

Multinational, Prospective Evaluation of a Commercial Pneumocystis Real-Time (RT) PCR test

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Background

- Pneumocystis jirovecii* is a fungal pathogen that causes Pneumocystis Pneumonia (PCP), a common and serious opportunistic infection in immuno-compromised patients.
- If undiagnosed and/or untreated, PCP leads to respiratory failure, and death.
- P. jirovecii* cannot be cultured; current diagnosis relies upon radiologic evidence and microscopic methods including immunofluorescence.
- PCR assays for the detection of *P. jirovecii* have been reported in the literature for nearly 20 years¹, with mixed results, and consequently little progress has been made to establish the technique in the differential diagnosis of PCP.
- MycAssay™ Pneumocystis (Myc-PCR) is the first commercially available PCR assay to be evaluated in a multi-center, multi-national prospective study looking at the at-risk population for this serious and potentially fatal infection.

Materials & Methods

Materials required: MycAssay™ Pneumocystis kits; MycXtra® Fungal DNA extraction kit; Vortex adaptor plate (Myconostica Ltd); BD BBL™ MycoPrep™ Specimen Digestion/ Decontamination Kit (BD Diagnostic Systems, Oxford, UK); Smartcycler® Real Time PCR platform (Cepheid, Sunnyvale, CA, USA);

Samples – see Table 1. The volume of sample available for DNA extraction ranged from <1ml to 60ml.

Merifluor Immunofluorescence: The comparator diagnostic test performed at the study sites. Manufacturers instructions were followed.

MycAssay™ Pneumocystis: Uses molecular beacons² for the detection of *P. jirovecii*. Target sequence is the Pneumocystis mitochondrial ribosomal large sub-unit (mLSU). The kit contains an Internal Amplification Control, to monitor the presence of PCR inhibitors, eliminating false negative results.

Threshold levels are established: The cycle (Ct) where the amplification signal cuts the threshold is diagnostic, see Figure 1

Limit of Blank studies conducted in house (data not shown) established that a Ct of 39 will differentiate between samples with and without target DNA.

Clinical Criteria

Subjects were enrolled into the study according to the following criteria:

- Moderate or Low probability of Pneumocystis jirovecii (PCP)
- Informed consent, if required.
- >18 years of age unless parental consent given
- Sample collection procedure was not unduly risky.

131 unique subjects were enrolled. Of these, 21 either withdrew consent (n=1), or insufficient sample for PCR testing (n=20), leaving 110 unique study subjects with analyzable data, see Table 1.

Clinical risk factors and final diagnosis

At study entry: clinical condition(s) were noted that contributed to the subject being at risk for the infection: 54 transplants (40 lung); 32 non malignant conditions; 13 leukemia; 11 solid tumours. 9 were HIV+

Clinical diagnosis was assigned by the enrolling clinician in accordance with his or her local procedures. The final diagnosis was assigned up to 10 weeks following study entry and the date that the respiratory sample was taken for analysis.

For selected subjects, follow up clinical data were requested.

	N	% of Samples
Male	59	54
Female	51	46
Caucasion	51	46
Black	19	17
No Data / Others	35 / 5	32 / 5
Pre Test Probability*		
Moderate	24	22
Low	86	78
BAL	101	92
Other LRT	7	6
Induced Sputum	1	1
Sputum	1	1
Processed Same Day	13	12
< 72 Hours	80	73
> 3 Days	14	12
Undetermined	3	3

Table 1: Demographics of study subjects and detail of sample type and processing.

*Pre-Test probability (PTP): Moderate: HIV/AIDS with bilateral infiltrates and/or hypoxaemia; or Low: patients undergoing BAL to diagnose pulmonary infiltrates of uncertain etiology or patients undergoing bronchoscopy for other reasons (e.g. lung cancer, solid organ transplantation, haematology patients, ICU patients, interstitial lung disease etc), or induced sputum samples from non HIV/AIDS patients (sputum if induced sputum is not available).

Results

- Thirteen of the 14 subjects with diagnosis of PCP were positive using the MycAssay™ Pneumocystis (Myc-PCR) kit.

- Five were HIV+ and recorded an average Ct of 25 (range 23.3 – 33.5). The remaining 8 subjects recorded an average Ct of 29.4 (range 24.6 – 38.9).

- Five of the 13 were judged moderate probability for the disease; 8 were low probability.

- One female subject aged 41, diagnosed with PCP, for whom Myc-PCR was negative, was HIV+, CD4 + 33, on corticosteroids, and admitted into the ICU. IF was positive,

LDH was 249 IU/L, bilateral ground glass noted on the CXR and lung function of 89% arterial O₂ on air. The sample obtained was induced sputum, < 1mL of poor quality.

- One subject with clinical diagnosis of PCP who was Myc-PCR positive, was negative by IF. A 63 year old male lung transplant subject, on corticosteroids and other immunosuppressants, LDH 527 IU/L, bilateral ground glass effects noted on chest CT, pO₂ of 44 mmHg on 35% O₂. Microscopy and IF were both negative.

- Nine subjects were positive by Myc-PCR, with a negative clinical diagnosis and IF. Ct values ranged from 29.1 to 38.9 (av. 34.2). All low probability for PCP at enrolment. See Table 2
- One subject (#2) received anti-PCP therapy of Cotrimoxazole / Septrin / Bactrim (data not shown)

- Follow up clinical information was obtained for all 9 subjects. The period of follow up was 3 months from study entry.

- One subject developed PCP within the follow up period. At the time of study entry, CXR and CT scans were normal, LDH and CRP levels were normal, some reduction in lung function noted (68 mm/Hg on 21% O₂).

Patient No. (Ct)	Age	Sex	Risk Factor	Episode Diagnosis	Merifluor IF	Microscopy	LDH	P _O (mmHG)	F _I O ₂ (%)	CxR	CT Scan	Follow Up
1 (32.4)	64	F	Lung Tx Corticosteroids	Lung Infection	-ve	-ve	220	68	21	Normal	Normal	Develop PCP within 3 months after diagnosis
2 (35.6)	62	M	Renal Tx Corticosteroids	Candida colonisation and Presumptive BOOP	-ve	-ve	423	50, 75	20, 60	Airspace opacities	Patchy air space opacities and small mediastinal lymph nodes	No relevant development
3 (34)	55	M	Renal Tx Corticosteroids	Pneumonia	-ve	-ve	251	93%	RA (21)	Bilateral infiltrates	Patchy foci of consolidation, calcified bilateral hilar lymph nodes	No relevant development
4 (39)	64	F	Lung Tx Corticosteroids	<i>P. aeruginosa</i> / <i>A. fumigatus</i> colonisation and infections	-ve	-ve	No data	No data	No data	No data	Bibasilar lung infiltrate and single nodule	No relevant development
5 (33.1)	69	M	Lung Tx Corticosteroids	Post lung transplant surveillance	-ve	-ve	204	70	21	Normal	No data	No relevant development
6 (34.1)	67	M	Lung Tx Corticosteroids	Post lung transplant surveillance	-ve	-ve	187	81	21	Normal	Normal	No relevant development
7 (36.1)	56	F	Lung Tx Corticosteroids	Viral pneumonia/pneumonitis	-ve	-ve	312	90	21	Multiple consolidations	No data	No relevant development
8 (29.1)	62	M	Other non malignant condition	Lung carcinoma	No data	-ve	154	59	21	Infiltrates right upper lobe	Right hilus, massive infiltrates	No relevant development
9 (34.5)	47	F	Other non malignant condition	Haemoptysis	No data	-ve	167	No data	No data	Normal	No data	No relevant development

Table 2: Details and disposition of the 9 subjects who were PCR positive and for whom clinical diagnosis and immunofluorescence were negative for Pneumocystis jirovecii.

- In total 132 samples were available for testing*, of which 2 (1.5%) failed to amplify (data not shown).

- For 110 subjects, Myc-PCR results were compared to final clinical diagnosis: See Table 3.

- Sensitivity and Specificity of the test were 93% and 91% respectively

- Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the test were 59% and 99% respectively

- 83 of these subjects had corresponding data from IF: See Table 4.

*Some subjects were enrolled more than once. The first documented visit with a PCR result and a clinical diagnosis was entered into the primary analysis, however all available samples were assayed.

Discussion

- The PCR assay was robust. 98.5% of samples tested gave a result.
- The Myc-PCR assay accurately identified 13/14 clinically positive cases of PCP, and the single case missed was potentially a result of poor or inadequate sampling.
- The clinical data for the 9 subjects (PCR positive, PCP negative) do not suggest PCP infection, however in one of the subjects,

progression did occur. These subjects were low probability for PCP at study entry.

- In this study, 9% of subjects enrolled with low probability for the infection had disease (8/86). 25% of subjects enrolled with moderate probability had the disease (6/24).

- Follow up where diagnosis and PCR result are mismatched, to better understand a potential link between pre-test risk for the disease and the PCR result, is warranted.

	Diagnosis +ve PCP	Diagnosis -ve Other than PCP
PCR +ve	13	9
PCR -ve	1*	87

Table 3: Comparison of results for 110 subjects with assigned clinical diagnosis and MycAssay™ Pneumocystis PCR results.

*A comment on the data collection form queried the quality of the sputum sample available for testing.

Clinical positives (n=14) IF vs PCR

	IF +	IF -
PCR +	12	1
PCR -	1	0

Clinical negatives (n=69) IF vs PCR

	IF +	IF -
PCR +	0	7
PCR -	0	62

Table 4: Comparison of the MycAssay™ Pneumocystis PCR results to Merifluor IF results in 83 subjects with confirmed clinical diagnosis

Conclusion

- MycAssay™ Pneumocystis is a robust, standardised and easy to use real time PCR assay which delivers a result within 4 hours of sample collection.

- The NPV of the Myc-PCR assay is high, matching the Merifluor IF assay results.

- The specificity and PPV is in line with other published data for PCR assays, indicative of the more sensitive molecular assays^{3,4}.

- Increased sensitivity may be suggestive of asymptomatic latent disease, as evidenced by the subject who developed PCP within a month of a positive PCR result.

- Results suggest potential link between pre-test risk for the disease and significance of PCR result.

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