DEVELOPMENT OF IN-HOUSE MULTIPLEX AND QUANTITATIVE PCR ASSAYS FOR DETECTION OF *PNEUMOCYSTIS JIROVECII* IN BRONCHOALVEOLAR LAVAGE AND SPUTUM SPECIMENS

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Abstract. A definite diagnosis of *Pneumocystis* pneumonia requires laboratory confirmation of the presence of *Pneumocystis jirovecii* in clinical specimens. In-house multiplex and quantitative (q)PCR assays were developed to detect *P. jirovecii mtLSU rRNA* in bronchoalveolar lavage (BAL) and sputum specimens in comparison with three standard cytological assays [Gomori's methenamine silver (GMS) staining, Giemsa staining and immunofluorescence assay (IFA)] and conventional PCR. Both in-house multiplex and qPCR assays demonstrated specific of *P. jirovecii* without cross-amplification with other organisms. In BAL samples (n = 131), median copies/μl of *P. jirovecii mtLSU rRNA* DNA (measured by in-house qPCR) in samples positive by GMS staining (37%), Giemsa staining (37%) and IFA samples (42%) were $4.5x10^5$, $3.8x10^5$ and $3.8x10^5$, respectively; and in sputum samples (n = 54), median value in 4, 4 and 7 % positives was $5.5x10^5$, $5.5x10^5$ and $2.2x10^5$ copies/μl, respectively. Using conventional PCR median copies/μl *P. jirovecii mtLSU rRNA* DNA in BAL (49% positive) samples compared to in-house multiplex PCR (43% positive) were $2.5x10^5$ and $3.1x10^5$ respectively, while in sputum samples (17 and 13% positives) were $4.3x10^4$ and $4.3x10^4$ respectively. PCR methods showed higher sensitivity in *P. jirovecii* detection in both type of specimens compared to the classical staining methods. Overall, qPCR was considered the most promising diagnostic method due to its sensitivity and quantitative ability.

Keywords: *Pneumocystis carinii*, *Pneumocystis jirovecii*, *Pneumocystis* pneumonia, cytological staining, immunofluorescence, multiplex PCR, qPCR

INTRODUCTION

Pneumocystis jirovecii (previously known as *P. carinii*) is one of the most common opportunistic fungal pathogens which causes life-threatening pneumonia in immunocompromised individuals, including those with advanced HIV-

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infection (Thomas and Limper, 2004). Accurate diagnosis relying heavily on laboratory testing to identify the causative agent in clinical specimens is highly warranted (Liu *et al*, 2017). Laboratory investigation of *P. jirovecii* is traditionally based on microscopic detection in respiratory samples of cysts or trophic forms of the organism stained with Giemsa or Gomori's methenamine silver (GMS) or by an immunofluorescence assay (IFA) using fluorescein-conjugated monoclonal antibodies (Linder *et al*, 1986; Elvin *et al*, 1988). However, sensitivity and specificity of these staining methods can vary widely depending on competence of the examiner, fungal load, and specimen quality and type (Calderón *et al*, 2010; Tomás and Matos, 2018).

Introduction of PCR-based assays has vastly improved detection sensitivity and specificity, especially employing such targets as mitochondrial large subunit ribosomal DNA (*mtLSU-rDNA*), internally transcribed spacer (*mt-ITS*), *5S rDNA* and *18S rDNA* (Durand-Joly *et al*, 2005; Tia *et al*, 2012; Wakefield *et al*, 1990; Wakefield *et al*, 1991). More recently, quantitative (q)PCR assay has allowed accurate measurement of *P. jirovecii* load in clinical specimens (Flori *et al*, 2004; McTaggart *et al*, 2012; Church *et al*, 2015; Moodley *et al*, 2017). PCR-based methods for detection of *P. jirovecii* in non-invasive respiratory specimens, such as nasopharyngeal aspiration, oral wash and sputum, have been reported (Lu *et al*, 2011). PCR analysis in sputum has been shown to be the most cost-effective diagnostic option and reduces the cost of invasive procedure of bronchoalveolar lavage (BAL) (Harris *et al*, 2011).

Hence, two novel PCR-based diagnostic methods to detect *P. jirovecii mtLSU-rRNA* in BAL and sputum specimens were developed, namely, a multiplex PCR and a qPCR. In order to evaluate the efficacy of the two methods, results were compared to other standard cytological staining methods, *ie* GMS staining, Giemsa staining and IFA, as well as a conventional PCR method (Wakefield *et al*, 1990).

MATERIALS AND METHODS

Samples collection

For BAL samples, the number of samples was calculated to enable estimation of true positive samples with 90% sensitivity, 95% specificity and a 95% confidence interval range (CIR) of 88-100%. An infinite population proportion method using an n4 Studies statistical program (Ngamjarus *et al*, 2017) indicated 55 and 73 samples for the expected sensitivity and specificity respectively, *ie* a total of 128 samples. For sputum samples, an nQuery Advisor Program, which compensates for a paucity of specimens (www.statsolusa.com) indicated to obtain 85% of samples producing the same results for the four tests (GMS staining, Giemsa staining, IFA, conventional PCR) with 95% confidence interval of 75-95%, 49 samples would suffice. BAL (n = 131) and sputum (n = 54) samples were obtained during January 2015 to June 2017 from patients attending Siriraj Hospital, Mahidol University, Bangkok, Thailand who were suspected of having *Pneumocystis* pneumonia (PcP). A 3-5 ml aliquot of BAL sample was centrifuged at 2,000 *g* for 5 minutes at 4°C and 1 ml aliquot of pellet was used for DNA isolation, IFA, Giemsa staining and GMS staining. A 200 μl aliquot of sputum sample was used for DNA isolation with a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). DNA purity and concentration were measured with a NanoDrop 2000

UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

The study protocol was approved by Siriraj Hospital Institutional Review Board for research involving human subjects (COA no. Si318/2016).

Staining procedures and IFA

Fifteen μl aliquot of BAL pelleted sample was smeared on a glass slide and left to dry in a biosafety cabinet at ambient temperature and then stained with Giemsa and GMS dyes as previously described (CDC, 2016). IFA was performed using mouse monoclonal antibodies (3F6; Dako, Glostrup, Denmark) as previously described (Linder *et al*, 1986; Elvin *et al*, 1988).

Development of in-house multiplex PCR assay

A primer pair (forward 5′-CAACAACTGTCTCCGCAATAAA-3′ and reverse 5′-GGCAAACTTCGTGGT CAATAC-3′) specifically targeting *mtLSU rRNA* was designed based on a comprehensive analysis and alignment of *P. jirovecii* genome (GenBank accession no. NC_020331.1) and confirmed using a nucleotide BLAST program (National Center for Biotechnology Information, Bethesda, MD) (Altschul *et al*, 1990). Primers targeting human *GAPDH* (internal control) were forward 5′-GGGACTGGCTTTCCCATAAT-3′ and reverse 5′-CCTGCTTCACCACCTTCTT-3′. Multiplex PCR mixture (20 μl) contained 10 ng of DNA, 0.2 μl of both sets of primer pairs (10 mM) and 10 μl of 2X KAPA *Taq* ReadyMix PCR kit (Kappa Biosystems, Wilmington, MA). Thermocycling was carried out in Applied Biosystems Thermocycler (Thermo Fisher Scientific, Waltham, MA) as follows: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 70°C for 60 seconds; and a final step of 70°C for 5 minutes. Amplicons (*mtLSU rRNA*, 535 bp and *GAPDH*, 361 bp) were separated by 2% agarose gel-electrophoresis, stained with Safeview™ dye (Applied Biological Materials, Richmond, British Columbia, Canada) and recorded by a Gel Doc™ EZ Gel Documentation System (Bio-Rad Lab, Hercules, CA). A band is considered positive for *mtLSU rRNA* if intensity is $\geq$ 1.8 background intensity and negative if < 1.8 background (Quantity One 1-D analysis software, Bio-Rad).

Restriction fragment length polymorphism (RFLP)-PCR

The *mtLSU rRNA* amplicons of conventional PCR and in-house PCR were confirmed for specificity by restriction fragment length polymorphism (RFLP) using PacI (AseI) enzyme (Thermo Fisher Scientific). RFLP reactions were performed in 30 μl reaction composing of 3 μl of 10X buffer O, 10 μl of PCR product, 16 μl of water and 1 μl of PacI enzyme. The reaction was set up at 37°C for 1 hour in Applied Biosystems Thermocycler (Thermo Fisher Scientific). The PCR products were separated by 2% agarose gel-electrophoresis. The RFLP fragments of the conventional PCR product yields 278 bp and 69 bp, respectively. The RFLP fragements of the in-house PCR product yields 204 bp and 331 bp, respectively.

Conventional PCR assay

Conventional PCR performed to detect *P. jirovecii mtLSU rRNA* was performed as previously described (Wakefield *et al*, 1900; Wakefield *et al*, 1991). Reaction mixture, thermocycling conditions and amplicon band (347 bp) evaluation were as described above using primers pAZ102-H (5′-GTGTACGTCGCAAAGTACTC-3′) and pAZ102-E (5′-GTGGGCTTGGAAACA GCCATC-3′) (Wakefield *et al*, 1900).

Development of in-house qPCR assay of *P. jirovecii mtLSU rRNA*

Primers used in qPCR assay of *P. jirovecii mtLSU rRNA* were forward 5′- GGCATAACTCATGCTTAACAGT-3′ and reverse primer used in multiplex PCR described above, and specificity of the amplicon was confirmed using the nucleotide BLAST program. Human *GAPDH* used as reference gene was amplified using forward 5′-GACAGGCAACTTGGCAAATC-3′ and reverse primer 5′- CCTTCTCTAAGT CCCTCCTACA-3′. Reaction mixture (10 μl) comprised of 2 μl of 2X LightCycler® 480 SYBR Green I Master Mix (Roche Life Science, Penzberg, Germany), 100 ng of DNA, 1 pmol of each primer, distilled H_2O and 0.25 U LightCycler® Uracil-DNA glycosylase (Roche Life Science) to prevent carryover contamination from the previous PCR products. Thermocycling was performed using a LightCycler® 480 Instrument II (Roche Life Science) as follows: 40°C for 10 minutes; 95°C for 10 minutes; 45 cycles of 95°C for 10 seconds, 55°C for 20 seconds and 72°C for 20 seconds; followed by a melting analysis from 95°C to 65°C.

A standard curve of log copy number *P. jirovecii mtLSU rRNA* versus threshold cycle was generated from recombinant plasmid carrying *mtLSU rRNA* insert. Recombinant plasmid was constructed using RBC TA Cloning System (Real Biotech Corporation, Taipei, Taiwan), transfected into NEB 10- beta Competent *Escherichia coli* (New England Biolabs, Ipswich, MA), extracted from transformant, purified and sequence checked. Eight solutions containing $1x10^1$-$1x10^8$ copies/μl *mtLSU rRNA* and three control samples (water, negative sample and positive sample) were included in each experiment conducted in duplicate and reported as number of *P. jirovecii mtLSU rRNA* DNA copies/μl.

Data analysis

Kruskal-Wallis test was employed for comparison of multiple variables and a p-value < 0.05 is considered statistically significant.

RESULTS

In-house single-step multiplex PCR assay

A total of six complete genome sequences of *P. jirovecii mtLSU rRNA* available in GenBank database (accession nos. CAKN01000002, JX855936, JX855937, JX855938, JX499143, and NC020331) were aligned using Clustal Omega multiple sequence alignment software (http://www.ebi.ac.uk/Tools/msa/clustalo/) and primers specific to *P. jirovecii* were designed to target a conserved region of *P. jirovecii mtLSU rRNA*. An additional set of primers was constructed to amplify a conserved portion of the human *GAPDH* as internal control as GAPDH is stably and constitutively expressed at high levels in most tissues (Caracausi *et al*, 2017). Both sets of PCR primers successfully annealed to their respective templates at the same temperature, with no observed cross-hybridization or target preference in the in-house multiplex PCR assay (Fig 1A). In negative samples, only *GAPDH* amplicon was observed, indicative of PCR amplification quality, whereas in a conventional PCR assay, a negative result may imply a true absence of *P. jirovecii* DNA in the sample, inadequate DNA quality, or inhibition of PCR, which requires another set of quality control assay (Fig 1A).

P. jirovecii mtLSU rRNA amplicons generated from the in-house multiplex PCR were confirmed by RFLP-PCR using PacI. Positive amplicon yielded two

fragments of 204 and 331 bp (Fig 1B). Some of the *P. jirovecii* amplicons were also sequenced to confirm presence of *P. jirovecii mtLSU rRNA* fragment (data not shown). No cross-amplifications were observed with *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Mycobacterium abscessus*, *M. avium* complex, *M. kansasii*, and *Nocardia* spp DNA (data not shown).

In-house qPCR for quantification of *P. jirovecii* DNA copy numbers

A conserved region of *P. jirovecii mtLSU rRNA* was selected as a target for the qPCR assay with human *GAPDH* as internal control to assure positive PCR. A standard curve with a dynamic range of log10-10^8 copies/µl *P. jirovecii mtLSU rRNA* DNA versus threshold cycle (R^2 = 0.9) was constructed (data not shown). All positive samples had an amplicon single melting temperature of 78.5 ± 0.5°C (data not shown).

Comparison of in-house multiplex PCR with conventional PCR assay and standard cytological staining methods for detection of *P. jirovecii* in BAL and sputum specimens

Regarding BAL specimens (*n* = 131), in-house multiplex PCR assay showed a lower number of samples with weak amplicon band intensities (18% *vs* 20%) compared to those observed with the conventional PCR assay, hence the number of true negative samples in the multiplex PCR assay was higher than conventional PCR (Table 1). This finding emphasize the limitation of conventional PCR technique in that it cannot distinguish between true negative result and PCR failure. Microscopically

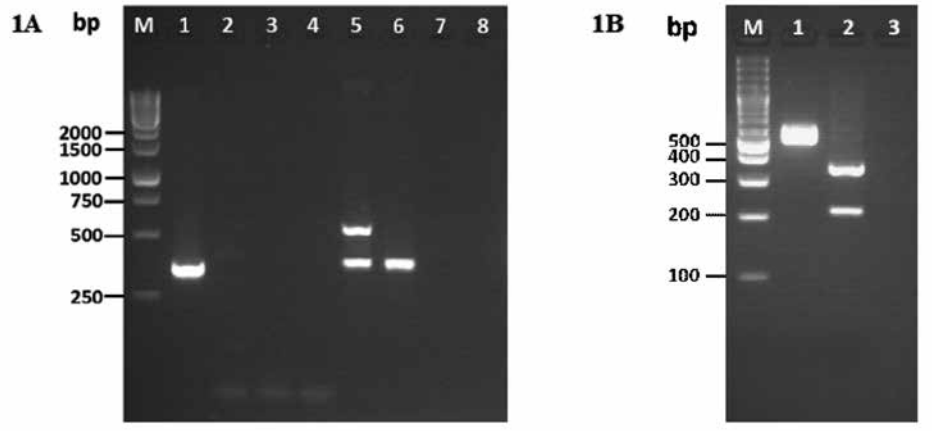

Fig 1-Gel-electropherogram of *Pneumocyctis jirovecii mtLSU rRNA* amplicon from (A) in-house and conventional PCR and (B) restriction fragment length polymorphism (RFLP)-PCR. Multiplex PCR was performed using in-house designed primers, conventional PCR using published primers (Wakefield *et al*, 1990) and RFLP-PCR using PacI. Amplicon bands were stained with Safeview™ dye. (A) Conventional PCR: Lane 1, *P. jirovecii* (347 bp); Lane 2, absence of *P. jirovecii* DNA; Lane 3: unsuccessful amplification of *P. jirovecii*; Lane 4, water (negative control). In-house multiplex PCR: Lane 5, *P. jirovecii* (535 bp) and human *GAPDH* (361 bp) (internal control); Lane 6, absence of *P. jirovecii* DNA; Lane 7, unsuccessful PCR amplification; Lane 8, water (negative control). (B) Lane 1, undigested *P. jirovecii* amplicon (535 bp); Lane 2, digested *P. jirovecii* amplicon (204 and 331 bp); Lane 3, water (negative control).

Table 1
Comparison of five diagnostic methods for *Pneumocystis jirovecii* detection in bronchoalveolar lavage (BAL) and sputum specimens from patients with suspected *Pneumocystis* pneumonia at Siriraj Hospital, Mahidol University, Bangkok, Thailand (January 2015 to June 2017).

	GMS		Giemsa		IFA		Conventional PCR			In-house multiplex PCR		
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Weak pos	Pos	Neg	Weak pos	Pos
BAL specimen												
Number (%) (n = 131)	83 (63)	48 (37)	82 (63)	49 (37)	76 (58)	55 (42)	41 (31)	26 (20)	64 (49)	51 (39)	23 (18)	57 (43)
Median copies/μl[a] (IQR)	<10 (UD-1,012)	$4.5x10^5$ ($1.1x10^5$-$7.8x10^5$)	<10 (UD-1,012)	$3.8x10^5$ ($7.9x10^4$-$7.8x10^5$)	<10 (UD-86)	$3.8x10^5$ ($7.1x10^4$-$7.7x10^5$)	<10 (UD-12)	<10 (UD-1,065)	$2.5x10^5$ ($3.1x10^4$-$6.6x10^5$)	<10 (UD-11)	1,970 (UD-4,340)	$3.1x10^5$ ($6.5x10^4$-$7.7x10^5$)
Sputum specimen												
Number (%) (n = 54) (%)	52 (96)	2 (4)	52 (96)	2 (4)	50 (93)	4 (7)	37 (68)	8 (15)	9 (17)	39 (72)	8 (15)	7 (13)
Median copies/μl[a] (IQR)	<10 (UD-95.7)	$5.5x10^5$ ($3.7x10^5$-$7.3x10^5$)	<10 (UD-95.7)	$5.5x10^5$ ($3.7x10^5$-$7.3x10^5$)	<10 (UD-34.8)	$2.2x10^5$ ($3.1x10^4$-$6.4x10^5$)	<10 (UD-11.8)	<10 (9.9-2,344)	$4.3x10^4$ ($1.6x10^4$-$8.8x10^5$)	<10 (UD-11.8)	1,396 (59.6-5,501)	$4.3x10^4$ ($2.6x10^4$-$8.8x10^5$)

GMS: Gomori's methenamine silver stain; IFA: immunofluorescence assay; IQR: interquartile range; Neg: negative; Pos: positive; UD: undetectable; Weak pos: weakly positive band intensity; [a]*P. jirovecii mtLSU rRNA* DNA measured by quantitative PCR.

P. jirovecii positive samples detected by GMS staining, Giemsa staining and IFA were not statistically different and were similar to those (with strong intensity band) observed with the PCR assays. Median *P. jirovecii mtLSU rRNA* DNA copies/μl are not statistically significant in samples positive by all five assays but significantly higher than those in negative samples, including those in the group categorized with weak intensity amplicon bands (p-value < 0.001) (Table 1). Concordant results were obtained with sputum specimens (n = 54), but percent positive samples in all five assays were lower than those of BAL specimens.

DISCUSSION

P. jirovecii pneumonia is a life-threating disease that mostly affects immunocompromised individuals (Thomas and Limper, 2004). The diagnosis of this type of pneumonia relies on clinical manifestation, chest radiological feature and laboratory testing (Calderón *et al*, 2010; Tomás and Matos, 2018). Two in-house PCR-based assays, multiplex and qPCRs, were developed to detect *P. jirovecii* in BAL and sputum specimens were validated by comparing with three standard cytological staining methods (GMS, Giemsa and IFA) and conventional PCR.

GMS and Giemsa staining methods, accepted gold standard tests, provide the most rapid, simple (only requiring an optical microscope) and inexpensive methods for *P. jirovecii* detection (Calderón et al, 2010; Tomás and Matos, 2018). These classical staining methods are considered to be the cheapest detection methods compared to IFA and PCR-based techniques (Harris *et al*, 2011; Esteves *et al*, 2015). Giemsa staining detects both *P. jirovecii* trophic and cystic forms without staining cystic wall, while GMS dye stains only the organism's cystic wall. However, sensitivity and specificity of *P. jirovecii* detection rely greatly on examiner's skill, fungal load and specimen type and quality (Esteves *et al*, 2015; Tomás and Matos, 2018). The staining have low sensitivity for detecting *P. jirovecii* in sputum compared to BAL specimens (Calderón *et al*, 2010; Tomás and Matos, 2018). High background staining in sputum specimens makes differentiation of organisms difficult (Pinlaor *et al*, 2004).

IFA employs fluorescein-labeled monoclonal antibodies to detect presence of an organism of interest viewed under an immunofluorescent microscope. It has been shown to have good sensitivity and specificity but requires expensive equipment and its specificity relies on antibody quality; operator's skill remains a critical issue (Midgley *et al*, 1991; Cruciani *et al*, 2002; Esteves *et al*, 2015; Tomás and Matos, 2018).

Conventional PCR has been shown to be effective in detecting *P. jirovecii* in various types of specimens, especially non-invasive specimens such as sputum and gastric wash (Harris *et al*, 2011; Esteves *et al*, 2015; Tomás and Matos, 2018). In addition, PCR technique allows genotyping of organisms (Esteves *et al*, 2015; Tomás and Matos, 2018). However, the technique is expensive, requires specialized equipment and reagents, needs experienced operator, and is qualitative (Esteves *et al*, 2015; Harris *et al*, 2011; Tomás and Matos, 2018). Among *P. jirovecii*-specific genes, *mtLSU rRNA* has emerged as one of the most acceptable and sensitive targets (Lu *et al*, 2011; Tomás and Matos, 2018). The *mtLSU rRNA* exits in multiple copies thereby contributing to its high success rate in *P. jirovecii*

detection compared to single copy genes (Matos and Esteves, 2010; Tomás and Matos, 2018). Although both conventional PCR and the in-house multiplex PCR methods amplify the same target gene but at different sequence sites, discordant results were observed with some of the specimens possibly due to differences in specificity of the primer binding region as polymorphism of the conventional PCR primer binding sites have been reported (Tia *et al*, 2012). Weak positive results were observed with both methods possibly due to low fungal burden, possible carry-over contamination or low DNA quality.

The advantages of qPCR for diagnosis other than its quantitative ability include less-time-consuming procedure and increased sensitivity (McTaggart *et al*, 2012; Moodley *et al*, 2017). In addition, the potential for carry-over contamination can be reduced by the use of uracil-DNA glycosylase. However, qPCR requires specialized equipment and reagents and a qualified operator.

BAL samples yield a higher probability of detecting *P. jirovecii* infection compared to sputum, due in part to difficulty of DNA extraction from sputum and lower fungal load (Caliendo *et al*, 1998; Bustamante and Levy, 1994). On the other hand, collection of BAL specimen requires invasive procedures which may not be practical in all clinical settings especially those with limited resources. It is important to note the overall sensitivity of the three staining methods for *P. jirovecii* detection of sputum specimens were reported to be remarkably lower than that of BAL specimen (Caliendo *et al*, 1998; Cruciani *et al*, 2002; Pinlaor *et al*, 2004).

A limitation of the study was the unavailability of clinical data and other laboratory findings, such as chest X-ray, serum (1-3)-B-D-glucan (BG) level, of the patients enrolled in the study. In further investigations inclusion of clinical data, radiologic findings, predisposing immune status, and additional laboratory data pertinent to PcP diagnosis should also be included in the diagnostic algorithm. This information is required in addition to qPCR data to define optimal cutoff values by receiver operating characteristic curve (ROC) analysis which could be used in laboratory practice to distinguish PcP from subclinical infection.

This study emphasizes the advantages and limitations of widely used techniques for PcP diagnosis. These findings may help to guide physicians when ordering and interpreting laboratory investigations for PcP diagnosis. The microscopic based methods including GMS stain, Giemsa stain and IFA are still appropriate for routine use in clinical laboratories with limited facilities. They show good detection sensitivity for BAL, but not for sputum analysis. The conventional PCR based methods seems to have higher sensitivity for *P. jirovecii* detection; however, weak positive results are uninterpretable. This emphasizes the limitation of using these techniques for routine analysis. Quantitative PCR demonstrates a better option for routine analysis of PcP since the quantitative amount/number of *P. jirovecii* can be assessed with carry-over contamination minimal. Definite diagnosis of PcP cannot/should not be contingent solely on laboratory results. It is still necessary to base the diagnosis on a combination of clinical manifestations, radiologic findings, and laboratory investigations.

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REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; 215: 403-10.

Bustamante EA, Levy H. Sputum induction compared with bronchoalveolar lavage by Ballard catheter to diagnose *Pneumocystis carinii* pneumonia. *Chest* 1994; 105: 816-22.

Calderón EJ, Gutiérrez-Rivero S, Durand-Joly I, Dei-Cas E. *Pneumocystis* infection in humans: diagnosis and treatment. *Expert Rev Anti Infect Ther* 2010; 8, 683-701.

Caliendo AM, Hewitt PL, Allega JM, Keen A, Ruoff KL, Ferraro MJ. Performance of a PCR assay for detection of *Pneumocystis carinii* from respiratory specimens. *J Clin Microbiol* 1998; 36, 979-82.

Caracausi M, Piovesan A, Antonaros F, Strippoli P, Vitale L, Pelleri MC. Systematic identification of human housekeeping genes possibly useful as references in gene expression studies. *Mol Med Rep* 2017; 16: 2397-410.

Centers of Disease Control and Prevention (CDC). Other specimens - sputum, induced sputum and bronchoalveolar lavage (BAL) for *Pneumocystis jirovecii* 2016 Mar [cited 2019 Jul 23]. Available from: URL: https://www.cdc.gov/dpdx/diagnosticprocedures/other/bal.html

Church DL, Ambasta A, Wilmer A, *et al*. Development and validation of a *Pneumocystis jirovecii* real-time polymerase chain reaction assay for diagnosis of *Pneumocystis* pneumonia. *Can J Infect Dis Med Microbiol* 2015; 26, 263-7.

Cruciani M, Marcati P, Malena M, Bosco O, Serpelloni G, Mengoli C. Meta-analysis of diagnostic procedures for *Pneumocystis carinii* pneumonia in HIV-1-infected patients. *Eur Respir J* 2002; 20, 982-9.

Durand-Joly I, Chabé M, Soula F, Delhaes L, Camus D, Dei-Cas E. Molecular diagnosis of *Pneumocystis* pneumonia. *FEMS Immunol Med Microbiol* 2005; 45, 405-10.

Elvin KM, Björkman A, Linder E, Heurlin N, Hjerpe A. *Pneumocystis carinii* pneumonia: detection of parasites in sputum and bronchoalveolar lavage fluid by monoclonal antibodies. *BMJ* 1988; 297: 381-4.

Esteves F, Calé SS, Badura R, *et al*. Diagnosis of *Pneumocystis* pneumonia: evaluation of four serologic biomarkers. *Clin Microbiol Infect* 2015; 21: 379. e1-10.

Flori P, Bellete B, Durand F, *et al*. Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing *Pneumocystis jiroveci* pneumonia from bronchoalveolar lavage specimens. *J Med Microbiol* 2004; 53: 603-7.

Harris JR, Marston BJ, Sangrujee N, DuPlessis D, Park B. Cost-effectiveness analysis of diagnostic options for *Pneumocystis* pneumonia (PCP). *PLoS One* 2011; 6: e23158.

Linder E, Elvin K, Bjorkman A, *et al*. Monoclonal antibody to detect *Pneumocystis carinii*. *Lancet* 1986; 2: 634.

Liu Y, Su L, Jiang SJ, Qu H. Risk factors for mortality from *Pneumocystis carinii* pneumonia (PCP) in non-HIV patients: a meta-analysis. *Oncotarget* 2017; 8: 59729-39.

Lu Y, Ling G, Qiang C, *et al*. PCR diagnosis of *Pneumocystis* pneumonia: a bivariate meta-analysis. *J Clin Microbiol* 2011; 49: 4361-3.

Matos O, Esteves F. *Pneumocystis jirovecii* multilocus gene sequencing: findings and implications. *Future Microbiol* 2010; 5: 1257-67.

McTaggart LR, Wengenack NL, Richardson SE. Validation of the MycAssay *Pneumocystis* kit for detection of *Pneumocystis jirovecii* in bronchoalveolar lavage specimens by comparison to a laboratory standard of direct immunofluorescence microscopy, real-time PCR, or conventional PCR. *J Clin Microbiol* 2012; 50: 1856-9.

Midgley J, Parsons PA, Shanson DC, Husain OA, Francis N. Monoclonal immunofluorescence compared with silver stain for investigating *Pneumocystis carinii* pneumonia. *J Clin Pathol* 1991; 44: 75-6.

Moodley B, Tempia S, Frean JA. Comparison of quantitative real-time PCR and direct immunofluorescence for the detection of *Pneumocystis jirovecii*. *PLoS One* 2017; 12:e0180589.

Ngamjarus C, Chongsuvivatwong V, McNeil E, Holling H. Enhancement of learning on sample size calculation with a smartphone application: a cluster-randomized controlled trial. *Southeast Asian J Trop Med Public Health* 2017; 48: 240-52.

Pinlaor S, Mootsikapun P, Pinlaor P, *et al.* PCR diagnosis of *Pneumocystis carinii* on sputum and bronchoalveolar lavage samples in immuno-compromised patients. *Parasitol Res* 2004; 94: 213-8.

Tia T, Putaporntip C, Kosuwin R, Kongpolprom N, Kawkitinarong K, Jongwutiwes S. A highly sensitive novel PCR assay for detection of *Pneumocystis jirovecii* DNA in bronchoalveloar lavage specimens from immunocompromised patients. *Clin Microbiol Infect* 2012; 18: 598-603.

Thomas CF Jr, Limper AH. *Pneumocystis* pneumonia. *N Engl J Med* 2004; 350: 2487-98.

Tomás AL, Matos O. *Pneumocystis jirovecii* pneumonia: current advances in laboratory diagnosis. *OBM Genetics* 2018; 2(4). doi: 10.21926/obm.genet.1804049.

Wakefield AE, Guiver L, Miller RF, Hopkin JM. DNA amplification on induced sputum samples for diagnosis of *Pneumocystis carinii* pneumonia. *Lancet* 1991; 337: 1378-9.

Wakefield AE, Pixley FJ, Banerji S, *et al.* Detection of *Pneumocystis carinii* with DNA amplification. *Lancet* 1990; 336: 451-3.