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Review Article

Review Article

Antibody testing in aspergillosis—quo vadis?

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Abstract

Humans are constantly exposed to airborne *Aspergillus* spores. Most develop *Aspergillus*-specific antibodies by adulthood. Persons with chronic lung disease or *Aspergillus* airway colonization often have raised levels of *Aspergillus*-specific immunoglobulin G (IgG). It is not known whether this signifies an increased risk of future aspergillosis.

Chronic and allergic forms of pulmonary aspergillosis are estimated to affect over three million people worldwide. Antibody testing is central to diagnosis of these conditions, with raised *Aspergillus*-specific IgG in chronic pulmonary aspergillosis and raised *Aspergillus*-specific IgE in allergic aspergillosis. Antibody levels are also used to monitor treatment response in these syndromes. Acute invasive disease is less common. There is a more limited role for antibody testing in this setting as immunosuppression often results in delayed or absent antibody response.

Many methods exist to detect *Aspergillus*-specific antibodies, but there are limited published data regarding comparative efficacy and reproducibility. We discuss the comparative merits of the available tests in the various clinical settings and their suitability for use in the resource-poor settings where the majority of cases of aspergillosis are thought to occur. We summarize the gaps in existing knowledge and opportunities for further study that could allow optimal use of antibody testing in this field.

Key words: *aspergillus*, aspergillosis, CPA, ABPA, serology.

Introduction

Aspergillus is a mould that causes disease in humans [1]. Infection can lead to a spectrum of clinical syndromes, ranging from rapidly fatal acute invasive infection to chronic debilitating pulmonary disease [2]. The latter can normally be characterized as either allergic airways disorders closely associated with asthma [3,4] or chronic lung infection that can be complicated by progressive fibrosis and massive haemoptysis [5–8]. Understanding of these conditions has improved significantly over the course of several decades, with associated changes in the case definitions and terminology used to describe disease [4,5].

It is likely that chronic and allergic forms of pulmonary aspergillosis are sufficiently common to be considered a public health issue on a global scale [9–13]. The most common form of aspergillosis is undoubtedly chronic

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pulmonary aspergillosis secondary to treated tuberculosis [7,8,14–16]. It is therefore likely that most patients with pulmonary aspergillosis will be living in the resource-poor settings where tuberculosis is most common.

Treatment with antifungal medication is associated with clinical and / or radiological stabilization or improvement in all common forms of aspergillosis [17–20]. It can be successfully delivered in resource-poor settings [18]. Surgery can cure chronic pulmonary aspergillosis in selected patients with localized disease [15,21] and can also be performed in resource poor settings [16].

Diagnosis of aspergillosis is challenging. Unfortunately the clinical presentation of chronic and allergic aspergillosis overlaps considerably with other, better-recognized conditions, and it is likely that the vast majority of cases go undiagnosed [5,14,22]. The development of assays to detect antigenaemia has led to improved ability to diagnose invasive infections promptly and the interpretation and efficacy of these antigen detection assays have been reviewed extensively [23–28]. Chronic and allergic forms of aspergillosis are much more common than invasive disease [11–13,29], but have been relatively neglected. Antibody testing is central to the diagnosis of these conditions.

It is the goal of this article to describe the antibody response that occurs in *Aspergillus* infection and its role in the diagnosis and management of aspergillosis. The strengths and limitations of the various techniques available to measure *Aspergillus*-specific antibodies will be described, together with a review of the evidence of their comparative efficacies.

Clinical syndromes due to *Aspergillus* infection

It is likely that human exposure to *Aspergillus* spp. is near universal, as *Aspergillus* spp. are consistently recovered from air samples in urban and rural areas throughout the year [30,31]. Human disease due to *Aspergillus* spp. has also been recorded worldwide [10]. The vast majority of patients with aspergillosis have one or more underlying disorders and the presentation of aspergillosis varies in line with the underlying disorder [2,14,22]. While there can be a significant degree of overlap between syndromes it is nonetheless useful to summarise the commonly observed syndromes. The antibody response to *Aspergillus* and thus the role of antibody measurement in diagnosis and management varies greatly from one syndrome to another.

Superficial aspergillosis

Cutaneous aspergillosis is uncommon as the physical barrier provided by the epidermis prevents *Aspergillus* inoculation. *Aspergillus* spp do cause keratitis, otitis externa, and onychomycosis in immunocompetent persons, but antibody response is not normally seen in these conditions and diagnosis relies on microscopy and culture [32–36].

Aspergillus bronchitis

Aspergillus can grow in the human respiratory tract. This can occur in asymptomatic patients and in these circumstances is termed colonisation [37,38]. However in some patients with no significant immune deficit, *Aspergillus* growth in the respiratory tract occurs and is associated with cough and recurrent chest infections, but without radiological evidence of pulmonary aspergillosis. These patients are considered to have *Aspergillus* bronchitis [39]. This is well described in persons with cystic fibrosis [40] but is not restricted to this group [39]. Evidence of *Aspergillus* growth is provided by either recurrent culture growth from respiratory samples or raised levels of *Aspergillus*-specific antibodies.

Acute invasive aspergillosis

Acute invasive disease can occur in immunocompromised persons and is termed invasive pulmonary aspergillosis, invasive rhinosinusitis, invasive tracheo-bronchial aspergillosis or disseminated aspergillosis depending on the site of the invasive infection [41–43]. These conditions are mostly associated with severe neutropaenia, but can also be seen in association with a large range of conditions including corticosteroid use, intensive care unit (ICU) admission, diabetes, liver failure, tuberculosis, chronic obstructive pulmonary disease (COPD), chronic granulomatous disease (CGD), graft versus host disease (GVHD), solid organ transplantation and acquired immunodeficiency syndrome (AIDS) [42–47].

Pneumonia is the most common initial presentation, but lesions involving the kidneys, cardiac valves, brain and skin have been documented [42,44,46]. Clear diagnostic guidelines have been published by the European Organization for Research and Treatment of Cancer (EORTC) [48]. Measurement of *Aspergillus*-specific antibodies do not form part of these criteria, with diagnosis resting on biopsy evidence for proven disease or a combination of risk factors, radiological change and microbiological evidence in the form of culture growth or antigen detection for probable disease.

Sub-acute invasive pulmonary aspergillosis

In addition to this well-recognized acute presentation of invasive disease, there can also be a more indolent presentation with progressive destruction of the lung over several weeks or months. This has been frequently referred

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to as chronic necrotizing pulmonary aspergillosis or semi-invasive aspergillosis in the past [6,49], but the term sub-acute invasive pulmonary aspergillosis has been adopted more recently [50] and will be used throughout this article. The condition is normally seen in patients with mild immunosuppression due to diabetes, steroid use, alcoholism, COPD, tuberculosis or AIDS [6,49,51–53]. A similar condition occurs in the sinuses, where is termed chronic invasive fungal rhinosinusitis [41].

Diagnosis of sub-acute invasive aspergillosis is based on a combination of symptoms, radiological changes and laboratory tests, including antibody and antigen tests or culture [6,53].

There is a large degree of overlap between sub-acute invasive pulmonary aspergillosis and chronic pulmonary aspergillosis [7]. The duration of symptoms is the main difference, over one month of symptoms considered appropriate for sub-acute invasive aspergillosis [6,53]. In the absence of treatment, death from progressive lung destruction and massive haemoptysis is common. Those who survive sub-acute invasive pulmonary aspergillosis can go on to develop chronic pulmonary aspergillosis [6].

Chronic pulmonary aspergillosis

The term aspergilloma refers to a fungal ball in a lung cavity. The cavity may be pre-existing or be created by *Aspergillus* as an aspergilloma forms. This can be an incidental radiological finding in an asymptomatic person and is termed simple aspergilloma in these cases [15]. Fungal balls are also well described in the sinuses [41].

Formation of new cavities and fibrosis of surrounding lung tissues often occurs in response to chronic *Aspergillus* infection. This process has been referred to as complex aspergilloma [15,54–56] but is now preferably referred to as chronic pulmonary aspergillosis (CPA) [5,7,8]. CPA occurs in patients with underlying lung conditions, including treated tuberculosis, atypical mycobacterial infection, sarcoidosis, COPD, pneumothorax, prior lung surgery, rheumatoid arthritis or lung cancer [7,8,14]. CPA can also complicate sub-acute invasive pulmonary aspergillosis [6] or allergic bronchopulmonary aspergillosis [13]. Progressive lung destruction due to fibrosis and cavitation occurs, with massive life-threatening haemoptysis complicating advanced disease [5,7,8]. CPA is estimated to affect 3 million people worldwide [11–13,57]. The five-year mortality of CPA is up to 85% [7].

Diagnosis is based on a combination of chronic symptoms, radiological changes and laboratory tests [5,7,8]. Unfortunately the symptoms of cough and breathlessness can overlap greatly with the underlying lung diseases. Radiological changes of cavitation, fibrosis and pleural thickening can also overlap greatly with underlying conditions, with distinctive aspergilloma detected only in a minority of patients [5,8,58]. Laboratory testing is therefore crucial in differentiating patients with CPA from those with underlying lung disease alone. Serum antigenaemia has been documented in up to 50% of CPA cases [8,59,60] and culture of sputum is positive in up to 44% of CPA cases [61], but raised levels of *Aspergillus*-specific immunglobulin g (IgG) antibodies are almost always present and are central to diagnosis [5,7,8].

Allergic aspergillosis

Sensitization to *Aspergillus* can occur in asthmatics and such patients are more likely to have severe asthma with life-threatening complications [9,62,63]. This is referred to as severe asthma with fungal sensitization (SAFS) [64]. Allergy to *Aspergillus* can also result in rhinosinusitis [41]. Diagnosis of sensitization can be achieved by skin testing or by detection of raised levels of *Aspergillus*-specific immunoglobulin e (IgE) antibodies [65].

Allergic broncho-pulmonary aspergillosis (ABPA) complicates 1–4% of adult asthma cases, many of whom have otherwise healthy lungs and no immunocompromise [22]. ABPA can also complicate cystic fibrosis [66] and occasional cases are also seen in persons with neither condition [67]. ABPA is characterized by recurrent exacerbations resulting in cough and breathlessness with lung infiltrates on chest x-ray and can be complicated by the development of bronchiectasis or CPA [22]. In contrast to other forms of aspergillosis, steroids are the main treatment, with antifungals used as steroid sparing agents in some cases [4].

The International Society for Human and Animal Mycology (ISHAM) has recently revised the diagnostic criteria for ABPA [4]. Diagnosis requires the presence of cystic fibrosis or asthma plus a total serum IgE of > 1000 IU/ml and evidence of *Aspergillus* sensitivity from either skin prick testing or raised *Aspergillus*-specific IgE antibodies. Two of the three following minor criteria must also be present; radiographic changes consistent with ABPA, raised eosinophil count or raised levels of *Aspergillus*-specific precipitating or IgG antibodies.

The diagnostic criteria for different clinical syndromes in aspergillosis are summarised in Table 1. Fig. 1 is a visual representation of the number of patients with each clinical syndrome overlaid with the bars showing the total number of patients where each test is diagnostic.

Antibody response to *Aspergillus*

Asymptomatic persons

While human airways are constantly exposed to *Aspergillus* spores present in the air [30,31], these spores are rendered immunologically inert by the presence of surface hydrophobin [68]. In healthy persons the innate immune

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Table 1. Abbreviated diagnostic criteria for acute pulmonary IA, sub acute pulmonary IA, CCPA and *Aspergillus* bronchitis.

	Proven invasive [48]	Probable invasive [48]	Sub-acute invasive (aka CNPA) [6]	CCPA [5,7,8,21]	*Aspergillus* bronchitis [39]	ABPA [4]
Clinical criteria	NOT REQUIRED	neutropaenia OR stem cell transplant OR high dose corticosteroids for >3 weeks OR immunosuppressant drugs OR CGD OR SCID	>1 MONTH SYMPTOMS; weight loss OR productive cough OR haemoptysis AND *absence* of host factors for acute invasive disease	>3 MONTHS SYMPTOMS; weight loss OR productive cough OR haemoptysis AND *absence* of host factors for invasive disease	persistent productive cough OR recurrent chest infections AND does not meet diagnostic criteria for chronic or allergic aspergillosis	asthma OR cystic fibrosis
Radiological criteria on CXR or CT scan	NOT REQUIRED	dense lesions +/− halo sign OR air-crescent sign OR one or more cavities	new cavitation OR expanding cavity OR paracavitary infiltrates	new cavitation OR expanding cavity OR paracavitary infiltrates	*absence* of changes consistent with CPA or ABPA	transient opacifications or permanent evidence of bronchiectasis of pleuropulmonary fibrosis (see other criteria below)
Laboratory criteria	culture from a sample from a normally sterile site OR histology (hyphae plus tissue damage on biopsy can diagnose invasive fungal infection but may not be able to differentiate *Aspergillus* from other fungi)	culture from sputum or BAL OR GM in blood or BAL OR ß(1,3)-D-glucan in blood	culture from sputum or BAL OR GM in blood or BAL OR ß(1,3)-D-glucan in blood OR raised *Aspergillus*-specific IgG OR histology	raised *Aspergillus*-specific IgG OR culture from sputum or BAL OR GM in blood or BAL* OR ß(1,3)-D-glucan in blood*	raised *Aspergillus*-specific IgG AND EITHER recurrent culture growth from sputum or BAL OR persistently positive PCR from sputum or BAL	Obligatory criteria total IgE > 1000 IU/ml AND raised *Aspergillus*-specific IgE (or positive skin prick test) Other criteria (2 of 3 needed) raised eosinophil count OR raised *Aspergillus*-specific IgG / precipitins OR radiological changes as above

Note: CNPA = chronic necrotizing pulmonary aspergillosis, CCPA = chronic pulmonary aspergillosis, ABPA = allergic bronchopulmonary aspergillosis, CGD = chronic granulomatous disease. SCID = severe combined immunodeficiency, CXR = chest x-ray, CT = computed tomography, BAL = bronchoalveolar lavage, GM = galactomannan antigen test, IgG = immunoglobulin g, IgE = immunoglublulin e, PCR = polymerase chain reaction. Unless stated otherwise patients must meet all 3 criteria for diagnosis of each condition. *GM and ß(1,3)-D-glucan are less sensitive than *Aspergillus* serology in CPA and so not included in all published case definitions but are consistent with CPA when present together with appropriate clinical and radiological features.

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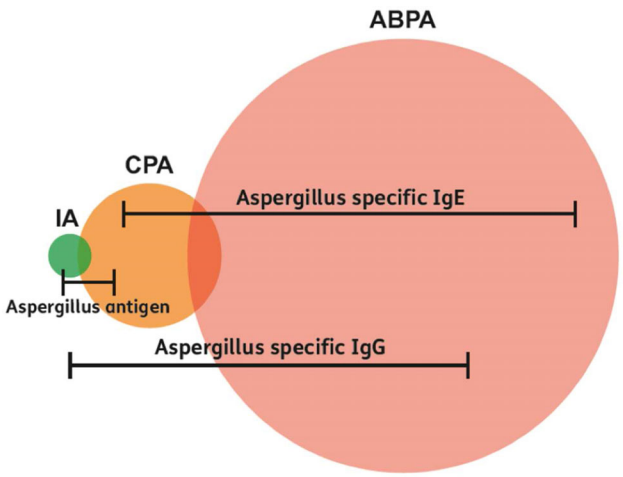

Figure 1. Visual representation of the number of patients with each condition and the number of patients where each test is diagnostic. *Note*: IA = invasive aspergillosis, CPA = chronic pulmonary aspergillosis, ABPA = allergic bronchopulmonary aspergillosis. The size of each circle is relative to the estimated European population affected by the disease, with prevalence used for the chronic conditions ABPA (887,000 cases) and CPA (240,000 cases) and incidence for the acute condition IA (63,000 cases) [29]. The length of the bars represents the total number of patients where each test is diagnostic by combining frequency of positive results annually for each condition. *Aspergillus*-specific IgE is raised in almost all cases of ABPA [4,22] and up to 66% of CPA cases [5]. *Aspergillus*-specific IgG is raised in 65% of ABPA (Smith and Denning, unpublished data), up to 100% of CPA [5,8] and up to 65% of cases of IA [95]. *Aspergillus* antigen tests are positive in around 62% of cases of IA in adults [24] and 23% of cases of CPA [8].

system ensures that most spores are promptly destroyed [69]. Those that germinate into hyphae are normally recognised and killed by neutrophils before they can invade host tissue [70].

Nonetheless, antibodies to *Aspergillus* are formed in healthy persons, with mean levels increasing from childhood into adulthood [71]. In accordance with this, tests for *Aspergillus*-specific antibodies are normally positive using sensitive methods such as enzyme-linked immunosorbent assay (ELISA), with abnormal results defined as a raised level above a cut-off related to range of antibody levels seen in healthy persons [38,72,73].

Asymptomatic persons with *Aspergillus* airway colonisation may develop raised levels of *Aspergillus*-specific antibodies and the correct interpretation of this is not clear. Some authors classify raised levels in this population as false positives [38]. However to our knowledge there are no published studies describing the long-term outcome in colonized patients. It is therefore not clear whether patients described in the literature as colonized are at higher risk of developing pulmonary aspergillosis in the future or not. If this were the case then raised levels of *Aspergillus*-specific antibodies in asymptomatic persons might be an indication of pre-clinical disease.

Aspergillosis normally develops in patients with underlying diseases [4,14]. The range of levels of *Aspergillus*-specific antibodies in persons with these diseases may not be the same as healthy persons. Up to 20% of patients with treated tuberculosis have positive tests for *Aspergillus*-specific antibodies [74], this rises to 25% when lung cavities are present [75] and 36% in those with haemoptysis [76]. Raised *Aspergillus*-specific IgG levels are also seen in 13% of Indian asthmatics [62], 24% of British cystic fibrosis patients [77], 25% of Indian children with thalassemia and human immunodeficiency virus (HIV) infection [78] and 8% of all patients attending a Brazilian tertiary respiratory clinic [79].

These surveys did not include further tests to diagnose pulmonary aspergillosis and some of these patients with raised antibody levels may therefore have undiagnosed aspergillosis. Nonetheless these results suggest that the range of *Aspergillus*-specific antibody levels in patients at risk of developing aspergillosis may be different from the ranges described in healthy individuals. Indeed the mean level of *Aspergillus*-specific IgG in cystic fibrosis patients without ABPA is higher than the manufacturers upper limit of normal [77].

Further work is needed to define the range of *Aspergillus*-specific antibody levels in other patient groups who are at risk of, but have not developed aspergillosis, as this is the population that is most likely to undergo testing for CPA or ABPA. It may be that existing diagnostic cut-offs for *Aspergillus*-specific antibody levels, which were defined using healthy persons as a control, are not appropriate for those at most risk of developing aspergillosis.

Aspergillus bronchitis

Seventy-one percent of patients with symptomatic *Aspergillus* bronchitis have raised *Aspergillus*-specific IgG and 29% have positive precipitins [39]. While raised levels of *Aspergillus*-specific IgE are not typical of *Aspergillus* bronchitis, there is considerable overlap between the clinical presentation of *Aspergillus* bronchitis and that of ABPA. Measurement of total and *Aspergillus*-specific IgE would therefore be appropriate in patients with symptoms of *Aspergillus* bronchitis, with the aim of identifying cases of serological ABPA [4].

Acute invasive aspergillosis

Acute invasive aspergillosis normally occurs in patients with profound immune dysfunction, meaning that antibody production may not occur in response to infection [80]. However *Aspergillus*-specific IgG antibodies are detectable by ELISA in 29–100% of patients during the course of acute

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invasive aspergillosis [81–88]. Sensitivity is higher in non-neutropaenic patients (48%) than neutropaenic patients (6%) [89].

When antibodies do develop in acute illness, they take a mean of 10.8 days to appear [85] and historically a majority of patients with invasive aspergillosis died without producing antibodies [82,89]. This greatly reduces their utility for diagnosis of acute disease as early treatment is crucial for survival [19]. Nonetheless when a patient with suspected invasive aspergillosis does develop newly raised *Aspergillus*-specific IgG antibodies this finding does provide evidence of acute infection [83].

There may be other uses for antibody testing in invasive aspergillosis other than diagnosis of acute disease. A retrospective survey described an increase in all-cause mortality in *Aspergillus* colonized lung transplant patients, with a hazard ratio of 2.2 [90]. Another similar study failed to show this association [47], but this cohort was complicated by the fact that colonized patients considered high risk for development to invasive aspergillosis were not included. This suggests that patients colonized with *Aspergillus* might then benefit from antifungal prophylaxis or early empirical antifungal treatment when immunosuppressed. Screening patients for raised *Aspergillus*-specific IgG antibodies prior to initiation of immunosuppressive therapy might be a convenient method of identifying such patients [88,91].

There can also be a role for serial measurement of *Aspergillus*-specific IgG antibodies after commencing treatment for presumed invasive aspergillosis. In this situation a fall in *Aspergillus*-specific IgG levels is a bad prognostic marker [92,93]. This most likely relates to failure of the immune system to mount a response to the infection. A rise in *Aspergillus*-specific IgG antibodies can retrospectively confirm the diagnosis in those who recover following empirical treatment for suspected invasive aspergillosis [23]. This knowledge might affect decisions about whether to forgo further immunosuppressive therapy or to provide antifungal prophylaxis with it.

Sub-acute invasive aspergillosis

Raised levels of *Aspergillus*-specific IgG antibodies are more likely to occur and thus are of greater use for diagnosis in this group than in acute disease [6,53]. In lung transplant recipients, invasive aspergillosis often develops months after transplantation and can evolve slowly. A rise in *Aspergillus*-specific IgG titers preceded radiological changes by 1–2 weeks and diagnosis of invasive aspergillosis by 2–20 weeks in this group [94]. Raised levels of *Aspergillus*-specific IgG antibodies were detected in 93% of 43 Korean patients [6] and 77% of 45 Japanese patients with sub-acute invasive pulmonary aspergillosis [53]. Sensitivities of serum (1,3)-ß-D glucan and galactomannan testing in the Japanese patients were 60% and 64%, respectively.

The sensitivity of galactomannan antigen testing is much lower when *Aspergillus*-specific antibodies are present than when they are absent [95]. This effect may be due to direct binding of anti-*Aspergillus* antibodies to the galactomannan antigen [96]. It is therefore possible that both antigen and antibody testing will both needed to achieve acceptable sensitivity for the diagnosis of sub-acute invasive aspergillosis in mildly immunosuppressed patients.

Chronic pulmonary aspergillosis

Raised levels of *Aspergillus*-specific IgG antibodies are almost always found in CPA [5,8,97]. Production of specific Immunoglobulin M (IgM) is also noted in up to 50% of CPA cases [87,98–102]. This might be considered unusual in a chronic disease as raised levels of specific IgM are typically associated with the acute phase of an infection.

Ongoing growth of *Aspergillus* produces numerous different antigens at different stages in its growth cycle that interact with the immune system in different ways [103]. IgM might therefore be repeatedly re-stimulated as an immune response develops to each new, individual *Aspergillus* antigen over time. An assay that detects IgM antibodies to a wide range of *Aspergillus* antigens could therefore remain positive for some time. The specificity of *Aspergillus*-specific IgM testing is poor, limiting its utility [88,98,100].

Persistently raised levels of specific Immunoglobulin A (IgA) are found in up to 76% of CPA cases [87,98–102]. This immunoglobulin type is normally associated with mucosal immunity and it may be persistently raised as the mucosa is constantly exposed to fungal growth. *Aspergillus*-specific IgE levels are also sometimes raised in CPA cases and may indicate the presence of underlying ABPA when present [5].

Measurement of *Aspergillus*-specific IgG antibodies had a higher sensitivity than either IgM, IgA, or IgE in all these studies and it should therefore be considered the most appropriate test for screening. However small numbers of cases of CPA have been identified which have normal *Aspergillus*-specific IgG, but raised *Aspergillus*-specific IgA or IgM [87,99,104]. This may be explained by the fact that *Aspergillus*-specific IgA and IgM can bind different *Aspergillus* antigens than *Aspergillus*-specific IgG [93,100]. Overall *Aspergillus*-specific IgM probably has little to offer due to poor specificity, but there may be a role for *Aspergillus*-specific IgA and IgE testing, in patients with symptoms and/or radiological changes of CPA, but normal *Aspergillus*-specific IgG levels.

Measurement of *Aspergillus*-specific IgG has additional uses beyond initial diagnosis of CPA. Precipitins titers

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fall following surgical resection of aspergilloma [105] and rise in correlation with clinical treatment failure [106]. *Aspergillus*-specific IgG levels have been successfully used to monitor response of CPA to medical therapy [8,58,107–109].

Allergic aspergillosis

In this context the patient may initially have healthy lungs and an intact immune function. However an exaggerated inflammatory response develops in response to fungal allergen exposure. This is characterized by overexpression of T helper (Th) 2 and Th17 CD4+ cells and down-regulation of T-regulatory cells (TREGs). This results in the high levels of both total and *Aspergillus*-specific IgE in serum in patients with SAFS and ABPA [64,110]. Raised total and *Aspergillus*-specific IgE in serum are also noted in patients with allergic fungal rhinosinusitis caused by *Aspergillus*. In this patient group raised levels of *Aspergillus*-specific IgE can also be found in the 'allergic mucin' extracted from the sinuses themselves [111,112].

Raised *Aspergillus*-specific IgG has been described as an exclusion criteria for the diagnosis of SAFS on the grounds it implies more complex disease and airways infection [110]. It should be noted though, that 10% of all asthmatics have raised *Aspergillus*-specific IgG or precipitins [22]. There are therefore likely to be some cases of asthma with *Aspergillus* sensitization where *Aspergillus*-specific IgG is raised in addition to IgE, but all diagnostic criteria for more severe conditions such as ABPA are not met.

Precipitating *Aspergillus*-specific antibodies were frequently found in ABPA cases in early studies [113,114]. They were then considered a mandatory diagnostic criteria for ABPA by some authors [115,116], whereas others regarded it only as a supporting criteria [117]. Reports on the frequency of raised *Aspergillus*-specific IgG or precipitins in ABPA will of course be heavily dependent on whether or not it is considered a mandatory diagnostic criteria, but 14% of ABPA cases have recently been reported as having a negative precipitins test [67].

ABPA can be complicated by the development of CPA, which is characterized by raised levels of *Aspergillus*-specific IgG. However elevated *Aspergillus*-specific IgG is much more common in ABPA than is the development of CPA [118,119]. Levels of *Aspergillus*-specific IgG are generally higher in CPA than ABPA. Unusually high levels in patients with ABPA may therefore suggest that CPA has developed and should prompt further investigation [4,5]. Raised *Aspergillus*-specific IgA has also been noted in ABPA [120], but it occurs only in a minority of patients and is of limited diagnostic value.

In patients with underlying cystic fibrosis (CF) quantitative measurement of *Aspergillus*-specific IgG has been suggested as a means to differentiate CF with ABPA from CF without APBA [77]. It was found that the mean *Aspergillus*-specific IgG concentration in CF patients without ABPA was 51.1 mg/L, compared to 132.5 mg/L in CF patients with ABPA. The authors of this study suggested an *Aspergillus*-specific IgG cut off of 90 mg/L to differentiate the two patient groups with a sensitivity of 91% and specificity of 88%.

Latent class analysis is a statistical technique used to find groups or subtypes of cases in multivariate categorical data. A recent publication used this technique to identify different disease groups in relation to *Aspergillus* infection in CF [66]. Four disease groups were identified; 1 – patients with no evidence of *Aspergillus* disease, 2 – patients with serological ABPA, 3 – patients sensitized to *Aspergillus* and 4 – patients with *Aspergillus* bronchitis.

Aspergillus-specific IgG could be used to differentiate between *Aspergillus* sensitization and serological ABPA with a sensitivity of 96% and a specificity of 90% when a cut off of 75 mg/L was used. *Aspergillus*-specific IgE could differentiate between *Aspergillus* bronchitis and serological ABPA with 100% sensitivity and specificity using a cut off 3.7 kUA/l. Patients with no *Aspergillus* disease could be differentiated from patients with *Aspergillus* sensitization using *Aspergillus*-specific IgE with a cut-off of 2 kUA/l. To our knowledge the efficacy of the diagnostic cut-offs suggested by these two studies have not been confirmed in populations other than the ones used to define the cut-offs.

Total IgE falls with effective treatment of ABPA [115,117,121–123]. *Aspergillus*-specific IgE can also fall with treatment [117], but this effect was noted later than the fall in total IgE in this study and was not reported in the majority of other treatment studies. *Aspergillus*-specific IgG has also been noted to fall in line with treatment [116], but this occurred at the same time as a fall in total IgE and provided no additional information. It therefore appears that total IgE is currently the most appropriate test for monitoring response to treatment in ABPA.

Laboratory methods for detection of *Aspergillus*-specific antibodies

Multiple techniques are available to measure levels of *Aspergillus*-specific antibodies in human serum in the laboratory in different ways. Since raised *Aspergillus*-specific IgG, IgE, IgA, and IgM all have different interpretations in different clinical scenarios it is important to understand which assays measure which antibody types when interpreting results.

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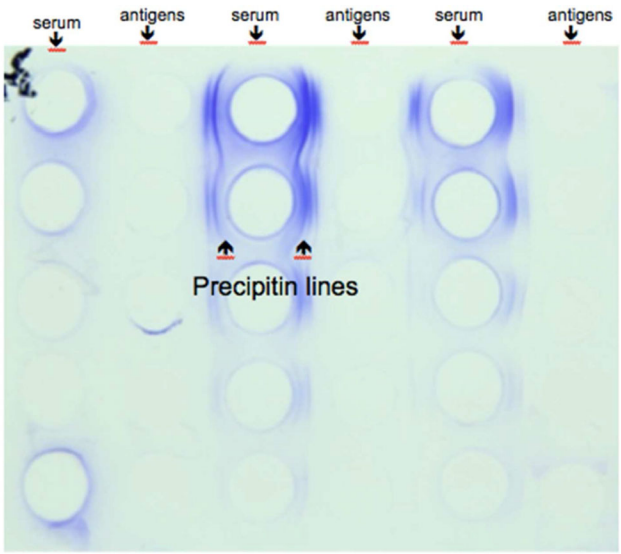

Figure 2. Picture of a CIE gel, with visible precipitin bands. *Note*: Stained precipitin lines are formed where antigens and antibodies meet and precipitation of antibody-antigen complexes occurs. They represent a positive result. Sera in the left hand column produced no lines and are negative.

Precipitation in gels

Detection of *Aspergillus*-specific antibodies in serum was first achieved by precipitation of antibody-antigen complexes in gels [124,125]. This method has also been referred to as double diffusion, immunodiffusion or the precipitins test. Antigens and antibodies are placed in separate wells within the gel and are allowed to diffuse towards one another. The presence of multiple binding sites on antibodies such as IgM [126] allows the formation of immune complexes that 'precipitate' when they become too large to pass through the gel. These 'precipitin bands' are visible to the naked eye with non-specific staining.

All antibody classes precipitate, but IgG predominates. The method takes around five days to perform, is labour intensive and relies on human interpretation of results. No complex equipment is needed. Commercial preparations of *A. fumigatus* antigens for use in precipitins tests are available from Microgen (UK), Bio-Rad/Platelia (France) and Immuno-Mycologics (IMMY) Inc. (USA).

Counterimmunoelectrophoresis

An improvement on the precipitation method was described with the development of counterimmunoelectrophoresis (CIE) [125]. Movement through the gel is accelerated by application of an electric current and precipitation occurs within a few hours [127].

Fig. 2 is a picture of a CIE gel with visible precipitin bands.

Haemagglutination

Haemagglutination tests use erythrocytes pre-coated with antigens. These erythrocytes clump together when antibodies cross-react with antigens on more than one cell. The resulting 'plaque' prevents erythrocytes from settling at the bottom of the test well. The difference in appearance between positive and negative wells is visible to the human eye [128,129]. This method produces a result in around two hours and does not require complex equipment, but does rely on human interpretation of results. It is commercially produced by ELITech Diagnostics (France). Antibody levels are considered raised if a positive reaction takes place at a dilutional titer greater than the manufacturers stated cut off level.

Fig. 3 is a picture of a haemagglutination plate showing positive and negative results.

Complement fixation

Complement fixation tests rely on the fact that human complement will both react with antibody-antigen complexes and also lyse sheep erythrocytes that are pre-bound to anti-sheep erythrocyte antibodies [130]. Complement is removed from human serum by heating. *Aspergillus* antigens, complement and sheep erythrocytes, pre-bound to anti-sheep erythrocyte antibodies are added in steps. In the absence of *Aspergillus*-specific antibodies a reaction takes place that results in lysis of the erythrocytes and thus color change visible to the naked eye [83]. The method is fairly labor intensive and relies on human interpretation of results. Kits are produced by and Serion (Germany) and IMMY (USA).

All of the above techniques can produce semi-quantitative results by following serial dilutions of serum.

ELISA

This well-described technique allows the detection of individual types of antibody (IgG, IgM, IgA, etc.). Antibodies from patient sera bind to antigens and are then detected by anti-human antibodies. Enzyme reactions produce a colour change that is measured with a spectrophotometer. ELISA has been used in diagnosing aspergillosis for decades [131,132]. It can be fully automated, which reduces labour costs and can produce results within two hours. The reaction can also be performed manually. ELISA produces a positive result in most sera, with a cut-off provided by the manufacturer to differentiate raised levels from normal ones.

Commercial *Aspergillus*-specific IgG plate ELISA tests are currently produced by Serion, (Germany), IBL

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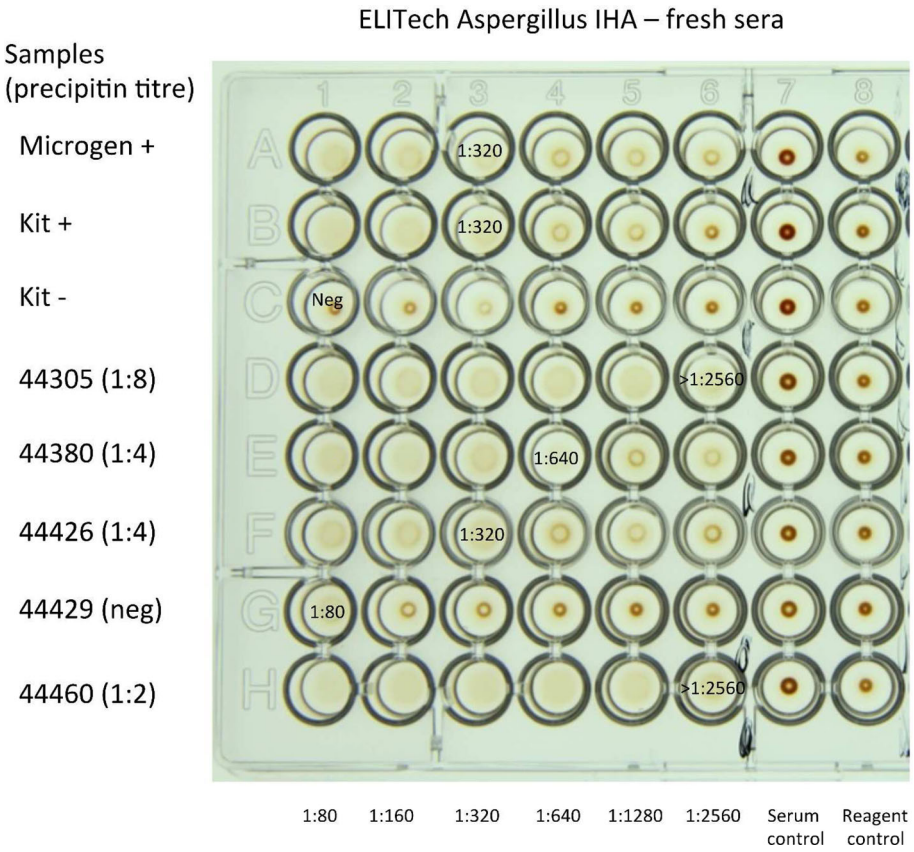

Figure 3. Haemagglutination assay. *Note*: The ELITech haemagglutination assay can be performed with no equipment other than a pipette. Results are visible to the naked eye. In the image above each row is a test sample with dilutional titres increasing from left to right. Result is the last titre at which a 'plaque' is still visible as shown.

(Germany/USA), Dynamiker/Bio-Enoche, (China), Bio-Rad (France), Bordier (Switzerland), and Omega/Genesis (UK). Siemens (Germany) supply an automated *Aspergillus*-specific IgG ELISA system (Immunolite) and ThermoFisher Scientific/Phadia (multi-national) supply an automated *Aspergillus*-specific IgG fluoroenzymeimmunoassay (FEIA) system (ImmunoCAP), which is an ELISA variant. The Serion and Bio-RAD *Aspergillus*-specific IgG assays can also be automated. Siemens and ThermoFisher Scientific both produce automated *Aspergillus*-specific IgE ELISA/FEIA tests. Serion and IBL produce commercial *Aspergillus*-specific IgA and IgM ELISA tests. The units of measurement often differ from one assay to another.

Immunoblot

Gel electrophoresis is used to separate *Aspergillus* antigens by molecular weight. Antigens are then transferred to a membrane to which human serum is added. An identical series of reactions to ELISA is then performed, producing a color change visible to the naked eye at the location of the antigen on the membrane when positive. It does not require complex equipment but is fairly labor intensive [82]. A commercial *A. fumigatus* immunoblot was released in 2012 by LDBIO diagnostics (France).

The attributes of a selection of commonly used methods for detection of *Aspergillus* antibodies are summarized in Table 2.

Sources of antigens for use in antibody detection assays

Extraction of antigens from fungal cultures

The traditional methods of antigen preparation for use in tests involves growth of *Aspergillus* culture in the laboratory, followed by either mechanical disintegration of intact cells to provide somatic antigens or culture filtration to provide extra-cellular antigens. The latter have often been referred to as 'metabolic' antigens in literature and product information sheets. This terminology is, however inaccurate as many of the antigens are not metabolites. These crude processes produce mixtures of many of the different antigens produced by *Aspergillus*. Up to 52 separate precipitins bands have been identified on double diffusion testing using this type of antigen preparation [133] and electrophoresis

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Table 2. Comparison of the features of selected commercial *Aspergillus* antibody assays.

Test	CIE	ThermoFisher Scientific IgG FEIA	Siemens IgG ELISA	Bio-Rad IgG ELISA	Serion IgG ELISA	Dynamiker IgG ELISA	ELITech HA	LDBIO Immuno blot
Antigen type	fungal extract	fungal extract	fungal extract	unspecified recombinant antigen	fungal extract	galactomannan	fungal extract	fungal extract
Volume (μL)	10	140 (dead volume = 100)	255 (dead volume = 250)	10	10	1	50	10
Dilutions	titres as required	1 if result > 200 mg/L.	1 if result > 200 mg/L.	1 pre-test and second in samples with high result	2 pre-test and third in samples with high result	1 pre-test and second in samples with high result	titres as required	none
Units	dilution titres	mg/L	mg/L	AU/ml	IU/ml or U/ml	AU/mL	dilution titres	n/a
No samples tested per batch	30 + 2 controls*	continuous testing	continuous testing	92 + 4 controls	92 + 4 controls.	92 + 6 controls	94 + 2 controls*	1
Equipment needed	gels antigens Coomassie blue stain, de-stain and washing solution CIE tank	Phadia 100 analyzer and antigen packs. test tubes. barcode labels	Siemens analyzer and antigen packs. test tubes barcode labels	kit pipettes test tubes incubator spectrophotometer/ ELISA reader OR automated analyzer	kit pipettes test tubes moist chamber incubator distilled water spectrophotometer/ ELISA reader OR automated analyzer	kit pipettes test tubes incubator distilled water spectrophotometer/ ELISA reader	kit pipette	pipette tweezers rocking tray
Suitable for resource poor laboratories	YES	NO	NO	YES (if manual)	YES (if manual)	YES	YES	YES
Total batch time	2 days	3 hours	2 hours	4 hours	4 hours	4 hours	2 $\frac{1}{2}$ hours	3 hours
Hands on time-approx	4 hours	30 mins	30 mins	2 hours	2 hours	2 hours	30 mins	1 hour

Note: CIE = counterimmunoelectrophoresis, IgG = immunoglobulin g, FEIA = fluoroenzyme immunoassay, ELISA = enzyme immunoassay, HA = haemagglutination, AU = arbitrary units. *Represents total number of sera wells per test. Can perform this many screening tests in one batch or use 1 well for each serial dilution if dilutional titres are required.

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of culture extracts has identified up to 200 bands, each representing a potential antigen that might react with human sera [134].

While the extraction of antigens from *Aspergillus* cultures has been taking place for decades there have been several difficulties encountered in attempts to provide consistent and reliable antigens for use in tests. It is clear that different laboratory strains of *Aspergillus fumigatus* produce different groups of antigens [129,135–139]. Even when a single strain is used somatic and culture filtrate methods produce different groups of antigens [135], which can produce different results when tested against patient sera [140]. Various factors such as the culture medium used, pH, and culture temperature have all been noted to affect the nature of antigens produced by cultures [141]. Antigens also vary with the age of the culture [97,136].

Even when identical methods are used, batch to batch variation from a single strain processed in the same lab has been noted [142]. In addition to antigens, culture extracts also contain enzymes and toxins, which might interfere with test performance [143]. When the same antigen extracts are used in different test formats they can produce widely variable results [144]. The antigen mixtures produced from culture extracts have also been shown to cross-react with antibodies produced against other fungi and bacteria [145,146].

As these traditional antigen extraction techniques can be performed in any mycology laboratory, reference laboratories often produce their own internally manufactured antigens for use in assays [59,85,95]. However the extensive difficulties noted above mean that quality control in *Aspergillus* antigen production is exceedingly challenging and by their nature internally manufactured assays in reference laboratories are not amenable to validation in interlaboratory studies. In contrast commercially manufactured assays can be performed and assessed across multiple laboratories and can also be compared to other assays under identical conditions in a single laboratory.

Measurement of antibodies in non-*fumigatus* aspergillosis

All the tests described above are designed to detect *A. fumigatus*. However, in India the most prevalent *Aspergillus* species causing fungal sinusitis is *A. flavus* [10]. This species also accounts for 38% of *Aspergillus* cultures from patients with chronic lung diseases in India [147]. In Brazil *Aspergillus niger* is a common cause of chronic pulmonary aspergillosis [148]. The frequency of growth of different *Aspergillus* species in association with human disease in selected countries is shown in Table 3.

Evidence on the efficacy of antibody detection assays in these cases is extremely limited. Culture filtrate antigens from *A. fumigatus* are positive in around 50% of cases with aspergilloma caused by *A. flavus* or *A. niger* [149]. *A. niger*-specific precipitins were positive in 78% of 23 patients with CPA due to *A. niger* in Brazil [148]. Other species-specific precipitins tests are available and might prove effective, but

Table 3. Frequency of different *Aspergillus* species grown in different respiratory conditions.

Paper	Country	Disease	No. of cases	*A. fumigatus* (%)	*A. niger* (%)	*A. flavus* (%)	*A. terreus* (%)
Baddley 2009 [191]	USA	invasive aspergillosis	274 isolates	66	10	10	9
Herbrecht 2002 [20]	International	invasive aspergillosis	110	77	8	6	5
Denning 2003 [5]	UK	CPA	10	100	none	none	none
Baxter 2013 [66]	UK	cystic fibrosis	39	100	none	3	none
Camuset 2007 [108]	France	CPA	21	95	none	5	none
Nam 2010 [6]	South Korea	sub-acute invasive aspergillosis + CPA	34	91	9	3	none
Jhun 2013 [8]	South Korea	CPA	18	78	22	17	none
Ohba 2012 [7]	Japan	CPA	75	68	15	4	none
Kurhade 2002 [192]	India	treated tuberculosis	14	79	14	7	none
Shahid 2001 [147]	India	'chronic lung diseases'	12	67	33	none	none
Michael 2008 [193]	India	allergic *Aspergillus* rhinosinusitis	125	11	3	79	1
		invasive *Aspergillus* rhinosinusitis	34	26	9	59	6
Prateek 2013 [194]	India	*Aspergillus* rhinosinusitis	16	19	none	75	6

Note: CPA = chronic pulmonary aspergillosis. Note multiple species identified in some cases.

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have been tested on very few patients [97]. Siemens produce ELISA tests for IgG specific to *Aspergillus niger, nidulans, terreus* and *flavus*, but to our knowledge there are no published data on the efficacy of these assays.

Detection of antibodies specific to individual *Aspergillus* antigens

Early experience with precipitins testing demonstrated that precipitin bands of consistent molecular weight appeared in many patients with aspergillosis and corresponded to enzymes associated with the fungus [133,150]. Individual antigens were identified, which had variable sensitivity and specificity for the diagnosis of aspergillosis. Many specific antigens reacting with human IgG and IgE have since been identified [151,152] and the genes relating to these antigens have been sequenced [153]. This has allowed the production of recombinant antigens by expressing these genes in genetically modified bacteria or fungi, which then produce pure extracts of single antigen.

Mitogillin-specific IgG is positive in 100% of aspergilloma cases, 64% of invasive pulmonary aspergillosis cases and only 1.3% of healthy volunteers in a single study [87]. Antibodies to purified recombinant Afmp1p, an *Aspergillus* cell wall galactomannoprotein, are positive in 100% of patients with aspergilloma and 33% of patients with invasive aspergillosis. To our knowledge the efficacy of these assays has not been confirmed in other laboratories and the assays have not been released commercially.

Testing for IgG specific to recombinant catalase, ribonuclease and dipeptidylpeptidase V showed sensitivity of 77%, 81% and 79% respectively for aspergilloma. This increased to 95% by using all three antigens together [154]. Bio-Rad (France) released a commercial recombinant assay following this study. It has shown good agreement with Serion culture filtrate ELISA in a retrospective survey [38]. Bio-Rad has not revealed which antigens are used in their commercialized test.

The Dynamiker *Aspergillus*-specific IgG ELISA assay utilizes purified galactomannan as its sole antigen. No data has yet been published on the efficacy of this test for the diagnosis of aspergillosis, but an earlier study detected antibodies to galactomannan in only 26% of aspergilloma cases [155].

Many efforts have been made to identify individual antigens against which specific IgE is formed in allergic aspergillosis [156,157]. These antigens are commonly referred to as allergens in this context. To date 23 *Aspergillus*-specific allergens have been recognized by the International Union of Immunology Societies [158]. This is likely to be an under-representation of the true number of *Aspergillus*-specific allergens as 81 IgE binding *Aspergillus* proteins have been identified using a highly sensitive phage display detection method [159].

Attempts have been made to develop individual allergen-specific IgE assays for use in allergic aspergillosis and to use them to differentiate between different diseases. IgE to allergen Asp f1 is found in 60–85% of ABPA cases [160,161] but has also been detected in the sera of *Aspergillus* sensitized asthmatics without ABPA [156]. Genomic studies have demonstrated that sensitization to this allergen is produced only by a small number of fungi [162], suggesting that there is likely to be limited cross-reactivity with this recombinant protein.

One study found IgE specific to allergens Asp f2/3/6 were all raised in both asthma and ABPA, but not in other forms of pulmonary aspergillosis. However another study found that IgE specific to allergens Asp f1/2/3/4/6 were all present at significantly higher concentrations in ABPA than asthma [152].

In patients with underlying cystic fibrosis, one study showed mean IgE to Asp f1 was ten times higher in those with ABPA than those without [163], but another study failed to show this differentiation [164]. IgE to Asp f4 and Asp f6 were found to differentiate CF with ABPA from CF without ABPA in this second study. A similar result was later found when these same antigens were used in skin prick testing [165].

A more recent study showed that no single allergen was absolutely effective in differentiating CF patients with and without ABPA [166]. IgE to Asp f1 showed non-specific binding with ABPA cases and controls, IgE to Asp f2 was consistently present in the sera of CF patients with ABPA, but was frequently also present in CF patients without ABPA. IgE to Asp f3 was highly specific for ABPA in CF but had poor sensitivity. *Aspergillus*-specific IgG subtypes and IgA were also analyzed and found not to differentiate CF with ABPA from CF without ABPA.

Attempts have also been made to identify single antigen-specific antibodies for the diagnosis of acute invasive aspergillosis [82,167], but to our knowledge no commercial assays have been released for this purpose and detection of serum antigenaemia is preferred in this patient group due to its superior sensitivity [48].

Overall, while the detection of antibodies specific to individual antigens might eventually result in more accurate and reproducible diagnosis of aspergillosis, existing study results are mostly inconsistent or unconfirmed. No individual antigen or group of antigens has been consitently shown to be more efficacious than traditional methods of antigen extraction for the diagnosis of any form of aspergillosis.

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Comparative efficacy of different laboratory methods

Invasive aspergillosis

Antibody measurement plays a peripheral role in the diagnosis of invasive aspergillosis and data on the comparative efficacy of different techniques is limited [23]. *Aspergillus*-specific IgG ELISA was more sensitive than precipitins or CIE in two studies with a total of 18 patients [81,86]. Comparison of haemagglutination and *Aspergillus*-specific IgG ELISA showed superior sensitivity for haemagglutination in one study with 14 patients [83], but superior sensitivity for ELISA in another study with 26 patients [85]. To our knowledge there are no comparisons of currently commercially produced *Aspergillus* antibody assays in this patient group, although the Serion *Aspergillus*-specific IgG ELISA formed part of a mix of methods for antibody detection that were less sensitive than galactomannan antigen test in one comparison [89].

Chronic pulmonary aspergillosis

The original reports of precipitins tests for diagnosis of aspergilloma reported sensitivity of 98% against patients with definite histological or radiological diagnosis of aspergillosis, with no positive results in healthy controls [97]. However it should be noted that the radiological methods available at the time did not include computed tomography (CT) scanning and would thus only have detected cases with fairly advanced disease.

Since then precipitins detection has been used as part of the diagnostic criteria for chronic forms of aspergillosis. The lack of a clear gold standard creates a difficulty in subsequent studies. Sensitivity is normally measured against clinical diagnosis recorded in the patients' notes. Precipitins will often have formed part of the diagnostic criteria. It is therefore difficult to prove that other tests are more sensitive than precipitins in study populations defined in this way.

In more recent studies the interpretation of reported sensitivity rates against diagnoses of CPA taken from case notes might be further complicated by the fact that many patients will be on antifungal treatment [58]. It is not known whether this would affect all tests equally or bias results in favor of one technique. Prospective studies comparing efficacy of tests in patients not yet diagnosed with aspergillosis would resolve these issues, but are difficult to conduct due to the low frequency of new diagnoses.

Many retrospective studies have shown equally excellent sensitivity when precipitins testing is compared to CIE or ELISA in cases of aspergilloma [168–173]. Precipitins were even reported as being more sensitive than other methods in one comparison [174]. However not all cases of aspergilloma or CPA are precipitins positive [7,175]. Negative precipitins results might occur as not all antibody-antigen complexes precipitate in gels [176].

The one prospective study comparing precipitins to CIE showed that CIE is more sensitive than traditional precipitins for detection of *Aspergillus*-specific antibodies [177]. However CIE has also been reported as producing more false positive results than precipitins [170,178,179]. A recent retrospective study suggested that the sensitivity of ELISA for the diagnosis of CPA was 30% higher than precipitins [73].

Unlike 'home-brew' assays using internally manufactured *Aspergillus* antigens, commercially available assays can be compared to one another in head-to-head comparisons. Commercial ELISAs with published efficacy data for the disgnosis of CPA include ThermoFisher Scientific *Aspergillus*-specific IgG FEIA, Serion culture filtrate *Aspergillus*-specific IgG ELISA and Bio-Rad recombinant *Aspergillus*-specific IgG ELISA [38,72]. Each showed good correlation with precipitins test results and superior reproducibility when automated. The Siemens (Immunolite) and ThermoFisher Scientific (ImmunoCAP) assays have good head-to-head correlation, but the Siemens assay produces a higher absolute result with a mean ratio of 1.78 [180]. This study also demonstrated acceptable inter-laboratory reproducibility for the ThermoFisher Scientific (ImmunoCAP) with a co-efficient of variation of 7.3–18.1%.

ThermoFisher Scientific *Aspergillus*-specific IgG FEIA, Bio-Rad recombinant *Aspergillus*-specific IgG ELISA and CIE using Microgen antigens were compared in 116 patients with CPA, who were mostly on antifungal treatment [73]. Sensitivity was 86% for ThermoFisher Scientific, 85% for Bio-Rad, and 56% for CIE. However 4% of cases were positive on precipitins testing only. This may be due to the ability of precipitins to detect IgM and IgA in addition to IgG. In the case of the Bio-Rad recombinant antigens assay, false negative results may also occur in patients who do not have antibodies to the selected antigens within their spectrum of anti-*Aspergillus* antibodies. These results suggests that while these ELISAs are more sensitive than precipitins testing for first line screening, there may be a role for precipitins testing in patients suspected of CPA with unexpectedly negative ELISA results.

The Bio-Rad test has also been directly compared to Serion ELISA in 51 cases with CPA [38]. Sensitivities of 94% and 92%, respectively, were noted. Specificity in healthy controls was 100% for Bio-Rad and 96% for Serion.

The published comparisons of the sensitivity of different methods of *Aspergillus*-specific antibody detection in patients with CPA are summarized in Table 4.

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Table 4. Direct comparisons of sensitivity of antibody tests in proven CPA / aspergilloma.

Paper	No. of patients	DD (%)	CIE (%)	HA (%)	Culture filtrate IgG ELISA (%)	ImmunoCAP FEIA (%)	Bio-Rad recombinant IgG ELISA (%)	Bio-Rad galactomannan antigen test (%)*
Dee 1975 [168]	9	89	89	–	–	–	–	–
Warnock 1977 [171]	5	100	100	–	–	–	–	–
Kurup 1978 [170]	23	87	91	100	–	–	–	–
Kauffman 1983 [169]	13	100	–	–	100	–	–	–
Mishra 1983 [86]	17	100	100	–	100	–	–	–
Gugnani 1990 [173]	5	100	–	–	100	–	–	–
Faux 1992 [172]	11	100	–	–	100	–	–	–
Kitasato 2009 [60]	28	89	–	–	–	–	–	50
Guitard 2012 [38]	51	–	–	–	92 (Serion)		94	–
Baxter 2013 [73]	116	56	–	–	–	86	85	–
Jhun 2013 [8]	47	–	–	–	99 (IBL)	–	–	23
Shin 2014 [59]	168	98	–	–	–	–	–	23

Note: CPA = chronic pulmonary aspergillosis, DD = double diffusion (precipitins), CIE = counterimmunoelectrophoresis, HA = haemagglutination, IgG = immunoglobulin g, ELISA = enzyme immunoassay, FEIA = fluoroenzyme immunoassay. *galactomannan positive when index ≥0.5.

Allergic pulmonary aspergillosis

A recent review compared the efficacy of different diagnostic tests for identifying new cases of ABPA in Indian asthmatics using latent class analysis [62]. *Aspergillus* skin prick testing was 95% sensitive and 80% specific, total IgE of >1000 IU/ml was 97% sensitive but only 40% specific, raised *Aspergillus* specific IgE was 100% sensitive and 70% specific, whereas *Aspergillus* precipitins testing was only 43% sensitive, but 97% specific.

These results suggest that *Aspergillus*-specific IgE testing is the most appropriate screening test for ABPA and can be used in place of skin prick testing where available. However, the high specificity of precipitins testing means that the diagnosis of ABPA can be made with high confidence in asthmatic patients with both raised *Aspergillus*-specific IgE and positive *Aspergillus* precipitins. Unfortunately most patients with ABPA in this study did not meet all of these criteria.

CIE has been reported as more sensitive than precipitins for the detection of precipitating antibodies in cases of allergic aspergillosis [181]. There are no published direct comparisons of the efficacy of the commercially available *Aspergillus*-specific IgE assays, but it should be noted that marked variation has been noted between *Aspergillus*-specific IgE levels and skin prick test results, with concordance of only 14–56% [65,182,183]. There is also marked variation between the Siemens and ThermoFisher Scientific assays in tests for peanut-specific IgE [184]. The Siemens system produces *Aspergillus*-specific IgG results roughly 2 fold higher than the ThermoFisher Scientific system [180]. Results of *Aspergillus*-specific IgE assays from different commercial assays should therefore be compared with caution.

The published comparisons of the sensitivity of different *Aspergillus*-specific antibody assays are summarized in Table 5.

Suitability of available laboratory techniques for resource-poor settings

As noted earlier the majority of patients suffering from pulmonary aspergillosis are likely to be located in resource-poor settings. We would suggest that many commonly used assays are not ideal for use in such settings. Automated ELISAs require equipment, which is expensive to purchase and requires both a reliable electricity supply and regular maintenance. Manual ELISAs might be suitable but still require a properly maintained spectrophotometer that may not be available in many resource poor settings. Such manual ELISAs have been described as having much poorer reproducibility than automated systems [73].

Precipitation in gels requires less high-tech equipment than ELISA but is time consuming, requires significant operator training, and produces subjective results. Complement fixation and immunoblot have similar difficulties. We consider haemagglutination assays a potentially attractive option as no complex equipment is required, but to our knowledge there are no published data describing the efficacy of the sole commercially available haemagglutination test (ELITech).

The lateral flow device (LFD) is well known for its use in point-of-care pregnancy tests. This format is also widely used for the diagnosis of human immunodeficiency virus (HIV) and malaria in resource-poor settings [185,186]. To our knowledge no LFD for the detection of *Aspergillus*-specific antibodies exists at this time. An LFD that

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Table 5. Direct comparisons of sensitivity of antibody and antigen tests in invasive aspergillosis.

Paper	Clinical group	No. of patients	DD (%)	CIE (%)	HA (%)	IgG ELISA (%)*	Serum GM (%)
Holmberg 1980 [81]	autopsy proven IA	10	–	70	–	80	–
Mishra 1983 [86]	IA	8	37	50	–	75	–
Manso 1994 [84]	mixed proven and probable IA	18	55	–	–	–	38 (LA)
Kappe 1996 [83]	biopsy proven IA	14	–	–	LD – 29 Roche – 36 Fumouze – 36	29	–
Kappe 2004 [85]	biopsy proven IA	26	–	–	8	22	–
Herbrecht 2002 [95]	definite IA	31				68	64
	probable IA	67				58	16
	possible IA	55				70	25
	all IA	133				64	29
Cornillet 2006 [89]	neutropaenic IA	52	6.25 *(mix of DD, CIE and Serion ELISA)*				64
	non-neutropaenic IA	36	48 *(mix of DD, CIE and Serion ELISA)*				65
	all IA patients	88	30 *(mix of DD, CIE and Serion ELISA)*				65

Note: DD = double diffusion (precipitins), CIE = counterimmunoelectrophoresis, HA = haemagglutination, IgG = immunoglobulin g, ELISA = enzyme immunoassay, GM = galactomannan antigen test (ELISA unless stated otherwise), LA = latex agglutination, IA = invasive aspergillosis. *IgG ELISA tests are internally manufactured by the research laboratory unless stated otherwise.

Figure 4. Suitability of tests in resource-poor settings. *Note*: The automated ELISA machine shown is of little use in settings with no regular electricity, whereas lateral flow devices, such as the *Aspergillus* antigen LFD above, are ideal.

detects an *Aspergillus* antigen has recently been developed and seems to perform well using serum for the diagnosis of acute invasive aspergillosis in mostly neutropaenic patients [187–189]. It is also effective using BAL fluid to diagnose invasive aspergillosis in non-neutropaenic patients with underlying lung disease [190]. However, to our knowledge there is no published evidence regarding its efficacy for the diagnosis of CPA. It is possible that in this context the sensitivity of this LFD will be low, as the alternative galactomannan antigen assay has poor sensitivity in this patient group [8,59].

Fig. 4 shows examples of tests that are unsuitable and potentially suitable for use in resource-poor settings.

Conclusions

Aspergillosis has been estimated to affect millions of persons worldwide, with CPA as the most common clinical syndrome. Many of these patients are likely to reside in resource-poor countries, given the current and previous prevalence of tuberculosis in these locations. Treatment of CPA is probably both affordable and deliverable in all healthcare settings. Improved diagnosis of CPA is a critical need in the battle to improve CPA outcomes and expanding access to *Aspergillus*-specific IgG testing in areas of high tuberculosis prevalence is key to achieving this goal.

Expanding the diagnosis of aspergillosis presents many challenges. The clinical and radiological signs of

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aspergillosis often overlap significantly with associated underlying diseases and so cannot be relied upon to diagnose aspergillosis. Culture can be helpful, but the sensitivity of culture for the diagnosis of aspergillosis is sub-optimal and access to reliable fungal culture is frequently challenging or even nonexistent in poorly resourced countries.

Serological testing is therefore of crucial importance. For acute invasive aspergillosis this mostly means antigen testing, which has been reviewed extensively elsewhere. However there may be a secondary role for antibody testing in this setting for retrospective diagnosis of recovering patients. The screening of patients for evidence of *Aspergillus* colonisation prior to immunosuppressive therapy may also be useful. Outside of this setting the interpretation of raised levels of *Aspergillus*-specific antibodies in asymptomatic colonized patients is not well described and follow-up studies of such patients that describe their risk of developing symptomatic forms of aspergillosis would be welcome.

In chronic and allergic aspergillosis measurement of *Aspergillus*-specific antibodies is central to diagnosis, with raised *Aspergillus*-specific IgG found mostly in chronic disease and raised total and *Aspergillus*-specific IgE found mostly in allergic disease. It is important to note, though, that there is a degree of overlap between these clinical syndromes, and many patients will have clinical and serological features of both.

Similarly sub-acute invasive aspergillosis occurs in mildly immunosuppressed patients with a presentation that overlaps acute invasive disease and CPA. Here patients may have positive antigen tests, raised *Aspergillus*-specific IgG or both simultaneously. As a result it is possible that this group of patients will need to be tested for both *Aspergillus*-specific IgG and *Aspergillus* antigens to achieve early diagnosis with good sensitivity.

Measurement of antibodies can also be used to monitor response to treatment. A falling *Aspergillus*-specific IgG indicates poor prognosis in acute invasive aspergillosis but a good response to therapy in CPA. For allergic aspergillosis, total IgE remains the best method for monitoring treatment response, although it is far from optimal.

Many methods exist for the measurement of *Aspergillus*-specific antibodies, with differing performance characteristics. It is thus unfortunate that they are frequently mislabeled in the literature with the term 'precipitins' often used to refer to *Aspergillus*-specific IgG ELISA rather than precipitation in a gel and 'RAST' often used to refer to *Aspergillus*-specific IgE ELISA rather than the older radioimmunoassay.

Evidence of comparative efficacy for different methods is sparse, but *Aspergillus*-specific IgG ELISA is likely to be more sensitive than precipitation in gels. However, there are some patients with CPA with normal *Aspergillus*-specific IgG ELISA results and positive precipitins tests or raised levels of *Aspergillus*-specific IgA. Performing these assays in patients suspected of CPA with negative *Aspergillus*-specific IgG ELISA would therefore probably result in better overall sensitivity.

Aspergillus-specific IgM ELISA is probably not useful for diagnosis of CPA due to poor specificity, although it should be noted that the specificity data come from studies of 'home-brew' assays. The commercially produced *Aspergillus*-specific IgM assays might have different performance characteristics, but to our knowledge there are no published data on this topic

The product inserts of most commercial ELISAs report good specificity at the manufacturers' diagnostic cut-offs, but the evidence for these statements is often not published in peer-reviewed journals. It should be noted that these cut-offs are normally calculated against the range of antibody levels found in a cohort of healthy volunteers. This is probably an appropriate comparator for most invasive aspergillosis patients. However, healthy volunteers may not be the ideal comparator for CPA or ABPA, as these conditions almost always occur in persons with underlying chronic lung disease or chronic immune dysfunction. Unfortunately, to our knowledge there is no published data on the distribution of *Aspergillus*-specific IgG levels in patients with these chronic underlying conditions, with the exception of cystic fibrosis. Our research team is undertaking a study measuring *Aspergillus*-specific IgG levels in patients with treated tuberculosis, COPD, and asthma using several assays. The diagnostic cut-offs for CPA and ABPA may need to be changed in response to this data.

Global standardization of assays has proved difficult, with many laboratories using assays derived from antigens manufactured 'in-house'. By their nature these assays are impossible to validate in other laboratories. Many commercially produced *Aspergillus*-specific IgG and IgE tests exist, but to our knowledge only one (ThermoFisher Scientific/ImmunoCAP) has published inter-laboratory variability data. The Bio-RAD recombinant *Aspergillus*-specific IgG has been tested against reasonable number of persons with CPA at more than one centre with good sensitivity reported. The IBL and ThermoFisher *Aspergillus*-specific IgG assays have been tested in reasonable numbers of patients with CPA at single sites. Most patients in all of these studies will have been on treatment and it is not known how this may have biased the results. Many other assays have no published efficacy or reliability data at all.

The publication of data from studies demonstrating the reliability of available assays both in terms of sensitivity and specificity in untreated patients and in terms inter-assay and inter-laboratory reliability is a prerequisite for their use in the large scale screening that will be necessary to achieve

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diagnosis of the predicted number of cases. Our unit is currently undertaking a single center study with this goal, but studies across multiple laboratories will be needed to determine inter-laboratory variability.

Many attempts have been made to develop ELISAs for the detection of antibodies specific to one or more individual *Aspergillus* antigens and commercially produced tests based on this principle do exist. In theory this should allow production of a reliable test and resolve the many problems that exist with traditional antigen extraction techniques. However, to our knowledge there is no published evidence that these assays are consistently either more reliable or efficacious than traditional techniques for the diagnosis of either allergic or chronic aspergillosis. Assays based on culture filtrate or somatic antigens remain in common usage.

As the majority of patients with pulmonary aspergillosis are predicted to live in resource-poor settings it will be necessary to identify a reliable test that is suitable for widespread use in such settings if such patients are to be diagnosed and treated. The haemagglutination assay may be suitable for use in this setting, but requires further validation. The *Aspergillus* antigen LFD is in the ideal test format, but is likely to have poor sensitivity for the diagnosis of CPA. An LFD that detects *Aspergillus*-specific IgG may need to be developed to allow widespread access to testing in resource poor settings.

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David Denning is a professor of Infectious Diseases in Global Health at the University of Manchester. He holds founder shares in F2G Ltd, a University of Manchester spin-out company and has current grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health Research, the European Union and the Fungal Infection Trust. He acts as a consultant to Trinity group, T2 Biosystems, GSK, as well as other companies over the last 5 years including Pfizer, Schering Plough (now Merck), Astellas and Gilead. In the last 3 years, he has been paid for talks on behalf of Astellas, GSK, Gilead and Pfizer.

Malcolm Richardson is a professor of mycology at the University of Manchester. He lectures on behalf of, and provides educational material and advice for Gilead Sciences Europe, Astellas Pharma. MSD and Pfizer.

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