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[Diagnostic Test Accuracy Review]

Polymerase chain reaction blood tests for the diagnosis of invasive aspergillosis in immunocompromised people

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ABSTRACT

Background

Invasive aspergillosis (IA) is the most common life-threatening opportunistic invasive mould infection in immunocompromised people. Early diagnosis of IA and prompt administration of appropriate antifungal treatment are critical to the survival of people with IA. Antifungal drugs can be given as prophylaxis or empirical therapy, instigated on the basis of a diagnostic strategy (the pre-emptive approach) or for treating established disease. Consequently there is an urgent need for research into both new diagnostic tools and drug treatment strategies. Newer methods such as polymerase chain reaction (PCR) to detect fungal nucleic acids are increasingly being investigated.

Objectives

To provide an overall summary of the diagnostic accuracy of PCR-based tests on blood specimens for the diagnosis of IA in immunocompromised people.

Search methods

We searched MEDLINE (1946 to June 2015) and EMBASE (1980 to June 2015). We also searched LILACS, DARE, Health Technology Assessment, Web of Science and Scopus to June 2015. We checked the reference lists of all the studies identified by the above methods and contacted relevant authors and researchers in the field.

Selection criteria

We included studies that: i) compared the results of blood PCR tests with the reference standard published by the European Organisation for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG); ii) reported data on false-positive, true-positive, false-negative and true-negative results of the diagnostic tests under investigation separately; and iii) evaluated the test(s) prospectively in

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cohorts of people from a relevant clinical population, defined as a group of individuals at high risk for invasive aspergillosis. Case-control studies were excluded from the analysis.

Data collection and analysis

Authors independently assessed quality and extracted data. For PCR assays, we evaluated the requirement for either one or two consecutive samples to be positive for diagnostic accuracy. We investigated heterogeneity by subgroup analyses. We plotted estimates of sensitivity and specificity from each study in receiver operating characteristics (ROC) space and constructed forest plots for visual examination of variation in test accuracy. We performed meta-analyses using the bivariate model to produce summary estimates of sensitivity and specificity.

Main results

Eighteen primary studies, corresponding to 19 cohorts and 22 data sets, published between 2000 and 2013 were included in the meta-analyses, with a median prevalence of IA (proven or probable) of 12.0% (range 2.5 to 30.8 %). The majority of people had received chemotherapy for a haematological malignancy or had undergone a hematopoietic stem cell transplant. Several PCR techniques were used among the included studies. The sensitivity and specificity of PCR for the diagnosis of IA varied according to the interpretative criteria used to define a test as positive. The mean sensitivity and specificity were 80.5% (95% CI; 73.0 to 86.3) and 78.5% (67.8 to 86.4) for a single positive test result, and 58.0% (36.5 to 76.8) and 96.2% (89.6 to 98.6) for two consecutive positive test results.

Authors' conclusions

PCR shows moderate diagnostic accuracy when used as screening tests for IA in high-risk patient groups. Importantly the sensitivity of the test confers a high negative predictive value (NPV) such that a negative test allows the diagnosis to be excluded. Consecutive positives show good specificity in diagnosis of IA and could be used to trigger radiological and other investigations or for pre-emptive therapy in the absence of specific radiological signs when the clinical suspicion of infection is high. When a single PCR positive test is used as diagnostic criterion for IA in a population of 100 people with a disease prevalence of 13.0% (overall mean prevalence), three people with IA would be missed (sensitivity 80.5%, 19.5% false negatives), and 19 people would be unnecessarily treated or referred for further tests (specificity of 78.5%, 21.5% false positives). If we use the two positive test requirement in a population with the same disease prevalence, it would mean that six IA people would be missed (sensitivity 58.0%, 42.1% false negatives) and three people would be unnecessarily treated or referred for further tests (specificity of 96.2%, 3.8% false positives). Galactomannan and PCR have good NPV for excluding disease but the low prevalence of disease limits the ability to rule in a diagnosis. The biomarkers are detecting different aspects of disease and the combination of both together is likely to be more useful.

PLAIN LANGUAGE SUMMARY

A new, non-invasive diagnostic blood test - polymerase chain reaction - for people at risk of an invasive mould infection (aspergillosis)

Review question. We planned to review the evidence about the accuracy of polymerase chain reaction (PCR) tests for diagnosing invasive aspergillosis (IA) in people with defective immune systems from medical treatment such as chemotherapy or following organ or bone marrow transplant**.**

Background: IA is a fungal disease caused by *Aspergillus*, a widespread mould. Most people breathe in *Aspergillus* spores every day without becoming ill, however people with weakened immune systems or lung diseases are at a higher risk of developing health problems due to *Aspergillus*. IA causes patient afflictions that are classically defined as invasive, saprophytic or allergic. Some types of IA are mild, but some of them are very serious. IA is the most common life-threatening, opportunistic, invasive fungal infection in people whose immune systems are compromised. Without treatment, most people with IA will die as a direct result, so early diagnosis and prompt administration of appropriate antifungal treatment are both critical factors to the survival of these people. As obtaining lung tissue is hampered by the risks involved, there is a need for new, non-invasive methods such as PCR to detect fungal nucleic acids in blood.

Study characteristics. The most recent search for studies was conducted in June 2015. Eighteen clinical studies reporting the evaluation of PCR tests prospectively in cohorts of people at high risk of IA were selected.

Study funding sources. None of the companies involved in the diagnosis of invasive fungal diseases funded any of the studies included in the review.

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Quality of the evidence. Most studies were at low risk of bias and low concern regarding applicability. However, differences in the reference standard may have contributed to differences we found in the distribution of cases as being classified as IA or not.

Key results. Several PCR techniques were used in the studies. Pooling the data from the studies showed that sensitivity and specificity of PCR for the diagnosis of IA varied (from 58 to 80.5 % and from 78.5 to 95.2 %, respectively) according to the interpretative criteria used to define a test as positive. When used as a diagnostic criterion for IA in a population of 100 people with a disease prevalence of 13.0% (overall mean prevalence), a single PCR positive test would have missed three people with the disease, and falsely classified 19 people as having the disease who would be treated unnecessarily or referred for further tests. A requirement of two positive tests as a diagnostic criterion in a population with the same disease prevalence would miss six people with the disease and falsely classify three people as having the disease. These numbers should, however, be interpreted with caution because of the limitations of the reference standard in allowing consistent assessment of cases as being IA or not. Overall, PCR shows moderate diagnostic accuracy when used as a screening test for IA in high-risk patient groups. Importantly the sensitivity of the tests confers, with the low prevalence of the disease, a high negative predictive value such that a negative test allows the diagnosis to be excluded.

BACKGROUND

Target condition being diagnosed

Invasive aspergillosis (IA) is a disease resulting from opportunistic fungal infection and mainly affects immunocompromised hosts, particularly neutropenic patients such as those undergoing cancer treatment and hematopoietic stem cell transplant (HSCT) and solid organ transplant recipients (Flückiger 2006; Marr 2002). The highest incidence (10% to 20%) and mortality rates (60% to 90%) of IA have been reported following allogeneic HSCT and heart, lung or heart/lung transplantation. The principle reason for such people developing IA is that the underlying disease and its treatment with chemotherapy induces bone marrow failure resulting in profound leucopenia and impaired cell-mediated immunity. The leucopenia is marked by a lack of functioning polymorphonuclear leukocytes (granulocytes) referred to as neutropenia which means that the patient lacks the phagocytic white blood cells that are needed to fight infections, including aspergillosis. The innate immunity is also impaired leading to a collapse of the local defences of the oral cavity, gastrointestinal tract and respiratory tract. Damage to the respiratory tract is poorly understood but prevents the lung from effectively clearing fungal spores, especially those of *Aspergillus fumigatus* which are small enough to lodge in the alveolar spaces. The lack of local and systemic immune defences means that any spores that germinate can infect lung tissue and progress to a full-blown infection. The disease that follows is characterised by invasion of the capillaries (angioinvasion) which can lead to further dissemination to other parts of the lung and indeed other organs, particularly the brain.

Early diagnosis of IA and prompt administration of appropriate antifungal treatment have been recognised as crucial to the survival of people with IA (Marr 2002; Walsh 2008). Antifungal drugs can be given as prophylaxis, as empirical therapy, instigated on the basis of a diagnostic strategy or for treating established disease. Clearly, the earlier that treatment is started the better the outcome. Consequently there is an urgent need for research into new diagnostic tools to detect infection before disease becomes manifest, to allow effective treatment strategies to be developed. The polymerase chain reaction (PCR) is becoming increasingly popular (Hope 2005; Donnelly 2006; Mengoli 2009; Tuon 2007), however it was not considered mature enough to be included in the international consensus definitions of the The European Organisation for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG); (Ascioglou 2002; De Pauw 2008). The prevalence of IA varies from 1 in 100 to about 1 in 6 depending upon the level of compromised immunity, the environmental exposure and preventative measures taken which can include protected isolation with filtered air and antifungal prophylaxis. The outcome depends upon the extent of infection, whether diagnosis is made and treatment with an effective drug is begun early and, importantly, on whether or not the immune systems begins to recover (Marr 2002; Walsh 2008). Unlike other infectious diseases, direct demonstration of *Aspergillus* infection is seldom possible by culture of sterile body fluids, and obtaining tissue is seldom possible as it requires lung biopsy which is a difficult and risky procedure; this has hampered proper diagnosis. Recently, advances have been made on several fronts. Firstly, the EORTC/MSG's published definitions of invasive fungal disease (IFD) allow for degrees of certainty of diagnosis: possible, probable and proven (Ascioglou 2002; De Pauw 2008). Demonstration of fungi in diseased tissue is still required for a proven diagnosis of IFD. Unfortunately, obtaining tissue from a live patient is seldom feasible because of the risks posed to the patient. Definitions of invasive fungal infection were devised in 2002 and revised in 2008 to focus on fungal dis-

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ease (Table 1). These are based on host factors, radiological features and mycological evidence. Probable and possible cases have to satisfy the same host and radiological criteria and they are only distinguished by the presence or absence of mycological evidence. Biomarkers have potential to detect infection before development of overt disease, allowing treatment to be initiated at an earlier stage.

These definitions were only made possible by other developments in the field. Computer assisted tomography (CT scan) became more widely available and allows lesions consistent with pulmonary iA to be detected at an early stage. This offered the possibility of performing bronchoscopy to obtain bronchoalveolar fluid in which to detect fungus by microscopy and culture. However the technique is not without risk and cannot always be performed when required. By contrast, blood is readily available which opens up the possibility of looking for fungi in an indirect fashion by detecting fungal cell components including the galactomannan (GM) of the cell wall of *Aspergillus* species (Leeflang 2008). The EORTC/MSG definitions help integrate all the clinical and laboratory information available in terms of host factor such as neutropenia, clinical features such as pulmonary nodules and mycological evidence such as detection of GM to allow a level of certainty of diagnosis to be assigned. These definitions have been adopted widely by government agencies such as the European Medicines Agency and the Food and Drug Administration of the USA for evaluating antifungal drug products and diagnostic tests, as well as by the scientific and medical community at large for investigating epidemiology and auditing antifungal stewardship.

There are circumstances when a host factor is present - for instance receipt of an allogeneic HSCT - and mycological evidence exists - such as *Aspergillus* being recovered from pulmonary secretions - without evidence of active disease. This may represent infection before disease becomes manifest. The range of potential drugs currently available allows treatment to be given for prevention (prophylaxis), for unexplained fever when IFD cannot be ruled out (empirical therapy), on the basis of non-specific clinical features or mycological evidence (pre-emptive therapy) and for possible, probable and proven IFD (directed therapy). However, the ability to identify who needs treatment, when, and with what, is sufficiently unreliable that many physicians continue to treat empirically. Not only does this lead to unnecessary costs but it is also not clear how many people are helped or harmed by this approach. Consequently there is an urgent need for new diagnostic tools and an assessment of their utility in the clinic.

Index test(s)

There are few direct diagnostic tests and those that are available are limited by the difficulties in obtaining tissue specimens to allow culture, microscopy and histology (Chamilos 2006). Blood in its various forms - whole blood, plasma and serum - is readily available, but only tests for antigens such as GM and beta-d-glucan have been deemed acceptable to support a diagnosis (Leeflang 2008; Pfeiffer 2006; Senn 2008). In neutropenic patients, pulmonary abnormalities consistent with invasive aspergillosis, such as nodules, often surrounded by a 'halo sign', can be detected using high-resolution computed tomography (Greene 2007). However, the 'halo sign' is transient and only detectable during early invasive aspergillosis, after which radiological signs become non-specific or appear too late to be therapeutically useful (Caillot 2001). Radiological signs also herald established disease so the opportunity to intervene early has been lost.

Molecular methods, such as the PCR, have being investigated in order to improve the diagnosis of IA (Donnelly 2006; Mengoli 2009; White 2010). PCR can amplify a single or a few copies of target DNA allowing target detection with great sensitivity and specificity. Moreover it can be quantitative, using the procedural variant called real-time PCR (qPCR). The sensitivity is based on the enormous potential for DNA target (the "amplicon") amplification due to repeated cycles of the polymerase reaction, where every cycle doubles the DNA sequence of interest. Real-time PCR continuously monitors the amplification of target DNA at every cycle. The threshold cycle number (preferred term Cq) is when the amplicon becomes detectable as an exponentially increasing signal, exceeding the background threshold, and is proportional to the amount of starting DNA in the reaction. A high initial DNA concentration will require fewer cycles to reach the threshold and has a lower cycle threshold value. The specificity of PCR resides in the DNA oligonucleotides used as primers, allowing the terminally stable variant of the enzyme DNA polymerase to initiate sequence duplication. These primers join to the DNA target ("annealing") in a very stringent way, allowing only minimal misfit possibility. Moreover, in real time PCR (RT-PCR), the use of reporter probes, hydrolysis probes or molecular beacons that bind to the central part of the target sequence increase the assay's specificity.

PCR has an enormous potential for diagnosing infectious diseases, particularly where traditional culture methods are less effective. The fungal genus *Aspergillus* is a good example of this kind of approach. The recovery of *Aspergillus* from blood cultures is rarely achieved even in overwhelming infection. Unlike the enzyme-linked immunosorbent assay (ELISA) test for GM, no commercial PCR has been validated for use on blood specimens, although standards for PCR performance have been developed. PCR based tests on blood specimens have gained popularity as the platforms become more automated and extraction methods and targets become commercially available (White 2010). However the technique was not included in either the original or the revised EORTC/MSG definitions because it had not been validated or standardised at that time. The European *Aspergillus* PCR Initiative (EAPCRI) was founded to address the issue of standardisation of PCR-based diagnostics for IA. It has published studies describing the critical stages in DNA isolation from blood samples (White 2010), and on the critical characteristics of a standardized *Aspergillus* PCR assay. These studies allied to the standardization of qPCR assays

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described in the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines. Bustin et al. (Bustin 2009) have helped pave the way for reliable and robust PCR assays for the diagnosis of IA in the clinical setting.

Clinical pathway

The range of antifungal drugs available allows treatment to be given for prevention (prophylaxis), for unexplained fever when IFD cannot be ruled out (empirical therapy), on the basis of non-specific clinical features or mycological evidence (pre-emptive therapy) and for possible, probable and proven IFD (directed therapy). However, many physicians persist in treating empirically, as the identification of who needs treatment, when, and with what, is uncertain. This may lead to unnecessary treatment, which incurs costs, and may or may not be harmful to some people. Diagnostics tests can be used to establish a diagnosis but can also be used to rule out a diagnosis. This is particularly useful for people at risk of IA where a highly sensitive test can deliver a high negative predictive value for disease, allowing empirical therapy to be safely withheld even on the basis of a single test. Conversely, a high positive predictive value is required to rule in the diagnosis. The use of PCR as a screening tool differs fundamentally from its use as confirmation of the diagnosis. Therefore, if prevalence is low (i.e., < 10%), invasive aspergillosis can be ruled out during the risk period for as long as any single PCR test is negative and, of course, there are no clinical signs of disease. Conversely, two or more PCR positive results could be used for mycological confirmation to allow a case of possible IA to be upgraded to probable IA.

Clinical pathways of managing patients can vary according to the risk of IA. High risk patients may be screened using GM and or PCR and positive results may trigger an intensive diagnostic workup with CT scanning and bronchoalveolar lavage (BAL) to look for disease (diagnostic driven) or used to initiate antifungal treatment to prevent development of disease (pre-emptive). Screening may occur throughout the period of risk or only when people develop fever. Alternatively patients may be tested when they develop symptoms suggestive of disease to confirm diagnosis.

Rationale

There is no single assay that has been validated for the early diagnosis of IA. Non-culture based methods, such as serial GM ELISA screening, hold most promise in establishing early diagnosis and may result in improved outcomes, but clinical utility is not yet established. Moreover, newer methods such as PCR are being investigated (Donnelly 2006). The utility of PCR as either a screening tool or a confirmatory test will depend on the population in which it is used. Prevalence of disease, use of prophylactic or empirical antifungal agents, availability of protective environments and other diagnostic tests will all influence how the test is used in clinical practice. It is not the aim of this present analysis to establish clinical outcomes but evaluate diagnostic accuracy so that rational use of PCR testing can be applied to different populations.

OBJECTIVES

To provide an overall summary of the diagnostic accuracy of PCR based tests on blood specimens for the diagnosis of IA in the immunocompromised host.

Secondary objectives

When studies included in the analysis also compared the diagnostic performance of PCR techniques and the GM ELISA assay, we comparatively evaluated the diagnostic performance of PCR based tests and GM ELISA assays. However, since the objective of this review is not to identify all studies dealing with GM ELISA assays and IA, only those within the study comparison were included in the review.

METHODS

Criteria for considering studies for this review

Types of studies

We included studies using PCR techniques on blood specimens for analysis if they:

1. compared the results of PCR tests with the diagnosis made following the published case definition criteria for invasive fungal disease proposed by the EORTC/MSG or, for studies published before the publication of these criteria in 2002, used comparable criteria as a reference standard (Ascioglou 2002; De Pauw 2008);
2. reported data on false-positive, true-positive, false-negative and true-negative results of the diagnostic tests under investigation separately; and
3. evaluated the tests prospectively in a cohort of people from a relevant clinical population, defined as a group of individuals at high risk of IA.

We classified studies, on the basis of the sampling method, as being consecutive or non-consecutive. We regarded studies evaluating specimens from a group of people known to have aspergillosis, and from a separate group of subjects without evidence of disease, as case-control studies (Lijmer 1999). These studies were included in the systematic review but excluded from the quantitative analysis.

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Aspergillus contamination and false positive PCR results with bronchoalveolar lavage (BAL) and sputum samples can follow inhalation of airborne spores or colonization of the lung (Lewis White 2006). Moreover, BAL is an invasive procedure performed only to confirm the aetiology in a subset of cases that already meet the clinical definitions of IA. Thus, to avoid bias related to the patient selection and specimen type, we analysed only studies evaluating PCR on blood, with exclusion of studies that analyse the accuracy of PCR tests on BAL only.

Participants

Patients at risk of IA, including neutropenic cancer patients and HSCT or solid organ transplant recipients.

Index tests

PCR methods on blood specimens (whole blood or serum). We considered different DNA extraction methods and PCR methods (e.g., nested, ELISA, qPCR).

Target conditions

The target condition of this review is IA (systemic aspergillosis).

Reference standards

Definitions for invasive fungal disease were first published in 2002 by the EORTC/MSG (Ascioglou 2002) and were revised in 2008 (De Pauw 2008; Table 1). These were used as a reference standard and comparable criteria were used for studies published before the publication of the definitions in 2002. The EORTC/MSG definitions divide the patient population into four categories: people with proven IA, people with probable IA, people with possible IA, and people without IA. In accordance with the previous *Aspergillus* review on *Aspergillus* GM detection (Leeflang 2008), sensitivity and specificity were assessed in each study considering the proven and probable cases of IA as having the disease, and the cases of possible IA and no IA as not having the disease.

Search methods for identification of studies

The search strategies for MEDLINE and EMBASE are listed in Appendix 1.

Electronic searches

We searched the following electronic databases to identify reports of relevant studies:

- MEDLINE, through Ovid (1946 to June week 2, 2015).
- EMBASE, through Ovid (1980 to June week 2, 2015).
- LILACS (1982 to June 2015).
- Database of Abstracts of Reviews of Effects to June 2015.
- Health Technology Assessment database to June 2015.
- Web of Science to June 2015.

Searching other resources

We also searched for unpublished material on Scopus (http://www.scopus.com). We checked the reference lists of all the studies identified by the above methods and contact other authors and trialists in the field.

Data collection and analysis

Selection of studies

Two review authors (PD, RB) independently assessed the abstract (if available) of each reference identified by the search against the inclusion criteria. Any disagreements that arose were resolved through discussion and consensus with a third author (MC). We retrieved those references that potentially met the inclusion criteria (based on their abstract or title) in full for further independent assessment.

Data extraction and management

We extracted the following data from each included study:

- Study design.
- Study population.
- Reference standard and performance of the reference standard.
- Performance of the index test.
- Technical details of the PCR methods used, including genetic target of PCR and nucleotide probe sequence, and any PCR testing methods; we classified the diagnostic modalities using PCR assays according to the sampling methods and how these relate to the definition of a positive result, namely either positive PCR in at least two consecutive blood samples drawn from the same patient, or a single sample yielding a PCR positive result. When we compared PCR based tests to GM, we assessed whether authors explicitly mention the exclusion of the GM ELISA test from the reference test definition (EORTC/MSG criteria). In this case, we performed a direct comparison of the index test and the comparator evaluated in the same study population towards the reference standard.
- QUADAS-2 items.
- Data for two-by-two table (false-positive, true-positive, false-negative and true-negative results of the diagnostic tests under investigation and reference standard).

Two review authors (RB, CM) extracted the data. Disagreements were resolved by discussion.

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Assessment of methodological quality

Assessment of the quality of diagnostic accuracy studies, as recommended in STARD (Standards for Reporting of Diagnostic Accuracy), is of absolute relevance in systematic reviews (Bossuyt 2003; Reitsma 2009; Whiting 2004). For this purpose, we used the QUADAS-2 tool , the current version of QUADAS that has been adopted for use by the Cochrane Collaboration and is recommended for use in all Cochrane diagnostic test accuracy reviews to evaluate the risk of bias and applicability of primary diagnostic accuracy studies. Pairs of authors independently assessed the methodological quality of the studies included, and disagreement were resolved by consensus with all of the authors.

QUADAS-2 consists of four key domains:

- patient selection;
- index test;
- reference standard;
- flow and timing.

Each is assessed in terms of risk of bias and the first three in terms of concerns regarding applicability. Signalling questions are included to assist in judgements about risk of bias. Risk of bias is judged as "low", "high", or "unclear". If all signalling questions for a domain are answered "yes" then risk of bias can be judged "low". If any signalling question is answered "no" this flags the potential for bias. The "unclear" category is used only when insufficient data are reported to permit a judgment.

Tabular and graphical displays are used to summarise QUADAS-2 assessments. We did not calculate a summary score estimating the overall quality of an article, since their interpretation is problematic and potentially misleading (Whiting 2005).

The items of the QUADAS-2 tool and their interpretation are reported in appendix (Appendix 2).

Statistical analysis and data synthesis

The values of sensitivity and specificity are automatically computed in RevMan 2014. Summary positive (LR+) and negative (LR-) likelihood ratios, and summary diagnostic odds ratio (DOR) were obtained from the bivariate analysis (see below). We evaluated different interpretive criteria for a PCR positive result in the two-by-two table, namely a single positive PCR result and two positive PCR results. We have presented individual study results graphically by plotting the estimates of sensitivity and specificity (and their 95% confidence intervals (CIs)) in both forest plots and receiver operating characteristics (ROC) space.

The diagnostic accuracy indexes and related 95% CIs were compared when studies compared PCR test and serum GM to the standard reference. The comparative analysis was undertaken by adding a binary covariate to the bivariate model.

We assessed the operating point sensitivity and specificity of the diagnostic test under scrutiny by a bivariate random-effects approach (Reitsma 2005). The original method was modified by using a random-effects bivariate logistic model (Chu 2006). The same procedure permits generation of a hierarchical summary receiver operating characteristic (HSROC) model (Rutter 2001). The parameters of the bivariate distribution can also be used to obtain a HSROC curve. Indeed, the bivariate analysis and the HSROC method without covariates are different parameterizations of the same model (Harbord 2007). These methods allow the meta-analysis of sensitivity and specificity in a single model, incorporating the amount of correlation between sensitivity and specificity across studies. Moreover, the random-effects approach allows the multilevel (within and between study) structure of the sources of variation to be coped with. The results of the bivariate model can be used to calculate likelihood ratios. To calculate (negative) predictive values, an estimate of prevalence in addition to values of sensitivity and specificity is required. One can then apply a Bayesian approach to obtain predictive values from these three parameters. Bivariate analysis was performed on STATA 11 software.

Investigations of heterogeneity

We assessed heterogeneity by visual inspection of forest plots of sensitivity and specificity, and through visual examination of ROC plot of the raw data. Heterogeneity was further investigated, exploring the effects of several study-level covariates. For this, we performed a multilevel mixed-effects logistic model using the probability of test positivity as a dependent variable; the group variable was the study, and the disease status was the first explanatory variable. This basic model admitted in turn several additional covariates. When available, we examined the following covariates:

- Distinctive groups of patients.
- Study size (< or > 100 patients).
- Children versus adults.
- Use of antifungal prophylaxis active against *Aspergillus* species.
- Variation in PCR techniques (RT-PCR versus other PCR methods).
- a single or two positive assay results requirement to define the test as positive.
- Quality item (e.g., blinding of the index test, blinding of the reference test).

We included the interaction between the disease status and the additional covariate into the model as well.

RESULTS

Results of the search

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Of the 1672 references identified, we selected 155 potentially relevant citations (Figure 1). After screening titles and abstracts, we selected 57 articles for full-text review. Of these, we excluded 39 studies for various reasons: patients were selected retrospectively in four studies (Auberger 2011; Bretagne 1998; Cesaro 2008; Challier 2004); 14 studies did not provide sensitivity and/or specificity data for 2 x 2 tables (Adhurti 2011; Armenian 2009; Badiee 2008; Badiee 2009; Bernal-Martinez 2011; Blennow 2010; Bucheidt 2004; Hasseine 2010; Hebart 2000; Kawazu 2004; Klingspor 2006; Lass-Florl 2001; Morrissey 2013; Teifoori 2011); eight were case-control studies (Bucheidt 2001; Bu Rong 2005; Kami 2001; Li 2013; Millon 2011; Scotter 2005; Skladny 1999; Springer 2013); two studies included BAL only (Bolehovska 2006; Jones 1998); one study included a subset af a previous (AMBILOAD) trial (Hummel 2010); the index test was inappropriate in five studies (Chryssanthou 1999; Halliday 2005; Jordanides 2005; Nakamura 2010; Yoo 2005); the reference standard was inappropriate in three studies (Johnson 2012; Mandhanija 2010; Teifoori 2011); two studies were in Chinese (Liu 2005; Sun 2010). Therefore, 18 studies published between 2000 and 2013 met the inclusion criteria and were included in the meta-analysis (Badie 2010; Barnes 2009; Cuenca-Estrella 2009; da Silva 2010; El Mahallawy 2006; Ferns 2002; Florent 2006; Halliday 2006; Hebart 2000a; Hummel 2009; Landlinger 2010; Ramírez 2009; Rogers 2013; Springer 2011; Suarez 2008; Sugawara 2013; von Lilienfeld-Toal 2009; White 2006). Two studies reported the diagnostic performance of PCR performed with different methodologies (Rogers 2013; Suarez 2008), and one in a different patient setting (Rogers 2013). Therefore data were analysed from 22 data sets.

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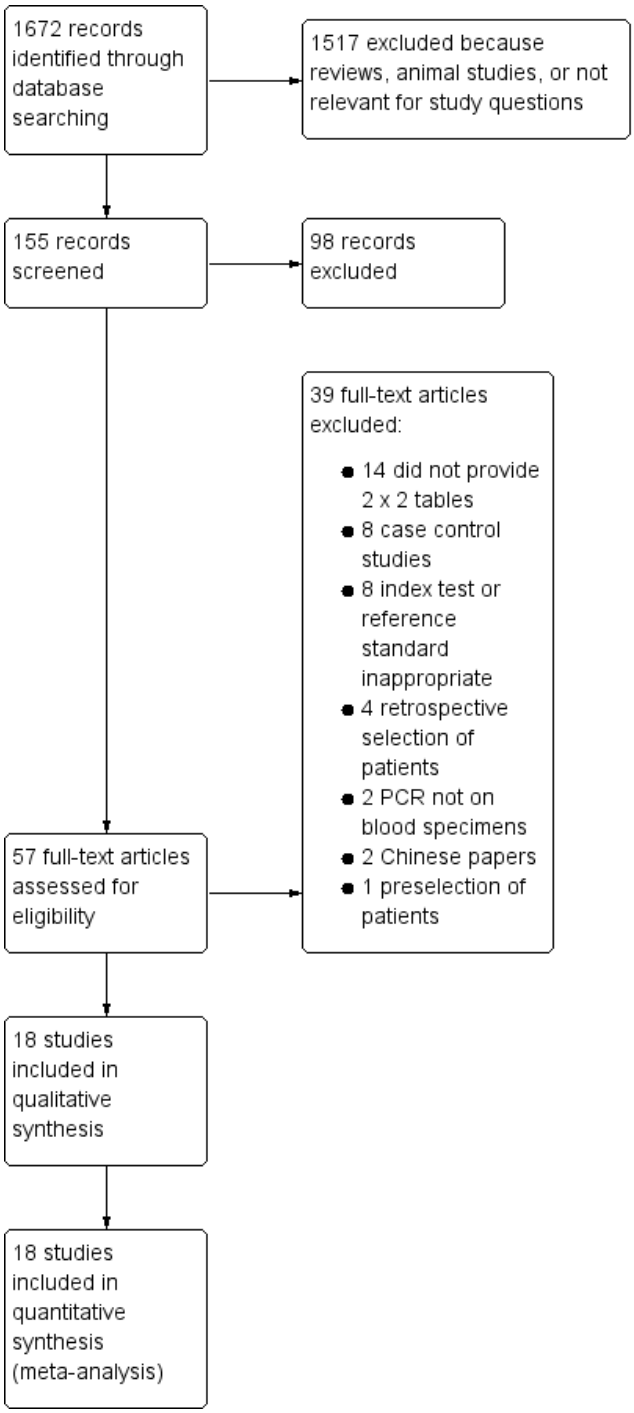
Figure 1. Study flow diagram.



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The main characteristics of the studies are summarized in the 'Characteristic of studies' tables. More than 24,000 clinical blood specimens from 1765 patients at risk of IA were included. The majority of people had received chemotherapy for a haematological malignancy or had been given an HSCT. The PCR techniques used are summarized in table Table 2. All the selected studies reported the results of a single PCR result, and seven studies reported using two PCR results (Figure 2; Figure 3). Eight of the studies included in the analysis also reported results of GM assay. The study by Rogers et al presented two cohorts of patients (one from the University Clinic of Wuzburg, and one from Saint James Hospital) according to the PCR test used: Internal Transcribed Spacer (ITS) qPCR and the 28S nested PCR; Rogers 2013]; the study by Suarez 2008 et al presented data according to the protocols for serum processing (large and small volume.

Figure 2. Forest plot of 1 PCR: single positive requirement. The study by Rogers et al. shows separately data according to PCR test used, and clinical centre participating in the study; in the study by Suarez et al, data are presented according to protocols for serum processing (large and small volume).

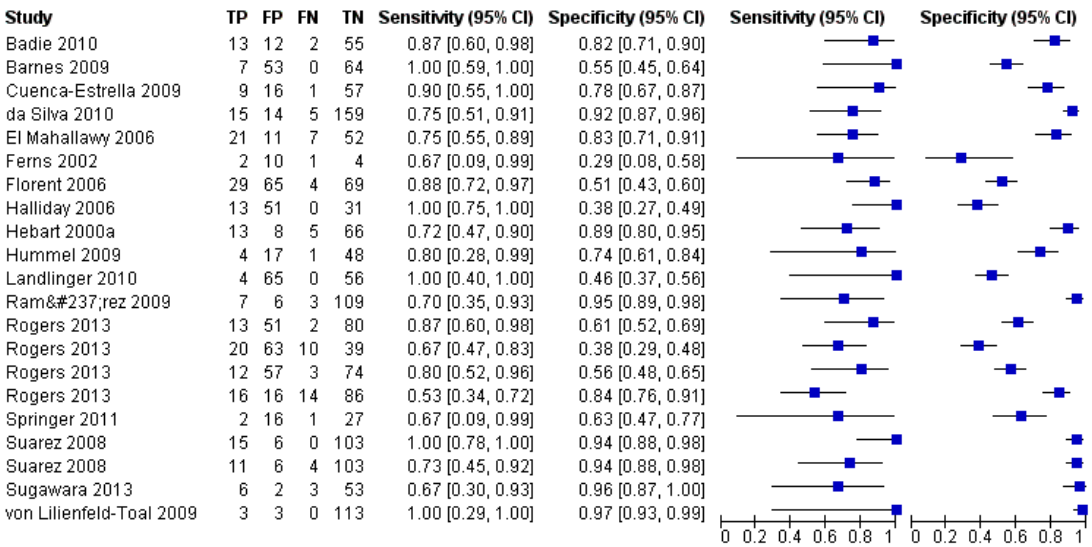

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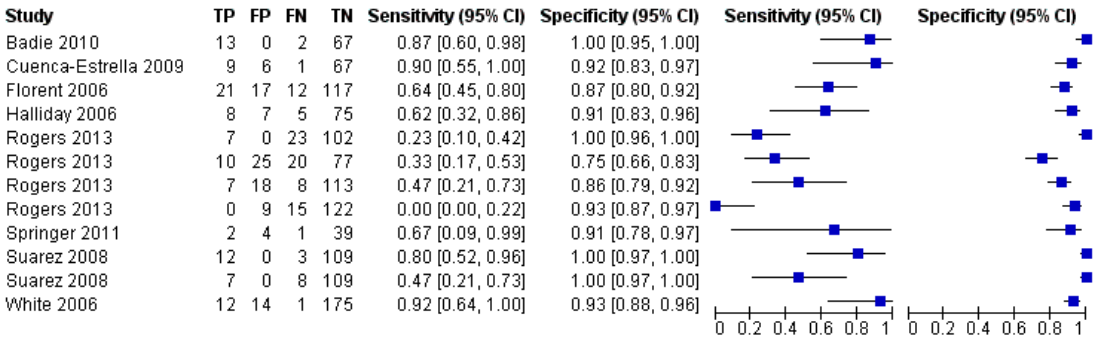
Figure 3. Forest plot of 2 PCR: two positive tests requirements



Thirteen further studies were identified in the latest search dated June 2015. These have been added to Studies awaiting classification and will be assessed in a future update.

Methodological quality of included studies

The quality of studies as assessed by the QUADAS-2 tool is summarized in tables and graphs. Figure 4 shows the overall risk of bias and applicability concerns for the 18 selected studies. Figure 5 presents the quality assessment results for the individual studies.

For all QUADAS-2 domains, most studies were at low risk of bias and low concern regarding applicability. In the patient selection domain, all the studies enrolled an homogenous and representative population of patients at risk of IA; 70% of studies were at low risk of bias because they enrolled participants consecutively and avoided inappropriate exclusions. The remaining studies were graded as being at unclear risk of bias because the manner of patient selection was not stated.

Figure 4. Risk of bias and applicability concerns graph: review authors' judgements about each domain presented as percentages across included studies

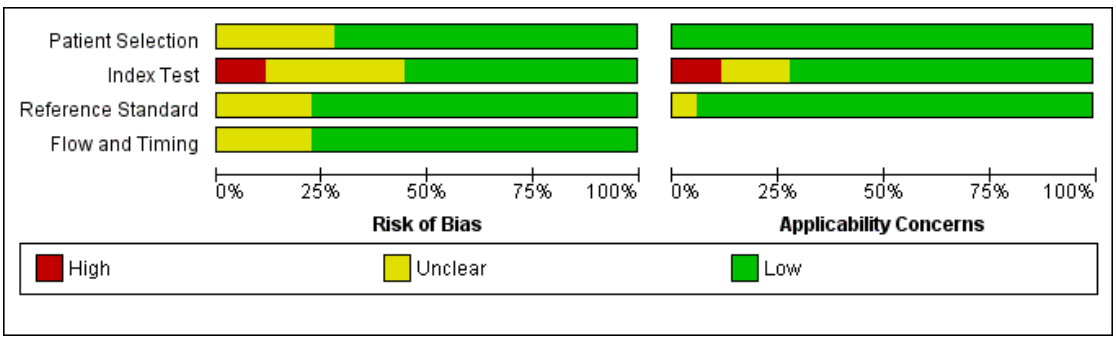


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Figure 5. Risk of bias and applicability concerns summary: review authors' judgements about each domain for each included study

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In the index test domain, we considered the majority of studies to be at low risk of bias (70%) and low concern regarding applicability (80%). The remaining studies were judged to be at high risk, because the index test was performed knowing the results of the reference standard, or at unclear risk of bias. In the reference standard domain, we judged 80% of studies to be at low risk of bias because it was stated that the reference standard results were interpreted without knowledge of the results of the index test, while in the remaining studies it was not specified. Applicability was of low concern for all studies in the reference standard domain. In the flow and timing domain, 75% of studies were judged to be at low risk of bias because all patients were accounted for in the analysis, the appropriate reference standard was used, and information about un interpretable results was provided. We had nearly complete information for all studies.

Findings

Results of the meta-analysis

Based on 22 data sets (18 primary studies, 19 cohorts), the median number of effectives (patients or episodes) per data set was 95 (range 17 to 202), and the median prevalence of proven or probable IA was 12.0% (range 2.5% to 30.8%); the mean was 108.3 for effectives, and 12.7% for study prevalence. The sensitivity and specificity of PCR for the diagnosis of IA varied according to the interpretative criteria used to define a test as positive. For PCR assays, the requirement for either one or two consecutive samples to be positive were evaluated for diagnostic accuracy. With the one positive requirement, the sensitivity reported in the studies ranged from 53% to 100%, and specificity from 29% to 97%. With the 2 positive requirements the summary estimates for the sensitivity ranged from 0% to 92%, and specificity from 75% to 100%. The mean sensitivity and specificity were 80.5% (95% CI 73.0% to 86.3%) and 78.5% (67.8% to 86.4%) for a single positive result requirement, and 58.0% (36.5% to 76.8%) and 96.2% (89.6% to 98.6%) for two positive results requirement. DORs were 15.1 (95% CI 7.9 to 28.6) for a single positive result, and 34.5 (95% CI 8.2 to 144.2) for two positive results LR+/LR- were 3.7 (2.4 to 5.7)/0.25 (0.18 to 0.35) for a single positive result, and 15.1 (4.9 to 46.1)/0.44 (0.26 to 0.73) for two positive results. When used in isolation, a single PCR positive test as diagnostic criterion for IA in a population of 100 people with a disease prevalence of 13.0% (overall mean prevalence), three people who have IA would be missed (sensitivity 80.5%, 19.5% false negatives), and 19 people would be unnecessarily treated or referred for further tests (specificity of 78.5%, 21.5% false negatives). If we use the two positive test requirement in a population with the same disease prevalence, it would mean that six IA people would be missed (sensitivity 58.0%, 42.1% false negatives) and three people would be unnecessarily treated or referred for further tests (specificity of 96.2%, 3.8% false negatives).

Heterogeneity

The appearance of the forest plots for PCR show a large dispersion of diagnostic indexes at study level; this was more apparent for specificity using the single positive requirement, and for sensitivity using the two positive requirement. Visual inspection of the prediction ellipses in the bivariate analysis show a large area occupying most of the full probabilistic space; the degree of eccentricity was more pronounced in the specificity direction for a single positive requirement, and in the sensitivity direction for two positive requirement (Figure 2; Figure 3).
Heterogeneity was investigated by subgroups analyses.

HSROC analysis and bivariate approach

Graphs (ellipses) of bivariate models for the 2 different criteria for PCR positivity are shown in Figure 6. Unpaired studies were excluded for the evaluation of the differential effect of the single positive/two positive criterion. The number of studies included in the paired analysis was reduced to seven, corresponding to 11 comparisons of PCR test (each paired for single positive and two positives criteria; Badie 2010; Cuenca-Estrella 2009; Florent 2006; Halliday 2006; Rogers 2013; Springer 2011; Suarez 2008).

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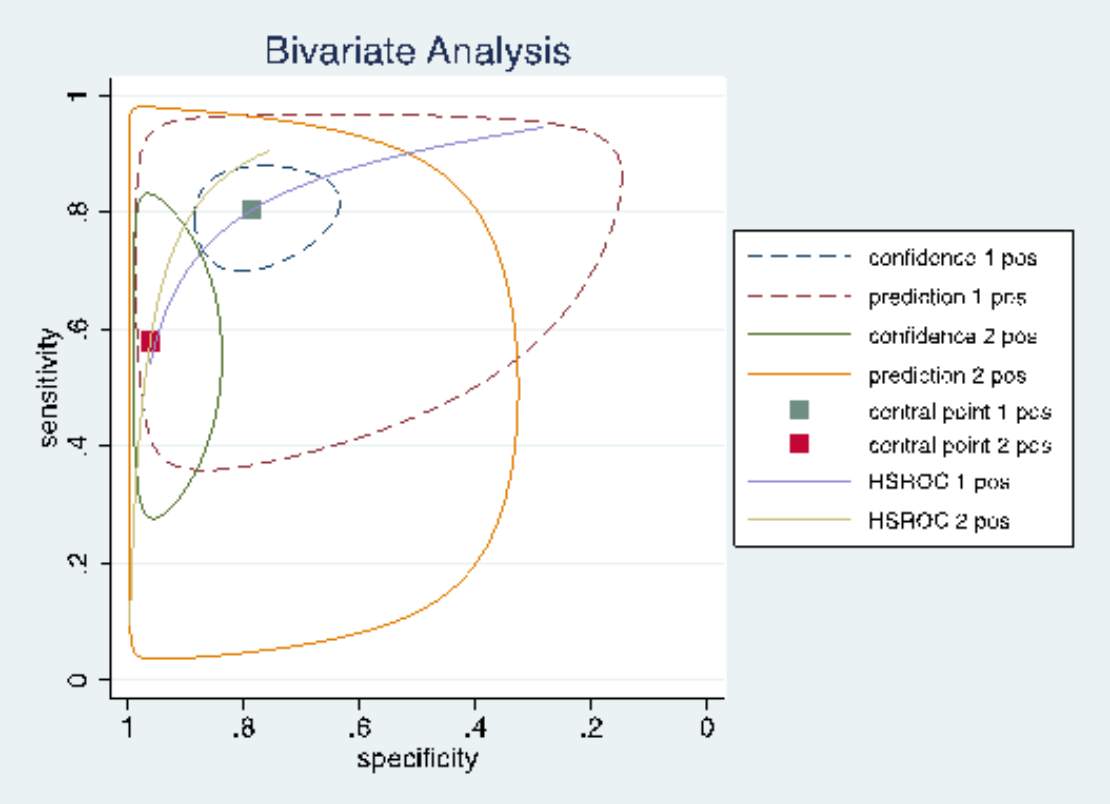
Figure 6. Bivariate analysis of the sensitivity and specificity of the PCR as a diagnostic tool for Aspergillus invasive infection. Two diagnostic criteria are compared: single positive PCR result ("1 pos") versus ≥ two positive consecutive PCR results ("2 pos"). The square dots indicate the means ("central point"). The smaller ellipses indicate the 95% confidence area of the means, the larger ellipses indicate the 95% forecast areas for a new observation. The hierarchical summary receiver-operator curves are depicted as well.



When sensitivity and specificity data from the bivariate model were compared, changing the positive results requirement from 1 to 2 increased significantly the specificity (from 78.5% to 96.2%, P value = 0.0000); by contrast, the sensitivity decreased significantly from 80.5% to 58.0% (P value < 0.0001). The joint effect on sensitivity and specificity was also significant (P value < 0.0001). The DORs changed from 15.1 with a single positive assay to 34.5 with two positive assays.

Subgroups analysis and bivariate analysis with covariates.

We carried out a subgroup analysis of adult and paediatric studies (El Mahallawy 2006; Halliday 2006; Hummel 2009; Landlinger 2010). The diagnostic yield did not differ significantly between adult and paediatric studies. However, the limited number of paediatric studies does not allow firm conclusion to be drawn regarding the diagnostic performance of PCR in paediatric patients. We also performed a subgroup analysis according to study size. Studies were defined as small or large size according to the number of enrolled people (< or > 100). Likewise study size did not have a significant impact on performance of PCR test.

A subgroup analysis of studies endorsing 2002 EORTC criteria (10 studies: El Mahallawy 2006; Ferns 2002; Florent 2006; Halliday 2006; Hebart 2000a; Hummel 2009; Ramírez 2009; Suarez 2008; von Lilienfeld-Toal 2009; White 2006) or 2008 criteria (seven studies: Badie 2010; Barnes 2009; Cuenca-Estrella 2009; da Silva 2010; Rogers 2013; Springer 2011; Sugawara 2013) was also performed using the bivariate method and considering the results of PCR test with the single positive criterion. One study stated the use of EORTC criteria but did not mention which criteria were employed (Landlinger 2010). Lower sensitivity and specificity values were found for studies using 2008 criteria compared to those using 2002 criteria (76.18% and 74.44% versus 82.82% and 79.50%, respectively), but these differences were probably driven by the low estimates of diagnostic accuracy found in some of the 2002 studies (Rogers 2013; Springer 2011).

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Eleven studies used antifungal prophylaxis in the entire population under investigation (Badie 2010; Barnes 2009; Cuenca-Estrella 2009; Ferns 2002; Florent 2006; Halliday 2006; Hebart 2000a; Rogers 2013; Springer 2011; Sugawara 2013; White 2006), four studies did not (da Silva 2010; El Mahallawy 2006; Suarez 2008; von Lilienfeld-Toal 2009), one study used prophylaxis (antifungal agent not specified) in a subset (50%) of patients (Landlinger 2010), and two studies provided no details on the use of prophylaxis (Hummel 2009; Ramírez 2009). Fluconazole was used as prophylaxis in four studies (Badie 2010; Halliday 2006; Hebart 2000a; Springer 2011), seven studies used prophylaxis with antifungal agents active against *Aspergillus* (itraconazole, voriconazole, amphotericins or caspofungin; Barnes 2009; Cuenca-Estrella 2009; Ferns 2002; Florent 2006; Rogers 2013; Sugawara 2013; White 2006). To evaluate the impact of prophylaxis on diagnostic accuracy of PCR, we used a mixed-model logistic analysis using the probability of the index test positivity as the dependent variable. When examining data under the criterion "single positive", the prophylaxis produced a large reduction in specificity (- 26.7%), whereas the change of sensitivity was small (- 2.9%), and not excluding the zero. When examining data under the criterion "two positives", the logistic regression was unfeasible because all "no prophylaxis" studies had no false positive results. When the analysis was conducted considering only the subgroup of seven studies with anti-*Aspergillus* prophylaxis versus "no-prophylaxis" studies (including fluconazole prophylaxis studies), there was again a reduction in specificity (- 20.7%), and a small, not statistically significant change in sensitivity (- 5.9%).

The PCR methods varied notably. Some studies were based on gel electrophoretic visualization after proper staining of the amplicons, whereas others were based on automated procedures, as real-time PCR, with substantial differences regarding the threshold of detection. We relied on the reported positive/negative results only, and the possible cut-point/threshold variation across studies was not considered. When the accuracy of the real-time PCR (quantitative PCR) was compared to other PCR methods, no significant effect was detected on sensitivity or specificity, separately or jointly.

Quality items that did have an effect on sensitivity or specificity were blinding of the index test (13% decrease in sensitivity and 9.4% decrease in specificity; P value = 0.0099) and blinding of the reference standard (10.6% decrease in sensitivity and 14.7% decrease in specificity; P value = 0.0087). In other words, failure of blinding produced a spurious increase in overall accuracy.

Predictive values

Positive and negative predictive value (PPV and NPV, respectively) of the *Aspergillus* PCR detection are shown in Figure 7. The predictive values were calculated by applying the Bayes rule. With a mean prevalence of invasive aspergillosis of 13%, the PPV is 36% with a single positive test criterion, and 70 % with two positive tests criterion; for NPV these figures were 96% and 94%, respectively.

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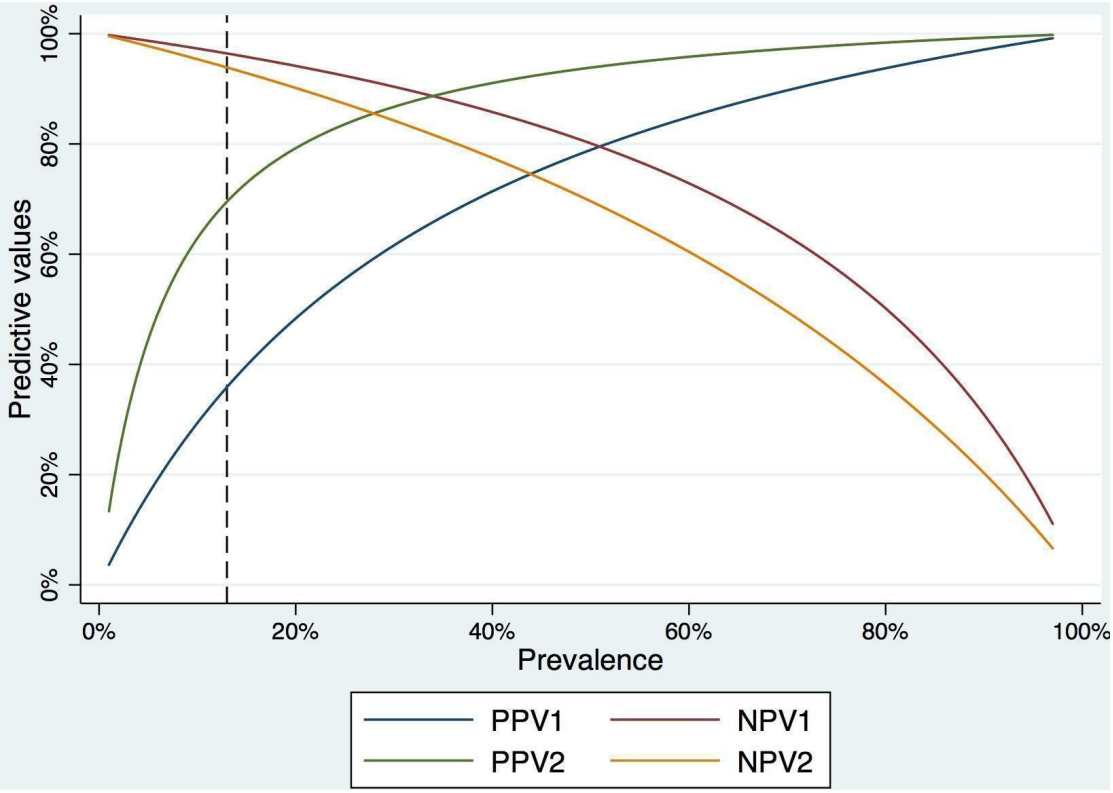
Figure 7. Predictive values. Positive and negative predictive value (PPV and NPV, respectively) of the Aspergillus PCR detection test (y-axis) as a function of the prevalence of the disease, invasive aspergillosis (x-axis). The curves are related to the diagnostic criterion (a single positive result or two consecutive positive PCR results). The PVs were calculated by applying the Bayes rule. The mean prevalence of invasive aspergillosis (13%) is indicated by the vertical dashed line. It corresponds to PPV1 = 36%, NPV1 = 96%, PPV2 = 70%, NPV2 = 94%.



Comparison between PCR techniques and GM assay

Nine studies (Barnes 2009; Cuenca-Estrella 2009; da Silva 2010; Florent 2006; Rogers 2013; Springer 2011; Suarez 2008; Sugawara 2013; White 2006) also evaluated GM assay, but in all studies but one GM was part of the reference standard (Suarez 2008). Thus, to avoid incorporation bias, data of GM assay were not compared to PCR, and not included in the current review.

In the study by Suarez 2008, sensitivity and specificity were 100% and 96.7% for RT-PCR using large sample volume (LSV), and 88.2% and 95.8% for GM. Thus the overall performance of RT-PCR using LSV was consistently higher than that of GM.

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Summary of findings

INTERPRETATIVE CRITERIA TO DEFINE THE TEST POSITIVE	EFFECT (95% CI)	NO. STUDIES	PREVALENCE: MEAN (95% CI)	WHAT DO THESE RESULTS MEAN
1 SINGLE PCR SPECIMEN	sensitivity: 80.5% (72.9% to 86.3%) specificity: 78.5% (67.8% to 86.4%)	17 studies	13.3% (11.9% to 14.8%)	With a prevalence of 13%, 13 out of 100 patients will develop IA. Of these, 3 will be missed by a single PCR test (19.5% of 13); of the 87 patients without IA, 18 will have a false positive result of the PCR test; repeating the test will reduced significantly rates of false positive results
≥2 PCR SPECIMENS	sensitivity: 57.9% (36.5% to 76.8%) specificity: 96.2% (89.6% to 98.6%)	8 studies	14.0% (12.3% to 15.9%)	With a prevalence of 14%, 14 out of 100 patients will develop IA. Of these, 6 will be missed using the 2 positive PCR test (42.0% of 14); of the 86 patients without IA, 3 will have a false positive result of the PCR test

The PCR methods varied notably across studies. Several covariates (in particular, the adoption of antifungal prophylaxis and blinding to the reference test or index test) were found to substantively affect the accuracy indexes under evaluation, mainly sensitivity and specificity.

CI: confidence interval

IA: invasive aspergillosis

PCR: polymerase chain reaction

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DISCUSSION

Summary of main results

Eighteen primary studies, corresponding to 19 cohorts and 22 data sets, were included in the meta-analyses, with a mean prevalence of IA (proven or probable) of 13.3%. The majority of patients had received chemotherapy for a haematological malignancy or had been given a hematopoietic stem cell transplant. Several PCR techniques were used among the included studies. The sensitivity and specificity of PCR for the diagnosis of IA varied according to the interpretative criteria used to define a test as positive. For PCR assays, the requirement for either one or two consecutive samples to be positive were evaluated for diagnostic accuracy. The mean sensitivity and specificity were 80.5% (95% CI 72.9% to 86.3%) and 78.5% (67.8% to 86.4%) for a single positive test result, and 57.9% (36.5% to 76.8%) and 96.2% (89.6% to 98.6%) for two positive test results. The findings indicate that PCR shows moderate diagnostic accuracy when used as a screening test for invasive aspergillosis in high risk patient groups. Several covariates (in particular, the adoption of antifungal prophylaxis and blinding to the reference test or index test) were found to substantially affect the accuracy indexes under evaluation, particularly sensitivity and specificity. The uneven distribution of these covariates may explain, at least partly, the large heterogeneity found in this analysis The subgroup analyses suggest that antifungal prophylaxis might impair performance and these conclusions may not be applicable to patients on concurrent antifungal therapy.

Strengths and weaknesses of the review

The findings of this review are based on comprehensive searching, strict inclusion criteria, and standardized data extraction. The strength of our review is that it enables an assessment of the diagnostic accuracy of PCR for detection of IA in a homogenous population of patients at risk of IA. The strict inclusion criteria (cohort of consecutive patients, including neutropenic cancer patients and hematopoietic stem cell or solid organ transplant recipients) were used to cover the spectrum of diseases likely to be encountered in the current or future use of this diagnostic test.

We only included studies that used the EORTC/MSG criteria or a similar reference standard. Differences in the reference standard may have contributed to differences we found in the distribution of patients with probable, possible and no invasive aspergillosis but not proven disease as this relies on demonstration of the fungus in tissue. For instance the clinical features in the revised definitions are based solely on radiological evidence of IA whereas the original 2002 definitions also included minor signs such as fever and cough as evidence of disease. Consequently employing the revised definitions to cases classified as possible IA by the 2002 definitions would only be retained as such if there was radiological evidence. Applying the 2008 definitions would have a similar effect on probable IA for the same reasons.

The impact of empirical antifungal usage has not been analysed. It is likely that PCR can detect infection before overt disease is radiologically detectible. Consequently, people with positive results who did not meet the criteria for proven or probable disease could have had early infection that resolved either with empirical or pre-emptive antifungal treatment or as a result of resolution of the underlying immunosuppression.

The lack of direct comparisons with other biomarkers including GM and beta-D-glucan could be a further shortcoming. Looking at our findings and at those of other reviews, the performance of PCR test is comparable to that reported for GM and superior to beta-D-glucan. It is likely that combinations of different biomarkers will provide the optimal diagnostic performance. Also it was difficult to distinguish between using PCR for screening purposes and for confirming the diagnosis as these are associated with low and high a priori likelihood respectively. Furthermore, screening requires testing at regular intervals during the period of risk (typically every 3 to 4 days) whereas tests for confirming the diagnosis of IFD will only be done once.

The molecular basis for azole resistance has been described, and the ability to detect *Aspergillus* DNA also raises the possibility of rapid detection of antifungal resistance using the same specimen. This could optimise patient management further and should be explored in future studies.

Applicability of findings to the review question

We noted that most studies performed PCR in high-level, reference laboratories. It is not clear whether intermediate/peripheral laboratories might be settings that match the review question due to the lack of commercially available PCR tests. Much has been done by the EAPCRI to establish a standard for PCR that should help laboratories offering the test (www.eapcri.eu). However incorporating PCR into routine practice also requires an explicit protocol indicating who should be tested, when and how frequently, as well as what action should be taken in the event of a given result. Moreover the process needs to be completed within 24 hours so that the results can be used to best advantage by the clinician. This requires an explicit care plan or pathway, a multidisciplinary approach and a clear understanding between the clinic and laboratory to ensure a smooth turnaround.

AUTHORS' CONCLUSIONS

Implications for practice

The findings indicate that PCR screening tests show moderately good diagnostic accuracy when used as screening tests for IA in

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high-risk patient groups. However, for a screening strategy, with the low prevalence of IA in the observed population and a low pre-test probability of disease, the moderate sensitivity of the PCR is sufficient to ensure a good negative predictive value, such that disease can be confidently excluded and the need for empiric therapy avoided. As such, screening strategies could replace empirical antifungal therapy in selected high-risk patients. Consecutive positives show moderate specificity in the diagnosis of IA and could be used to trigger radiological and other investigations or for pre-emptive therapy in the absence of specific radiological signs when the clinical suspicion of infection is high. The subgroup analyses suggest that antifungal prophylaxis could impair performance and these conclusions may not be applicable to people on concurrent antifungal therapy. With the observed prevalence of disease (13 %), repetition of the PCR test increase considerably the positive predictive values, with a modest decline of the negative predictive values. Therefore, the repetition of the PCR assay is recommended in order to increase the diagnostic accuracy.

Implications for research

It is clear that PCR holds a lot of promise as a useful test for detecting *Aspergillus* infection although the diagnostic accuracy might be improved further by combining the test with other biomarkers such as GM, and this should be explored in future studies. Further validation is also need to determine whether using PCR for screening high-risk patients could become the standard of care. Future studies that validate PCR for aspergillosis clearly need to distinguish between use of the test to screen for the presence or absence of IA in high-risk patients if there are no signs of illness, and its use to confirm or exclude the disease when it becomes manifest. IA can be ruled out during the risk period for as long as any single PCR test is negative and there are no clinical signs of disease. Conversely when prevalence of aspergillosis is around 10%, two or more PCR positive results can be used for mycological confirmation to allow a case of possible IA to be upgraded to probable.

The tests need to be incorporated into patient care pathways that compare prophylactic, empirical, pre-emptive and targeted antifungal drug use looking at impacts on patient management.

It was not possible to investigate the diagnostic utility of combinations of biomarkers (e.g. PCR and GM) because the GM is incorporated into the EORTC/MSG definitions and would introduce incorporation bias. Hence, cases would have to be classified by omitting GM. Further studies are needed to assess clinical utility and cost effectiveness.

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CHARACTERISTICS OF STUDIES

Characteristics of included studies *[ordered by study ID]*

Badie 2010

Study characteristics	
Patient sampling	Prospective study, samples collected Sep 2004 - June 2006. Patients with haematological malignancies (who had received chemotherapy)
Patient characteristics and setting	Sample size: 194 Males/females: 133/61 Mean age: 33.7 years (range 14 to 80) Presentation: patients with haematological malignancies and solid organ transplantation at risk for IFD Setting: Nemazi Hospital, Shiraz, Iran
Index tests	DNA extracted through lysis of blood and fungal cells (van Burik 1998) followed by purification using the QIAamp DNA Mini Kit. Standard PCR was used as well as PCR-ELISA. *Aspergillus* specific assays (Aquirre 2004). Presence or absence of bands indicated a positive result; positive results were retested with species specific probes
Target condition and reference standard(s)	Patients were evaluated for IA; patient samples (urine, cerebrospinal fluid, pleural and abdominal tap, BAL and sputum) were examined for signs of infection. Cases of IA were defined according to the EORTC/MSG 2002 criteria
Flow and timing	Samples were collected from 209 patients between September 2004 and June 2006; 985 samples collected from 194 patients were analysed. Blood samples (EDTA) were collected once per week and frozen prior to analysis. Patients were excluded if they did not attend follow-up for more than two weeks. No indication that patients with possible IA were excluded from 2x2 analysis
Comparative	
Notes	This study describes the performance of standard PCR and PCR-ELISA

Methodological quality			
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**
DOMAIN 1: Patient Selection			
Was a consecutive or random sample of patients enrolled?	Yes		
Was a case-control design avoided?	Yes		
Did the study avoid inappropriate exclusions?	Yes		

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Badie 2010 (*Continued*)

		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	No			
		High	**High**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear			
		Unclear	**Unclear**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear			
Did all patients receive the same reference standard?	Unclear			
Were all patients included in the analysis?	No			
		Unclear		

Barnes 2009

Study characteristics		**Study character**
Patient sampling	Prospective study between October 2005 and March 2006; at risk febrile patients or SCT patients with graft-versus-host disease were tested	

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Patient characteristics and setting	Sample size: 125 patients Males/females: 1.4/1 Mean age: 56.2 years (range 16 to 83) Presentation: haematology patients at risk for IFD including SCT, acute myeloid leukemia Setting: University Hospital of Wales
Index tests	DNA extracted from 2 ml blood, red cell lysis, white cell lysis, bead beating and Magna Pure (Roche) DNA purification (White 2006). Nested PCR with second round on lightcycler (Roche) targeting 28S, 60 cycles all together. All positive samples were repeated
Target condition and reference standard(s)	IFD was the target condition for PCR assays; GM antigen testing was performed on patient samples, EORTC/MSG 2008 criteria (including GM) were used to define cases of IFD
Flow and timing	1028 specimens collected from 125 patients over a six month period. 130 patients were screened but 125 were evaluable. No indication that patients were excluded from 2 x 2 analysis; this analysis was performed for "single non-reproducible positive PCR", "Single reproducible positive PCR" and "multiple positive PCR" results
Comparative	
Notes	Report examines diagnostic driven care pathway, limited empirical treatment. Data provided for interpretation of single and reproducible results. Very relevant to this review

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			

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If a threshold was used, was it pre-specified?	Unclear		
		Unclear	**Unclear**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	No		
		Low	

Cuenca-Estrella 2009

Study characteristics		**Study character**
Patient sampling	Patients with febrile neutropenia considered at risk from IA were studied prospectively between October 2004 and November 2005	
Patient characteristics and setting	Sample size: 83 patients Males/females: 48/35 Mean age: 52 years Presentation: patients with haematological malignancies and febrile neutropenia at risk for IA Setting: Hospital Universitario 12 de Octubre in Madrid, Spain	
Index tests	DNA extraction: DNA was extracted from the samples using the QiampDNA Mini Kit (Qiagen, Izasa, Madrid, Spain) DNA detection: 2 µl of DNA from each sample were used for each RT-PCR, which contained a final volume of 20 µl with 3 mM of Cl2Mg, 0.5 µM from each primer, and 0.4 µM of molecular beacon	

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	probe. Preincubation was at 95°C, followed by 45 denaturation cycles (15 s at 95°C), annealing (30 s at 56°C), and extension (5 s at 72°C). Each experiment was run twice Definition of positive assay: the results were considered positive when an exponential increase in fluorescence was detected compared with that of the negative controls before cycle 40 of amplification. The detection limit was 10 fg of DNA per µl of sample (cycle 42 of amplification) *Aspergillus*-specific: analyses for at least 1, 2 or 3 positive PCR tests retesting. 2244 specimens tested
Target condition and reference standard(s)	The definitions of proven, probable and possible IA were set according to the definitions of the EORTC/MSG. HRCT and GM testing were also performed as a part of reference standard
Flow and timing	Four weekly samples (two blood and two serum) were taken during episodes of febrile neutropenia Time interval sampling: 2004-05 Selection/exclusion for analysis: excluding patient 10, for whom the PCR result was negative, it was possible to calculate the time gain in diagnosis for the PCR technique compared to that for HRCT and GM for the other 11patients with IA Sampling/storage: years (range) Analysis type: at least 2 consecutive positive PCR results missing/uninterpretable results: N
Comparative	
Notes	Prophylaxis: itraconazole; proven/probable/possible/no IA: 1/9/2; PCR effectiveness (replica/eluat into PCR volume): 2 x 2 of 200 µl. The information collected on each patient, as well as the PCR results, were entered in a database

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	No			

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Cuenca-Estrella 2009 (*Continued*)

If a threshold was used, was it pre-specified?	Yes		
		High	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

da Silva 2010

Study characteristics		**Study character**
Patient sampling	From October 2000 to August 2003, 172 patients with haematologic malignancies and 27 patients receiving high dose chemotherapy in an autologous hematopoietic stem cell transplantation setting were studied prospectively. All patients were screened by PCR twice a week since admitted in the ward	
Patient characteristics and setting	Patients with haematological malignancies and febrile neutropenia at risk for IA Median age 50 years Male/female: 102/70 Setting: Hospital dos Capuchos, Lisbon, Portugal	
Index tests	Blood samples, BAL samples, fungal DNA extraction and PCR conditions were performed as described in van Burik 1998. The whole process of amplification was done using Taq polymerase	

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	(Gibco BRL) and pan-fungal primers that bind to the conserved regions of the fungal 18S rRNA gene sequence. Established PCR negative and positive controls were used in every assay. 1311 blood specimens tested
Target condition and reference standard(s)	Fungal infections were classified according to EORTC/MSG revised consensus
Flow and timing	Peripheral blood samples from patients were screened twice weekly for both methods since admission to the ward. If a positive value was obtained the patient would be screened every day for 3 consecutive days in the first week and then twice weekly again
Comparative	
Notes	The study also evaluated GM assay, but due to incorporation bias (GM is part of the reference standard), these data were not included in the current review

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	Unclear			
		Unclear	**Unclear**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target	Yes			

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da Silva 2010 (*Continued*)

condition?			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Unclear	**Low**

DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Yes		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

El Mahallawy 2006

Study characteristics		**Study character**
Patient sampling	Febrile, neutropenic paediatric cancer patients were prospectively sampled between April 2003 and April 2004. Patients were included if they had antibiotic-resistant fever. Patients were given full diagnostic work-ups for any signs of IFD	
Patient characteristics and setting	Sample size: 91 patients Males/females: 37:25 Mean age: 8 (range 2 to 18) Presentation: "at risk" for IA including febrile neutropenic cancer patients and fever not responding to antibiotics Setting: National Cancer Institute, Cairo University	
Index tests	Serum samples (unknown volume) were treated with Lyticase, then extracted using QIAamp DNA Mini Kit (Qiagen), PCR amplified 420 bp products from 18S gene (universal fungal assay). Single round conventional PCR with 30 cycles. Products detected on agarose gel	
Target condition and reference standard(s)	Target condition was IFD; CT scan, blood culture and *Aspergillus* antigen detection were used to aid in defining cases of IFD according to the EORTC/MSG (2002) criteria	
Flow and timing	91 patients tested, unknown sample numbers during 1 year period. All patients were included in 2 x 2 analysis to calculate sensitivity, etc	

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Comparative				
Notes	Pan-fungal conventional PCR used with low cycles, lack of specific IA information may be a problem for inclusion			
Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Yes			
If a threshold was used, was it pre-specified?	No			
		Low	**High**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes			
		Low	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**

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El Mahallawy 2006 (*Continued*)

Was there an appropriate interval between index test and reference standard?	Yes		
Did all patients receive the same reference standard?	Unclear		
Were all patients included in the analysis?	Yes		
		Unclear	

Ferns 2002

Study characteristics				**Study character**
Patient sampling	Ninety-four blood samples from 17 patients at high risk of IA undergoing chemotherapy for acute leukaemia (10) or undergoing allogenic BMT (7) on the haematology unit at the University College London Hospital Trust were screened			
Patient characteristics and setting	Gender and age: not specified Setting: University College London Hospital Trust			
Index tests	*Aspergillus* DNA, from whole blood samples, was amplified by nested PCR to detect a 135 bp fragment in the mitochondrial region of *Aspergillus fumigatus* or *Aspergillus flavus* (121 bp).			
Target condition and reference standard(s)	IA in haematologic patients. The diagnosis of aspergillosis was classified into proven, probable or possible on the basis of EORTC/MSG criteria			
Flow and timing	PCR results were retrospectively compared with clinical data and antifungal treatment			
Comparative				
Notes	None of the 94 samples from the 17 patients were above the cut-off value when tested as serum in the Platelia *Aspergillus* antigen ELISA			
Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Unclear			
Was a case-control design avoided?	Yes			

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Did the study avoid inappropriate exclusions?	Yes			
		Unclear	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	Unclear			
		Unclear	**Unclear**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes			
		Low	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear			
Did all patients receive the same reference standard?	Unclear			
Were all patients included in the analysis?	Yes			
		Unclear		

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Florent 2006

Study characteristics				**Study character**
Patient sampling	From April 2001 through November 2002, all patients (> 15 years) with hematological malignancies who were routinely screened for GM detection were included in the study. Gender and age were not specified. Setting was Hopital Saint-Louis and Hotel-Dieu, Paris			
Patient characteristics and setting	A total of 201 patients were enrolled in the study and had 256 consecutive episodes of neutropenia (neutrophil count fewer than 500 cells/mL). During the high-risk periods for infection and until absolute neutrophil counts increased to greater than 500 cells/mL, all patients were hospitalised in protected facilities with high-efficiency particulate air filtration associated with laminar air flow for patients undergoing allogeneic stem cell transplantation			
Index tests	DNA was extracted from both serum and fungal cultures by use of the QIAamp DNA Mini Kit (Qiagen), in accordance with the manufacturer's recommendations. Two negative controls were used in each DNA extraction experiment. The PCR-ELISA was performed using the serum sample that was collected for GM detection, which was stored at -20°C until processing. 1205 specimens tested			
Target condition and reference standard(s)	the criteria proposed by the EORTC/MSG were used. To evaluate the performance of the GM assay either alone or in combination with the PCR-ELISA, the results of the GM assay were not included in the microbiological criteria for the diagnosis of probable IA			
Flow and timing	Single-positive results were defined as at least a single positive result, and consecutive positive results were defined as at least two positive results obtained consecutively within 1 week. Thirty-four patients did not have consecutive serum samples that were collected within 1 week, and they were excluded from the final analysis. Because of the uncertainty of the diagnosis in patients with possible IA, 3 separate analyses were performed: the first included only proven and probable IA cases; the second included proven and probable IA cases, and possible cases were considered to be proven IA cases; and the third included proven and probable IA cases, and possible cases were not considered to be IA. Inhibitors were detected in 18 serum samples, and these samples were excluded from the analysis			
Comparative				
Notes	PCR-ELISA precocity in diagnosing IA was assessed in comparison with the timing of the clinical suspicion of IA, the results of CT, and histological and microbiological criteria as defined by the EORTC/MSG			

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			

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Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Yes			
If a threshold was used, was it pre-specified?	Yes			
		Low	**Low**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear			
		Low	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Yes			
Did all patients receive the same reference standard?	Yes			
Were all patients included in the analysis?	No			
		Unclear		

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Halliday 2006

Study characteristics				**Study character**
Patient sampling	Prospective collection of samples from patients undergoing chemotherapy or HSCT who had developed febrile neutropenia between Aug 2002 and July 2003. Blood samples collected from consecutive patients twice weekly; only patients from whom three samples were obtained per febrile episode were analysed			
Patient characteristics and setting	Sample size: 65 patients Males/females: 23:6 Mean age: 37 (range 16 to 62) Presentation: Episodes of febrile neutropenia in patients undergoing chemotherapy or HSCT Setting: Westmead Hospital, NSW, Australia			
Index tests	Blood collected twice weekly; DNA extracted from 500 µl EDTA blood using the GenElute Mammalian Genomic DNA Kit (Sigma-Aldrich) with modified protocol that included RCLB, followed by lyticase treatment; no bead beating. Conventional nested PCR no qPCR assay modified from (Skladny 1999). *Aspergillus* specific targeting 18S. Sensitivity of 10 CFU/ml			
Target condition and reference standard(s)	Target condition was IA, classified according to the EORTC/MSG criteria (2002). IA defined at the end of "at risk" episodes			
Flow and timing	998 blood samples from 65 patients (29 adults and 36 children) were collected between August 2002 and July 2003. Separate 2 x 2 analyses were carried out to calculate sensitivity, etc, with possible cases excluded, or with possible cases included as true negatives or true positives			
Comparative				
Notes				
Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Unclear			
		Unclear	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**

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Halliday 2006 (*Continued*)

Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear		
If a threshold was used, was it pre-specified?	No		
		Low	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Yes		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

Hebart 2000a

Study characteristics		**Study character**
Patient sampling	Prospective sample collection from patients who had undergone allogenic SCT between 1996 and 1997. Five ml EDTA was collected 2 to 4 times weekly from the time of admission until discharge or death. Samples from multiple centres were analysed in Tübingen	
Patient characteristics and setting	Sample size: 84 patients Males/females: not specified Mean age: 35 years (range 17 to 57) Presentation: patients had undergone allogeneic SCT	

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Hebart 2000a (*Continued*)

	Setting: University Hospital Würzburg
Index tests	DNA extracted from 5 ml blood as described by Einsele et al 1997 (JCM); PCR targeting 18S with *Aspergillus* specific probe (*Aspergillus fumigatus*, *flavus* and *versicolour*) for slot blot testing (not qPCR)
Target condition and reference standard(s)	IA was the target condition; cases of proven IA were defined as recovery of *Aspergillus* from normally sterile sites, positive culture or demonstration of hyphae from deep tissue biopsy and autopsy specimens along with clinical symptoms. Probable IA was defined as the presence of clinical signs and symptoms together with radiographic evidence compatible with IA and isolation of *Aspergillus* from respiratory specimens.
Flow and timing	1193 samples from 84 patients collected twice weekly and processed twice weekly. 2 x 2 analysis to calculate sensitivity, etc. Included all patients (possible was not defined). Parameters were calculated for both early and late onset IA
Comparative	
Notes	This study utilises definitions of IA that are pre-EORTC/MSG. Generally seems a compatible study

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Yes			
If a threshold was used, was it pre-specified?	No			
		Low	**Low**	

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Hebart 2000a (*Continued*)

DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes			
		Unclear	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear			
Did all patients receive the same reference standard?	Yes			
Were all patients included in the analysis?	Yes			
		Low		

Hummel 2009

Study characteristics		**Study character**
Patient sampling	PCR results from all consecutive patients from three university children's hospitals investigated between November 2000 and January 2007 were evaluated in this study	
Patient characteristics and setting	The majority of patients had malignant haematological diseases. Patients from three university children's hospitals	
Index tests	*Aspergillus* DNA was detected in clinical samples by an experimentally and clinically validated nested PCR assay as described previously (Bucheidt 2001; Bucheidt 2004; Skladny 1999).	
Target condition and reference standard(s)	IA; EORTC/MSG criteria	
Flow and timing	between November 2000 and January 2007	
Comparative		

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Notes	Results of serological diagnostic techniques (GM assay, Platelia *Aspergillus* enzyme immunoassay; Bio-Rad) and post-mortem histological examination were included for clinical classifications			
Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	No			
If a threshold was used, was it pre-specified?	Unclear			
		Low	**Low**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes			
		Low	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**

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Hummel 2009 (*Continued*)

Was there an appropriate interval between index test and reference standard?	Yes		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

Landlinger 2010

Study characteristics				**Study character**
Patient sampling	Clinical specimens from consecutive patients were prospectively collected			
Patient characteristics and setting	125 paediatric haemato-oncological patients undergoing intensive chemotherapy (65) or allogeneic stem cell transplantation (60) were analysed during 150 episodes of febrile neutropenia			
Index tests	Pan-fungal RT-PCR			
Target condition and reference standard(s)	IA; EORTC/MSG criteria			
Flow and timing	Whenever possible, specimens were collected at first onset of fever, within 48 hours thereafter, and at subsequent time points in the course of the febrile episode, upon availability			
Comparative				
Notes				
Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			

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Landlinger 2010 (*Continued*)

		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Yes			
If a threshold was used, was it pre-specified?	Unclear			
		Low	**Low**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes			
		Low	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Yes			
Did all patients receive the same reference standard?	Yes			
Were all patients included in the analysis?	Yes			
		Low		

Ramírez 2009

Study characteristics		**Study character**
Patient sampling	Prospective sampling of "at risk" patients for IFD between June 2004 and July 2006. Samples also taken from patients for whom confirmation of IFD before, during and after treatment was required	

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Ramírez 2009 (*Continued*)

Patient characteristics and setting	Sample size: 127 patients Males/females: 64/63 Mean age: 45 years (range 30 to 58) Presentation: patients at risk for IA and those requiring confirmation of IFD Setting: Hospital Universitario de Valme, Seville, Spain
Index tests	DNA extracted from 5 ml blood (EDTA); used RCLB, glass bead disruption and QiaAmp DNA Mini Kit. Light cycler assay as described by (Loeffler 2000). Twenty µl PCR included 10 µl template DNA; 50 cycles; followed by melt-curve analysis. DNA extraction control included, no internal control
Target condition and reference standard(s)	IA was the target condition; cases were defined according to the EORTC/MSG criteria (2002)
Flow and timing	948 clinical samples from 127 patients collected between June 2004 and July 2006. Samples processed immediately or stored prior to processing. 2 x 2 analysis was not conducted. Study focused on analytical sensitivity (60 fg *Aspergillus* DNA, or 5 to 20 conidia); 1% of the samples were PCR positive
Comparative	
Notes	This study had 5 proven/probable cases, 17 possible

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Unclear			
		Unclear	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	No			

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Ramírez 2009 (*Continued*)

		Low	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

Rogers 2013

Study characteristics		**Study character**
Patient sampling	Consecutive patients at risk of IA. Age not specified	
Patient characteristics and setting	Patients undergoing remission-induction chemotherapy for acute leukaemia, lymphoma, or myeloma, autologous or allogeneic bone marrow or stem cell transplant were eligible for inclusion. Over the course of the study 146 patients were recruited from Trinity College Dublin & St. James's Hospital, Dublin, and 132 from the Department of Internal Medicine, University of Würzburg Medical Centre, Würzburg, Germany	
Index tests	ITS qPCR assay targeting the ITS 1/5.8S ribosomal operon was performed as previously described (Springer 2011)	
Target condition and reference standard(s)	The EORTC/MSG definitions were used for categorization of patients with IFD including IA	

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Flow and timing	Patient blood samples were collected twice weekly; in UKW the EDTA blood samples were logged and processed prospectively while, in SJH, they were frozen at 80°C and processed in retrospective batches. DNA extracts were stored at 20°C until they were processed by the second PCR assay		
Comparative			
Notes	GM was part of the EORTC/MSG criteria for IFD		
Methodological quality			**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**
DOMAIN 1: Patient Selection			**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes		
Was a case-control design avoided?	Yes		
Did the study avoid inappropriate exclusions?	Yes		
		Low	**Low**
DOMAIN 2: Index Test All tests			**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Yes		
If a threshold was used, was it pre-specified?	Yes		
		Low	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes		
		Low	**Low**

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Rogers 2013 (*Continued*)

DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear			
Did all patients receive the same reference standard?	Yes			
Were all patients included in the analysis?	Yes			
		Low		

Springer 2011

Study characteristics				**Study character**
Patient sampling	Consecutive patients at high risk of IA. Five hundred thirty-six specimens from 46 patients at high risk for invasive fungal infection were collected			
Patient characteristics and setting	Patients at risk of IA after allogeneic SCT and patients receiving myeloablative chemotherapy with an expected duration of neutropenia (leukocyte count of 1,000/L) of at least 10 days. Nineteen males (mean age 51 years), 17 females (mean age 58 years)			
Index tests	Quantitative PCR and ITS semi quantitative RT-PCR assay			
Target condition and reference standard(s)	EORTC/MSG criteria			
Flow and timing	Between January and August 2009, blood samples from patients with a high risk of IFD, together with clinical data, were collected			
Comparative				
Notes	GM performed as a part of EORTC/MSG criteria for IA			
Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			

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Springer 2011 (*Continued*)

Was a case-control design avoided?	Yes		
Did the study avoid inappropriate exclusions?	Yes		
		Low	**Low**
DOMAIN 2: Index Test All tests			**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear		
If a threshold was used, was it pre-specified?	Unclear		
		Unclear	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

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Suarez 2008

Study characteristics				**Study character**
Patient sampling	All adult patients receiving allogeneic or autologous hematopoietic SCT, or intensive (induction, consolidation, or salvage) chemotherapy for hematological malignancies were included in the study			
Patient characteristics and setting	124 patients (138 treatment episodes) at risk of IA in the adult hematology and bone marrow transplant unit at Necker-Enfants Malades hospital, a tertiary-care university hospital (Paris, France)			
Index tests	RT-PCR on 1342 specimens			
Target condition and reference standard(s)	EORTC/MSG-documented IA. The diagnosis of IA (proven, probable, or possible) was defined for a given patient as the day on which the first clinical, radiological and/or microbiological EORTC/MSG criteria, other than a GM-positive result, appeared			
Flow and timing	This study was conducted prospectively from February 2006 to March 2007. The dates of diagnosis and the dates on which the first positive test results for *Aspergillus fumigatus* DNA and GM were recorded.			
Comparative				
Notes	for GM, incorporation bias avoided			

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	Unclear			

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Suarez 2008 (*Continued*)

		Unclear	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

Sugawara 2013

Study characteristics		**Study character**
Patient sampling	Prospective analysis of consecutive blood samples from patients at risk for IFD	
Patient characteristics and setting	51 patients with haematologic disorders at high risk for IFD who were treated at Mie University Hospital, Japan. Median age in years (range) 57.5 (17 to 78). Sex (male/female) 37/14	
Index tests	pan-fungal PCR assay on 273 specimens	
Target condition and reference standard(s)	revised criteria of the EORTC/MSG	
Flow and timing	The study was conducted between April 2007 and October 2010. Two hundred seventy-three consecutive blood samples from 64 risk episodes in 51 patients with haematologic disorders were analysed	
Comparative		

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Sugawara 2013 (*Continued*)

Notes	IFD was documented in 14 episodes (21.9%, 9 probable IFDs and 5 possible IFDs). PCR was positive in all of these 14 episodes, and in 4 of the 50 episodes with no IFD category. In this study, a considerable number of fungi (44.4%) other than major ones such as *Aspergillus* and *Candida* species were positive by PCR. Non-major fungi identified were *Cunninghamella* species, *Fusarium* species, *Scedosporium apiospermum*, *Rhodotorula* species, *Rhizopus* species, *Paecilomyces lilacinus*, and *Penicillium sclerotiorum*. In 10 of the 18 PCR-positive episodes, continued PCR screenings disclosed the clearance of the fungal DNA during antifungal therapy. The study also evaluated the diagnostic performance of GM, but GM was also part of the reference standard

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Yes			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Yes			
		Low	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	Unclear			
		Low	**Low**	
DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear			

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Sugawara 2013 (*Continued*)

		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Yes		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

von Lilienfeld-Toal 2009

Study characteristics		**Study character**
Patient sampling	70 patients with febrile neutropenia (median leukocyte count 420/mm^3) after chemotherapy	
Patient characteristics and setting	patients treated between September 2001 and February 2002 and between April 2003 and January 2004 on the hematology ward of the University Hospital Bonn, Germany. Median age in years (IQR) was 60 (49 to 66). Nunber of males (%) was 38 (54)	
Index tests	Commercial PCR-based kit to detect the DNA of 20 different pathogens (SeptiFast), including IFD. PCR testing was performed retrospectively	
Target condition and reference standard(s)	IFD according to the standards of the EORTC/MSG	
Flow and timing	784 serum samples of 119 febrile neutropenic episodes in 70 patients with hematological malignancies were analysed	
Comparative		
Notes	The only patient with proven IFD (*Candida glabrata* in one blood culture which also grew *Klebsiella pneumoniae* and *Enterococcus faecium*) yielded a negative result for fungus in the PCR, although the PCR did detect *Enterococcus faecium*. All of the patients with probable IFDs had positive results for *Aspergillus* in the PCR.	

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**

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Was a consecutive or random sample of patients enrolled?	Unclear		
Was a case-control design avoided?	Yes		
Did the study avoid inappropriate exclusions?	Unclear		
		Unclear	**Low**
DOMAIN 2: Index Test All tests			**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear		
If a threshold was used, was it pre-specified?	Unclear		
		Unclear	**Low**
DOMAIN 3: Reference Standard			**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes		
Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes		
		Low	**Low**
DOMAIN 4: Flow and Timing			**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	Unclear		
Did all patients receive the same reference standard?	Yes		
Were all patients included in the analysis?	Yes		
		Low	

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White 2006

Study characteristics				**Study character**
Patient sampling	a group of patients at risk of IA			
Patient characteristics and setting	A group of 203 patients at risk of IFD were tested by RT-PCR over a 13-month period (November 2003 to December 2004). The majority (176) were hematology patients, with 133 receiving remission-induction therapy for acute leukaemia (68 patients) or undergoing SCT (65 patients). The mean age of patients was 48 years			
Index tests	RT-PCR			
Target condition and reference standard(s)	IA. The EORTC-MSG criteria			
Flow and timing	Patients at risk of IFD were tested by RT-PCR over a 13-month period (November 2003 to December 2004)			
Comparative				
Notes				

Methodological quality				**Methodological**
Item	**Authors' judgement**	**Risk of bias**	**Applicability concerns**	
DOMAIN 1: Patient Selection				**DOMAIN 1: Pa**
Was a consecutive or random sample of patients enrolled?	Unclear			
Was a case-control design avoided?	Yes			
Did the study avoid inappropriate exclusions?	Unclear			
		Unclear	**Low**	
DOMAIN 2: Index Test All tests				**DOMAIN 2: In**
Were the index test results interpreted without knowledge of the results of the reference standard?	Unclear			
If a threshold was used, was it pre-specified?	Yes			
		Low	**Low**	

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White 2006 (*Continued*)

DOMAIN 3: Reference Standard				**DOMAIN 3: Re**
Is the reference standards likely to correctly classify the target condition?	Yes			
Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear			
		Unclear	**Low**	
DOMAIN 4: Flow and Timing				**DOMAIN 4: Fl**
Was there an appropriate interval between index test and reference standard?	No			
Did all patients receive the same reference standard?	Yes			
Were all patients included in the analysis?	Yes			
		Low		

BAL: broncho-alveolar lavage
EDTA: ethylenediaminetetraacetic acid
EORTC/MSG: European Organisation for Research and Treatment of Cancer/Mycoses Study Group
GM: galactomannan
HRCT: high-resolution computed tomography
IA: invasive aspergillosis
IFD: invasive fungal disease
ITS: Internal transcribed spacer
PCR: polymerase chain reaction
ELISA: enzyme-linked immunosorbent assay
RCLB: red cell lysis buffer
RT-PCR: real time polymerase chain reaction
SCT: stem cell transplant

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Characteristics of excluded studies *[ordered by study ID]*

Study	Reason for exclusion
Adhurti 2011	no 2x2 data provided
Armenian 2009	no 2x2 data provided
Auberger 2011	Retrospective study
Badiee 2008	no 2x2 data provided
Badiee 2009	no 2x2 data provided
Bernal-Martinez 2011	only sensitivity data provided
Blennow 2010	no 2x2 data provided
Bolehovska 2006	Include several materials and at risk patients (not only haematologic)
Bretagne 1998	retrospective selection of patients at risk of IA from a cohort of haematologic patients
Bu Rong 2005	Case control, not consecutive pts
Bucheidt 2001	case control (control group healthy control)
Bucheidt 2004	no 2x2 data provided
Cesaro 2008	no 2x2 data provided
Challier 2004	retrospective selection
Chryssanthou 1999	Candida PCR
Halliday 2005	Methodological, assay procedure
Hasseine 2010	no 2x2 data provided (published only as abstract)
Hebart 2000	no 2x2 data provided
Hummel 2010	no 2x2 data provided; preliminary selection of patients
Johnson 2012	gold standard different from EORTC; 3 cases only
Jones 1998	BAL only
Jordanides 2005	doesn't distinguish Aspergillus from Candida

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(*Continued*)

Kalkank 2010	no 2x2 data provided (published only as abstract)
Kami 2001	This study has combined patient samples from both a non-random sampling strategy and from prospective sampling. The authors suggest a case-control approach. The study does not follow EORTC/MSG criteria for defining IA
Kawazu 2004	no 2x2 data provided
Klingspor 2006	only sensitivity data provided
Lass-Florl 2001	only sensitivity data provided
Li 2013	case-control
Liu 2005	Chinese
Mandhanija 2010	terms not according EORTC criteria (e.g., suspected cases)
Millon 2011	case control (retrospective selection of patients GM-posiitive from a cohort of haematologic patients)
Morrissey 2013	efficacy end-points, not diagnostic performance
Nakamura 2010	PCR for bacteria and fungi, one positive case
Scotter 2005	retrospective, case control
Skladny 1999	retrospective, case control
Springer 2013	retrospective, case control
Sun 2010	Chinese
Teifoori 2011	No reference standard; no 2x2 tables; not clear if pts were consecutive and when PCR was performed
Yoo 2005	NASBA

Characteristics of studies awaiting classification *[ordered by study ID]*

Aguado 2015

Study characteristics		Study character
Patient sampling		
Patient characteristics and setting		

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Aguado 2015 (*Continued*)

Index tests	
Target condition and reference standard(s)	
Flow and timing	
Comparative	
Notes	To be assessed

Amirrajab 2015

Study characteristics	**Study character**
Patient sampling	
Patient characteristics and setting	
Index tests	
Target condition and reference standard(s)	
Flow and timing	
Comparative	
Notes	To be assessed

Chanza 2014

Study characteristics	**Study character**
Patient sampling	
Patient characteristics and setting	
Index tests	
Target condition and reference standard(s)	
Flow and timing	

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Chanza 2014 (*Continued*)

Comparative	
Notes	To be assessed

da Silva 2014

Study characteristics		**Study character**
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

Danylo 2014

Study characteristics		**Study character**
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

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Golas 2013

Study characteristics		Study character
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

Heng 2014

Study characteristics		Study character
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

Reinwald 2013

Study characteristics		Study character
Patient sampling		
Patient characteristics and setting		

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Reinwald 2013 (*Continued*)

Index tests	
Target condition and reference standard(s)	
Flow and timing	
Comparative	
Notes	To be assessed

Reinwald 2014

Study characteristics	**Study character**
Patient sampling	
Patient characteristics and setting	
Index tests	
Target condition and reference standard(s)	
Flow and timing	
Comparative	
Notes	To be assessed

Shah 2015

Study characteristics	**Study character**
Patient sampling	
Patient characteristics and setting	
Index tests	
Target condition and reference standard(s)	
Flow and timing	

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Shah 2015 (*Continued*)

Comparative	
Notes	To be assessed

Theel 2013

Study characteristics		**Study character**
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

White 2013

Study characteristics		**Study character**
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

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Zarrinfar 2013

Study characteristics		Study character
Patient sampling		
Patient characteristics and setting		
Index tests		
Target condition and reference standard(s)		
Flow and timing		
Comparative		
Notes	To be assessed	

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DATA

Presented below are all the data for all of the tests entered into the review.

Tests. Data tables by test

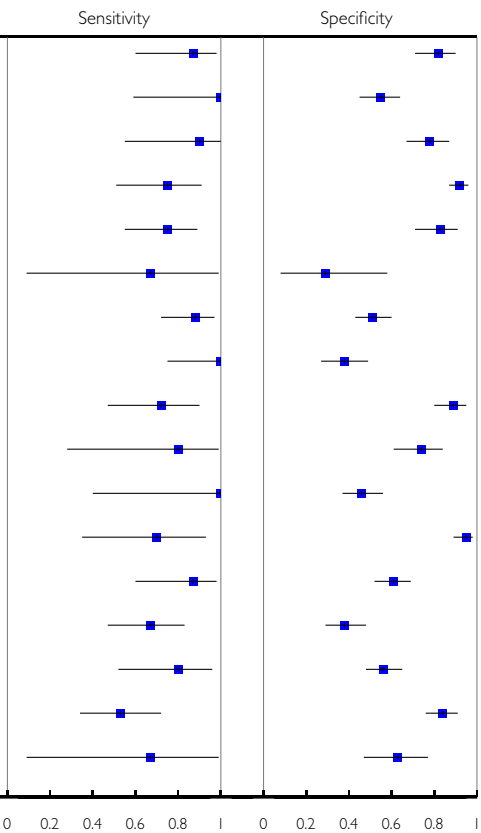
Test	No. of studies	No. of participants
1 PCR: single positive requirement	17	2297
2 PCR: two positive requirement	8	1479

Test 1. PCR: single positive requirement.

Review: Polymerase chain reaction blood tests for the diagnosis of invasive aspergillosis in immunocompromised people

Test: 1 PCR: single positive requirement

Study	TP	FP	FN	TN	Sensitivity	Specificity
Badie 2010	13	12	2	55	0.87 [0.60, 0.98]	0.82 [0.71, 0.90]
Barnes 2009	7	53	0	64	1.00 [0.59, 1.00]	0.55 [0.45, 0.64]
Cuenca-Estrella 2009	9	16	1	57	0.90 [0.55, 1.00]	0.78 [0.67, 0.87]
da Silva 2010	15	14	5	159	0.75 [0.51, 0.91]	0.92 [0.87, 0.96]
El Mahallawy 2006	21	11	7	52	0.75 [0.55, 0.89]	0.83 [0.71, 0.91]
Ferns 2002	2	10	1	4	0.67 [0.09, 0.99]	0.29 [0.08, 0.58]
Florent 2006	29	65	4	69	0.88 [0.72, 0.97]	0.51 [0.43, 0.60]
Halliday 2006	13	51	0	31	1.00 [0.75, 1.00]	0.38 [0.27, 0.49]
Hebart 2000a	13	8	5	66	0.72 [0.47, 0.90]	0.89 [0.80, 0.95]
Hummel 2009	4	17	1	48	0.80 [0.28, 0.99]	0.74 [0.61, 0.84]
Landlinger 2010	4	65	0	56	1.00 [0.40, 1.00]	0.46 [0.37, 0.56]
Ram rez 2009	7	6	3	109	0.70 [0.35, 0.93]	0.95 [0.89, 0.98]
Rogers 2013	13	51	2	80	0.87 [0.60, 0.98]	0.61 [0.52, 0.69]
Rogers 2013	20	63	10	39	0.67 [0.47, 0.83]	0.38 [0.29, 0.48]
Rogers 2013	12	57	3	74	0.80 [0.52, 0.96]	0.56 [0.48, 0.65]
Rogers 2013	16	16	14	86	0.53 [0.34, 0.72]	0.84 [0.76, 0.91]
Springer 2011	2	16	1	27	0.67 [0.09, 0.99]	0.63 [0.47, 0.77]



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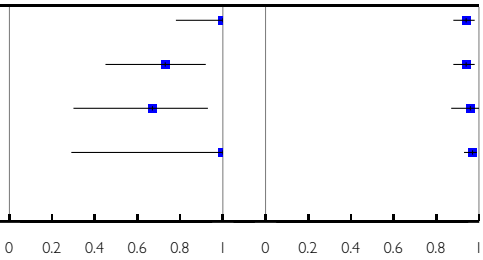
(. . . *Continued*)

Study	TP	FP	FN	TN	Sensitivity	Specificity
Suarez 2008	15	6	0	103	1.00 [0.78, 1.00]	0.94 [0.88, 0.98]
Suarez 2008	11	6	4	103	0.73 [0.45, 0.92]	0.94 [0.88, 0.98]
Sugawara 2013	6	2	3	53	0.67 [0.30, 0.93]	0.96 [0.87, 1.00]
von Lilienfeld-Toal 2009	3	3	0	113	1.00 [0.29, 1.00]	0.97 [0.93, 0.99]

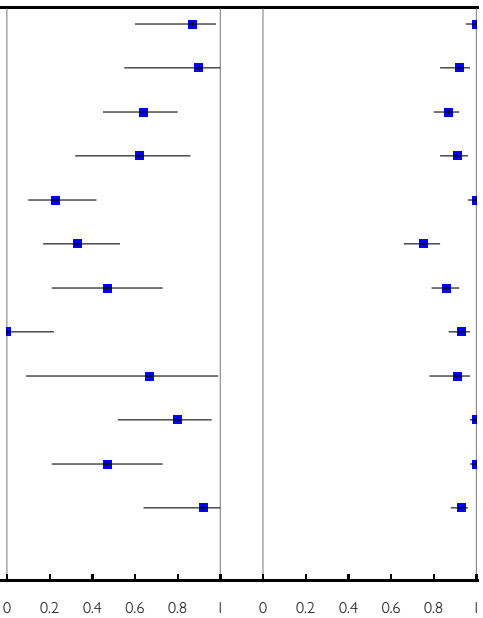


Test 2. PCR: two positive requirement.

Review: Polymerase chain reaction blood tests for the diagnosis of invasive aspergillosis in immunocompromised people

Test: 2 PCR: two positive requirement

Study	TP	FP	FN	TN	Sensitivity	Specificity
Badie 2010	13	0	2	67	0.87 [0.60, 0.98]	1.00 [0.95, 1.00]
Cuenca-Estrella 2009	9	6	1	67	0.90 [0.55, 1.00]	0.92 [0.83, 0.97]
Florent 2006	21	17	12	117	0.64 [0.45, 0.80]	0.87 [0.80, 0.92]
Halliday 2006	8	7	5	75	0.62 [0.32, 0.86]	0.91 [0.83, 0.96]
Rogers 2013	7	0	23	102	0.23 [0.10, 0.42]	1.00 [0.96, 1.00]
Rogers 2013	10	25	20	77	0.33 [0.17, 0.53]	0.75 [0.66, 0.83]
Rogers 2013	7	18	8	113	0.47 [0.21, 0.73]	0.86 [0.79, 0.92]
Rogers 2013	0	9	15	122	0.0 [0.0, 0.22]	0.93 [0.87, 0.97]
Springer 2011	2	4	1	39	0.67 [0.09, 0.99]	0.91 [0.78, 0.97]
Suarez 2008	12	0	3	109	0.80 [0.52, 0.96]	1.00 [0.97, 1.00]
Suarez 2008	7	0	8	109	0.47 [0.21, 0.73]	1.00 [0.97, 1.00]
White 2006	12	14	1	175	0.92 [0.64, 1.00]	0.93 [0.88, 0.96]



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ADDITIONAL TABLES

Table 1. European Organisation for Research and Treatment of Cancer/Mycoses Study Group definitions of invasive aspergillosis

	Original definitions of Ascioglou 2002	Revised definitions of De Pauw 2008
PROVEN IA	Specimen obtained by needle aspiration or biopsy from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process**AND** ***EITHER*** histopathological, cytopathological, or direct microscopic examination of the specimen in which hyphae are seen accompanied by evidence of associated tissue damage ***OR*** recovery of *Aspergillus* species by culture from the specimen obtained by a sterile procedure excluding bronchoalveolar lavage, cranial sinus cavity, and urine	
PROBABLE IA	At least 1 host factor criterion **PLUS** 1 major (or 2 minor) clinical criteria from abnormal site consistent with infection**PLUS** 1 microbiological criterion	At least 1 host factor **PLUS** 1 clinical feature **PLUS** 1 microbiological criterion
POSSIBLE IA	At least 1 host factor criterion **PLUS** ***EITHER*** 1 major (or 2 minor) clinical criterion from abnormal site consistent with infection ***OR*** 1 microbiological criterion	At least 1 host factor **PLUS** 1 clinical feature

Host factor criteria will include the temporal relationship between the onset of fungal disease and the receipt of an allogeneic stem cell transplant.

Clinical features include for example neutropenia, persistent fever, predisposing conditions, prolonged use of corticosteroids; in the case of lower respiratory tract infection, the presence of one of the following signs on CT: dense well circumscribed lesions(s) with or without a halo sign or an air crescent sign, cavity.

Microbiological criteria consist of a positive culture including the presence of fungal elements indicating a mould on microscopy or recovery by culture of *Aspergillus* species from sputum, bronchoalveolar lavage (BAL) fluid, bronchial brush or sinus aspirate samples; positive result for *Aspergillus* detection of galactomannan antigen in specimens of plasma, serum, BAL, cerebrospinal fluid or two or more blood samples. Major clinical criteria are, for example, new infiltrates on computerized tomography imaging (e.g. halo sign) or suggestive radiological findings.

Minor clinical criteria are suggestive symptoms and signs.

The exact definitions of the European Organisation for Research and Treatment of Cancer/Mycoses Study Group criteria and their host factor, microbiological or clinical criteria can be found in Ascioglou 2002 and De Pauw 2008.

Table 2. Technical details of the PCR methods used in the studies analysed in this review

Study	Sample type	Sample volume	DNA extraction m		PCR method[C]	Target gene	Appropriate controls			Requirements for positive by PCR	Methods used (refs)	
			Cell wall disruption[B]	DNA isolation kit/ protocol			Negative[D]	Positive[E]	PCR inhibition			

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Table 2. Technical details of the PCR methods used in the studies analysed in this review (*Continued*)

							Ex	PCR	Ex	PCR				
Hebart 2000a	Whole blood	5 ml	Zymolase and NaOH lysis buffer	Protein precipitation and DNA precipitation	PCR-slot blot	18S	-	Yes	-	Yes	Yes	Single Positive	Einsele 1997	
Ferns 2002	Whole blood	2 ml	Lyticase	QIAamp	Nested PCR	mtDNA	Yes	Yes	Yes	Yes	-	Positive on two occasion	Bretagne 1998 Tang 1993	
Florent 2006	Serum	200 µl	-	QIAamp	PCR-ELISA	mtDNA	-	Yes	-	Yes	Yes	Two Consecutive Positives	Bretagne 1998	
Halliday 2006	Whole blood	500 µl	Lyticase	GenElute	Nested PCR	18S	Yes	Yes	-	Yes	Yes	Two consecutive positives	Skladny 1999	
El Mahallawy 2006	Serum	-	Lyticase	QIAamp	Standard PCR	18S	-	Yes	-	Yes	-	Single positive	Williams 2000	
White 2006	Whole blood	2 ml	Glass beads	MagNA Pure	Nested qPCR	28S	Yes	Yes	Yes	Yes	Yes	Serial positives in single episode	Loeffler 2002; William 2000	
Suarez 2008	Serum	1 ml or 200 µl	-	MagNA Pure	qPCR	28S	-	Yes	-	Yes	-	Single positive	Challier 2004	

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Table 2. Technical details of the PCR methods used in the studies analysed in this review (*Continued*)

Hummel 2009	Blood	5 ml	Lysicase	Phenol-chloroform	Nested PCR	18S	-	Yes	-	Yes	-	Single positive	Skladny 1999
Ramírez 2009	Whole blood	5 ml	Lysicase and glass beads	QIAamp	qPCR	18S	-	Yes	-	Yes	-	Single positive	Loeffler 2000
Barnes 2009	Whole blood	2 ml	Glass beads	MagNA Pure	Nested qPCR	28S	Yes	Yes	Yes	Yes	Yes	Confirmed positive[F]	White 2006
Cuenca-Estrella 2009	Whole blood and serum	-	-	QIAamp	qPCR	ITS1	-	Yes	-	Yes	Yes	Two consecutive positives	Yoo 2008
von Lilienfeld-Toal 2009	Whole blood	10 ml	Ceramic beads	Septifast	qPCR	18S	-	Yes	-	Yes	Yes	-	Lehmann 2008
Landlinger 2010	Whole blood	3 ml	Lysicase	MagNA Pure	qPCR	28S	-	Yes	-	Yes	Yes	Single positive	Baskova 2007; Watzinger 2004
Badie 2010	Whole blood	3 to 5 ml	Lysicase	QIAamp	qPCR	18S	Yes	Yes	-	Yes	-	Single positive	van Burik 1998; Kami 2001;
da Silva 2010	Serum	5 ml Blood	Lysicase	Protein pre-	Standard PCR	18S	-	Yes	-	Yes	-	Two consecu-	Ribeiro 2006;

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Table 2. Technical details of the PCR methods used in the studies analysed in this review (*Continued*)

				cipitation and DNA precipitation							tive positives	van Burik 1998	
Springer 2011 [G]	Whole blood	3 ml	Glass beads	High Pure PCR Template Preparation Kit (Roche)	qPCR	ITS	-	Yes	-	Yes	-	Single positive[H]	-	
				Fast-Prep-24 MP (Biomedicals)										
	Whole blood	5 ml	Glass beads		Standard PCR	-	-	Yes	Yes	Yes	Yes	-	Sachse 2009	
Rogers 2013 [G]	Whole blood	3 ml	Glass beads	High Pure PCR Template Preparation Kit (Roche)	Nested qPCR	28S	Yes	Yes	Yes	Yes	Yes	Single positive[I]	White 2006	
													Springer 2011	
					qPCR	ITS1		Yes		Yes		Single positive[I]		
Sugawara 2013	Whole blood	1 ml	Beads and lysis buffer	Phenol-chloroform	Nested PCR and se-	18S	-	Yes	-	Yes	-	Single positive	Nakamura 2010	

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Table 2. Technical details of the PCR methods used in the studies analysed in this review (*Continued*)

					quenc-ing								

-: not reported; MagNA Pure: an automated DNA isolation system manufactured by Roche; mtDNA: mitochondrial DNA; PCR: polymerase chain reaction; QIAamp: QIAamp DNA isolation kit manufactured by Qiagen; Ex: extraction; ITS: Internal Trascribed Spacer; RCLB: red cell lysis buffer.

A DNA isolation protocols may include steps to remove red and white blood cells, fungal cell wall disruption and DNA purification kits.

B Lyticase/Zymolase enzymatically digest fungal cells walls; ceramic or glass beads cause mechanical disruption of the cell wall.

C PCR methods used vary between standard PCR where products are resolved on agarose gels to detect positive or negative reactions or quantitative PCR (qPCR) which allows real time monitoring of the reaction. Nested qPCR involves first round standard PCR and second round qPCR.

D Negative DNA extraction controls feature a sample blank, e.g. blood or sterile solution, that allows detection of any contamination in the DNA isolation protocol.

E Positive DNA extraction controls are a sample blank that is spiked with fungal or specific bacterial spores to ensure that the DNA isolation protocol is working optimally.

F The confirmed positive requires that any single positive sample is confirmed with an additional sample from the same patient. Barnes 2009 also used multiple analyses to determine the effectiveness of single versus multiple positives to yield diagnostic accuracy.

G Studies assessed the effectiveness of more than one assay.

H The study analysed the effect of both single and multiple positives.

I The effects of both single and multiple positives were analysed as well as analyses of combined PCR and galactomannan tests.

APPENDICES

Appendix 1. Search strategies

MEDLINE

1 exp Aspergillosis/
2 exp Pulmonary Aspergillosis/
3 exp Aspergillus/
4 (aspergillosis or aspergillus or aspergilloma or “A.fumigatus” or “A. flavus” or “A. clavatus” or “A. terreus” or “A. niger”).ti,ab.
5 or/1-4
6 exp Nucleic Acid Amplification Techniques/
7 pcr.ti,ab.
8 “polymerase chain reaction*”.ti,ab.
9 or/6-8
10 5 and 9
11 exp Animals/ not Humans/
12 10 not 11
key: ti,ab. = title,abstract

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EMBASE

1 Aspergillosis/
2 Lung Aspergillosis/
3 exp Aspergillus/
4 (aspergillosis or aspergillus or aspergilloma or "A.fumigatus" or "A. flavus" or "A. clavatus" or "A. terreus" or "A. niger").ti,ab.
5 or/1-4
6 nucleic acid amplification/
7 Polymerase Chain Reaction/
8 pcr.ti,ab.
9 "polymerase chain reaction*".ti,ab.
10 or/6-9
11 5 and 10
12 (exp Animal/ or Nonhuman/ or exp Animal Experiment/) not Human/
13 11 not 12
key: ti,ab =title,abstract

WEB of Science, LILACS, Database of Abstracts of Reviews of Effects, Health Technology Assessment, Scopus

(Aspergillus or Aspergillosis) AND (Polymerase Chain Reaction or Nucleic Acid Amplification) in title, abstracts and keywords

Appendix 2. QUADAS2 Items

DOMAIN	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING
Description	Describe methods of patient selection: Describe included patients (prior testing, presentation, intended use of index test and setting):	Describe the index test and how it was conducted and interpreted:	Describe the reference standard and how it was conducted and interpreted:	Describe any patients who did not receive the index test(s) and/or reference standard or who were excluded from the 2 x 2 table (refer to flow diagram): Describe the time interval and any interventions between index test(s) and reference standard:
Signalling questions (yes/no/unclear)	Was a consecutive or random sample of patients enrolled?	Were the index test results interpreted without knowledge of the results of the reference standard?	Is the reference standard likely to correctly classify the target condition?	Was there an appropriate interval between index test(s) and reference standard?
Was a case-control design avoided?	If a threshold was used, was it pre-specified?	Were the reference standard results interpreted without knowledge of the results of the index test?	Did all patients receive a reference standard?	

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(*Continued*)

Did the study avoid inappropriate exclusions?	Did all patients receive the same reference standard?			
Were all patients included in the analysis?				
Risk of bias: high/low/unclear	Could the selection of patients have introduced bias?	Could the conduct or interpretation of the index test have introduced bias?	Could the reference standard, its conduct, or its interpretation have introduced bias?	Could the patient flow have introduced bias?
Concerns regarding applicability: high/low/unclear	Are there concerns that the included patients do not match the review question?	Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Are there concerns that the target condition as defined by the reference standard does not match the review question?	

Item Patient selection. Code this item: Yes. If the characteristics of the spectrum of patients fulfilled the pre-stated requirements and the method of recruitment was consecutive, or random samples were taken from consecutive series. No. If the sample does not fit with what was pre-specified as acceptable or if groups with and without the target disorder were recruited separately, particularly with healthy controls. Unclear. If there is insufficient information available to make a judgment. **Independent index and reference test (incorporation).** Yes.If the index test did not form part of the reference standard. No. If the reference standard formally included the result of the index test. Unclear If it is unclear whether the results of the index test were used in the final diagnosis. .**Index test blind for reference test results and vice versa.** Yes. If test results (index or reference standard) were interpreted blind to the results of the other test, or blinding is dictated by the test order, or meets the pre-stated assumptions. No.If it is clear that one set of test results was interpreted with knowledge of the other. Unclear. If it is unclear whether blinding took place. **Item Reference Standard** Yes. All reference standards used meet the pre-stated criteria. No. One or more reference standards used do not meet the pre-stated criteria. Unclear. It is unclear exactly what reference standard was used. **Were partial verification and differential verification prevented?** Yes. If all patients, or a random selection of patients, who received the index test went on to receive verification of their disease status using a reference standard, even if the reference standard was not the same for all patients. No. If some of the patients who received the index test did not receive verification of their true disease state, and the selection of patients to receive the reference standard was not random. Unclear. If this information is not reported by the study. **Item Flow and timing.** Yes.If the time between tests was shorter than that required, at least for an acceptably high proportion of patients. No. If the time between tests was longer than that required for an unacceptably high proportion of patients. Unclear. If information on timing of tests is not provided.

WHAT'S NEW

Date	Event	Description
14 September 2015	Amended	Errors in text corrected
14 September 2015	New citation required but conclusions have not changed	Errors in text corrected

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CONTRIBUTIONS OF AUTHORS

MC, CM, RAB and PD conceived the original idea for the review and wrote the protocol. MC, CM, RAB, PD, OM, LK, JL, BLJ reviewed articles for inclusion and extracted data. MC and CM analysed the data and drafted the methodological and statistical analysis part of the draft. All authors reviewed, commented on and approved the final version.

DECLARATIONS OF INTEREST

RAB has been a consultant to Astellas Pharma, Gilead Sciences, Merck, Sharpe & Dohme, Pfizer and Schering-Plough, has received a research grant from Pfizer, has been a member of a speaker's bureau for Astellas Pharma, Gilead Sciences, Merck, Sharpe & Dohme, Pfizer and Schering-Plough, and has received travel grants from Astellas Pharma, Gilead Sciences, Merck, Sharpe & Dohme, Pfizer and Schering-Plough.

JL has received research grants from Novartis and travel grants from Pfizer and Cephalon.

PD has been a consultant to Gilead Sciences, Ipsat Therapies and Pfizer, has received research grants from AM-Pharma, Basilea Pharmaceutica and Schering-Plough, has been a member of a speaker's bureau for Gilead Sciences, Janssen Pharmaceuticals, Pfizer, Schering-Plough, and Xian-Janssen and has received travel grants from Merck, Sharpe & Dohme and UCB Pharma.

LK has been an adviser to Astellas, Gilead, Schering-Plough, has received research grants from Gilead, Merck, Sharpe & Dohme, Schering-Plough and has received honoraria for educational lectures from Gilead, Pfizer, Merck, Sharpe & Dohme, Schering-Plough and Janssen.

BLJ has been an advisor to Gilead, MSD, Astellas and Pfizer; has received research grants from Gilead, Astellas, Pfizer, Janssen and MSD; has received honoraria for educational lectures from Gilead, MSD and Pfizer; and owns stock in Gilead, MSD and Pfizer.

JM has served as consultant to Schering-Plough, Gilead Sciences, Merck, Sharp & Dohme, Pfizer, Bio-Rad, Fujisawa healthcare, Astellas, Nextar and Zeneus (Cephalon), has received research funding from Bio-Rad, Merck, Sharp & Dohme, and Pfizer, and has been on the speaker's bureau for Schering-Plough, Gilead Sciences, Merck, Sharp & Dohme, Pfizer, Bio-Rad, Fujisawa healthcare, Astellas and Zeneus (Cephalon).

MC, CM, OM: no conflicts of interest to declare with regard to the content of the article.

RAB, JL and PD are founder members of the European *Aspergillus* PCR Initiative Working Group of the International Society for Human and Animal Mycology, and board members of the EAPCRI, which is registered with the Dutch Chamber of Commerce, number 09165918.

None of the authors has any interests, financial or otherwise, in any of the companies involved in the diagnosis of IFD. The authors' disclosures are on public record to ensure their independence and integrity and to help avoid potential conflicts of interest.

DIFFERENCES BETWEEN PROTOCOL AND REVIEW

We intended to use QUADAS, as described in the protocol, but switched to QUADAS-2 for the review.

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INDEX TERMS

Medical Subject Headings (MeSH)

*Immunocompromised Host; Aspergillosis [blood; *diagnosis]; Cohort Studies; Data Accuracy; False Negative Reactions; False Positive Reactions; Opportunistic Infections [blood; *diagnosis]; Polymerase Chain Reaction [*methods]; Predictive Value of Tests; Sensitivity and Specificity

MeSH check words

Humans

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