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Conventional Methods for the Diagnosis of *Pneumocystis jirovecii* in Immunocompromised Iraqi Patients

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Abstract

Background *Pneumocystis jirovecii* is the causative agent of *pneumocystis* pneumonia, one of the most frequent and severe opportunistic infections in immunocompromised patients.

Objectives To determine the possible implication of *pneumocystis jirovecii* in immunocompromised Iraqi patients with pulmonary infections, and investigate the efficiency of indirect qualitative immunofluorescence diagnosis in compared with conventional stains for the detection of this agent.

Methods A total of 200 clinical samples from 100 immunocompromised patients (70 bronchoalveolar lavage, 21 sputum samples and 9 pleural fluids). One hundred samples from immunocompetent individuals (50 bronchoalveolar lavage, 30 sputum samples and 20 pleural fluids). Detection of pneumocystosis was done by conventional satins and indirect qualitative immunofluorescence technique.

Results Fourteen samples gave positive results by indirect immunofluorescence monoclonal antibody test. Twelve out of 100 samples were positive by each Gomori methenamine silver, modified toluidine blue O stains and Diff-Quik stain (modified Giemsa).

Conclusion *Pneumocystis jirovecii* is the fundamental opportunistic infection among immunocompromised patients. The indirect qualitative immunofluorescence method gives a promise for use as a primary method for diagnosis of *pneumocystis jirovecii* pneumonia or as rapid screen to exclude the presence of *pneumocystis jirovecii* in sputum and bronchoalveolar lavage samples.

Keywords *Pneumocystis jirovecii (carinii), pneumocystis jirovecii* pneumonia, immunocompromised, Iraqi patients.

List of abbreviation: *p. jirovecii = pneumocystis jirovecii*, PCP = *Pneumocystis* pneumonia, BAL = bronchoalveolar lavage, GMS = Grocott-Gomori methenamine silver stain, DQS = differential Quik stain, TOB = Toluidine blue, PE = pleural effusion, IS = induced sputum, IFA = indirect Immunofluorescence antibody.

Introduction

Pneumocystis jirovecii (*p. jirovecii*), previously known as *Pneumocystis carinii*) is an unusual opportunistic organism. *P. jirovecii* most commonly causes *Pneumocystis* pneumonia (PCP) in patients with acquired immune deficiency syndrome and patients receiving intensive or prolonged immune suppressive treatment for malignancy, transplantation and immune disorders [1-3] which causes a severe and often fatal pneumonia in immunocompromised individuals [4].

The organism has a unique tropism for the lungs, where it exists primarily as an alveolar pathogen. Individuals with intact immunity control this primary infection, there are no apparent clinical manifestations of primary infection in immunocompetent individuals, and the organism likely remains latent in the lungs for long periods of time, clinically apparent pneumonia occurs when cellular or humoral immunity becomes severely deficient, the organisms proliferate, evoking a mononuclear cell response, alveoli become filled with

proteinaceous material and intact and degenerating organisms [5,6].

P. jirovecii inability to culture suggests that it has evolved to require a very specific environment that is not easy to reproduce outside its host [7]. The diagnosis of *P. jirovecii* disease requires the demonstration of cysts or trophozoites within tissue or body fluids via colorimetric or immunofluorescent stains since the human organism cannot be cultured in *vitro* and *vivo* [8].

The aims of this study was to determine the possible implication of *P. jirovecii* in a sample of immunocompromised Iraqi patients, and investigate the efficiency of indirect qualitative immunofluorescence diagnosis in comparing with conventional stains for the detection of this agent.

Methods

Patient's selection

This study included a total of 200 clinical samples from 100 immunocompromised patients (43 men, 57 women; average age 16-90 years) as 70 bronchoalveolar lavage (BAL), 21 sputum samples and 9 pleural fluids, with different underlying immunocompromised diseases including 22 (22%) leukemia, 17(17%) solid tumor, 15(15%) lymphomas, 12(12%) chronic obstructive pulmonary disease, 10(10%) asthma (steroid therapy), 10(10%) rheumatoid arthritis (cytotoxic therapy), 8(8%) solid-organ transplantation and 6(6%) Multiple myeloma with suspected of pneumocystosis. Control group included 100 samples (50 BAL, 30 sputum samples and 20 pleural fluids) from immunocompetent individuals were collected from in-and out patients who attended of Al-Imammian Al-Kadhmain Medical City, Baghdad teaching Hospital, Baghdad, and Al-Zahra Teaching Hospital, Wasit province and other private laboratories, during the period from May 2014 to March 2015. The ethical aspects of this study have been approved by the Institute Review Board of the College of Medicine, Al-Nahrain University.

Samples collection

Bronchialveolar lavage (BAL) was performed by a bronchofibroscope (STORZ, Germany) wedged in segmental orifice of sedated spontaneous breathing patients or intubated patients, in most cases, 20-50 ml warmed saline was infused into targeted segment followed by gentle suction by specialist physician. BAL fluids were directly collected by sterile syringe. About 10-15 ml were dispensed into sterile test tube and immediately placed on ice then transmitted to the laboratory for processing.

Induced sputum samples (IS) were obtained by induction in patients involved in the study. Sputum induction was done using an ultrasonic nebulizer (serial No. 2000, England). This was done in an open space using a 3ml saline as an inducing fluid, from each induced patient by nurse practitioner; this sputum sample (10-15ml) was directly collected by sterile screw cup bottles and immediately placed on ice then transmitted to the laboratory for processing. Induced sputum were divided into two portions and treated with either 0.1% Dithiothreitol or with 0.9% NaCl alone.

Pleural effusion samples (PE) was done by aspirating pleural fluid with 25G needle after marking, cleaning the suspected area with antiseptic and then local anesthetic (5-10 ml of 2% lidocaine) was injected locally; this method was done by specialist physician. Ten-15ml pleural fluid was aspirated into sterile test tube and immediately placed on ice then transmitted to the laboratory for further processing.

Samples processing

BAL, sputum and pleural fluid containing mucous martial were added to a 2-fold volume of 0.9% NaCl and were mixed vigorously vortexes for 5 minutes. Samples centrifuged at 3000 rpm for 5 minutes, supernatants were discarded and the precipitated pellets were placed into a 1.5 ml microcentrifuge tubes according to Alexander *et al* [9] several slides

were prepared simultaneously, depending on the number of stains to be employed, with a few spare slides prepared for any repeat stains which might be needed.

A portion of precipitate pellets (100µl) was used to prepare smears for each Diff-Quik stain (SYRBIO®, Syria), Grocott-Gomori methenamine silver stain (abcam®, UK), Toluidine blue stain (BHD, England) and for fluorescent monoclonal antibody test (IF: Detect IF PC, Axis-Shield Diagnostics, Dundee, UK).

Results

Descriptive data on study subjects

Among those 100 immunocompromised patients, 43 were males and 57 were females whereas in the control group each group comprised of 50 subjects. The age of the patients ranges from 16-90 years (Mean± S.D = 54.56±16.46 years) as compared to 16-66 years (Mean± S.D = 37.33±13.24 years) of the control group ($p < 0.001$).

Laboratory Diagnosis of *P. jirovecii*

Gomori's Methenamine Silver stain (GMS)

Under light microscope, cysts of *P. jirovecii,* the diagnostic form, had a characteristic appearance as a spherical, cup-shaped, or crescent-shape object stained by GMS which usually crowded in foamy alveolar casts in BAL. GMS used for staining carbohydrates producing gray to black and *green* background counterstained with light green, this stain is useful for identifying cyst form 12(12%) out 100 samples were positive (Fig. 1).

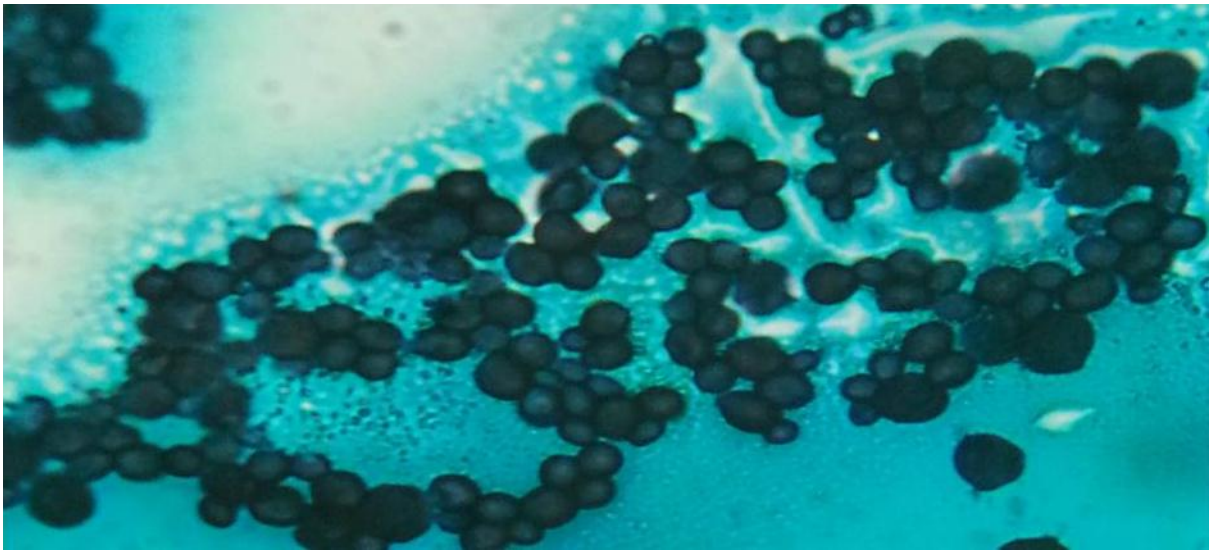

Fig. 1. Gomori's methenamine silver (GMS) stained smear of BAL from leukemia gray to black color of the fungus is seen against a light green background, the cysts of *P. jirovecii* were crowed in foamy alveolar casts in BAL. The cyst appears as a spherical, cup-shaped, or crescent-shaped object. Some cysts are empty and collapsed; others contain dark bodies or dots, which acquire different positions in relation to the cyst depending on the angle of visualization (X1000).

Modified toluidine blue O Stain (MTolB)

Cyst forms of *P. jirovecii* are cup-shaped appear as violet to purple. The cyst outline is distinct, and the internal region stains uniformly. The cysts were frequently observed in clusters, while trophic form cannot be stained 12(12%) out 100 samples were positive (Fig. 2).

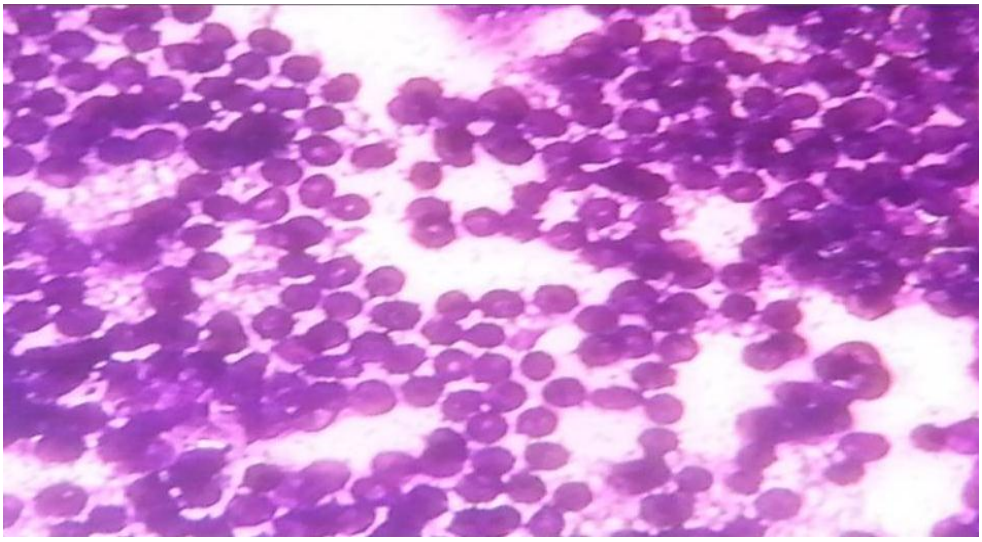

Fig. 2. Toludine blue (TOB) stained smear from BAL of solid tumor, the cyst of *P. jirovecii* forms appear as violet to purple.(X1000).

Differential Quik Stain Kit (Modified Giemsa)
Specimens were collected and stained with Diff-Quik stain (modified Giemsa). By this technique, it was possible to demonstrate cystic and trophic forms of *P. jirovecii* and confirm the diagnosis of *Pneumocystis* pneumonia in this patient. Stained slides were examined using a light microscope 12(12%) out 100 samples were positive (Fig. 3).

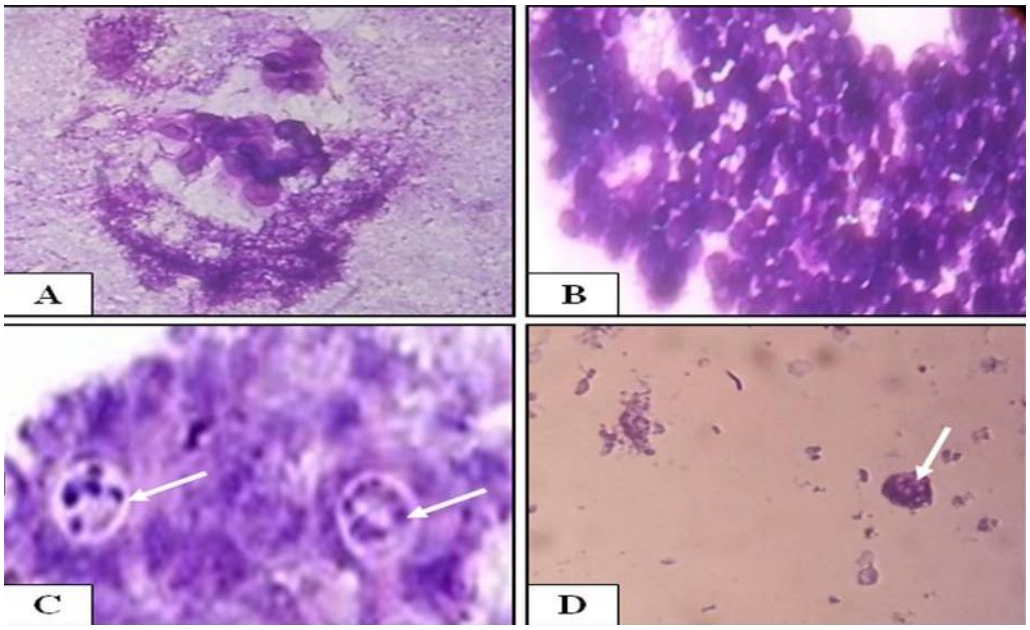

Fig. 3. Diff-Quik stain (modified Giemsa). Stained sputum smear from leukemia, the cysts of *P. jirovecii* appear as spherical dark blue (A). Stained BAL smear from asthma, the cysts of *P. jirovecii* appear as aggregated spherical dark blue forms (B). Stained BAL smear from solid tumor (intracystic bodies were arrowed) (C). Stained sputum smear lymphoma, (intracystic bodies were arrowed) (D) X1000).

Indirect qualitative immunofluorescence
The pellets from (BAL, IS or PE) specimens were placed on slides and fixed for the detection of *P. jirovecii* by a monoclonal antibodies technique. This technique was performed according to manufacturer's instructions.

Stained slides were examined by a fluorescence microscope, oocysts show as medium bright to bright apple green color they may be evenly or unevenly labeled 14(14%) out 100 samples were positive (Fig. 4).

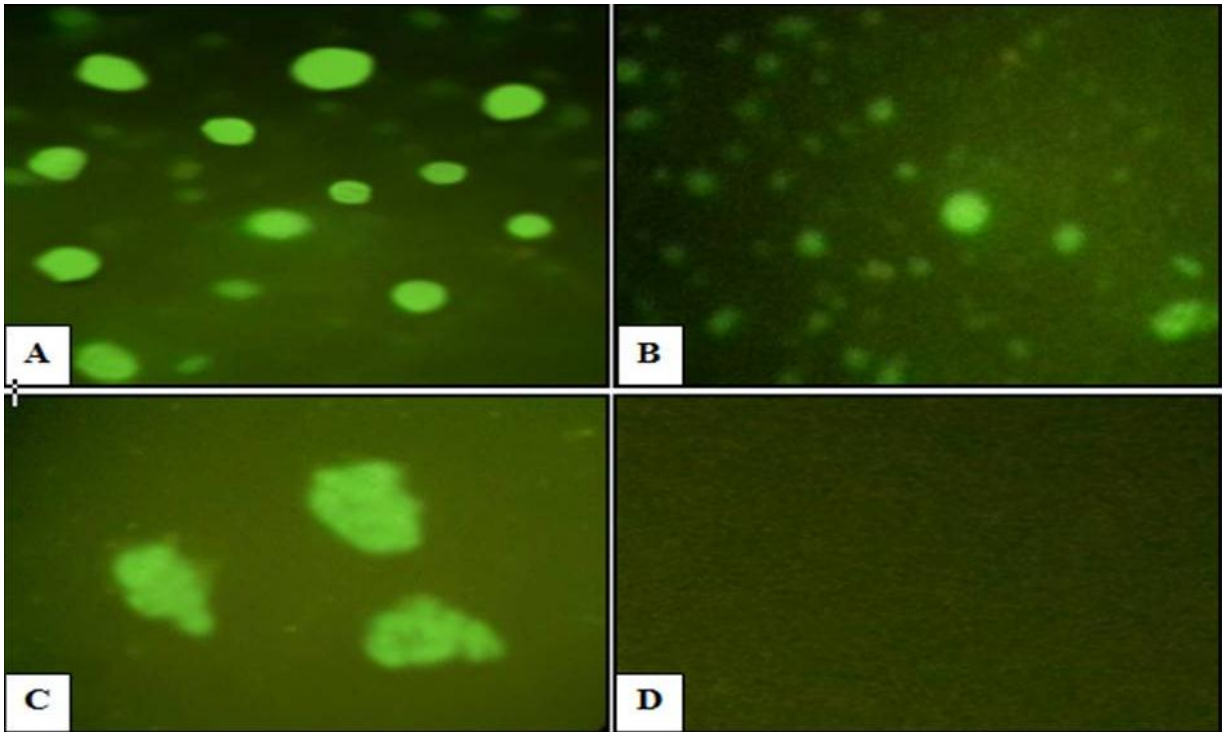

Fig. 4. Immunofluorescent staining using monoclonal antibodies against *P jirovecii*. (A) stained smear from BAL of lymphoma disease, the cyst of *P. jirovecii* appears bright to bright apple green (X1000). (B) stained smear from BAL of leukemia disease showing bright to bright apple green cysts with different size (X400). (C) stained smear from sputum of kidney transplantation , many aggregated cysts of *P jirovecii* which appear bright to bright apple green (X1000). (D) Negative control (X1000).

Overall results of conventional methods

The organism was detected in BAL, and/or sputum of immunocompromised patients only. Fourteen samples gave positive results by indirect immunofluorescence monoclonal antibody test within this number only 12 (12%) were positive by Gomori methenamine silver (GMS), 12 (12%) were positive by modified toluidine blue O stains (TOB), and 12 (12%) were positive by Diff-Quik stain (DQS) modified Giemsa (Fig. 5).

These samples were obtained from 14 immunocompromised patients including, four with leukemia, three with solid tumor, two with lymphoma, and only one for each chronic pulmonary obstructive disease, asthma (steroid therapy), rheumatoid arthritis (cytotoxic therapy), solid-organ transplantation and multiple myeloma disease. Eighty six immunocompromised patients were initially negative by both indirect immunofluorescence and staining methods (GMS, TOB and DQS), while only 12 samples were positive by both methods.

Discussion

P. jirovecii is the causative agent of *Pneumocystis* pneumonia, one of the most frequent and severe opportunistic infections in immunocompromised patients. As *P. jirovecii* cannot be grown in culture from clinical specimens [10,11]. The current laboratory diagnosis of *Pneumocystis* pneumonia has relied mainly upon microscopic techniques, for

detection of cysts and trophozoites of the organism by cytological staining or by immune-fluorescent assay (IFA) with monoclonal or polyclonal antibodies [12,13].

In this study the positive results of 12 samples by three staining methods (GMS, TOB and DQS), is in consistent with Ng *et al.* [14] who proved, that the specimens were considered to contain *P. jirovecii* (i.e., a truly positive specimen) if this organism was detected by two or more of the staining methods. Conversely, specimens were considered to not contain the organism (i.e., a truly negative specimen) if all stains were negative or if only one stain was positive that could not be corroborated.

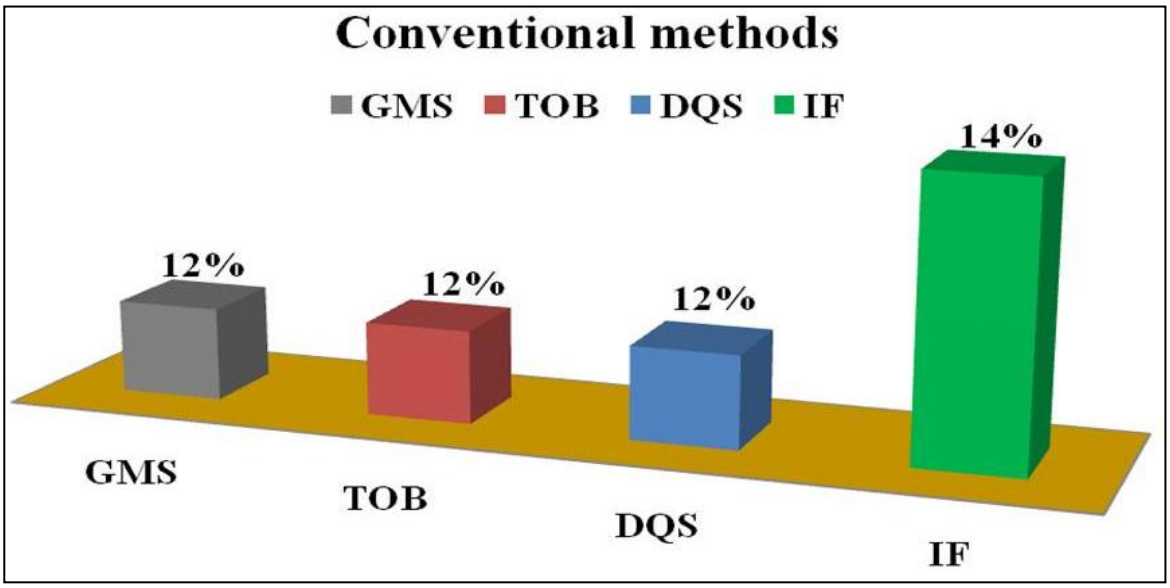

Fig. 5. *Pneumocystis jirovecii* detection methods using Gomori's methenamine silver, Diff-Quik stain (modified Giemsa), Toludine blue and Immunofluorescent staining in bronchoalveolar lavage and sputum samples.

LaRocque et *al* [15] found that the choice of the optimal staining method for the detection of *Pneumocystis* was also important for patients with other immune-compromising conditions who were at risk for infection. In fact, the choice of the optimal staining method may be more important for the detection of *Pneumocystis* in the non-HIV-infected, immunocomp-romised patients, since it has been shown that respiratory specimens from these patients have a lower burden of organisms than those from HIV-infected, immune-compromised patients.

Results of the current study is in consistent with Yehia *et al* [16] who diagnosed *P. jirovecii* infection by conventional methods only from lower respiratory tract of both immune-competent and immunocomp-romised Iraq patients. The organism was detected in alveolar lavage and/or sputum of immune-compromised patients only. *P. jirovecii* was diagnosed by direct microscopical examination with different stains included calcofluor stain, Giemsa and TOB was identified in 8 cases out 150 samples with immune-compromised patients with malignant diseases under radiation and/or cytotoxic therapy

In this study the results of the detection of *P. jirovecii* in respiratory samples BAL and sputum agree with this by Turner *et al* [17] who diagnosed the diseases by using three different staining techniques, included silver stain, Diff-Quik (a modified Giemsa stain) modified toluidine blue, and found that Induced sputum (IS) has been shown to be a reliable tool in terms of sensitivity and specificity comparable to BAL sample in diagnosing PCP.

In another study, John *et al* [18] who used direct immunofluorescence monoclonal antibody (DFA) method for identification of *P. jirovecii* in induced sputum and BAL specimens was compared in a blinded study with an established Giemsa stain method, for the 67 patients (64%) infected with the human immunodeficiency virus 49 were initially negative by both the DFA and Giemsa methods, none were negative by DFA and positive by Giemsa, 6 were positive by DFA and negative by Giemsa, and 12 were positive by both methods, were indicates that the DFA method represents an advance as a sensitive, simple, and rapid way to identify *P. jirovecii* in induced sputum and BAL specimens from HIV-infected patients and suggests greater sensitivity of the DFA than the Giemsa method in this patient population. This result in line with the present study concerning immunofluorescence mono-clonal method compared with staining methods.

Procop *et al* [19] who used four staining methods on replicate smears of respiratory specimens submitted for *Pneumocystis jirovecii* examination, he found that the indirect immunofluorescent antibody stain is the more sensitive than silver stain (GMS), Diff-Quik stain and Merifluor *Pneumocystis* stain. Baughman *et al* [20] who described the sensitivity of an indirect immunofluorescent antibody stain it was the superior in comparison with a modified Wright stain and GMS stain. In another studies when compare the feasibility of different stain methods were applied in respiratory secretions to stain the *P. jirovecii* (Methenamine Silver stain, TOB, Acridine Orange, Diff-Quik, Gram-Weigert, etc.) they found the low sensitivity although these stains are cheap and easily applicable [21-24]. However, commercial immunofluorescence (IF) kit which contains monoclonal antibodies has, subsequently, increased the specificity and sensitivity for diagnosis [25-27].

It is concluded from this study that the *P. jirovecii*, is the fundamental opportunistic infection among immunocompromised patients. GMS staining may have the best overall predictive values for routine clinical use when monoclonal antibody staining is not available, Diff-Quik is a good diagnostic tool in the diagnosis of, *P. jirovecii* because of its cost-effectiveness and because of its rapid diagnosis of severe pneumocystosis. In the present study showed the indirect qualitative immune-fluorescence method give a promise for use as a primary method for diagnosis of *P. jirovecii* pneumonia or as rapid screen to exclude the presence of *P. jirovecii* in sputum and BAL samples.

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Author contributions

Touhali conducted the sampling, isolation, and staining, the molecular work and writing the manuscript. Dr. Ibrahim and Dawood supervised the work, edit and finalize the writing of the study.

Conflict of interest

The authors declare no conflict of interest.

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