Infect Chemother. 2025 Mar;57(1):45-62
https://doi.org/10.3947/ic.2024.0148
pISSN 2093-2340·eISSN 2092-6448

Infection & Chemotherapy

Review Article

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Diagnosis of *Pneumocystis jirovecii* Pneumonia in Non-HIV Immunocompromised Patient in Korea: A Review and Algorithm Proposed by Expert Consensus Group

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ABSTRACT

Pneumocystis jirovecii pneumonia (PJP) is a life-threatening infection commonly observed in immunocompromised patients, necessitating prompt diagnosis and treatment. This review evaluates the diagnostic performance of various tests used for PJP diagnosis through a comprehensive literature review. Additionally, we propose a diagnostic algorithm tailored to non-human immunodeficiency virus immunocompromised patients, considering the specific characteristics of current medical resources in Korea.

Keywords:*Pneumocystis jirovecii* pneumonia; Diagnosis; Polymerase chain reaction; β-D glucan

Received: Dec 17, 2024
Accepted: Jan 14, 2025
Published online: Jan 20, 2025

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BACKGROUND AND PURPOSE

Pneumocystis jirovecii pneumonia (PJP) is a form of pneumonia in which the fungus *P. jirovecii* affects the lung interstitium. In the 1980s, PJP was reported to be an opportunistic infection that affects individuals infected with the human immunodeficiency virus (HIV). However, in recent years, there has been a notable increase of PJP in immunocompromised patients without HIV infection [1-5]. Patients with suppressed cell-mediated immunity or those on long-term immunosuppressive therapy, including those with hematologic malignancies, organ transplant recipients, and patients with autoimmune diseases, have an elevated risk of developing PJP [6-12].

Early diagnosis and subsequent treatment are essential for improving the prognosis of PJP. Traditionally, PJP is diagnosed in high-risk patients using a two-pronged approach: first, suspicion based on observation of characteristic clinical symptoms and imaging findings, and second, identification of the fungus through microscopic examination [6, 13, 14]. Classical microscopy methods used for the detection of *P. jirovecii* include Wright-Giemsa staining, Calcofluor white staining, and Gomori's methenamine silver staining. However, these methods are limited by their low sensitivity and specificity. Immunofluorescence staining recently emerged as a prominent diagnostic technique [6, 13-16]. Notably, it has been documented that microscopic techniques exhibit diminished sensitivity for respiratory specimens obtained from non-HIV-infected patients due to the low number of asci present within their respiratory tracts [14, 17-19].

To address these limitations, several recent international guidelines recommend utilizing molecular diagnostic techniques, such as quantitative polymerase chain reaction (PCR), to detect *P. jirovecii* [6, 13, 14, 16]. PCR has greater sensitivity and a higher negative predictive value for the detection of *P. jirovecii* than microscopic examination with fluorescent staining. In addition, it is considered a particularly useful diagnostic tool in cases where the fungal burden of non-HIV-infected patients is low [18, 20-23]. Furthermore, the test can identify genetic variants that are resistant to sulfa drugs, which could be used not only for diagnostic purposes but also for therapeutic interventions [24]. Nevertheless, the diagnostic efficacy of the prevailing real-time PCR test in Korea has not been fully elucidated. Moreover, the implementation of immunofluorescence microscopy in Korea is constrained by the scarcity of the requisite reagents.

The aim of this review was to evaluate recent research findings and guidelines related to the diagnosis of PJP and assess the status of the testing methods for PJP available in Korea.

Also, we aimed to propose suitable practice guidelines for the diagnosis of PJP that can be easily implemented in the current Korean healthcare setting.

SCOPE

This was a comprehensive review of the available diagnostic methods used for the detection of PJP in non-HIV-infected patients. This review covers risk assessment, imaging findings, microscopic examination, molecular biological testing, and biochemical testing methods, with a particular focus on the diagnosis of PJP in the Korean healthcare setting. In addition, a literature review was conducted to analyze and identify additional considerations for pediatric patients. Furthermore, a survey of the members of the Korean Society of Clinical Microbiology was conducted in March 2024 to ascertain the actual testing methods used to diagnose PJP in Korea. Based on survey findings, a diagnostic algorithm for PJP was proposed for implementation in clinical practice in Korea.

ORGANIZATION OF COMMITTEES

In March 2024, the Korean Society of Infectious Diseases organized a clinical guideline committee composed of experts from the Departments of Infectious Diseases, Diagnostic Laboratory Medicine, Radiology, and Pediatric Infectious Diseases to establish diagnostic criteria for non-HIV-infected patients in Korea. The Korean Society of Clinical Microbiology participated in and assisted in a survey on the current status of the use of diagnostic tests for PJP in Korea.

AGENDA SETTING AND LITERATURE REVIEW PROCESS

The core agenda for this review was developed through a synthesis of national and international literature and guidelines reflecting Korean clinical realities. Expert consensus was achieved during multiple committee meetings, focusing on the diagnostic process from the perspectives of patients, imaging, and diagnostic tests.

https://doi.org/10.3947/ic.2024.0148

Table 1. Summary of non-HIV-infected patient groups with a high risk for *Pneumocystis jirovecii* pneumonia

Risk factor	Comments
Disease or condition	
Primary immunodeficiency	Increased risk for PJP in patients with severe combined immunodeficiency, complete DiGeorge syndrome, partial DiGeorge syndrome, Wiskott-Aldrich syndrome, X-linked hyper-IgM, X-linked agammaglobulinemia, and ataxia-telangiectasia
Hematologic malignancy	Risk for PJP is highest in patients with acute lymphoblastic leukemia, with an increased risk in patients with non-Hodgkin's lymphoma, chronic lymphoblastic leukemia, and multiple myeloma
Solid organ malignancy	PJP is commonly diagnosed in patients with metastatic brain tumors, lung cancer, and breast cancer, and the risk varies depending on the type of anticancer drug administered and whether the patient uses steroids
Hematopoietic stem cell transplantation	Increased risk for PJP in patients who underwent allogenic hematopoietic stem cell transplantation, particularly those with graft versus host disease
Solid organ transplantation	Risk for PJP is highest in patients who underwent lung and heart-lung transplants, and relatively low in those who underwent kidney and liver transplants
Therapeutics	
Glucocorticoids	Use of prednisolone 20 mg/day (2 mg/kg in pediatric patients) or more for more than 4 weeks increases the risk for PJP
Chemotherapeutic drugs	Use of methotrexate, fluorouracil, bleomycin, fludarabine, cytarabine, or temozolomide increases the risk for PJP
Monoclonal antibodies	Administration of an anti-CD20 antibody (rituximab), anti-CD52 antibody (alemtuzumab), or anti-TNF antibody (infliximab) increases the risk for PJP
CAR-T cell therapy	Prophylaxis is recommended due to the high incidence of PJP in patients who underwent CAR-T therapy The risk for PJP is particularly high once cytokine release syndrome occurs following CAR-T treatment and glucocorticoids are administered

HIV, human immunodeficiency virus; PJP, *Pneumocystis jirovecii* pneumonia; TNF, tumor necrosis factor; CAR-T, chimeric antigen receptor T-cell.

High-risk patient groups, excluding HIV-positive patients, were defined, and imaging findings were summarized. Diagnostic tests, including microscopic tests (standard), real-time quantitative PCR (recently recognized), and serum (1-3)-β-D-glucan tests (serological), were categorized by clinical specimens and their diagnostic significance for PJP.

The review incorporated domestic and international guidelines and literature published in Korean or English since 2000, integrating the latest research. Data sources included PubMed, EMBASE, the Cochrane Database, and KMBASE.

HIGH RISK GROUP FOR PJP AMONG NON-HIV-INFECTED PATIENTS

PJP in non-HIV-infected patients was first reported as plasmacytoid pneumonia at the end of World War II, particularly in malnourished infants in orphanages [25]. Presently, PJP is most prevalent in patients with underlying medical conditions that impair cell-mediated immunity, or in patients undergoing immunosuppressive therapy. The most well-known underlying conditions that increase the risk for PJP are hematopoietic stem cell transplantation and solid organ transplantation. Autoimmune diseases and solid cancers are also associated with an increased risk of PJP (**Table 1**). Patients receiving immunosuppressive medications, such as steroids, chemotherapy, or immunosuppressants, are at increased risk of developing PJP. This risk is further elevated in those taking multiple immunosuppressive drugs for underlying conditions [26-28].

Acute lymphocytic leukemia is associated with the highest risk for PJP among all hematologic malignancies. Additionally, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and multiple myeloma are linked to a high risk of developing PJP, whereas myelodysplastic syndromes and acute myeloid leukemia are associated with a relatively low risk for PJP [27]. These discrepancies in the risk for PJP in patients with hematologic malignancies may be attributed to the use of steroids. However, the precise mechanism underlying the correlations between hematologic malignancies and PJP remains unclear. Allogeneic hematopoietic stem cell transplantation markedly elevates the likelihood of developing PJP. It has been reported that in the absence of PJP prophylaxis, 9–16% of patients develop PJP within six months after transplantation [29-31]. Notably, the incidence of PJP has significantly decreased since the introduction of trimethoprim/sulfamethoxazole for prophylactic therapy. Most cases of PJP occur in cases of graft versus host diseases or disease relapse [32].

The recent introduction of chimeric antigen receptor T cell (CAR-T) therapy has led to its use in cases

of treatment failure, particularly in patients with lymphocytic leukemia, multiple myeloma, and lymphoma, for whom PJP prophylaxis is recommended [33, 34]. Patients undergoing CAR-T therapy frequently exhibit an immunocompromised status due to the presence of underlying medical conditions and treatment with cytotoxic anticancer drugs. The development of cytokine release syndrome during CAR-T therapy may elevate the risk of PJP due to treatments such as use of high-dose steroids or IL-6 receptor-blocking antibodies (tocilizumab). In a recent study, 1.7% of 1,107 patients treated with CD-19 CAR-T cells and 1.4% of 280 patients treated with B-cell maturation antigen CAR-T cells developed PJP. In the study, the patients who developed PJP were found to be significantly more likely to receive dexamethasone than those who did not develop PJP. Furthermore, those who developed PJP had a shorter duration of PJP prophylaxis, with a median of 9 weeks compared to 19 weeks for those who did not develop PJP [35].

In the absence of PJP prophylaxis, 5–15% of patients who underwent solid organ transplantation develop PJP within six months post-transplant. The risk for PJP is higher in lung and heart-lung transplant recipients and relatively lower in kidney and liver transplant recipients [36]. With the implementation of an appropriate prophylactic regimen, PJP typically manifests at least 12 months post-transplant. The presence of additional risk factors, including advanced age, allograft rejection, low CD4 T-cell count, and cytomegalovirus infection, may increase susceptibility to PJP [12, 37].

PJP has been diagnosed in patients undergoing chemotherapy for different types of solid tumors, most commonly primary or metastatic brain tumors, lung cancer, and breast cancer [38]. In particular, PJP has been identified as a concomitant phenomenon in approximately 1% of patients with brain tumors [29, 32]. High-dose steroid use is strongly associated with the development of PJP in patients with solid tumors [39]. Temozolomide, an alkylating agent and anticancer drug commonly used to treat glioblastoma, can cause lymphopenia, which increases the risk of developing PJP [40].

The use of immunosuppressive drugs increases the risk of developing PJP, with steroids being most commonly associated with this phenomenon. Several studies have demonstrated that most patients diagnosed with PJP have a history of steroid treatment within the previous month. Generally, the risk of PJP is increased in patients with underlying medical conditions, particularly if they receive steroids co-administered with other immunosuppressive drugs. Conversely, the risk of PJP is relatively low when steroids are used alone for the treatment of patients without underlying medical conditions. The risk of developing PJP may vary depending on the patient's underlying medical conditions and the immunosuppressant used for treatment. Notably, the reported risk threshold for steroid dose and duration of therapy varies across studies. However, the generally accepted threshold is prednisolone 20 mg or more for more than four weeks (2 mg/kg or more in pediatric patients under 10 kg) [26-28, 41].

Anticancer drugs that cause lymphopenia are known to increase the risk of PJP. Methotrexate, fluorouracil, bleomycin, fludarabine, cytarabine, and temozolomide are associated with the development of PJP [39, 42-45]. It is noteworthy that a history of steroid use has been observed in most cases of PJP following the administration of anticancer drugs. However, fludarabine may independently increase the risk of PJP development, irrespective of whether steroids were used [42].

Various immunosuppressive monoclonal antibodies are associated with the development of PJP. Rituximab, an anti-CD20 monoclonal antibody, has been reported to elevate the risk of PJP, even in the absence of concomitant corticosteroid administration [46, 47]. Alemtuzumab, an anti-CD52 monoclonal antibody, elevates the risk of PJP as a consequence of prolonged T cell suppression. This is evidenced by reports of the occurrence of PJP in early-phase clinical trials. It is therefore recommended that prophylaxis be administered subsequent cases [40, 48, 49]. Several cases of PJP in patients undergoing treatment with infliximab, an anti-TNF monoclonal antibody, have also been documented. However, since high-dose steroids or other immunosuppressive drugs were co-administered in these cases, strong evidence is lacking to confirm that infliximab independently increases the risk of PJP [50].

There is currently no tangible evidence to suggest that mycophenolate mofetil and calcineurin inhibitors, which are commonly used after transplantation, are associated with an increased risk of PJP [39]. One study indicated that the use of tacrolimus in lieu of cyclosporine increases the risk of PJP in renal transplant recipients; however, a subsequent investigation did not reveal a statistically significant difference in immunosuppressant categories

https://doi.org/10.3947/ic.2024.0148

and the incidence of PJP between solid organ transplant patients with and without PJP [37, 51].

Primary immunodeficiencies that increase the risk of PJP in pediatric patients include severe combined immunodeficiency with complete T lymphocyte deficiency, complete DiGeorge syndrome, and partial DiGeorge syndrome with partial T lymphocyte deficiency. Other conditions that may increase the risk of PJP include Wiskott-Aldrich syndrome, X-linked hyper-IgM syndrome, X-linked agammaglobulinaemia, and ataxia-telangiectasia [52].

IMAGING FINDINGS OF PJP IN NON-HIV-INFECTED PATIENTS

Chest radiographs and computed tomography serve as initial, non-invasive diagnostic tools for screening patients with risk factors who present with symptoms suggestive of PJP. The immediate availability of results offers a significant advantage in clinical decision-making. However, the imaging findings of PJP overlap with those seen in other infections and inflammatory conditions, including viral pneumonias and organizing pneumonia, making pathognomonic radiological diagnosis challenging. The characteristics of these imaging modalities are delineated below (**Table 2**).

1. Chest radiography

In non-HIV-infected patients presenting with dyspnea, hypoxia, and fever without sputum, chest radiographs typically demonstrate features consistent with interstitial pneumonia. It is important to note that chest radiographs of early PJP may appear normal, necessitating careful clinical correlation and potentially more advanced imaging. The typical imaging findings of PJP consist of bilateral patchy opacities with perihilar distribution, which often extend to the peripheral lung fields and bases. The opacities may gradually worsen for 3–5 days after treatment initiation, leading to the formation of distinct consolidation. In addition to the typical presentation, atypical patterns, including pulmonary nodules, unilateral involvement, pleural effusion, pneumothorax, lymphadenopathy, or lobar consolidation, may also be observed [53].

2. Computed tomography (CT)

When chest radiographs are normal or equivocal despite clinical suspicion for PJP, CT serves as a more sensitive imaging modality that can detect subtle parenchymal abnormalities not visible on conventional radiography [54]. CT imaging not only contributes to establishing the diagnosis of PJP but also functions as a powerful tool for evaluating disease severity and predicting clinical outcomes [55-57].

The cardinal CT finding of PJP in non-HIV-infected patients is extensive ground-glass opacities (GGOs). These GGOs indicate alveolitis caused by the accumulation of fibrin or microbial debris in the alveoli. The opacities demonstrate a characteristic distribution pattern marked by bilateral symmetry, predominantly affecting the central and perihilar regions while showing relative sparing of the peripheral lungs [56]. Notably, a mosaic pattern, representing heterogeneous involvement of lung parenchyma, is observed in approximately 57% of patients [57].

Approximately 20% of non-HIV-infected patients with PJP demonstrate nonspecific imaging findings on CT. These variant patterns may manifest focally or show predilection for the lower lung zones, where rapid progression from GGOs to frank consolidation can occur. This swift evolution to consolidation likely reflects the intense immune-mediated inflammatory response in non-HIV-infected patients [58]. Other atypical findings such as pulmonary nodules, lymphadenopathy, and pleural effusions remain rare on both CT and chest radiographs, highlighting an important pattern recognition principle that aids in diagnostic confidence [59].

Table 2. Summary of *Pneumocystis jirovecii* pneumonia imaging findings in non-HIV-infected patients

Imaging modality	Findings in the early stage	Findings after the mid-stage
Chest X-ray	Normal or nearly normal	• Bilateral infiltrates in the mid-stage • Bilateral consolidation in the late stage
Chest CT	Finding: Ground glass opacity, Distribution: Perihilar, sparing of the lower lung zones and subpleural regions	• Evolution in cases of ineffective therapy; resolution in weeks to months after specific treatment in most cases • Finding: Mosaic pattern and architectural distortion, with increasing density of infiltrates • Distribution: Same as the acute phase

HIV, human immunodeficiency virus; CT, computed tomography.

https://doi.org/10.3947/ic.2024.0148

Pulmonary cysts predominantly occur in the upper lobes and are exceedingly uncommon in non-HIV-infected patients, with an incidence of approximately 3%. This stands in stark contrast to HIV-infected patients, where pulmonary cysts are observed in approximately 56% of cases [56]. When present, these cysts may be complicated by pneumothorax or pneumomediastinum [60]. Multiple pulmonary nodules represent another uncommon imaging finding, characteristically displaying random distribution and reflecting underlying granulomatous inflammation [61]. This granulomatous form of PJP shows a particular association with hematologic malignancies [62], with nodules varying in size from millimeters to over a centimeter in diameter. The identification of centrilobular or branching opacities (tree-in-bud pattern) should direct diagnostic consideration toward infectious bronchiolitis rather than PJP. In cases of severe PJP, patients may develop acute respiratory distress syndrome, which carries a higher mortality burden in the non-HIV-infected population.

MICROBIOLOGICAL DIAGNOSTICS OF PJP IN NON-HIV-INFECTED PATIENTS

Microscopic examination, PCR, and serum β-D-glucan assays are commonly used for the diagnosis of PJP in non-HIV-infected patients. A summary of the characteristics of these diagnostic methods is provided, with detailed considerations as follows (**Table 3**).

1. Microscopic examination

P. jirovecii cannot be cultured *in vitro*; therefore, a microbiological diagnosis is made by confirming the presence of *P. jirovecii* through microscopy or molecular testing. Stains used for microscopic examination of *P. jirovecii* include Giemsa, rapid Giemsa-like stains, fluorescein-conjugated monoclonal antibody kits, methenamine silver (Gomori/Grocott), toluidine blue O/cresyl echt violet, calcofluor white, Gram-Weigert, and Papanicolaou (**Table 4)** [63, 64].

Table 3. Summary of diagnostic microbiological features of *Pneumocystis jirovecii* pneumonia in non-HIV-infected patients

Diagnostic techniques	Specimen	Advantages	Disadvantages	Indications
Microscopic examination	• BALF is preferred. • Induced sputum and airway aspirate can also be used. • Normal sputum is not recommended	• High specificity (visual identification of pathogens) • Relatively consistent results • Rapid testing • Low cost	• Low sensitivity • Requires skilled testers • Varying accuracy of results based on the type of specimen used (BALF recommended) • High workload for testers	• Methenamine silver staining is used for staining asci and is a standard test for diagnosing PJP. • Rapid Giemsa-like stain and immunofluorescence stain are commonly used to diagnose PJP due to their high sensitivity and specificity.
Real-time quantitative PCR	• BALF is generally preferred, but induced or normal sputum is acceptable.	• Low workload • High sensitivity and specificity • Increased diagnosis rates compared to microscopy alone	• High sensitivity may result in false positives caused by reporting simple colonization as positive results. • The use of induced sputum for diagnostic purposes may not be sufficient to definitively exclude the presence of PJP, even in the absence of a positive PCR result.	• Recommended due to its high sensitivity and specificity, coupled with a relatively low workload. • BALF is a more accurate diagnostic specimen; however, induced sputum can also be used for testing. • Results obtained from induced sputum specimens should be interpreted cautiously, particularly in the context of false negatives and false positives.
Serum β-D-glucan	• Performed using serum • Use of BALF not sufficiently evaluated	• High negative predictive value	• False positives can be caused by a variety of factors. • Diagnostic performance of the Goldstream β-D-glucan test for domestic use has not been sufficiently assessed	• Used as a supportive diagnostic method to help rule out PJP owing to its high predictability of negativity

HIV, human immunodeficiency virus; BALF, bronchoalveolar lavage fluid; PCR, polymerase chain reaction; PJP, *Pneumocystis jirovecii* pneumonia

https://doi.org/10.3947/ic.2024.0148

Table 4. Characteristics of various respiratory specimen smear stains for the diagnosis of *Pneumocystis jirovecii* pneumonia

Staining method	Testing time	Stained asci walls	Trophic and other stained forms	Common specimens	Advantages	Disadvantages
Giemsa Rapid Giemsa-like stains	30–60 minutes Within 5 minutes	Not stained	Core: Reddish-purple Cytoplasm: Blue (darker depending on how clumpy it is)	BALF, induced sputum	Low cost; easy staining; staining at all life cycle stages; staining of the host or other pathogens	Proficiency required for interpretation of results
Fluorescein-conjugated monoclonal antibody	15–30 minutes	Apple green fluorescence, shaped like a wrinkled raisin	Polygonal or spherical with an apple green, fluorescent rim; nucleoli also stained; masses stained throughout	Lung tissue, BALF, sputum	Recommended for inexperienced users; high sensitivity and specificity	Requires a fluorescence microscope and expensive reagents
Methenamine silver	30 minutes (microwave), 1–2 hours (expedited), and 6–24 hours (conventional)	Brown-black	Not stained	Lung tissue, BALF	Easy detection of asci; no staining of the host cell	Relatively long staining time; high cost; strong acid used; stains only asci; stains other fungi as well
Toluidine blue O/cresyl echt violet	1–6 hours	Purple	Not stained	Lung tissue, BALF	Easy detection of asci; does not stain host cells	Relatively long staining time; strong acid used; stains only asci; stains other fungi as well
Calcofluor white	Within 5 minutes	Blue-white or green, depending on the filter	Not stained	Lung tissue, BALF, sputum	Bright asci fluorescence; easy to perform; low cost	Requires a fluorescence microscope; strong base used; stains only asci; stains other fungi as well; proficiency required for interpreting results
Gram-Weigert	Within 5 minutes	No staining; asci is purple	Faint trophic forms	BALF, sputum	Easy to perform in cytopathology laboratories	Faint staining; proficiency required
Papanicolaou	1–6 hours	No staining; asci is purple	Faint trophic forms	BALF, sputum	Easy to perform in cytopathology laboratories	Faint staining; proficiency required

BALF, bronchoalveolar lavage fluid.

Methenamine silver stain is a staining method capable of staining other fungal species. This process relies on oxidizing polysaccharides in the fungal cell wall and membrane into aldehydes using periodic acid. Then, aldehydes reduce silver ions to metallic silver under basic conditions. One type of methenamine silver stain is the Grocott-Gomori stain. This stain can be applied to tissues and fluids, including bronchoalveolar lavage fluid (BALF) and induced sputum. The disadvantages of this staining technique include the chemical instability of the dye, potential degradation of the dyed metal, and the relatively long dyeing time. Such disadvantages can be mitigated to some extent with standardized protocols and reagents. With Grocott-Gomori stain the dye stains asci (the sexual spore-bearing cell produced in ascomycete fungi) without staining spores. The silver-stained asci exhibit a distinctive black cup-shaped morphology and contrast with the green-stained host cell structure. The walls of asci adopt a dark brown to black hue, resembling wrinkled raisins.

Other reagents that stain the walls of asci include periodic acid-Schiff, toluidine blue, cresyl echt violet, and calcofluor white. The results obtained with cresyl echt violet and toluidine blue O staining are comparable to those obtained with methenamine silver staining. Toluidine blue O produces a bright purple stain, whereas calcofluor white produces blue fluorescence under ultraviolet light. Calcofluor white frequently exhibits a distinctive chromosome in the form of a double bracket within the ascus. In contrast, Giemsa and rapid Giemsa-like stains do not stain the walls of asci; instead, they stain the nuclei of *P. jirovecii* reddish-purple and the cytoplasm light blue at all life cycle stages of the fungus (**Fig. 1**). The walls of asci are not stained, appearing as transparent areas. The nuclei of the host's lung cells, which can be stained together, are markedly larger than those of *Pneumocystis* and exhibit a deep reddish-purple color.

Rapid Giemsa-like stains are recommended for the diagnosis of PJP using BALF and induced sputum due

https://doi.org/10.3947/ic.2024.0148

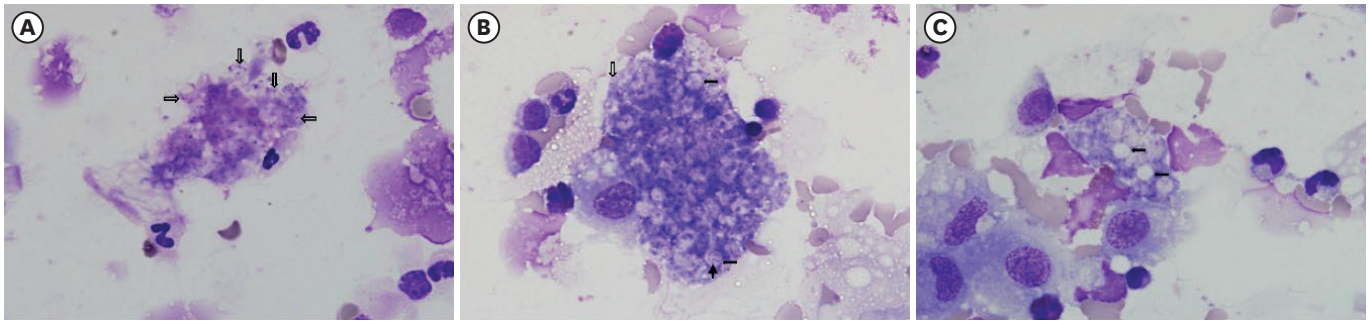


Figure 1. Diff-Quick (rapid Giemsa-like stain) staining (×1,000). (A) Trophic forms (open arrows) are predominant. (B) Both trophic forms (open arrows) and cystic forms (closed arrows) are observed. Eight asci, one of which is indicated by a line arrow, are visible within a cystic form. (C) Mature cystic forms (open arrows) are unstained and appear as empty circles.

to their low cost, ease of use, and speed. The staining is completed in less than one minute and can detect all forms of *Pneumocystis*. Given that trophic forms are typically 10 times more abundant than asci, rapid Giemsa-like stains may enhance the sensitivity of detection compared to other techniques that only stain asci. An additional benefit is that rapid Giemsa-like stains also stain alveolar macrophages, thus enabling the assessment of specimen quality. Furthermore, other pathogens are also stained, facilitating the rapid diagnosis of other pulmonary infections. A limitation of this method is that host cells are also stained; therefore, training is necessary for accurate interpretation of results.

Immunofluorescent assays employ direct or indirect fluorescein-conjugated monoclonal anti-*P. jirovecii* antibodies, targeting either asci or all forms of *Pneumocystis*. Given that trophic forms are more numerous than asci, kits that utilize antibodies capable of targeting all forms of *Pneumocystis* are more sensitive in diagnosing PJP. Fluorescein isothiocyanate, a fluorophore frequently employed for antibody-binding or indirect detection of substances, produces a bright apple-green color. The staining is distributed extensively across the surface of the aggregated pathogen, often manifesting on the matrix within which *Pneumocystis* is embedded (**Fig. 2**). Fluorescent staining is typically observed on the periphery of individual asci, exhibiting a somewhat indistinct appearance within. Immunocytochemical staining can also be utilized for the diagnosis of PJP (**Fig. 3**).

Papanicolaou stain colors extracellular pathogen masses green. Thicker clumps of pathogens may exhibit heterochromatic characteristics, displaying a range of colors from pink or purple to green or turquoise. Gram stain fails to stain *Pneumocystis* distinctively.

Smear staining of respiratory specimens has historically been the primary diagnostic test for PJP. However, immunofluorescence staining has been widely utilized since the 1990s. This is due to the relatively low sensitivity and the need for skilled interpretation of smear staining. A recent meta-analysis reported a sensitivity of 50% (95% confidence interval [CI], 39–61%) for conventional staining of induced sputum and 74% (95% CI, 62–87%) for immunofluorescence staining [65]. When the studies using BALF were pooled, the sensitivity of microscopic diagnosis compared to PCR as the gold standard was

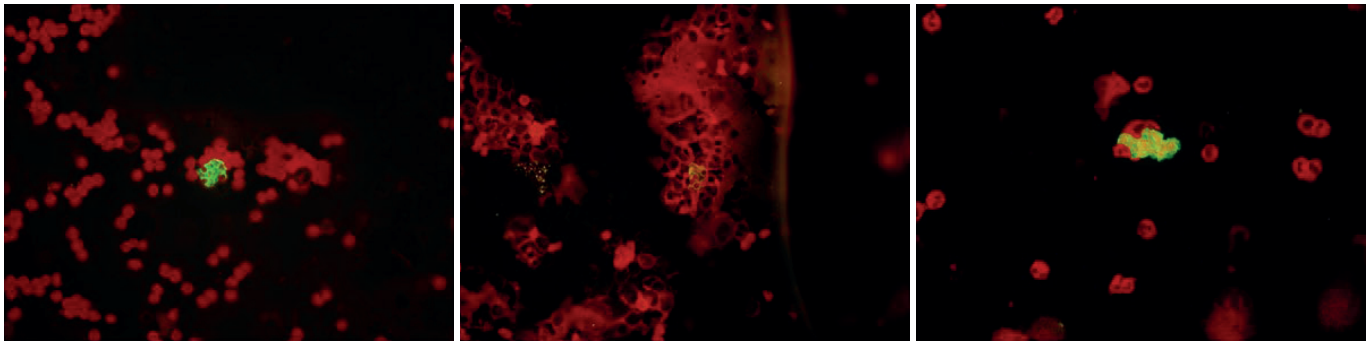


Figure 2. Direct immunofluorescence staining using monoclonal antibody 2G2 (×400). Trophic forms and cystic forms appear apple green due to fluorescein isothiocyanate fluorescence.

https://doi.org/10.3947/ic.2024.0148

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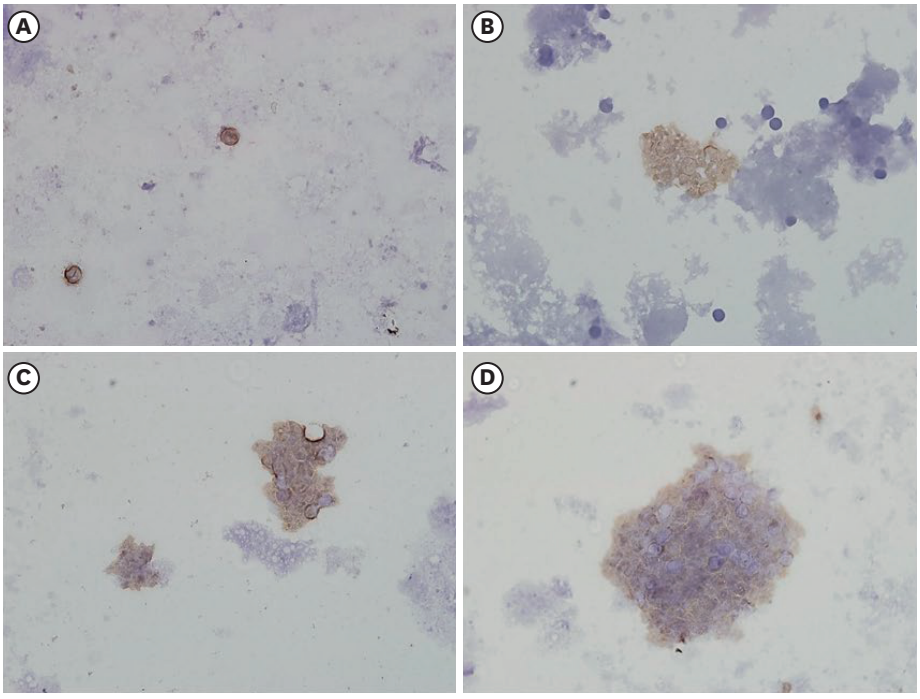


Figure 3. Immunocytochemistry using monoclonal antibody 3F6 (×1,000).
(A) The image shows two cystic forms. (B–D) The images display a mixture of trophic forms and cystic forms. Trophic forms are predominantly observed at the 8 o'clock position on (C). The remaining images display a mixture of trophic forms and cystic forms.

65% (95% CI, 21–100%) [14]. However, the studies on diagnostic techniques for PJP are limited by the fact that most of the study populations comprised HIV-infected patients, the staining methods used varied, and PCR was often included as a diagnostic criterion. Consequently, the positive and negative predictive values of the tests are contingent upon the patient's comorbidities, degree of immunosuppression, clinical course, and imaging findings. In non-HIV-infected patients, it is crucial to consider histopathological findings whenever feasible, in conjunction with the comprehensive clinical picture. This is because the low pathogen abundance in this population limits the sensitivity of microscopy and may result in false negatives.

In a retrospective study conducted at the Brigham and Women's Hospital/Dana-Farber Cancer Institute, diagnostic characteristics of immunofluorescent staining for induced sputum, BALF, and video-assisted thoracoscopic surgery biopsies were evaluated in 71 HIV-positive and 156 HIV-negative patients [66]. *P. jirovecii* infection was confirmed in 14% of the HIV-positive patients after the first induced sputum test, with no subsequent cases confirmed on further testing. In contrast, only 4% of the HIV-negative patients were confirmed to have *P. jirovecii* infection after the first induced sputum test, making additional testing necessary. Of the nine cases of *P. jirovecii* infection, three (33.3%) were confirmed after additional testing, two of which were confirmed in second induced sputum tests. The authors concluded that for HIV-negative patients, an initial sputum test for *P. jirovecii* should be performed and repeated if negative, followed by more invasive testing if PJP remains suspected.

In conclusion, immunofluorescence staining is the preferred method for microscopic diagnosis of PJP due to its superior sensitivity over traditional cell staining techniques [14]. Furthermore, the ratio of asci produced by sexual reproduction to trophic forms produced by asexual reproduction is approximately 1:10; therefore, the use of a method capable of staining both forms or a combination of methods that can stain each form can enhance the sensitivity of testing [14, 67]. In non-HIV-infected patients, a single induced sputum may be insufficient for diagnosis due to the low number of pathogens. Consequently, repeated induced sputum or invasive tests are often required.

2. Polymerase chain reaction test

1) Usage status and availability

Real-time quantitative PCR is not being used in Korea despite its several advantages over microscopic examination, including better reliability, the ability to quantify fungal loads, and a reduced workload. Instead, real-time PCR followed by reporting cycle threshold (Ct) is commonly used in Korea. However, the lack of standardization of the specimens due to the nature of respiratory samples and insufficient validation of the test kits resulted in a mix of qualitative and semi-quantitative reporting of results. In addition, despite the growing use of real-time quantitative PCR for diagnosing PJP globally [68], the threshold for differentiating colonization from infection remains poorly defined. Moreover, testing remains unavailable in many institutions due to technical or economic constraints.

https://doi.org/10.3947/ic.2024.0148

Commercial PCR kits have been developed to detect *P. jirovecii* DNA in a variety of specimens, including sputum, nasopharyngeal swab, BALF, and lung biopsy tissue. The test may be conducted at a medical institution or through a contractor. The entire process, from sample preparation to PCR, takes approximately four to five hours. However, each laboratory has its own testing days, and most take 1-3 days from the receipt of specimens to report the results.

2) Methods and meaning

PCR test for PJP is performed by extracting DNA from the specimen and subsequently utilizing primers that target the gene region that codes the large-subunit ribosomal DNA of *P. jirovecii* mitochondria. BALF is generally preferred for PCR for the diagnosis of PJP; however, induced sputum, sputum, mouthwash, or nasopharyngeal aspirate can also be used [69]. The use of blood in PCR test has been studied; however, it has not been adopted for clinical use [70].

PCR tests can be divided into qualitative PCR and quantitative PCR. Qualitative PCR performed using BALF was first proposed by Wakefield et al. in 1990 [71]. Subsequently, PCR methods performed using induced sputum were proposed [72]. The technique for real-time quantitative PCR was first described in 2002 by Larsen et al. [73]. In 2004, Flori et al. reported that real-time quantitative PCR is as sensitive as qualitative PCR and has the advantage of being able to distinguish colonization from true infection by defining thresholds [74]. In 2012, Mühlethaler et al. evaluated the diagnostic performance of quantitative PCR performed using BALF for the diagnosis of PJP in non-HIV-infected immunocompromised patients [20]. In the study, quantitative PCR test results were categorized into three levels based on the number of fungi per unit volume: positive, moderate, and negative. A positive result was considered a reliable diagnosis, whereas a negative result suggested that PJP could be virtually ruled out. The authors suggested that patients with moderate results required microscopic examination and further clinical evaluation.

Real-time PCR tests, both qualitative and quantitative PCR, are highly sensitive. PCR tests are more sensitive than microscopy in both HIV-infected and non-infected individuals. Real-time PCR using BALF has a sensitivity of 94–99% and a specificity of 82–100% [22, 23, 75]. PCR is an important diagnostic method, especially for non-HIV-infected patients who may present with a relatively low fungal load, and is reported to increase the diagnosis rate for PJP by about 7% compared to microscopy alone [76]. However, PCR can detect very small amounts of fungi and may lead to false positive results that detect *Pneumocystis* in a colonized state [16]. Real-time quantitative PCR is known to have a low false-positive rate because its results can be reported based on a cut-off value, unlike qualitative PCR. Currently, most international guidelines recommend using real-time quantitative PCR for differentiating between simple colonization and infection [14, 68]. However, it should be noted that real-time quantitative PCR for the diagnosis of PJP remains unstandardized, as researchers utilize various target genes and self-developed calibration materials, making it challenging to compare results across assays and limiting its validity as a truly quantitative method [20, 71]. Therefore, a negative microscopy result with a positive PCR result should be interpreted with caution, as both active infection and simple colonization are possible. Additional tests, such as the β-D-glucan, and careful consideration should be undertaken in such cases.

3) Interpretation of PCR results by types of respiratory specimens

Examination of respiratory specimens from infected patients revealed that the greatest amount of *P. jirovecii* is detected in specimens obtained from the alveoli [77]. Therefore, BALF is preferred over sputum samples, as negative PCR of BALF enables the exclusion of diagnosis [14]. BALF offers the additional benefit of enabling the evaluation of a range of infectious and non-infectious pulmonary conditions [13]. However, BAL is semi-invasive and requires trained professionals, which may not be feasible for certain patients and settings. In these cases, PCR testing with induced sputum may be considered. The sensitivity of induced sputum is high in patients with HIV infection due to their high fungal load but is lower in non-HIV-infected patients due to their relatively low fungal load. Thus, a negative PCR of induced sputum in non-HIV-infected patients may not be sufficient to rule out the diagnosis of PJP [14]. In non-HIV-infected patients, PCR tests performed using upper respiratory tract specimens, such as sputum or nasopharyngeal swabs, exhibit even lower sensitivity and should be interpreted with caution (**Table 5**) [13].

BALF is also preferred for the diagnosis of PJP in pediatric patients [41]. Induced sputum can also be an effective diagnostic specimen in pediatric patients; however, narrow airways and difficulty expectorating sputum in

https://doi.org/10.3947/ic.2024.0148

Table 5. Interpretations of PCR results by types of respiratory specimens

Respiratory specimens	Advantages	Disadvantages	Interpretations
BALF	• High sensitivity	• Semi-invasive	• A positive result in patients with typical features of PJP confirms the diagnosis
	• High negative predictive value	• Requires trained professionals	• Due to its high negative predictive value, a negative result can rule out PJP
Induced sputum	• Non-invasive • Ease of testing	• Lower sensitivity and negative predictive value compared to BALF	• A positive result in patients with typical features of PJP confirms the diagnosis, but a negative result cannot rule out PJP
Sputum or nasopharyngeal swabs	• Non-invasive • Ease of testing	• Lower sensitivity and negative predictive value compared to BALF and induced sputum	• A positive result in patients with typical features of PJP confirms the diagnosis, but a negative result cannot rule out PJP

PCR, polymerase chain reaction; BALF, bronchoalveolar lavage fluid; PJP, *Pneumocystis jirovecii pneumonia*.

children may impact the sensitivity and specificity of the test. Given that non-HIV-infected patients exhibit a lower fungal load than HIV-infected patients, testing before antibiotic treatment can increase the sensitivity of PCR tests, when possible. Nevertheless, it is not advisable to defer treatment to conduct diagnostic testing, as *P. jirovecii* can be detected in BALF up to 72 h after the commencement of treatment [78].

3. Serum (1-3)-β-D-glucan test

The β-D-glucan test can detect (1,3)-β-D-glucan, a polysaccharide found in the cell walls of various fungal species, including *P. jirovecii* [79]. The diagnostic performance of this test for candidiasis and aspergillosis has been well established. In addition, it is also useful for the diagnosis of PJP. However, the kinetics of the secretion and clearance of β-D-glucan remain poorly understood. In addition, the potential for interference by other substances complicates the interpretation of results [80, 81]. False positives may result from a number of factors, including intravenous immunoglobulin, packed red blood cells, fresh frozen plasma, fungal colonization, use of surgical gauze containing glucan, mucositis or mucosal injury of the gastrointestinal tract, cardiopulmonary bypass, intravenous amoxicillin/clavulanate, colistin, ertapenem, meropenem, cefazolin, trimethoprim-sulfamethoxazole, cefotaxime, cefepime, ampicillin/sulbactam, piperacillin/tazobactam, aqueous penicillin G, polyethylene glycol-asparaginase, and defibrotide. Furthermore, bacteria possess β-D-glucan, which can result in β-D-glucan positivity in cases of gram-positive or gram-negative bacteremia.

Serum β-D-glucan test is generally performed as an adjunctive diagnostic test for PJP. Given its high sensitivity and negative predictive value, a negative β-D-glucan test result indicates a low likelihood of PJP [82, 83]. A recent meta-analysis of 23 studies by Del Corpo et al. evaluated the diagnostic accuracy of the serum β-D-glucan test for PJP in HIV-infected and non-HIV-infected patients using 23 studies published up to September 2019 [84]; they reported that the sensitivity and specificity of the serum β-D-glucan test in non-HIV-infected patients is 86% (95% CI, 78–91%) and 83% (95% CI, 72–90%), respectively. In addition, the positive and negative likelihood ratios were 4.78 (95% CI, 2.80–8.17) and 0.18 (95% CI, 0.11–0.29), respectively. The pre- and post-test probability curves showed that a negative β-D-glucan result was associated with a low post-test probability of ≤5% only if the pre-test probability in non-HIV-infected patients was ≤20%.

The current United States guidelines indicate that the serum β-D-glucan test is not a highly specific method for diagnosing PJP. However, it can be a valuable supplementary diagnostic tool when used in conjunction with other techniques (strength of recommendation: weak, quality of evidence: moderate) [53]. The European Conference On Infections In Leukemia guidelines also suggested that the β-D-glucan test can be used as an adjunctive diagnostic method to rule out PJP (strength of recommendation: A, quality of evidence: II) [14]. Notably, the potential utility of serum or BALF β-D-glucan tests for evaluating treatment response or predicting clinical prognosis remains unexamined, and no recommendations were made.

Some commercially available β-D-glucan tests include Wako β-D-glucan (Wako Pure Chemical Industries, Osaka, Japan), Fungitec-G-test MK (Seikagaku Corporation, Japan), and Dynamiker Fungus (1,3)-β-D-glucan (Dynamiker Biotechnology Co., Ltd., Tianjin, China), Goldstream β-D-glucan (Gold Mountain River Tech Development, Beijing, China), and Fungitell® (Associates of Cape Cod, Falmouth, MA, USA). Of these, Fungitell® (Associates of Cape Cod) is the most extensively

https://doi.org/10.3947/ic.2024.0148

researched, as it is the only test approved by the United States Food and Drug Administration. In Korea, most institutions currently use the Goldstream β-D-glucan test. This test utilizes horseshoe crab blood from a species that is different from the one used for Fungitell® (Associates of Cape Cod). Furthermore, the diagnostic efficacy of the Goldstream test for PJP has not been evaluated. Therefore, it requires further validation, and its results should be interpreted with caution.

Most clinical guidelines do not recommend β-D-glucan testing for pediatric patients due to its low sensitivity and specificity [41, 85-87]. Notably, the number of studies on β-D-glucan testing in pediatric patients is considerably smaller than that on adult patients. In addition, most of these studies were performed using the Fungitell® test, with the results demonstrating inconsistency compared to those observed in adult patients. The median sensitivity of the β-D-glucan test as a screening test is reported to be 75% (range, 0–100%), whereas its specificity is 55% (range, 29–95%). Additionally, its positive predictive value is 17% (range, 0–25%), whereas the negative predictive value is 95% (range, 84–100%). In diagnostic applications, the test demonstrated a sensitivity of 80%, a specificity of 47%, a positive predictive value of 69%, and a negative predictive value of 71% [88]. An important consideration is that thresholds for the β-D-glucan test have not been established for pediatric patients. Notably, healthy children have a higher blood concentration of β-D-glucan than adults, with an average of 85 pg/mL determined using the Fungitell® test; this is higher than the FDA-approved threshold for adults of 80 pg/mL [81].

CURRENT STATUS OF DIAGNOSTIC TESTING FOR PJP IN KOREA

A survey of the members of the Korean Society of Clinical Microbiology was conducted to collate information on diagnostic testing for PJP in Korea. Of 403 hospitals (general hospitals or higher-tier hospitals) contacted for the survey, 50 (12.4%) responded. Of the 50 hospitals that participated in the survey, 47 (94%) reported offering diagnostic tests for PJP (11.7% of 403). Of the 47 institutions, eight (17.0%) reported using nonspecific stains that do not utilize *Pneumocystis*-specific fluorescent antibody. The most common was silver stain (four institutions), followed by rapid Giemsa-like stain and Calcofluor white stain (three institutions each), Giemsa stain and fluorescein blue stain (two institutions each),

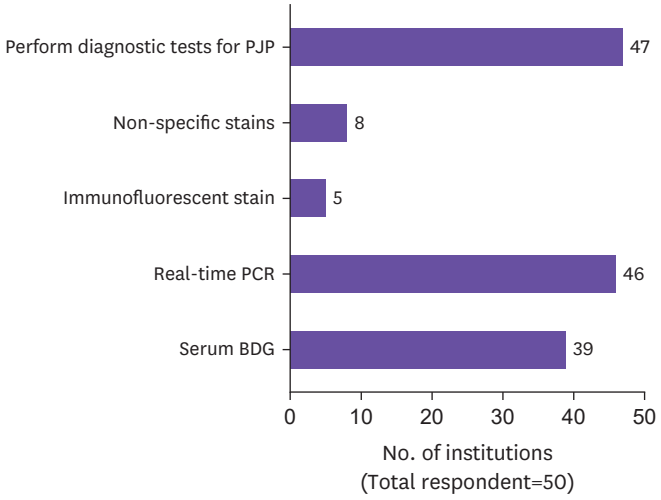


Figure 4. Current status of the use of diagnostic tests for *Pneumocystis jirovecii* pneumonia in Korea.
BDG, β-D-glucan; PCR, polymerase chain reaction; PJP, *Pneumocystis jirovecii* pneumonia.

and Wright stain (one institution). Seven institutions reported using nonspecific staining with BALF, whereas five (10.6%) conducted immunofluorescence staining using sputum, bronchoscopy, and lung biopsies. Of the five institutions, four reported that they perform tests using BALF, whereas all five reported that they test specimens other than BALF.

All but one institution (46, 97.9%) reported performing real-time PCR. All the institutions that perform real-time PCR reported that they test BALF, whereas 45 (97.8%) reported that they also test specimens other than BALF. Regarding real-time PCR test results, only five institutions (10.9%) provide semi-quantitative values based on Ct values. The remaining 41 institutions (89.1%) employ qualitative reporting (positive/negative). Thirty-nine institutions (83.0%) reported conducting serum β-D-glucan testing (**Fig. 4**).

DIAGNOSTIC APPROACHES

Based on the aforementioned facts, a series of pivotal elements have been identified for developing a diagnostic flowchart for non-HIV-infected patients in Korea. First, the risk factors for PJP should be sought for and identified in each patient suspected of PJP, as PJP is an infection that occurs in individuals with risk factors. Second, given the low abundance of *P. jirovecii* in the alveoli of non-HIV-infected patients, BAL should be prioritized whenever possible. Third, the survey on the current state of diagnostic testing

https://doi.org/10.3947/ic.2024.0148

for PJP in Korea demonstrated that only a few institutions perform immunofluorescence staining (five institutions, 10.6%); most institutions surveyed perform PCR, particularly real-time PCR. Consequently, real-time PCR is recommended as the principal diagnostic test for PJP in Korea, with β-D-glucan considered an adjunctive test.

For patients who present with symptoms suggestive of PJP, such as dyspnea, it is of utmost importance to ascertain whether they have any underlying risk factors for PJP. In the absence of any risk factors, other potential causes should be evaluated. The presence of risk factors for PJP warrants a chest CT. In the absence of medical contraindications, a respiratory specimen for real-time PCR should be obtained via bronchoscopy. If bronchoscopy is not a viable option, respiratory specimens can be obtained through sputum expectoration or induced sputum testing. A positive real-time PCR result with compatible clinical course and imaging findings is indicative of the diagnosis of PJP. Conversely, a negative result from real-time PCR using BALF can rule out PJP. If real-time PCR using sputum or induced sputum returns a negative result, blood β-D-glucan testing can be performed as an adjunctive test as the sensitivity of the specimen may be inferior to that of BALF. A negative β-D-glucan test result in this setting excludes PJP. Nevertheless, if the β-D-glucan test yields a positive result, it would be prudent to either reperform bronchoscopy or obtain a second induced sputum sample for quantitative PCR. A positive result on the second test indicates a PJP diagnosis, whereas a negative result excludes PJP (**Fig. 5**).

LIMITATIONS & SUGGESTIONS

Diagnosis of PJP in non-HIV-infected patients differs from that in HIV-infected patients in several aspects. First, false negatives may be more common due to the lower number of microorganisms in the alveoli of non-HIV-infected patients. Second, PJP in non-HIV-infected patients occurs in the presence of underlying medical conditions that lead to nonspecific imaging findings, making it challenging to differentiate PJP from other lung diseases that mimic PJP. Third, accurate diagnosis of PJP in non-HIV-infected patients requires more complex or invasive tests such as bronchoscopy, which may be difficult to perform due to the patient's underlying medical conditions. Ironically, expert consultation had to be suggested in the current guidelines that aimed to guide the diagnosis of PJP without the assistance of an expert.

Diagnosis of PJP in non-HIV-infected patients undergoes the following steps. If an individual with risk factors for PJP presents with symptoms consistent with PJP such as shortness of breath, imaging studies and the testing of respiratory specimens should be performed to confirm the diagnosis of PJP. Additionally, serum β-D-glucan testing may be performed as an adjunctive test. In the diagnosis of PJP in non-HIV-infected patients, symptoms, imaging findings, and β-D-glucan test results are nonspecific and cannot be used alone. Although staining is a conventional diagnostic approach, its use in Korea remains constrained by the limited supply of requisite reagents, the need for trained professionals to interpret the results, and the low sensitivity of the method.

Therefore, PCR is the most important tool for the diagnosis of PJP, particularly in non-HIV-infected patients who exhibit a lower concentration of microorganisms in the alveoli than in HIV-infected patients. The national survey on the status of diagnostic testing for PJP in Korea revealed that PCR is performed in 94% of laboratories that perform diagnostic tests for PJP. However, it should be noted that the only PCR test kit available in Korea is from a sole manufacturer that does not market in the United States and Western Europe, and the precise threshold for the diagnosis of PJP using this test remains unclear. Furthermore, further studies are warranted on the comparative diagnostic performance of BALF versus induced sputum and the optimal threshold of real-time PCR to distinguish between colonization and infection.

In addition, further research is warranted to determine whether the incorporation of β-D-glucan testing as an adjunct to PCR can assist the diagnosis, as recommended by the guidelines from other countries. In Korea, the β-D-glucan test is conducted using a kit distinct from the one that has been extensively studied in the United States and Europe. Therefore, further research is required to determine whether the results from the studies conducted in other countries can be applied to Korea.

https://doi.org/10.3947/ic.2024.0148

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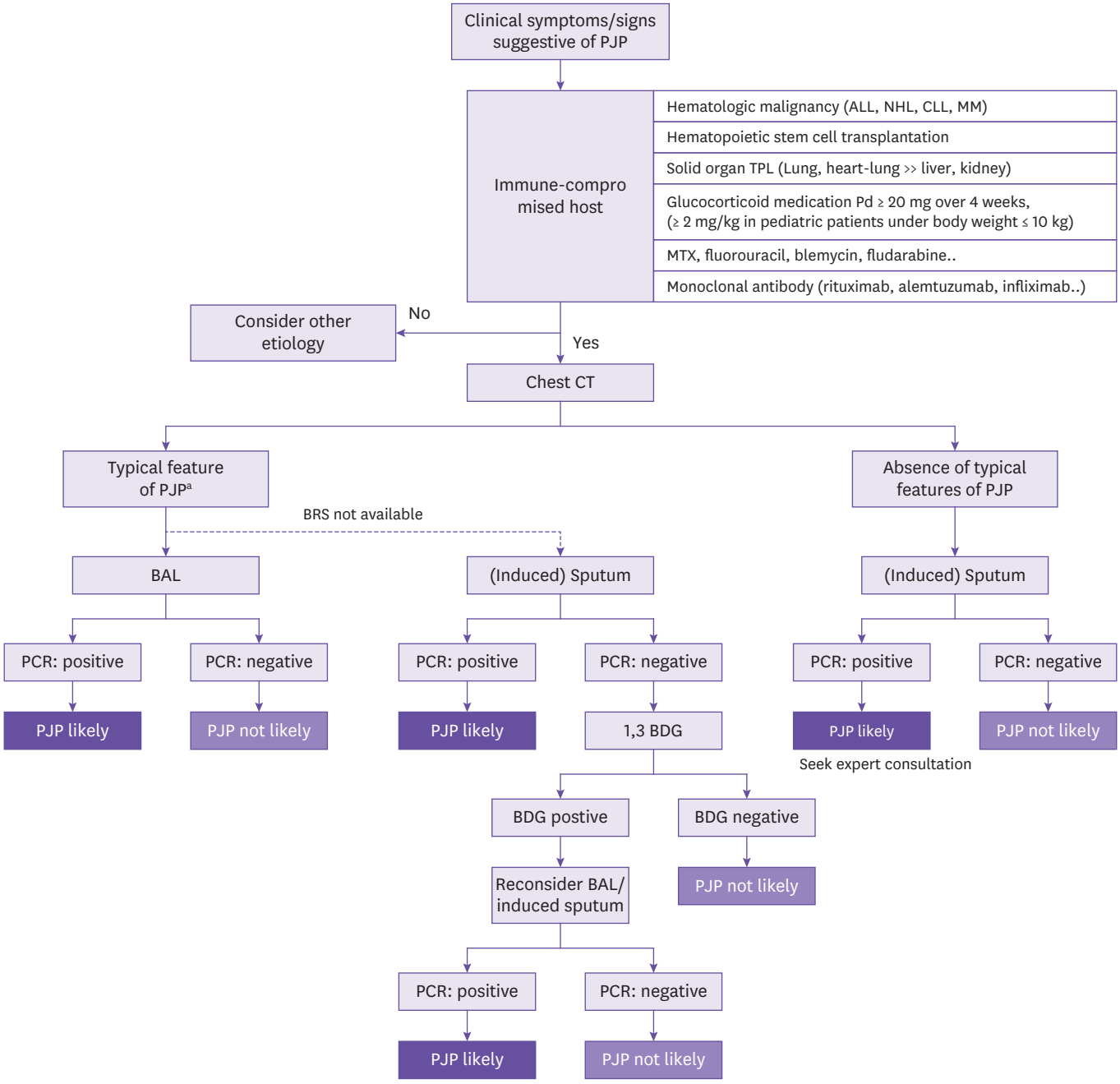


Figure 5. Diagnostic algorithm for *Pneumocystis jirovecii* pneumonia applicable in clinical practice in Korea.
[a]Bilateral ground glass opacities.
PJP, *Pneumocystis jirovecii* pneumonia; ALL, acute lymphoblastic leukemia; NHL, non-hodgikin lymphoma; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; TPL, transplantation; Pd, prednisolone; MTX, methotrexate; CT, computed tomography; BRS, bronchoscopy; BAL, bronchoalveolar lavage; PCR, polymerase chain reaction; BDG, β-D-glucan.

https://doi.org/10.3947/ic.2024.0148

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Funding
None.

Conflict of Interest
HS is ethics editor of *Infect Chemother*; however, he did not involve in the peer reviewer selection, evaluation, and decision process of this article. Otherwise, no potential conflicts of interest relevant to this article was reported.

Author Contributions
Conceptualization: RL, KH, CJK, KHS. Data curation: RL, KH, CKK, YCK, JHK, HK, JSP, JYP, HS, JJ. Methodology: HK, JSP, HS, CJK, KHS. Project administration: RL, KHm CJK, KHS. Investigation: RL, KH, CKK, YCK, JHK, HK, JSP, JYP, HS, JJ, CJK, KHS. Supervision: HK, HS, CJK, KHS. Validation: RL, KH, CJK, KHS. Writing - original draft: RL, KH, CKK, YCK, JHK, HK, JSP, JYP, HS, JJ, CJK, KHS. Writing - review & editing: RL, KH, CKK, YCK, JHK, HK, JSP, JYP, HS, JJ, CJK, KHS.

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