pneumonia is labeled with a 6-carboxyfluorescein (FAM) beacon, while the IAC sequence is labeled with a HEX beacon. The fluorescence emitted by each beacon was monitored using CFX96 touch real-time PCR system. In this method, the negative cut-off value of cycle threshold (CT) of 39 was used as per manufacturer's instruction. A CT value lower than this was considered positive (Myconostica Ltd, 2010). The semiquantitative manner was used to interpret CT values, which explains the correlation between fungal burden and CT values (Hauser et al., 2011).

2.4. Merifluor DFA immunofluorescence testing

The reference diagnostic test was Immunofluorescent staining (IFAT) using Merifluor DFA immunofluorescence test, according to the manufacturer's instructions (Meridian Bioscience, Inc., Cincinnati, Ohio).

2.5. Standard diagnostic tests

Special staining diagnostic techniques, including Gomorimethanamine silver staining (GMSS), Giemsa staining, and Toluidine blue O (TBO), were used. Both trophozoite forms and cyst can be detected by using Giemsa staining, while TBO detects the cyst form only.

The results of *Pneumocystis* RT-PCR were compared with GMSS, TBO, and Giemsa staining results. We have shown that IFAT positive cases responded to treatment. Moreover, all cases, which were positive by other staining techniques, were IFAT positive. Therefore, in our study, the other techniques were compared to IFAT, as it is considered a standard gold method. The specimens were reported truly positive for *Pneumocystis jirovecii* if the pathogen was detected by any of the previously mentioned methods accompanying with clinical presentations of *Pneumocystis jirovecii*.

2.6. Statistical analysis

Means and standard deviations (SD) were calculated for participants' age, and frequencies were calculated for gender and test results. The sensitivity, positive and negative predictive values, and specificity were calculated using the Statistical Package for the Social Sciences (version 17.0; SPSS S.L., Madrid, Spain). The significance level was set at $p \leq 0.05$.

3. Results

The diagnostic examination of *Pneumocystis jirovecii* from BAL was performed on 100 patients. The patients' mean age was 52 (SD \pm 16) years ranged from 16 to 78 years. 41% were female, and 59% of the patients were male. Thirty-five patients were positive for *Pneumocystis jirovecii* in our study by using several detection methods.

As shown in Table 1, cough (100%), fever (92%), weight loss (80%), and dyspnea (76%) night sweats (54%) were the most common clinical manifestations. Crepts and wheezing were seen in

Table 1
Chest X-ray findings of PCP respiratory patients.

S.No.	Chest X-Ray findings	Number	Percentage (35)
1	Central Perihilar infiltrates	19	54.28
2	Patchy infiltrates	8	22.86
3	Consolidation	3	8.57
4	Hilar Lymphadenopathy	3	8.57
5	Pneumothorax	1	2.86
6	Pleural effusion	1	2.86

62% and 75% PCP patients, respectively. Chest X-ray showed pulmonary infiltrates, which was the most common finding.

Generally, out of 100 specimens, 35 were positive, whereas 65 of 100 specimens were negative. 35 of 35 (100%) of positive specimens were detected by IFAT stain, 15 of 35 (42.86%) by GMSS, 8 of 35 (17.6%) by Giemsa stain, and 1 of 35 (2.8%) was detected by TBO stains. RT-PCR showed that 39 (39%) of patients were found to be positive for PCP. 35 of the 39 patients were positive IFAT (89.74%); the IFAT of 4 patients was either negative or undefined (Fig. 1). All 39 patients (100%) showed the clinical symptoms of *Pneumocystis jirovecii* pneumonia.

The calculation of positive predictive values (PPV), sensitivity, disease prevalence (95% CI), negative predictive values (NPV), and specificity is shown in Table 2. Sensitivity and specificity values are showed RT-PCR (Fig. 2) to be the best method, followed by IFAT, GMSS, Giemsa, and TBO methods. Positive sample amplification as shown in Fig. 2 representing the positive amplification of *Pneumocystis jirovecii* infected patients and negative sample did not showed any amplification. The disease prevalence value for PCP positive cases ranged from 25.73% to 45.18% at 95% confidence interval (95% CI). Figs. 3 and 4 showed the cysts forms of *Pneumocystis jirovecii* stained with IFAT and GMSS, respectively. Fig. 3 showed immunofluorescence of antibody used against *Pneumocystis jirovecii* in infected patients.

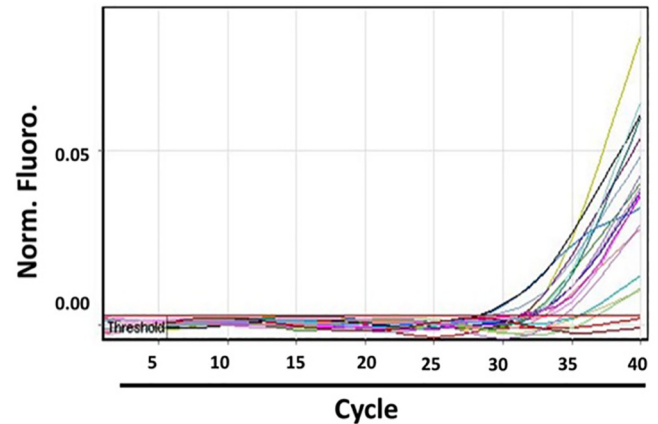


Fig. 2. Real time PCR amplification for *Pneumocystis jirovecii* positive patients.

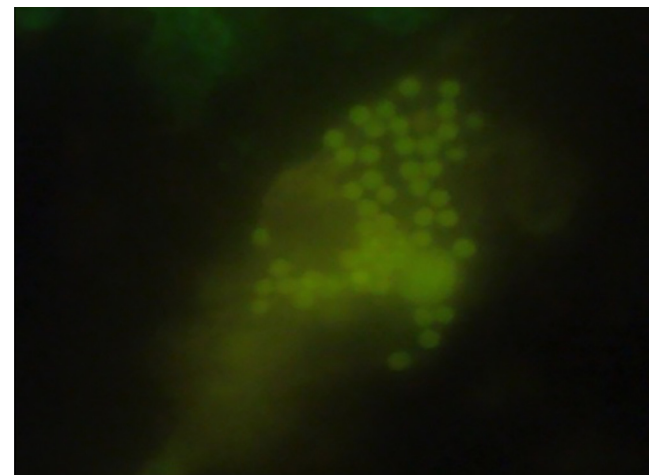


Fig. 3. *Pneumocystis jirovecii* stained with IFAT seen in BAL sample.

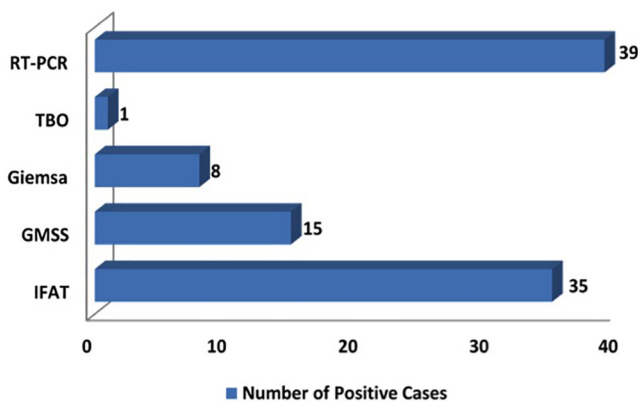


Fig. 1. Comparison of different techniques among respiratory PCP patients.

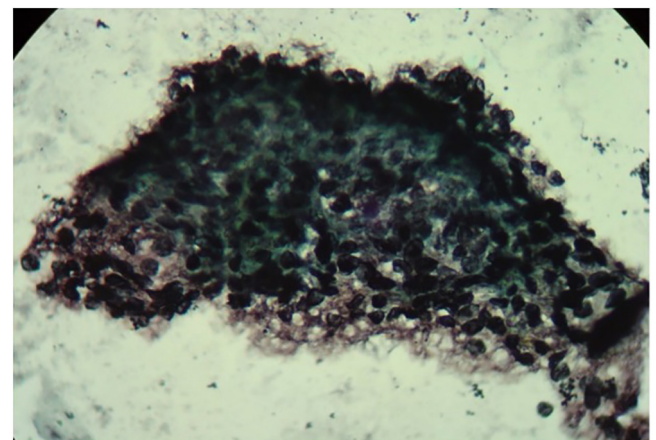


Fig. 4. *Pneumocystis jirovecii* stained with GMSS in BAL sample.

Table 2
Statistical parameters of different techniques positive for *Pneumocystis jirovecii* pneumonia.

No.	Techniques	Number of positive cases	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	95% CI
1	IFAT	35	100.00	100.00	100.00	100.00	25.73–45.18
2	GMSS	15	42.86	100.00	100.00	76.47	
3	Giemsa	08	22.86	98.46	88.89	70.33	
4	TBO	01	02.86	96.92	33.33	64.95	
5	RT-PCR	39	100.00	93.85	89.74	100.00	

4. Discussion

Carlos Chagas discovered *Pneumocystis Jirovecii* in 1909, while investigating interstitial pneumonitis in Brazilian railroad workers (Chagas, 1909), but he misinterpreted the diagnosis and classified it as *Trypanosoma cruzi*. Previously *Pneumocystis Jirovecii* considered to be a protozoa and taxonomically has been a matter of controversy. Currently, the new studies have reclassified the *Pneumocystis Jirovecii* as an ascomycete fungus (Ravinder et al., 2015). The pleomorphic trophozoite form and the cystic stage are major developmental stages of *Pneumocystis Jirovecii*. (Ravinder et al., 2015). Colonization of *Pneumocystis Jirovecii* has been described in adult patients with chronic obstructive airway disease, making them reservoir for the infection (Helweg-Larsen et al., 2002). In our study, clinical symptoms and findings like cough (100%), fever (92%), unintentional weight loss (80%), dyspnea (76%) were associated with risk of PCP similar to which has been seen in studies conducted in the United States (Stansell et al., 1997). In chest X-ray findings, 30/35 (85.7%) patients of PCP positive patients had typical interstitial pulmonary infiltrates consistently similar to the study which reported that pulmonary infiltrates present in 17 out of 19 (89.5%) patients, with 16 out of 19 (84.2%) patients presenting bilateral pulmonary infiltrates (Doyle et al., 2017).

In our study, we enrolled 100 patients with atypical pneumonia, 35% of patients were positive for *Pneumocystis Jirovecii*. PCP was detected using IFAT in all the 35 specimens, 15 of 35 (42.9%) were detected by GMSS, 8 of 35 (17.6%) by Giemsa stain, and 1 of 35 (2.8%) was detected by TBO stains. Nowadays, PCR for detection of *Pneumocystis*, especially Real Time-PCR, is commonly used in diagnosing PCP (Muhlethaler et al., 2012). Our study showed excellent sensitivity (100%) and strong NPV (100%) for RT-PCR, which were also confirmed by several other studies (Alanio et al., 2011; Flori et al., 2004).

In most studies that compared PCR methods with microscopy for PCP diagnosis, PCR exhibited better sensitivity for *Pneumocystis Jirovecii* detection in patients with chronic lung disease (Lucia et al., 2018). Meta-analysis showed a very high accuracy of PCR in BAL samples for the diagnosis of *Pneumocystis Jirovecii* in patients who are at risk and a pooled sensitivity of 98.3% and a specificity of 91.0% (Lucia et al., 2018; Fan et al., 2013). The specificity and sensitivity of nested-PCR were 93% and 100%, respectively (Lucia et al., 2018). Moreover, the quick and reliable detection of the organism using the PCR test remains an advantage (Samuel et al., 2011).

Our RT-PCR showed that 39 (39%) patients were found to be positive for PCP. Thirty-five of these patients had a positive IFAT (89.74%), with negative or undefined IFAT in 4 (10.26%) samples. The probable reason for this over diagnosis might be due to the fact that PCR does not discriminate between pneumocystis colonization and infection as this highly sensitive method detects pneumocystis DNA regardless of the clinical presentation (Unnewehr et al., 2016). Therefore, this differentiation remains the most relevant clinical issue.

Contamination in PCR and the lack of a suitable culturing system to confirm the PCR results are the main problems of this technique (Medrano et al., 2005). Nowadays, PCR assays for *Pneumocystis* are commonly used for diagnosis (Muhlethaler et al., 2012), even though they are not usually presented clinically but are a lot used in research settings.

Many studies have suggested that BAL is the ideal sample for *Pneumocystis Jirovecii* detection. The sample collection is delicate and requires expensive items and very-well trained specialists, arising the need for alternative, less harmful methods to detect this organism (Ravinder et al., 2015).

The detection of *Pneumocystis Jirovecii* by PCR is highly sensitive; however, the diagnosis should not rely only on PCR. Thus, the decision of PCP diagnosis should be concluded according to the clinical symptoms, signs, radiological imaging, and advanced microbiology techniques, especially if the PCR result strongly positive (Robert et al., 2014), with which we agree entirely.

5. Conclusion

As revealed in our study, BAL is the ideal sample, considering the significant harmful due to the procedures of collecting the sample. Although *Pneumocystis* RT-PCR has been demonstrated to be more sensitive than commonly used morphologic methods, although RT-PCR & IFAT are expensive methods for *Pneumocystis Jirovecii* detection in countries with limited resources. In lab settings where RT-PCR & IFAT are not available, GMSS staining might be the best option for *Pneumocystis Jirovecii* detection.

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