Recent Advances in the Diagnosis of \textit{Pneumocystis} Pneumonia

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\textbf{ABSTRACT}

\textit{Pneumocystis jirovecii} is a prototypical opportunistic pathogen, causing an asymptomatic or mild infection in normal hosts and fulminating pneumonia (\textit{Pneumocystis} pneumonia, PCP) in immunocompromised hosts. PCP is a leading cause of morbidity and mortality in immunocompromised patients such as AIDS patients. Microscopic detection of cysts and trophic forms of \textit{P. jirovecii} in respiratory secretions is simple and useful but may underestimate the \textit{P. jirovecii} infection. Conventional polymerase chain reaction (PCR) and nested PCR increase the sensitivity and specificity to identify PCP and provide an approach to discriminate PCP from pulmonary \textit{P. jirovecii} colonization, but the targeted genes and cut-off value from quantitative real-time PCR remain to be determined. Serum (1-3)-\textit{β}-D-glucan level and the specific serum antibody titer are ancillary indicators for PCP diagnosis. The successful cultivation of \textit{P. jirovecii in vitro} is an important progress for PCP research. The diagnosis of PCP relies on the combination of these laboratory examinations as well as the clinical presentations.

Key words: (1-3)-\textit{β}-D-glucan, CuFi-8 cells, ELISA, major surface glycoprotein, \textit{Pneumocystis jirovecii}, \textit{Pneumocystis} pneumonia, polymerase chain reaction

\textbf{Introduction}

\textit{Pneumocystis} spp. are extracellular, host-specific, and yeast-like parasitic fungi virtually restricted to lung tissues of many species of rodents, horses, and primates. \textit{Pneumocystis} was discovered in 1909, detected in rats in 1910, and classified as \textit{Pneumocystis carinii} in 1912. In the first decade of the 20th century, the human pathogen of \textit{Pneumocystis} was classified as a separate species from the rodent form (\textit{P. carinii}), and was renamed as \textit{Pneumocystis jirovecii}\textsuperscript{1}. \textit{Pneumocystis} pneumonia (PCP) is an opportunistic infection caused by \textit{P. jirovecii} in immunocompromised humans, especially in individuals infected with HIV or undergoing chemotherapy. Epidemiologically, ~95% of the worldwide population are believed to be infected with this fungus during childhood, but healthy adults are asymptomatic carriers of the fungus\textsuperscript{2}. PCP may develop from the reactivation of latent infection, but \textit{de novo} exposure to PCP patients or \textit{P. jirovecii} carriers through person-to-person transmission may also occur\textsuperscript{3-6}. PCP remains one of the important complications in AIDS patients and HIV-uninfected immunocompromised patients including patients with malignancies and organ transplantation. Better diagnostic tools are needed. In recent years, the promising diagnostic methods such as molecular biology allowed detection of \textit{P. jirovecii} in sputum and serologic test for the differentiation between PCP and pulmonary \textit{P. jirovecii} colonization have made the diagnosis of PCP less invasive and more accurate\textsuperscript{3,7,8}. This article will review the recent advances in the laboratory diagnosis of PCP.
Microscopic detection of *P. jirovecii* in respiratory tract specimens

Microscopic examination of *Pneumocystis* in respiratory tract specimens after staining with dyes or antibodies is an important approach for PCP diagnosis. Currently, the most commonly employed staining techniques include the methenamine silver stain which stains the cyst wall, the modified Giemsa stain which stains the parasite at all life cycle stages, the nonspecific fluorescent stain with calcofluor white, and the immunofluorescent stain using specific monoclonal antibodies.

The sensitivity of staining methods for the induced sputum samples ranges 55-78%. Positive results in bronchoalveolar lavage fluid (BALF) and lung biopsy are considered as the “gold standard” for PCP diagnosis. The sensitivities of immunofluorescent antibodies against surface antigens of *P. carinii* or *P. jirovecii* are higher than those using dye staining methods.

The accuracy of microscopic approaches is closely dependent on the skill of observers. The diagnosis can also be hampered in patients using highly active antiretroviral therapy and PCP chemoprophylaxis which may lead to low burden of *P. jirovecii*.

**In vitro culture of *P. jirovecii***

Culturing *P. jirovecii* has been attempted for several decades but without successful results. The lack of a reliable *in vitro* culture system hampered the comprehension of *P. jirovecii* pathogenicity and epidemiology. In 2014, Schildgen et al. reported that *P. jirovecii* could be cultured in a permanent three-dimensional air-liquid interface culture system formed by CuFi-8 cells. CuFi-8 cell is a differentiated pseudostratified airway epithelial cell line. When the pseudostratified epithelia grow and a mucous layer on the cell surface forms, the cells become polarized. The cultured CuFi-8 cells on the air side of the air-liquid interface are inoculated with 10-150 μl BALF. Five days later, the cells and basal medium are harvested and tested for *P. jirovecii*. *P. jirovecii* can be detected in the basal culture medium by quantitative real-time PCR (qPCR), which indicates that *P. jirovecii* is able to grow in this culture system, and must have actively passed through CuFi-8 cells, collagen, and a pore size of 4 μm in filter membrane into the culture medium. The culture of *P. jirovecii* will enable us to study its life cycle, drug sensitivity and environmental factors on its growth.

**The role of serum (1-3)-β-D-Glucan (BG) in the diagnosis of PCP**

Serum BG may be one of the useful serologic markers for establishing PCP diagnosis. Serum measurement of BG is based on the level of this polysaccharide that is present within the cell wall of *Pneumocystis* and other fungi. BG is one of the best characterized host response factors of *Pneumocystis*. Numerous studies have demonstrated that purified *Pneumocystis* BG stimulates expression of inflammatory cytokines and chemokines *in vitro*. In addition, BG in the cell wall of *Pneumocystis* stimulates pulmonary inflammation. The clinical usefulness of BG in serum and BALF as a potential marker of PCP has been evaluated. Treatment of PCP with BG synthase inhibitors such as anidulafungin in animal models provides an alternative therapeutic strategy for PCP.

Several studies using BG assay obtained a sensitivity of 90-100% and a specificity of 88-96% for PCP at various cutoff values depending on the BG kit and the population. There is a higher cutoff value for the diagnosis of PCP than for the diagnosis of invasive aspergillosis or candidiasis, but the optimal cutoff values have not been determined for these diseases. Damiani et al. found that infants with PCP had higher serum BG of 184~2,710 pg/ml, and that the serum BG levels (applying a threshold of 100 pg/ml) combined with qPCR for mtLSU rRNA gene in BALF (applying lower cutoff value of 1.6 × 10³ and upper cut off value of 2 × 10⁴ copies/l) could differentiate PCP from *P. jirovecii* colonization. One study indicated that BG level correlated with organism burden, but most others did not find a relationship of serum BG with organism burden, PCP severity, and response to therapy. Serum BG is also increased in other fungal infections such as Aspergillus, it is therefore used as an ancillary test in patients with a high suspicion of PCP. In patients who have a clinical presentation and radiological findings compatible with PCP, an elevated serum BG together with an appropriate copy number of *P. jirovecii* DNA in respiratory samples is the stronger indication for the diagnosis of PCP.
Serum anti-major surface glycoprotein (Msg) antibody level

Msg protein is a highly glycosylated protein of 90-120 kDa encoded by multiple genes with an estimation of 50-100 copies/P. jirovecii. Switch of Msg gene expression gives rise to multiple Msg isoforms and antigenic variations. Msg contains shared and specific antigenic determinants, elicits humoral and cellular protective immune responses, and plays a central role in the interaction between Pneumocystis and host. Recombinant Msg proteins offer valuable antigens to develop novel immunological assays. In a series of studies utilizing recombinant Msg fragments that cover several parts of the polypeptide, MsgA (amino acid residues 15-119), MsgB (amino acid residue 729-2282), and MsgC (amino acid residues 2015-3332), Walzer et al. developed western blot and ELISA that suggested an infection of observation, suggesting that they had experienced an infection of epidemiological significance.

Conventional and nested PCR for the detection of P. jirovecii DNA

Amplification of P. jirovecii specific DNA sequences by PCR may increase the detection sensitivity. The targeted P. jirovecii DNA segments for amplification include internally transcribed spacer (ITS), and the genes of 18S rRNA, 5S rRNA, mitochondrial large subunit rRNA (mtLSUrRNA), dihydrofolate reductase, Msg, cdc2, and heat shock protein 70 (HSP70). The targeted sequence for PCR is preferentially the mtLSUrRNA gene using pAZ102-E and pAZ102-H primers. Fan et al. conducted a systematic review and found that the sensitivity and specificity values of PCR in BALF for diagnosis of PCP were 98.3% and 91.0%, respectively, and the positive and negative likelihood ratios were 10.894 and 0.018, respectively.

Nested PCR increases the sensitivity without loss of specificity. The nested PCR for mtLSUrRNA gene was found to be more sensitive and useful than conventional PCR targeting Msg gene in detecting PCP. The primer pairs for the nested PCR targeting mtLSUrRNA gene included the outer pair of primers pAZ102-E and pAZ102-H and the inner pair of primers pAZ102-X and pAZ102-Y. Nested PCR could detect P. jirovecii DNA in BALF two to three orders of magnitude more dilute than conventional PCR.

Sequencing of amplified DNA fragments is useful to identify P. jirovecii strain and genotype, providing a unique way for tracking the fungus in human populations. Amplification of selected polymorphic sequences such as ITS in P. jirovecii genome has become a method to genotype P. jirovecii strain that may relate to its virulence. ITS can be used as a genetic marker to distinguish new infection and reactivation of latent infection. However, changes of ITS have been detected in a single episode of disease. Mutations in P. jirovecii dihydropteroate synthase gene detected by PCR are associated with sulfa drug resistance and has been used for monitoring the treatment effectiveness and also for typing P. jirovecii isolates in AIDS patients. Microsatellite genotyping on noninvasive samples (oropharyngeal washes) may aid in studying the molecular epidemiology of this pathogen.

qPCR for the detection of P. jirovecii DNA

qPCR is a new and cost-effective method for the detection and quantification of P. jirovecii in samples, monitoring therapeutic effectiveness, and distinguishing colonization from pneumonia in P. jirovecii infections. For BALF from HIV-infected patients with or without PCP, qPCR targeting HSP70 gene in P. jirovecii to quantify P. jirovecii DNA showed a clinical sensitivity of 98% and specificity of 96%; by contrast, the clinical sensitivity was 97% and the specificity was 68% when conventional PCR targeting mtLSUrRNA gene was used. Matsumura et al. found that the sensitivity and specificity for discriminating defi-
nite PCP from colonization was 100.0% and 80.0%, respectively, at a cutoff value of 1,300 copies/ml(3). Juliano et al. reported that the specificity was increased to 100% with the combination of the two qPCR assays targeting dihydropteroate synthase and Mgs in oropharyngeal washes(2). However, Flori et al. found overlapped cutoff values between samples obtained from potential carriers and proven PCP(3). In most cases, qPCR could greatly increase specificity with a good sensitivity to perform a diagnosis of PCP, however, qPCR cannot diagnose all cases of PCP for the presence of grey zone(4, 5). Further studies should focus on standardization of qPCR and determination of the optimal cutoff value to discriminate PCP from colonization for wide use of this method in clinical practice.

In summary, the accurate diagnosis of PCP is still a challenge. It is crucial to develop a less invasive and more accurate diagnosis strategy for PCP. The combination of clinical diagnosis, etiological diagnosis, molecular diagnosis and serological tests is necessary. The successful cultivation of P. jirovecii in vitro will influence the fields of diagnostic microbiology and clinical treatment of PCP.

Conflict of Interest

All authors declare no conflict of interest.

References

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