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Evaluation of a new commercial real time PCR for the detection of *Aspergillus spp*. in serum and respiratory samples

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Abstract

Objectives. Diagnosis of invasive aspergillosis is still disappointing and often delayed because of the lack of sensitivity of diagnostic tools. DNA detection based-methods have been developed, but differ widely and comparisons are difficult to assess. The objective of the study is to compare a new commercial real-time PCR kit, affigene® Aspergillus tracer assay, with an in house nested PCR targeting 18S rRNA *Aspergillus sp.* gene.
Methods. Twelve patients at risk for invasive aspergillosis were included in the study. They were classified to have possible (5 cases), probable (1 case) or proven (6 cases) invasive aspergillosis following E.O.R.T.C. criteria. Fifteen serum and respiratory paired samples were collected. The DNA extraction was performed by using the QIAmp DNA mini kit® (Qiagen, Germany). All samples were tested by both PCR assays and respiratory samples were cultured.
Results. **Respiratory samples**. *A. fumigatus*, *A. niger* and *A. flavus* were isolated from 10/15 samples; both PCR methods were positive for these samples except one that was positive for affigene® and equivocal for the nested PCR. The real-time PCR assay reported cycle thresholds ranging from 25 to 38. Three of the five culture-negative samples were negative by both PCR methods; one of three was negative in affigene® assay and equivocal by nested PCR; the last sample was positive in affigene® assay and negative by nested PCR. **Serum**. Thirteen of fifteen blood samples were negative by both PCR methods. One sample was equivocal by nested PCR and was inhibited in affigene® assay despite a culture-positive paired respiratory sample. The last case was inhibited by the real-time PCR assay and negative by nested PCR. Nor the nested PCR, nor affigene® assay could detect any *Aspergillus* DNA in serum. In total, there was 87% of agreement between the two PCR assays.
Conclusion. Both methods are in good agreement and can detect at least three different species of *Aspergillus*. However, the sensitivity of both assays does not permit the detection of *Aspergillus* DNA in serum. affigene® assay can easy replace the "in house" assay: it allows a fast and standardized detection of *Aspergillus* sp. DNA in respiratory samples without inconvenient due to the handling of PCR products.

Background

Diagnosis of invasive aspergillosis is still disappointing and often delayed because of the lack of sensitivity of diagnostic tools. Galactomannan detection in serum is know widely used for the diagnosis of IA in neutropenic patients with haematological malignancies. DNA detection based-methods have been developed, but differ widely for a centre to another and comparisons are difficult to assess.

It is important to develop commercial techniques in order to develop the use of molecular tests by the laboratories involved in the diagnosis of fungal opportunistic infection as invasive aspergillosis.

Objectives

- to compare a new commercial real-time PCR kit, affigene® Aspergillus tracer assay, with an "in house" nested PCR targeting 18S rRNA *Aspergillus sp.* gene
- to evaluate the test feasibility in a routine laboratory practice

Methods

Patients

The study was performed at the University Hospital of Liège from November 2005 to November 2006. Twelve patients at risk for invasive aspergillosis were included in the study. They were classified to have possible (5 cases), probable (1 case) or proven (6 cases) invasive aspergillosis following E.O.R.T.C. criteria (1). Fifteen serum and respiratory paired samples were collected in total. They were frozen at –20°C until performing the assays.

Fungal cultures

The cultures were performed on sabouraud agar supplemented with antibiotics (Biomérieux, France). The tubes were incubated for 28 days at 30°C.

Galactomannan detection test (Platelia® Aspergillus, Biorad)

The test was performed on serum samples following the manufacturer's recommendations

PCR methods

DNA extraction. The DNA extraction was performed by using the QIAmp DNA mini kit® (tissue protocol, Qiagen, Germany). All samples were tested by both PCR assays and respiratory samples were cultured. For both serum and respiratory samples, the input volume was 200 µl and the elution volume 100 µl.

"In house" PCR. This method is a nested PCR adapted from Yamakami et al. (2). The targeted sequence is the 18S rRNA gene of several *Aspergillus* species.
Briefly, the PCR mixtures were identical for both PCR except for MgCl2. The PCR were carried out in a 50-µl volume containing 10 mM Tris-HCl at pH 8.3, 50mM KCl and 1,5 mM $MgCl_2$ (2,25 mM for the nested PCR) (GeneAmp 10 x PCR buffer II, Applied Biosystems, USA) with 0.4 µM of both primers (Eurogentec, Belgium), 2,5 mM dNTP (Promega, The Netherlands), and 1,25 U of *Taq* polymerase (Amplitaq DNA polymerase, ABI). A 5-µl volume of DNA was added to the mixture. Positive and negative controls were amplified in parallel to validate the run. Thermal cycling conditions (Thermohydaid, UK) were as follow: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 50°C (65°C for the nested PCR) and 1 min at 72°C followed by a final extension step of 10 min at 72°C. For the nested PCR, 2 µl of the first amplified product was added to the new reaction mixture. The final amplified products (357bp) were analyzed on 2% agarose gels stained with ethidium bromide and visualized by UV transillumination. Each sample was investigated for the presence of inhibitors by amplification of the ß-globin gene. All samples were performed in duplicate. The equivocal results (one positive test, one negative test) were rerun.

affigene® Aspergillus tracer kit

The kit utilizes the real-time PCR technology. All required reagents are included for the amplification: a mastermix containing nucleotides, primers/probes, UDG and DNA polymerase in a buffered solution. Positive, negative and internal control are also included.

The amplification has been conducted on the Mx3000P™ instrument (Stratagene, La Jolla, USA). The fluorescence was collected in the FAM (targeted gene) and ROX (IC) chanels. The results are automatically calculated by a specific software and reported as negative or positive (with a Ct value). Only qualitative results are obtained. The run is completed after 2,5 hours.

The kits and the RT-PCR instrument were provided by Sangtec molecular diagnostics.

Results

Table 1. Comparison between the « in house » nested PCR and affigene Aspergillus assay.

Case	Underlying	Serum			Respiratory sample			Invasive
		"in house"	GM[c]	affigene	"in house"	Fungal	affigene	aspergillosis
N°	disease	PCR	Antigen	PCR	PCR	culture	PCR	group
1	Aplastic anemia	NEG	1,172	NEG	NEG	NEG	NEG	Proved
		NEG	0,383	NEG	NEG	NEG	NEG	
		equivocal	1,784	INH[b]	POS	A. fumigatus	Ct=27	
2	Cirrhosis	NEG	3,662	NEG	NEG	NEG	NEG	Proved
		NEG	4,402	NEG	POS	A. fumigatus	Ct=32	
3	Lymphoma	NEG	0,21	NEG	POS	A. niger	Ct=36	Proved
4	Rheumatoid arthritis	NEG	2,021	NEG	POS	A. fumigatus	Ct=30	Probable
5	Myelodysplasia	NEG	0,632	NEG	POS	A. fumigatus	Ct=29	possible
6	COPD[a]	NEG	0,244	INH[b]	equivocal	NEG	NEG	possible
7	Lymphoma	NEG	0,576	NEG	POS	A. flavus	Ct=34	possible
8	Tuberculosis	NEG	1,734	NEG	POS	A. fumigatus	Ct=25	Proved
9	COPD[a]	NEG	0,139	NEG	POS	A. fumigatus	Ct=38	possible
10	Lung carcinoma	NEG	0,812	NEG	POS	A. fumigatus	Ct=35	possible
11	COPD[a]	NEG	0,34	NEG	equivocal	A. fumigatus	Ct=38	Proved
12	COPD[a]	NEG	1,87	NEG	NEG	NEG	Ct=34	Proved

[a] corticodependent; [b] inhibited after rerun; [c] galactomananne

In total, there was 87% of agreement between the two PCR assays.

Conclusions

- Both PCR are in good agreement for the detection of *Aspergillus* DNA in clinical samples.
- Both PCR assays are able to detect at least three different *Aspergillus* species: *A. fumigatus*, *A. flavus* and *A. niger* . These tests allow the detection of aspergillosis due to other species than A. fumigatus the most frequently targeted species.
- affigene® test is more sensitive to the presence of inhibitors in serum than the conventional test.
- None of these PCR methods could detect *Aspergillus* DNA in serum even in proved aspergillosis despite the positivity of the galactomannan detection test. These data suggest that blood sample is not an appropriate material to be tested by PCR for the diagnosis of invasive aspergillosis.
- affigene® assay can easy replace the "in house" assay: it allows a fast and standardized detection of *Aspergillus* sp. DNA in respiratory samples including the screening for inhibitors in the same run. The real-time technology avoids the inconvenient due to the handling of PCR products and allows the implementation of several runs in the same week. The major inconvenient of this method is the higher cost in comparison with a "in house" PCR test.

References

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