Supplementary Material.
Mühlethaler et al.

Bronchoalveolar lavage (BAL) processing and polymerase chain reaction (PCR) methods

The BAL procedure was performed following a standardized protocol by an experienced pulmonologist at Bern University Hospital. Briefly, 150 mL of sterile saline solution was instilled and recovered, either at the site of the radiographic abnormality, or in the middle lobe when no radiographic abnormalities were detected.

For pneumocystis diagnostic tests, 10-mL BAL fluid aliquots were centrifuged. Twenty-five microliters of the resuspended pellet was used for direct immunofluorescence (IF) testing and the remaining pellet was stored at −80°C for further *Pneumocystis jirovecii* (Pj) PCR.

Direct IF testing

Methods

Direct IF Pj testing was performed with the IF antibody test Monofluo *P. jirovecii* IFA Test Kit (Bio-Rad, Redmond, WA, USA). Semiquantitative microscopy was performed (number of cysts/trophozoites per field of vision, magnitude 200×) with the following score: + = few (<1), ++ = many (1-10), +++ = abundant (>10). According to manufacturer specifications, sensitivity was 100% and specificity 95.8% when compared with reference staining methods (Wright-Giemsa stain, toluidine blue O).

Real-time PCR

Methods

Standards. Live plasmid containing a *P. jirovecii* major surface glycoprotein (MSG) gene insert was kindly provided by J. A. Kovacs (Larsen et al., 2002). This plasmid was linearized and 2×10^5, 2×10^4, 2×10^3, 10^3, 2×10^2, and 10^2 copies per reaction were used in triplicate as standards.

Controls. Three negative controls were included in each run: (1) water (NTC = no template control); (2) 10× Exo IPC Block (Applied Biosystems [ABI], Foster City, CA, USA; NAC = no amplification control); and (3) a negative extraction control.

Different plasmid concentrations were used as positive controls, as described earlier. In addition, a positive extraction control (a negative BAL sediment spiked with 2×10^4 copies of linearized plasmid) was included in each PCR run.

To detect inhibitors in the patient specimens, we included an Exo IPC DNA (ABI) in each real-time PCR reaction.

DNA extraction. Nucleic acids were extracted from 100 µL of BAL sediments with the NucliSense easyMAG platform (bioMérieux, Boxtel, the Netherlands). Purified DNA was resuspended in a final volume of 25 µL. Each extracted DNA was analyzed in duplicate by real-time PCR.

DNA amplification. Real-time PCR that targeted the MSG gene (Linssen et al., 2006) was performed. Assays were run in 96-well optical reaction plates (ABI). The 25-µL real-time PCR reaction contained 0.6 µM of each primer, PCPFor and PCPRev (Table 1); 0.15 µM PCPProbe (Table 1); 1× TaqMan Universal Master Mix (ABI); 1× Exo IPC Mix (ABI); 1× Exo IPC DNA; nuclease free water; and 5 µL purified DNA. The following amplification protocol was performed on an ABI PRISM 7000 Sequence Detection system (ABI): 2 min at 50°C; 10 min at 95°C, followed by 42 cycles of 15 s at 95°C; and 1 min at 60°C.

Data analysis. Quantification was performed by using the ABI PRISM software and based on extrapolation of data to standard curves.

The sample was interpreted as positive if the duplicates were positive by this assay. A positive result in only one well warranted a retest of the sample. If the retest showed at least one of the two wells as positive, the sample was considered positive for *P. jirovecii*. A negative MSG result had to be positive for Exo IPC DNA to be valid and to exclude inhibitors in the specimen. If inhibition was present, samples were retested by real-time PCR.

Table 1. Primers and Probe for Real-time PCR (5' – 3')

	Sequence (5' – 3')	Publication
PCPFor	CAA AAA TAA CAY TSA CAT CAA CRA GG	Larsen et al., 2002; Linssen et al., 2006
PCPRev	AAA TCA TGA ACG AAA TAA CCA TTG C	Larsen et al., 2002; Linssen et al., 2006
PCPProbe	FAM-TGC AAA CCA ACC AAG TGT ACG ACA GG-TAMRA	Larsen et al., 2002; Linssen et al., 2006

Literature

Larsen HH, Masur H, Kovacs JA, Gill VJ, Silcott VA, Kogulan P, Maenza J, Smith M, Lucey DR, Fischer SH. Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing Pneumocystis carinii pneumonia. *J Clin Microbiol.* 2002 Feb;40(2):490-494.

Linssen CF, Jacobs JA, Beckers P, Templeton KE, Bakkers J, Kuijper EJ, Melchers WJ, Drent M, Vink C. Inter-laboratory comparison of three different real-time PCR assays for the detection of Pneumocystis jiroveci in bronchoalveolar lavage fluid samples. *J Med Microbiol*. 2006 Sep;55(Pt 9):1229-1235.

e-Table 1. Clinical characteristics of patients with positive PCR and negative IF testing.

Testing of 171 randomly selected BAL samples (group 2, Table 1) showed 18 patients with a positive PCR value. Patients are grouped according to real-time Pj PCR values (>1450 / 1450-81 / 81-1 pathogens/mL) and results of the PCP probability algorithm. Note that only 3 patients of 18 were retrospectively judged as having a possible PCP according to both the PCP probability algorithm and retrospective clinical judgment (patients P2, P3, P11). See text for more details.

Abbreviations: AB: antibiotic therapy; AML: acute myeloid leukemia; BAL: bronchoalveolar lavage; CML: chronic myeloid leukemia; CMV: human cytomegalovirus; Comp.: compatible with; DD: differential diagnosis; Excl.: excluded; F: female; ILD: interstitial lung disease; M: male; N: no; Nr.: number; p-ANCA: p-antineutrophil cytoplasmic antibody; Pat.: patient; PCP: pneumocystis pneumonia; PCR: polymerase chain reaction; Pj: *Pneumocystis jirovecii*; Poss.: possible; TB: *M. tuberculosis*; TMP/SMX: trimethoprim-sulfamethoxazole; tx: transplantation; Y: yes.

PCR value (pathogens/mL)/"PCP probability algorithm"	Pat Nr.	Age	Sex	Cause of immuno-suppression	Pj PCR value (pathogens/mL)	Classification "PCP probability algorithm"	Retrospective diagnosis - data review by 2 physicians	Definitive diagnose acute lung disease	Definitive diagnosis of PCP	TMP/SMX prophylaxis before BAL	Complete Pj therapy	Outcome
>1450 / PCP excl.	P1	75	M	Myositis (unclassified autoimmune disease)	53285.14	Excl.	Excl.	Aspiration pneumonia	Colonized	N	N	Alive
1450-81 / PCP poss.	P2	66	M	CML	164.57	Poss.	Poss.	CML, aplasia. Diffuse interstitial and alveolar infiltrates. Culture: TB positive.	Possible PCP	N	N	Dead
	P3	56	M	Kidney tx	474.61	Poss.	Poss. but unlikely	Received broad spectrum AB for pneumonia, including TMP-SMX	Possible PCP	Y	Y	Alive
1450-81 / PCP excl.	P4	62	M	Kidney tx	308.56	Excl.	Excl.	Suspicion of bacterial pneumonia	Colonized	Y	N	Alive
	P5	48	F	Kidney tx	1424.14	Excl.	Excl.	Suspicion of bacterial pneumonia, DD CMV	Colonized	N	N	Alive
	P6	63	M	Purpura Schönlein-Henoch	468.23	Excl.	Excl.	Suspicion of bacterial pneumonia	Colonized	N	N	Alive
	P7	71	M	Rheumatoid arthritis	248.68	Excl.	Excl.	ILD flare	Colonized	N	N	Alive
	P8	61	F	AML	179.14	Excl.	Excl.	Pneumonia in aplasia. All cultures negative (DD bacterial, fungal)	Colonized	N	N	Alive
	P9	59	F	AML	231.80	Excl.	Excl.	Pneumonia in aplasia. All cultures negative (DD bacterial, fungal)	Colonized	N	N	Alive
	P10	25	M	ALL	143.57	Excl.	Excl.	Pneumonia in aplasia. All cultures negative (DD bacterial, fungal)	Colonized	N	N	Alive
1-80 / PCP poss.	P11	38	M	Non-Hodgkin lymphoma	24.21	Poss.	Poss.	Diffuse interstitial + alveolar pneumonia in aplasia. Cultures negative. Complete PCP therapy together with AB and antifungal therapy	Possible PCP	N	Y	Alive
1-80 / PCP excl.	P12	48	M	Interstitial lung disease	76.40	Excl.	Excl.	ILD flare	Colonized	N	N	Alive
	P13	66	M	Non-Hodgkin lymphoma / heart tx	45.15	Excl.	Excl.	Bacterial pneumonia (Pseudomonas) and possible fungal pneumonia	Colonized	N	N	Alive
	P14	71	M	Autoimmune hepatitis	29.39	Excl.	Excl.	Aspiration pneumonia	Colonized	N	N	Alive
	P15	81	F	p-ANCA positive vasculitis	42.35	Excl.	Excl.	Suspicion of bacterial pneumonia	Colonized	N	N	Alive
	P16	75	F	Connective tissue disease (not more closely defined)	55.02	Excl.	Excl.	Lung neoplasia	Colonized	N	N	Alive
	P17	47	M	Liver tx	24.50	Excl.	Excl.	Suspicion of bacterial pneumonia, DD CMV	Colonized	N	N	Alive
	P18	69	M	Interstitial lung disease	14.35	Excl.	Excl.	Amiodarone-induced ILD (DD idiopathic lung fibrosis)	Colonized	N	N	Alive