A systematic literature review on the diagnosis of invasive aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples

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Summary

Polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples (BAL) has been used to assist in the diagnosis of invasive aspergillosis. Several studies have been published regarding the utility of this test, although no systematic review of the literature has been performed to date.
The objective of this systematic review was to evaluate the efficacy of PCR from BAL for the diagnosis of invasive aspergillosis in high risk patients. MEDLINE and LILACS databases (1980-2006) searches to identify articles related to PCR in diagnosis of invasive aspergillosis. For inclusion, the study had to report sufficient data to calculate sensitivity, specificity and diagnostic odds ratio of the PCR-based technique. Patients with proven and probably invasive aspergillosis were considered. Forty-five articles met our initial inclusion criteria of which 15 articles were selected. Combining the results from the different reports, the overall sensitivity and specificity values of PCR-based techniques were 79% and 94%, respectively. Contamination, specific primers and method of PCR were important variables that could complicate interpretation of these tests. The present study showed support for the clinical value of PCR from BAL for the diagnosis of invasive aspergillosis in patients with risk factors for this disease.

Key words *Aspergillus*, Polymerase chain reaction, Diagnosis, Bronchoalveolar lavage fluid

Revisión sistemática de las publicaciones sobre diagnóstico de la aspergilosis invasora mediante reacción en cadena de la polimerasa (PCR) de lavados bronco-alveolares

Resumen

La amplificación de ADN en lavados broncoalveolares por medio de la reacción en cadena de la polimerasa, (PCR), ha sido utilizada para ayudar al diagnóstico de la aspergilosis invasora. Se han publicado varios estudios sobre la utilidad de esta prueba pero no se ha realizado ninguna revisión sistemática de estas publicaciones. El objetivo de la presente revisión ha sido evaluar la eficacia de la PCR de lavados broncoalveolares en el diagnóstico de la aspergilosis invasora de pacientes de alto riesgo. Las bases de datos MEDLINE y LILACS (1980-2006) han sido utilizadas para identificar los artículos relacionados con PCR y diagnóstico de la aspergilosis invasora. Como criterios de inclusión los artículos tenían que ofrecer datos suficientes para poder calcular la sensibilidad, la especificidad y los resultados diagnósticos de las técnicas de PCR. Cuarenta y cinco artículos cumplieron los criterios de inclusión de los que se seleccionaron quince artículos.
Los resultados obtenidos para la PCR combinando los de los diferentes estudios, mostraban unos valores globales de sensibilidad y especificidad del 79% y 94% respectivamente. La contaminación, el uso de cebadores específicos y el método de PCR empleado eran variables importantes que complicaban la interpretación de las pruebas. Este estudio muestra que la PCR con muestras de lavado broncoalveolar tiene valor clínico para el diagnóstico de la aspergilosis invasora en pacientes con factores de riesgo predisponentes para esta enfermedad.

Palabras clave *Aspergillus*, Reacción en cadena de la polimerasa, Diagnóstico, Lavado broncoalveolar

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Invasive aspergillosis (IA) is caused by several pathogenic *Aspergillus* species, mainly *Aspergillus fumigatus* and *Aspergillus flavus* [3]. Immunocompromised patients, particularly leukemic patients and bone marrow/organ transplant recipients, are vulnerable to this pathogen [29]. AIDS and high-dose corticosteroid therapy are other considerable risk factors for IA.

The incidence of IA is increasing and it is only exceeded by *Candida* infections in neutropenic patients with nosocomial fungal infections [2,18]. However, the real incidence is difficult to assess since as far as 30% of the cases remain undiagnosed [4]. It has been shown previously that up to 41% of patients who died of acute leukemia had evidence of aspergillosis [21]. IA has become a major cause of death in immunocompromised patients such as allogenic bone marrow transplant recipients.

Early diagnosis of IA is fundamental in controlling the progress of this fungal infection, since antifungal therapy could reduce mortality rate [22]. Thus, rapid and more sensitive novel diagnostic tests are constantly under development. A traditional diagnosis of IA is achieved by histological examination of tissue samples, often obtained during open lung biopsy or transbronchial lung biopsy [11]. Unfortunately, severe thrombocytopenia is commonly present in this group of patients, delaying invasive procedures. *Aspergillus* infection is difficultly to confirmed by blood culture and the detection of antibodies is not helpful due to the immune status of these patients [7]. Circulating *Aspergillus* antigen (galactomannan) detected by ELISA and 1,3-ß-D-glucan, were recently described method in the diagnosis of IA, but its value is doubtful for the early diagnosis of the infection [13,20,24,28,32].

Although isolation of the fungus from sputum and bronchoalveolar lavage (BAL) fluid is indicative of infection, both specimens lack sufficient specificity and sensitivity [23]. A positive culture from BAL samples is obtained in only 50% of patients with proven IA, and it can take several days [16].

The diagnosis of IA by molecular methods has been performed successfully with different type of clinical samples. The application of this test on fixed or fresh biopsy material shows high sensitivity, but it needs invasive procedure. When applied to clinical samples which were obtained from noninvasive procedure, as in BAL fluid, nucleic acid amplification (NAA) tests showed high sensitivity too. The role of polymerase chain reaction (PCR) assay of the BAL fluid for the diagnosis of IA has yet to be determined. Several published studies have showed contradictory results with using different aims and methods. Thus, the aim of this study was to review the literature on this topic to evaluate the many published studies and the efficacy of PCR in BAL fluid for the diagnosis of IA in high risk patients.

Methods

Search strategy. A systematic search was performed on two medical databases - PubMed (1966–June 2006) and LILACS (1990–June 2006). In addition, the Journal of Clinical Microbiology, a high-yield journal for molecular methods in infectious diseases, was also searched. The search strategy used was: aspergillosis, *Aspergillus*, nucleic acid amplification techniques, NAA, polymerase chain reaction, PCR, bronchoalveolar and BAL. The reference section of primary studies and narrative reviews were reviewed to search for any additional primary articles that could have been overlooked by the electronic search [12]. No language restrictions were imposed.

Study selection and methodological quality. The published studies were initially selected by one author, and then reviewed by two independent assessors to verify the pertinence of their inclusion. Any discrepancies were discussed and a consensus opinion was reached.

All articles must provide 1) data necessary for the computation of sensitivity and specificity of the PCR; 2) include at least 10 BAL fluid specimens to avoid selection bias [8]; 3) diagnosis and classification of IA by EORTC system once data of patients studied was often lacking [1]; 4) patients characteristics of control group (degree of risk of IA). Articles published before EORTC criteria of IA were included if sufficient clinical data were available.

The methodological quality of published paper was assessed by using previously described criteria [38]. Quality assessment was performed using methods adapted from guidelines on systematic reviews of diagnostic studies (Table 1). If no data on the above criteria were avai-

Table 1. List of validity criteria operationalised for papers reporting on the accuracy of NAA tests * in the diagnosis of invasive aspergillosis.

	Criteria of internal validity (IV)	***Possibilities***
1	Description of primer, probe and product of NAA	Complete; parcial; None
2	Blind measurement of test	Yes; No
3	Description of extraction & amplification	Yes; No
4	Use of Internal control/probe	Yes; No
5	Valid reference standard	Proven; probable; possible
6	Prevention of contamination	Yes; No
7	Design	Prospective or retrospective
8	Use a control group with same risk	Same risk factor or colonization; different risk factor; healthy control
	Criteria of external validity (EV)	
1	Spectrum of disease	In- and/or exclusion criteria mentioned
2	Setting	Enough information to identify setting
3	Previous tests/referral filter	Clinical details to which the NAA test is being evaluated (symptomatic or asymptomatic patients
4	Duration of illness before diagnosis	Duration mentioned
5	Co-morbid conditions	Details given (type of population)
6	Demographic information	Age and gender data provided
7	Execution of NAA test	Information about standard procedure directly or indirectly available: BAL collection procedure, frozen of samples, time of transportation of the BAL sample, way of reading NAA test, persons reading NAA test
8	Data of contamination control	If available
9	Setting of NAA tests	If available
10	Reproducibility of NAA test	Reproducibility studied or reference mentioned

* NAA – Nucleic acid amplification

lable, the given value was zero. "A" quality articles were arbitrarily defined as that which met at least 9/11 or 10/12.5 of the internal and external validity criteria, respectively; "B" quality met at least 5/11 or 6/12.5 of the internal and external validity criteria, respectively; and "C" quality study met less than 5/11 or 6/12.5 criteria.

Data extraction. Data extraction was performed by two independent assessors. The selected data included the following: methodological quality, patients characteristics (age, gender, form of disease, underlying conditions, treatment of underlying condition, antifungal therapy), test methods and outcome. Characteristics of primers, probes, DNA extraction and amplification methods of NAA were included.

Statistical analysis. The validation of this methodology of meta-analysis was based on guidelines to guarantee quality, correct article selection and data extraction.

The chi-square test was performed to determine the variability between studies (heterogeneity) and the significance was established at $p < 0.05$ [26].

The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic odds ratio (DOR) were calculated with a confidence interval (CI) of 95%. A graph of SROC (summary receiver operating characteristic) curve was constructed from the sensitivity and specificity from each article. Positive likelihood ratio (LR+) and the negative likelihood ratio (LR-) were calculated with the aim of improving the significance of the NAA tests, from a certain pre-test probability, in a clinical investigation. Publication bias was analyzed using the Egger test [27]. The software program Meta-Disc 1.4 was used to assist with calculation, graph construction and determination of heterogeneity.

Results

Description of the published studies selected in this investigation. This study found 883 articles and using the above guidelines, excluded 838 during the initial screening. Of the 45 remaining articles selected, thirty were not reports from human BAL or did not provide sufficient data for sensitivity and specificity calculation. Four publications were from the same medical services, but none of them had overlapped data. At the end, fifteen articles were included in this systematic review (Figure 1) [4,5,9,10,14,19,25,30,31,33-37,39]. All articles evaluated

Figure 1. Study selection process.

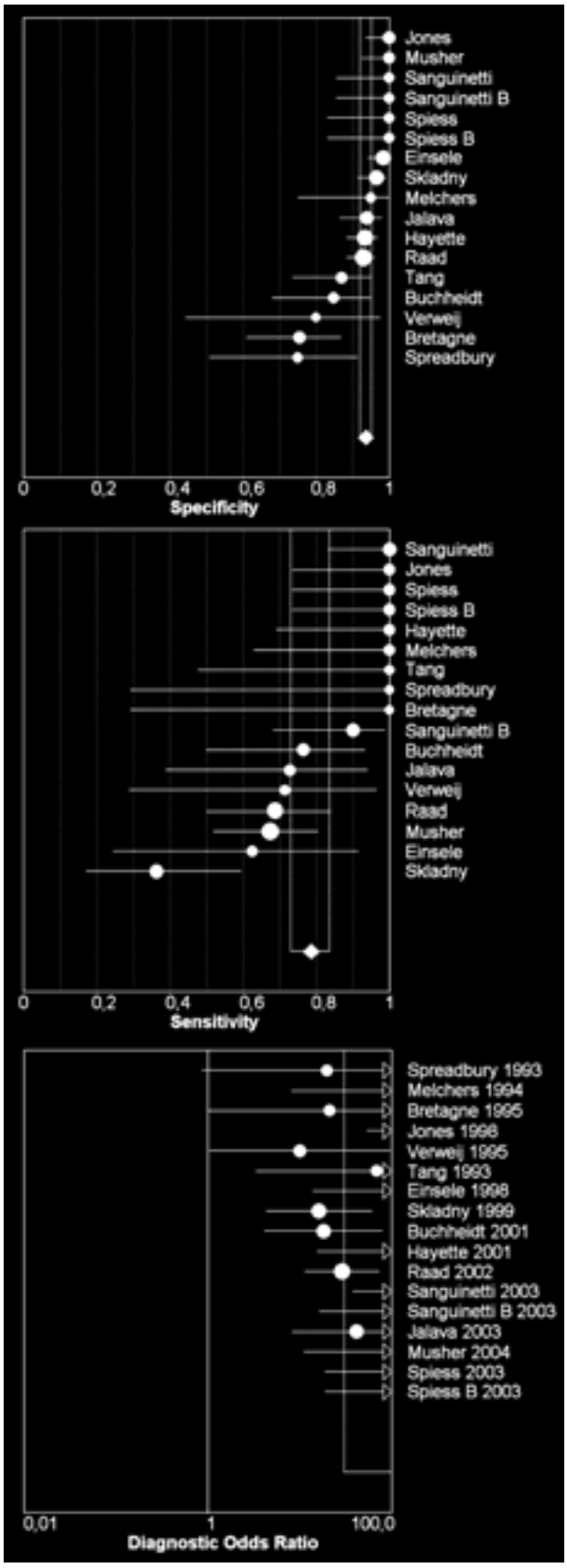

Figure 2. Sensitivity, specificity and DOR plotted on Forest graphs for bronchoalveolar fluid polymerase chain reaction in the invasive aspergillosis diagnosis. The central point determines the mean and the line determines the extremes with a confidence interval of 95%. The lozenge in the lower portion of each graph represents the sum of the mean of the works and the respective CI of 95%. *B - Different method (another group) in the same publication.

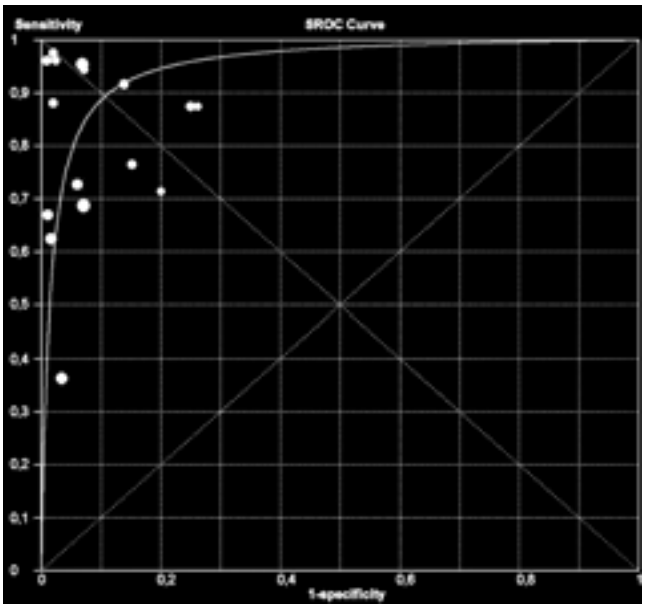

Figure 3. SROC curve graph for bronchoalveolar fluid polymerase chain reaction in the invasive aspergillosis diagnosis.

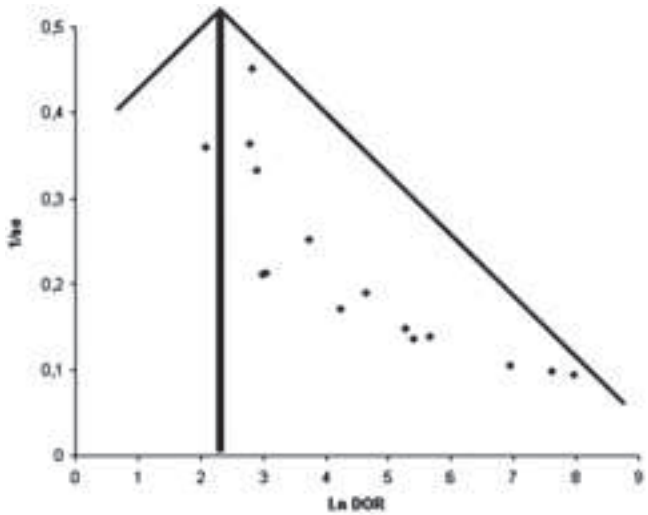

Figure 4. Publication bias assessment plotted in ln DOR.

in-house methods of amplification and rarely commercial kits for DNA extraction. The average (median) sample size was 51 BAL specimens/study, ranging from 19 to 249. When more than one BAL was performed by patient, it was evaluated the last sample before IA diagnosis.

Study characteristics and quality. More than 90% of studies had a B quality of external and internal validity (Table 2). The mean inter-rater agreement between the two reviewers for items in the quality checklist of internal and external validity was 0.86. Data from some articles were affected by incomplete reporting. The authors from original articles were not contacted to obtain additional data because some trials showed unreliable information. Descriptive results of this meta-analysis are shown in table 2, including internal and external validity, heterogeneity, technique of NAA, sensitivity and specificity. Seven studies were prospective (46%). The BAL sample of retrospective studies (54%) used frozen BAL sample. The preferred method of PCR was the nested-PCR because its known increased sensitivity. Four studies, published after 2001, evaluated the real time PCR.

Results of the variables. Sensitivity and specificity were calculated with different variables. Sensitivity was calculated using proven and probable IA. Possible IA cases were discharged from database analysis. Different control groups were used: 1) healthy adults or patients without risk factors for IA; 2) patients with high risk for IA; 3) patients with low risk for IA and; 4) all control groups together. The sensitivity and specificity are displayed on figure 2.

Specificity from several studies was close to 100%. The pooled specificity value was 94%. Sensitivity value was lower and also more heterogeneous than specificity, with a mean value of 79%. Figure 3 shows the SROC curve for the PCR. All measures were highly heterogeneous. Heterogeneity analysis calculated for sensitivity and specificity were 88.16 ($p < 0.001$) and 80.85 ($p < 0.001$), respectively. These numbers showed a greater dispersion values, suggesting an unreliable data. Although heterogeneous values, the sensitivity and specificity were high and the area under the curve from SROC curve presents a tendency towards 1 (0.9569). The tendency of SROC curve corroborated for the diagnostic value of the PCR test for IA using BAL samples. The result for the LR+ was 10.41 (6.40-16.95) and for the LR- was 0.22 (0.14-0.36). These data, when transferred to a Bayesian normogram, demonstrate the enormous value of a positive PCR, as post-test probability, for IA.

Publication bias. The publication bias, using the Egger test, was not presented in this meta-analysis. There was no statistical difference among the studies, despite the small number of articles ($p = 0.1433$) and a non-significant statistical value corroborates the absence of publication bias (Figure 4).

Discussion

This meta-analysis summarizes the evidence on accuracy of in-house PCR tests in the diagnosis of IA using BAL. The results of this study indicate that NAA tests have higher specificity and sensitivity than culture. Although different PCR assays were applied, none improve the sensitivity. Real time PCR was not different of nested-PCR or conventional assay. The high values of sensitivity and specificity in several assays were related to the efficacy of all methods of PCR. Probably, real time PCR will replace old assays due to high efficiency with quantitative technique.

The inability of the PCR assay to differentiate *Aspergillus* conidial colonization from true tissue invasion was confirmed by Hayette et al. [10]. This author performed PCR on BAL fluid of previous colonized patients and all samples were amplified. Unfortunately, the articles were heterogeneous and the plot of the sensitivity values with CI revealed six outlier studies on figure 2. In many cases, the interpretation of heterogeneity proved to be the most fascinating product of the meta-analysis. The low number of patients with proven IA was responsible for the heterogeneity in this study. All articles evaluated in this study showed specificity values higher than 0.75. Although heterogeneity was described, the plotted forest graph of specificity did not really showed dispersion of mean values and CI. These data should be carefully observed because control groups varied in several studies although specificity found was the same with different control groups.

Aspergillus antigens detected by EIA, recently accepted method for the diagnosis of IA, shows lower sensitivity than PCR tests [6,24]. This assay was not accepted because there is no standardization of so many methods of

Table 2. Sensitivity, specificity and quality grade of studies with bronchoalveolar fluid polymerase chain reaction in the invasive aspergillosis diagnosis.

			Invasive Aspergillosis										
First author (Country)	Year	Study Quality* IV / EV	Type of NAA	Proven PCR+/ Total	Probable PCR+/ Total	Control PCR+/ Total	Sensitivity (CI 95%)	Sensitivity Proven IA	Sensitivity Probable IA	Specificity (CI 95%)	Specificity At risk of IA	Specificity less risk of IA	Specificity without risk of IA
Spreadbury (England)	1993	B/B	PCR		3/3	5/20	1.00 (0.31-1.00)		1.00	0.75 (0.50-0.90)	0.66	0.80	0.71
Melchers (The Netherlands)	1994	B/B	PCR	4/4	2/2	1/20	1.00 (0.52-1.00)	1.00	1.00	0.95 (0.73-0.99)	0.83		1.00
Bretagne (France)	1995	A/A	Competitive PCR	3/3		12/49	1.00 (0.31-1.00)	1.00		0.75 (0.60-0.86)	0.88	0.66	0.55
Jones (England)	1998	B/B	PCR-ELISA	3/3	9/9	0/57	1.00 (0.70-1.00)	1.00	1.00	1.00 (0.92-1.00)	1.00		
Verweij (France, The Netherlands)	1995	B/B	PCR		5/7	2/10	0.71 (0.30-0.94)		0.71	0.80 (0.44-0.96)	0.80		
Tang (England)	1993	C/B	PCR	2/2	2/2	6/46	1.00 (0.39-1.00)	1.00	1.00	0.87 (0.73-0.94)	0.94		0.82
Einsele (Germany)	1998	B/C	PCR	5/8		2/126	0.66 (0.25-0.89)	0.66		0.98 (0.94-1.00)	0.98		
Skladny (Germany)	1999	B/B	Nested-PCR	3/7	5/15	4/118	0.36 (0.18-0.59)	0.43	0.33	0.97 (0.91-0.99)	1.00		0.91
Buchheidt (Germany)	2001	A/A	Nested-PCR	7/7	6/10	5/33	0.76 (0.50-0.92)	1.00	0.60	0.84 (0.67-0.94)	0.84		
Hayette (Belgium)	2001	A/C	Nested-PCR	7/7	3/3	11/167	1.00 (0.66-1.00)	1.00	1.00	0.93 (0.88-0.97)	0.00**		0.96
Raad (USA)	2002	A/B	PCR	8/10	14/22	14/199	0.69 (0.50-0.83)	0.80	0.64	0.9 (0.88-0.96)	0.93		
Sanguinetti (Italy)	2003	B/B	RT-PCR	5/5	15/15	0/24	1.00 (0.80-1.00)	1.00	1.00	1.00 (0.83-1.00)	1.00		
Sanguinetti (Italy)	2003	B/B	Nested-PCR	5/5	13/15	0/24	0.90 (0.67-98)	1.00	0.87	1.00 (0.83-1.00)	1.00		
Jalava (Finland)	2003	B/C	RT-PCR	6/7	2/4	5/83	0.74 (0.39-0.93)	0.86	0.50	0.94 (0.86-0.98)	0.92	0.96	
Musher (USA)	2004	A/C	RT-PCR	31/46		0/47	0.67 (0.52-0.80)	0.67		1.00 (0.91-1.00)		1.00	
Spiess (Germany)	2003	A/B	RT-PCR	8/8	4/4	0/20	1.00 (0.70-1.00)	1.00	1.00	1.00 (0.80-1.00)			1.00
Spiess (Germany)	2003	A/B	Nested-PCR	8/8	4/4	0/20	1.00 (0.70-1.00)	1.00	1.00	1.00 (0.80-1.00)			1.00

* The quality grade of studies is described in the methods; ** Colonized patients.

NAA. No commercial kit has been developed to perform a semi-automated NAA assay which could improve the sensitivity and further diagnostic efficacy.

The threshold for detection of fungal DNA was variable in different studies. Some PCR assays were able to detect 1 fg of fungal DNA and others 40 fg [19,25,31,34,36]. The threshold of the method for detection of fungal DNA on clinical samples was indirectly related to sensitivity for IA diagnosis (data not showed).

False positive results in control groups can be secondary to colonization of the patients´ respiratory tract or contamination of the samples during processing. Loeffler et al. found 8% of contamination with 2,800 specimens [17]. There are limited potential of contamination of fungal samples and it originates from airborne spore inoculation or carryover contamination. A positive PCR result should be considered only in patients with risk factors and clinical findings suggesting IA. A positive result of the NAA tests probably boosts the decision in favor of therapy, but a negative result should not delay the treatment. False negatives were found mainly by Musher et al. using a real time technique [25]. Skladny et al. also found false negative PCR tests on clinical samples of BAL, despite of nested-PCR with an assay able to detect 10 fg of fungal DNA [34].

Only six studies used species-specific primers [9,10,30,33,36,37]. Some primers used for the detection of *Aspergillus* spp. DNA can also amplified *Penicillium* spp. contamination. This was described by Spreadbury et al. and must be considered when the diagnosis of IA is on doubt [36]. The primers used for amplification were different in all studies. This is one of the subjects this study did not call meta-analysis. An interesting data found in this review showed that sensitivity of NAA tests for proven IA is always higher than for probably IA. It suggests more differential diagnosis in the probably IA group or less fungal DNA in the BAL. *Aspergillus fumigatus* is the most common agent of IA, but other species can be found (*Aspergillus flavus, Aspergillus niger, Aspergillus terreus*). As discussed above, a species-specific primer increases false-negative results which decreases the sensitivity.

Recently, several studies have been performed with blood samples and the results are improving, including pre-symptomatic diagnosis [15]. More studies are needed to confirm these results once bronchoscopy is an "invasive" procedure with some complications (pneumothorax and acute respiratory failure). The PCR in the BAL samples should be performed in all patients submitted to this procedure to improve the diagnosis of IA.

With the understanding that earlier diagnosis of IA may facilitate higher cure rates, newer diagnostic platforms that are not based on culture have been proposed and investigated. Diagnostic assays that detect fungal nucleic acids by PCR have been investigated for use with BAL fluid and these studies reported that PCR assay has reasonable sensitivity and specificity when used to test samples from patients at high risk for IA.

Sensitivity has been highly variable because of differences in assay protocols characteristics of patients evaluated, and form of IA diagnosis (proven or probable). More studies are needed to evaluate the use NAA tests, including real-time PCR assays, applied to BAL fluid. A standard assay platform has to be used and larger studies performed.

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