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Comparison of six *Aspergillus*-specific IgG assays for the diagnosis of chronic pulmonary aspergillosis (CPA)

Iain D. Page [a,b,c,*], **Malcolm D. Richardson** [a,b,d], **David W. Denning** [a,b,c]

[a] *The University of Manchester, Oxford Road, Manchester M13 9PL, UK*
[b] *Manchester Academic Health Science Centre, 46 Grafton Street, Manchester M13 9NT, UK*
[c] *National Aspergillosis Centre, 2nd Floor Education and Research Centre, University Hospital of South Manchester, Southmoor Road, Manchester M23 9LT, UK*
[d] *Mycology Reference Centre, Manchester and National Aspergillosis Centre, 2nd Floor Education and Research Centre, University Hospital of South Manchester, Southmoor Road, Manchester M23 9LT, UK*

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Summary *Objectives:* Chronic pulmonary aspergillosis (CPA) is estimated to affect 3 million persons worldwide. *Aspergillus*-specific IgG is a key component in CPA diagnosis. We aimed to establish the optimal diagnostic cut offs for CPA and the comparative performance of six assays in this context.
Methods: Sera from 241 patients with CPA and 100 healthy blood donors were tested using five *Aspergillus*-specific IgG assays plus precipitin testing using Microgen *Aspergillus* antigens.
Results: Receiver operating characteristic (ROC) curve area under the curve (AUC) results were as follows: ThermoFisher Scientific ImmunoCAP 0.996 (95% confidence interval 0.992–1), Siemens Immulite 0.991 (0.982–1), Serion 0.973 (0.960–0.987), Dynamiker 0.918 (0.89–0.946) and Genesis 0.902 (0.871–0.933).

Optimal CPA diagnostic cut-offs were; ImmunoCAP 20 mg/L (96% sensitivity, 98% specificity), Immulite 10 mg/L (96% sensitivity, 98% specificity), Serion 35 U/ml (90% sensitivity, 98% specificity), Dynamiker 65 AU/ml (77% sensitivity, 97% specificity) and Genesis 20 U/ml (75% sensitivity, 99% specificity). The precipitin test was 59% sensitive and 100% specific.

* Corresponding author. Present address: Department of Infectious Diseases, North Manchester General Hospital, Delaunays Road, Manchester M8 5RB, UK. Tel.: +44 7974805378.
E-mail addresses: iain.page@pat.nhs.uk (I.D. Page), malcolm.ricahrdson@manchester.ac.uk (M.D. Richardson), ddenning@manchester.ac.uk (D.W. Denning).

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Conclusions: ImmunoCAP and Immulite were statistically significantly superior to the other assays. Precipitins testing performed poorly. The currently accepted ImmunoCAP cut-off of 40 mg/L appears sub-optimal for CPA diagnosis and may require revision in this context.

Introduction

CPA is a serious disease that leads to severe disability and death, but which can be treated effectively with existing drugs and surgery.[1–7] There is growing evidence that it is much more common than previously perceived, with an estimated global prevalence of 3 million cases.[8–10] The majority of these cases are predicted to occur as a complication of pulmonary tuberculosis,[11] especially in resource-poor countries where tuberculosis is common.[9]

Diagnosis of CPA requires the presence of chronic symptoms, plus appropriate radiological findings, raised inflammatory markers and microbiological evidence of disease.[1–3,12] The latter can be provided from biopsy or by culture of either broncho-alveolar lavage (BAL) fluid or sputum, but these samples are more challenging to acquire than blood and culture sensitivity is poor with current methods.[13] Raised levels of *Aspergillus*-specific IgG (or precipitins) also fulfil the 'microbiological evidence' criteria for CPA diagnosis. In a recent large CPA case series only 26% of patients had positive culture, but 99% had raised *Aspergillus*-specific IgG.[3]

Many specialist laboratories have developed their own in-house precipitins assays using poorly defined antigens. However, replication of such techniques in other laboratories is challenging[11] and therefore unsuitable for resource poor settings where most CPA is predicted to occur, but few specialist mycology laboratories exist.[9]

Commercial ELISAs assays provide an attractive alternative that could be used in most laboratories, including resource poor settings where mycology laboratories are rare. Multiple commercial tests for *Aspergillus*-specific IgG are available, but the optimal cut-offs for use in CPA diagnosis have never been properly assessed.

The current UK approved cut-off (40 mg/L) for the commonly used ThermoFisher Scientific ImmunoCAP method is derived from a study of just 20 European patients with either allergic or chronic aspergillosis.[14] The Siemens Immulite (Germany), Genesis (UK) and Dynamiker (China) have no published data to justify the diagnostic cut-offs suggested by the manufacturers. No studies have considered the appropriate cut-offs for any assay for use in Africa, where a large proportion of the global burden of CPA is predicted to occur.[8]

Two studies have reported the efficacy of commercial *Aspergillus*-specific IgG assays for the diagnosis of CPA. One noted that the Bio-Rad (France) and Serion (Germany) assays had respective sensitivity of 94% and 92% for the diagnosis of CPA in 51 cases.[15] Specificity was 87% and 76% respectively. The other compared the Bio-Rad assay, ImmunoCAP assay and precipitins detection using Microgen (UK) FSK1 antigens.[16] The latter study showed respective sensitivity of 85%, 86% and 56% for the diagnosis of CPA in 116 cases.

The Siemens Immulite assay has been shown to have good correlation with the ImmunoCAP assay,[17] but its efficacy for the diagnosis of CPA has not been directly measured. The published comparisons are probably too small to detect differences in test efficacy for high performing assays and are potentially biased due to the presence of long-term antifungal therapy, which lowers *Aspergillus*-specific IgG levels, in many patients.[18]

We have performed a retrospective comparison of six methods in a large cohort of CPA patients who were not taking long-term antifungal medication. Receiver operating characteristic (ROC) curve analysis was used to define optimal diagnostic cut offs and compare test performance.[19,20]

Patients

241 CPA patients were identified at the UK National Aspergillosis Centre with a stored sample of serum that had been used for *Aspergillus*-specific IgG measurement as part of routine clinical care when either off treatment or within three months of starting treatment. Samples were stored at minus 80 °C between 2004 and 2014.

CPA diagnosis was confirmed in each patient following review by an experienced specialist clinician. Diagnosis required over three months of relevant symptoms plus radiological features of CPA as described in recent ESCMID/ERS guidelines.[21] 'Microbiological evidence' of CPA was also required. This normally consisted of either positive *Aspergillus* precipitins detected by the Ouchterlony method[22] or an ImmunoCAP *Aspergillus*-specific IgG level of over 40 mg/ml. Positive *Aspergillus*-specific IgG or precipitins from referring laboratories, histological evidence of *Aspergillus* infection following biopsy or resection of lung tissue or *Aspergillus* culture growth from a normally sterile site were also accepted as 'microbiological evidence' of CPA. Rare patients with clear clinical and radiological evidence of CPA and either repeated *Aspergillus* growth from sputum culture or repeated strongly positive sputum *Aspergillus* PCR in the absence of antibody response were also accepted as CPA cases. Raised inflammatory markers were often present, but were not required for CPA diagnosis. Appropriate investigations were conducted to exclude other conditions before diagnosing CPA as described in ESCMID guidelines.[21]

Healthy control samples were collected from 100 Ugandan blood donors. Samples were tested for *Aspergillus*-specific IgG by all methods.

Methods

Aspergillus-specific IgG levels were measured on all samples from CPA patients and controls using the automated Immulite 2000 and ImmunoCAP systems. Where a sample produced a result of >200 mg/L a 1 in 10 dilution was performed and the sample was retested.

Plate ELISAs were performed on all samples using kits supplied by Serion, Genesis and Dynamiker in accordance with the manufacturers' instructions with manual pipetting and washing. Optical density was measured with a PolarStar Omega spectrophotometer (BMG Labtech, UK). Readings were converted to arbitrary units using the formulae or software provided by each test manufacturer. If the manufacturers' stated quality control criteria were not met for an individual test plate it was repeated. Where a result was greater than a threshold specified by the manufacturer a 1 in 10 dilution was performed and the assay was repeated.

Precipitation in gel (precipitins) testing was performed on all samples by counterimmunoelectrophoresis (CIE) using Microgen FSK1 cytopasmic and culture filtrate *Aspergillus* antigens (Microgen Bioproducts Limited, Camberly, UK). Briefly, 10 ml low-EEO/multi-purpose agarose (Fisher Scientific, Pittsburgh, USA) was dissolved into 0.75% veronal (sodium barbital) buffer and poured onto a hydrophobic gel bond film (GE Healthcare, USA) and set. Three mm diameter test wells were cut. Twenty μL sera were placed in one row of wells and 20 μL antigens at 2 mg/ml in the adjacent row. The gel was placed above a CIE tank containing 0.75% veronal buffer and blotting paper wicks were used to connect either end of the gel to the buffer tanks before applying 34 V for 90 mins. The gel was placed in 0.95% sodium chloride washing solution overnight. After drying it was placed in a 5% Coomassie Blue stain (Coomassie Blue, methanol, glacial acetic acid) for 15 min, followed by two serial de-stain solutions of 10 min each in stain-free solvent. After further drying the gels were read on a light box with the assistance of a magnifying glass. The presence of any precipitins bands was reported as a positive result. Serial dilution to a maximum 1 in 32 dilution was performed to provide titres. The same technician performed all the above tests.

Where a sample produced the same result (positive or negative by manufacturer's instructions) on a single test by all test methods this result was accepted. Where a sample produced divergent results on different assays it was repeated. If this result produced the same result (positive or negative) as the first run the result of the first run was accepted. If it produced the opposite result (positive or negative) a decisive third run was performed. If this produced the same result (positive or negative) as the first run the original result was accepted. If the third run produced the same result as the second run (positive or negative) then the mean result from runs two and three replaced the original result in the final analysis.

To calculate intra-assay variability two samples were selected for each assay, except ImmunoCAP, with high and low results respectively. Each assay was repeated 20 times per sample. Outliers were identified by study team consensus and removed from the final analysis.

Intra-assay variation (IAV) is reported for the Immulite, Serion, Genesis and Dynamiker assays. Result range, mean, standard deviation and co-efficient of variation are reported for each assay. Co-efficient of variation was calculated as (standard deviation/mean) $\times$ 100.

Descriptive statistics are reported for each assay in each patient group, including the frequency of positive, negative and intermediate results by manufacturers' guidelines. ImmunoCAP and Immulite both produce results in mg/L. Correlation between these two assays is measured by Spearman's rank analysis. Results in arbitrary units from Serion, Genesis and Dynamiker assays cannot be directly compared.

ROC curve analysis was performed for each ELISA assay. ROC area under the curve (AUC) is shown with 95% confidence intervals (95% CI). Wald's statistic was used to compare the significance of differences in ROC AUC between assays. A significant result is defined as $p < 0.005$.

Optimal diagnostic cut-offs for each assay are calculated using Youden's J statistic (sensitivity + specificity − 1). Sensitivity and specificity are described for these cut-offs. Statistical analyses were performed using SPSS version 20 (IBM, USA) under license to the University of Manchester, UK.

Results

All kits produced results within the manufacturers' stated quality control criteria for the tests included in the analysis, with the exception of Dynamiker. In this case the high concentration control serum was slightly below the stated range in all runs.

Intra-assay variation is shown in Table 1. Four low level Serion samples and one low level Dynamiker sample with readings lower than the substrate blank were removed as outliers, as was one low level Genesis sample with apparent cross-well contamination. No high level samples were removed as outliers. All low level precipitins repeats were negative and all high level repeats were positive, but with dilutional titre results as follows; neat = 1 sample, 1:2 = 2 samples, 1:4 = 11 samples, 1:8 = 4 samples, 1:16 = 1 sample. The Serion IAV results were repeated three times with similar results each time.

Characteristics of the 100 healthy controls and 241 CPA patients are shown in Table 2. A patient is defined as having positive sputum culture or precipitins if they have had a positive result from one or more samples taken at any time during their care. Forty-two (17%) of CPA patients

Table 1 Intra-assay variation.

Test (unit)	Low range	Low mean	Low co-efficient of variation	High range	High mean	High co-efficient of variation
Dynamiker (AU/mL)	45.5—66.2	55.8	10.1%	240.7—372.7 (132)	287.5	11.1%
Genesis (U/mL)	4.6—6.3	5.2	8.2%	55.4—96.5 (41.1)	83.4	12.1%
Serion (U/mL)	6—42.5	24	43.7%	59.9—122.1 (62.2)	75	23.2%
Immulite (mg/L)	58.4—67.8	62.6	3.6%	95.3—107 (11.7)	99.6	3.4%

Table 2 Patient and control characteristics.

Characteristic	CPA patients (n = 241)	Healthy controls (n = 100)
Female gender	101 (42%)	55 (55%)
Mean age (years)	65	19
Age range (years)	23–92	17–39
Chronic cavitary pulmonary aspergillosis	238 (99%)	–
Aspergillus nodule disease	3 (1%)	–
ImmunoCAP *Aspergillus*-specific IgG > 40 mg/L	211 (88%)	–
Positive precipitins	138 (57%)	–
Aspergillus fumigatus growth on sputum culture	89 (37%)	–
Aspergillus niger growth on culture	3 (0.5%)	–
HIV	0	2 (2%)
Prior tuberculosis	37 (15%)	–
Non-tuberculous mycobacterial infection	28 (12%)	–
COPD	85 (35%)	–
Bronchiectasis	60 (25%)	–
ABPA	35 (15%)	–
Sarcoidosis	9 (4%)	–
Malignancy (active or in remission)	33 (14%)	–
Autoimmune disease	33 (14%)	–
Diabetes	7 (3%)	–

HIV = human immunodeficiency virus seropositive; COPD = chronic obstructive pulmonary disease; ABPA = allergic bronchopulmonary aspergillosis.

included in the study had received up to three months antifungal therapy at the time of sampling. The remaining 199 (83%) patients were not on any antifungal therapy at the time of sampling. Median levels of *Aspergillus*-specific IgG were higher in those that received up to three months antifungal therapy than those that received no antifungal therapy (Appendix 1).

Results are presented in Table 3, together with the manufacturers' interpretations, where available. Dynamiker, Genesis and Serion advise reporting of results as positive, intermediate or negative. ImmunoCAP is interpreted with a single diagnostic cut-off (40 mg/L) in line with current UK practice. Results to 200 mg/L are CE marked; above that a dilution step is required, which has been internally validated in our laboratory, but not CE marked. 21% of the samples required this dilution step (median level 500 mg/L). Immulite does not currently have a recommended diagnostic cut-off. Box and whisker plots of results in controls and cases for each assay are shown in Figs. 1–5.

Precipitins tests produced the following results in CPA cases; negative = 102 cases (42%), neat = 23 cases (10%), 1:2 = 34 cases (14%), 1:4 = 29 cases (12%), 1:8 = 26 cases (11%), 1:16 = 23 cases (10%), 1:32 = 4 cases (2%).

A significant correlation was found between the ImmunoCAP and Immulite assay results (Spearman's rank analysis 0.894, p = 0.000). ROC curves are shown in Fig. 6. We report the specificity and sensitivity of potential cut-offs

Table 3 Results in CPA cases and healthy controls.

Test (Unit)	Controls range (n = 100)	CPA range (n = 241)	Controls mean	CPA mean	Controls median	CPA median	Frequency of positive results by manufacturer's guidelines in controls (intermediate results)	Frequency of positive results by manufacturer's guidelines in CPA (intermediate results)	ROC AUC	95% CI
Dynamiker (AU/ml)	16–88	23–6118	37	341	34	124	6% (11%)	78% (5%)	0.918	0.89–0.946
Genesis (U/ml)	0–20	1–930	7	111	6	60	22% (13%)	82% (5%)	0.902	0.871–0.933
Immulite (mg/L)	0–35	3–7660	5	678	4	392	n/a[a]	n/a[a]	0.991	0.982–1
ImmunoCAP mg/L	0–36	9–1707	6	216	5	126	0%[b]	88%[b]	0.996	0.992–1
Serion (U/ml)	0–40	4–3436	10	232	6	131	0% (0%)	74% (10%)	0.973	0.96–0.987
Precipitins	All negative	Negative to 1:32	–	–	–	–	0%	59%	–	–

[a] There is no recommended cut off for Immulite at the time of publication.
[b] Results for ImmunoCAP reported against the cutoff of 40 mg/L recommended for use in UK labs at the time of publication.

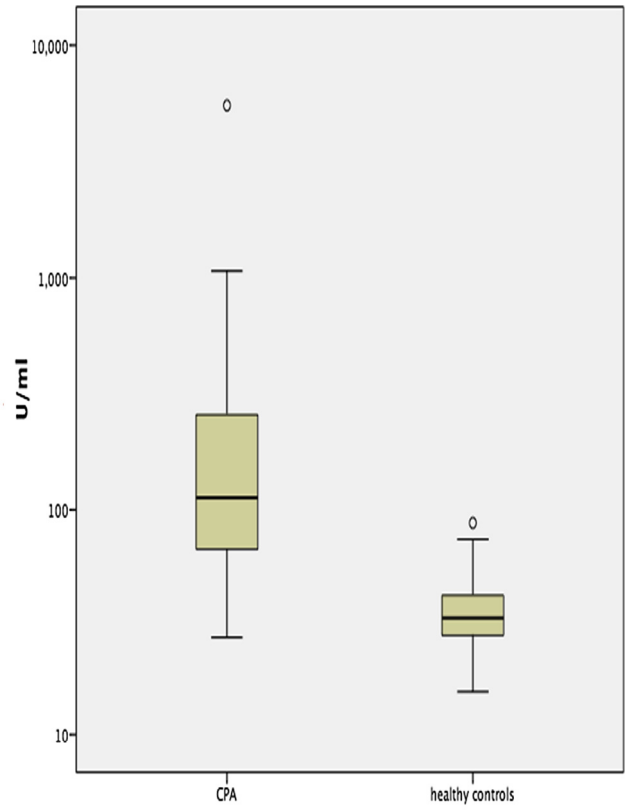

Figure 1 Dynamiker levels in cases and controls.

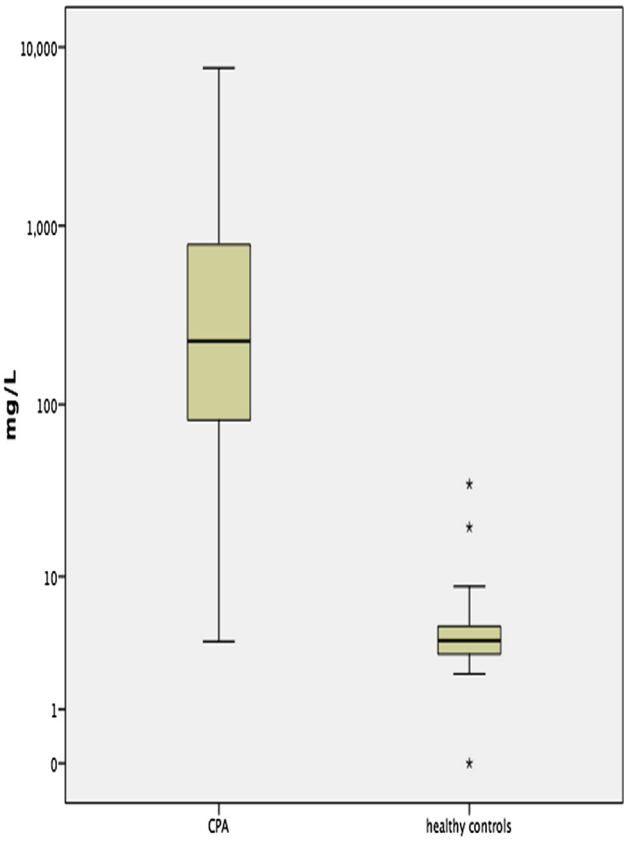

Figure 3 Immulite levels in cases and controls.

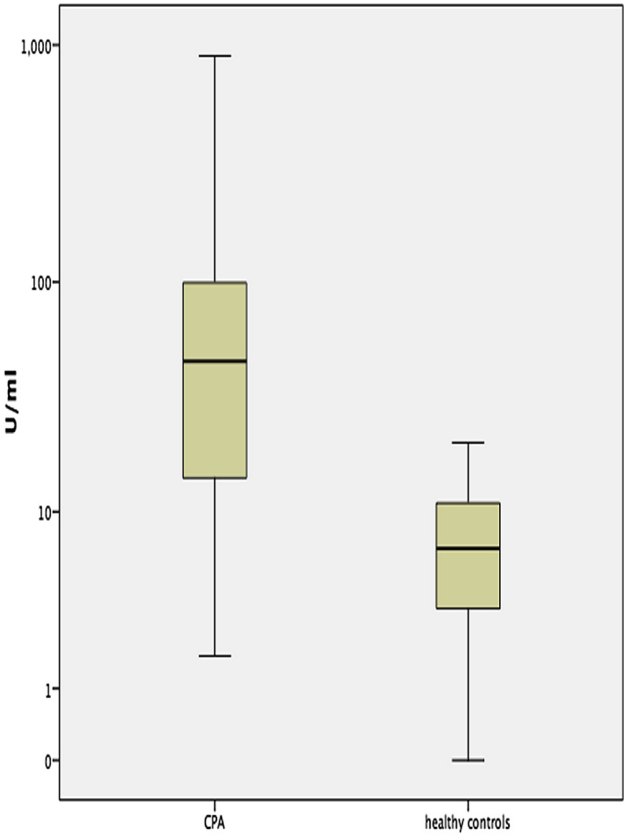

Figure 2 Genesis levels in cases and controls.

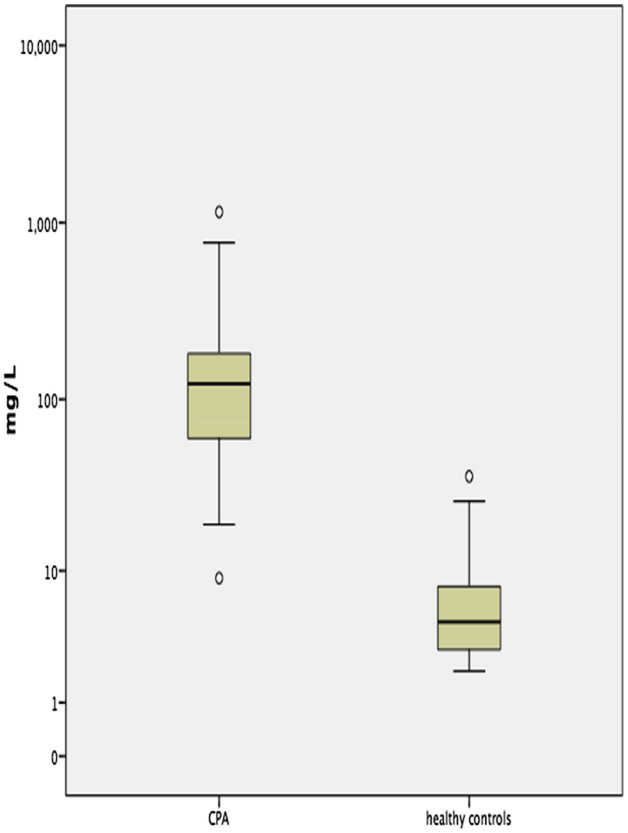

Figure 4 ImmunoCAP levels in cases and controls.

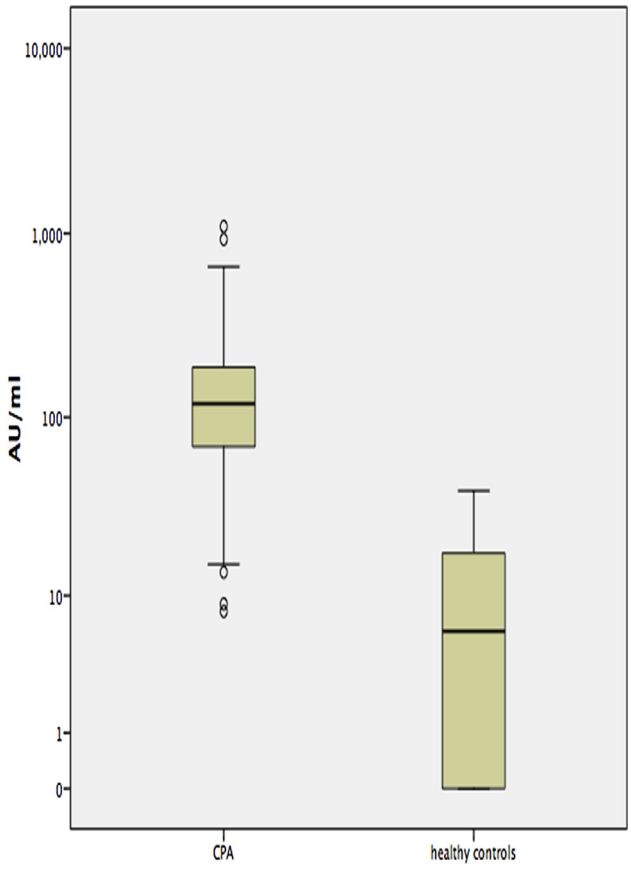

Figure 5 Serion levels in cases and controls.

for the diagnosis of CPA in Table 4. The suggested optimal cut off for each assay is highlighted in bold.

Wald's statistic confirmed the overall difference in performance across the five assays is statistically significant ($p < 0.0001$). ImmunoCAP ROC AUC is equivalent to Immulite ($p = 0.32$). ImmunoCAP and Immulite both have significantly superior ROC AUCs to the other three ELISA assays ($p = 0.0006$ for Immulite vs. Serion). Serion has a superior ROC AUC to both Dynamiker and Genesis ($p < 0.0001$ for Serion vs. Dynamiker). Dynamiker and Genesis have equivalent ROC AUCs ($p = 0.38$).

Nine of 241 sera from CPA cases were negative (<20 mg/L) on testing with the ImmunoCAP assay. Using the new diagnostic cut-offs suggested above 6 of these 9 (67%) were positive on Immulite testing (mean level in positives 93 mg/mL), 3 of 9 (33%) samples were positive on Serion testing (mean level in positives 217 U/ml), 1 sample (11%) was positive by Genesis at 130 U/ml, 1 sample (11%) was positive by Dynamiker at 614 AU/ml and 3 (33%) were positive by CIE (two neat, one at 1 in 2). Two samples were negative on all assays.

Discussion

This is the first study to compare the performance of *Aspergillus*-specific IgG assays in a large population of well-characterised patients with untreated CPA. ImmunoCAP and Immulite assays both have excellent performance in terms of ROC AUC, which is statistically superior to the other assays tested. They should be adopted as the tests of choice wherever possible. However, regulatory approvals for each assay for *Aspergillus* IgG are not universal, with the ImmunoCAP being much more extensively used currently.

This is also the first study to assess the performance of an *Aspergillus*-specific IgG assay using purified galactomannan as its sole antigen (the Dynamiker assay). The ROC AUC performance of this assay was reasonable and statistically equivalent to one of the two manual plate ELISAS included in the comparison that used traditional culture based antigen extraction techniques.

Precipitins performed very poorly, in keeping with an earlier study.[16] This manual technique is time consuming, provides subjective results, is very difficult to replicate reliably[23] and has no advantages over ELISA when diagnosing CPA. It should no longer be considered a first line test or a necessary diagnostic criterion. Authors should clearly specify whether precipitins testing or *Aspergillus*-specific IgG testing is used in future studies.

The median Immulite level was three times higher than the median ImmunoCAP level in CPA cases. This confirms earlier findings[17] and should be taken into consideration when comparing results. Laboratories should identify the assay used when reporting test results.

The IAV for Serion was unexpectedly high at 43.7% for the low level sample and 23.2% for the high level sample. This is considerably higher than the CV of 9.8% published from an earlier study.[15] There is no clear explanation for this, although different batches of tests will have been used in these studies.

We were unable to obtain funding to assess intra-assay variability for ImmunoCAP, but three existing studies from separate laboratories have shown between-run CV results from <5% to 23% for this assay.[14,17,24] The inter-laboratory CV for ImmunoCAP is 7.3—18.1%.[17] Our study was limited to testing in a single laboratory with tests kits from a single batch used for all assays except ImmunoCAP. Additional work will be required to better evaluate batch-to-batch and inter-laboratory variation for all the available assays.

A small number of CPA sera were negative on ImmunoCAP, the assay with the best ROC AUC result. Most were strongly positive by at least one other assay. This may be explained by variation in the mixes of antigens between assays. An underlying immune deficit preventing an effective antibody response to *Aspergillus* infection may explain the two cases with negative results by all tests. Some of our patients have profoundly low B cell and/or NK cell circulating cell numbers (CD19 and CD56), and some have been found to have hypogammaglobulinaemia or low post-immunisation pneumococcal antibody titres (unpublished observations). Such patients with otherwise overt CPA and negative *Aspergillus* antibody levels could be termed 'sero-negative CPA'.

ImmunoCAP testing was performed on fresh samples from CPA patients, but other tests were performed on the same samples after frozen storage. However, this should not bias the comparison as long term storage does not appear to significantly reduce antibody levels in serum.[25,26] We note that *Aspergillus*-specific IgG levels in study patients who had received up to three months antifungal

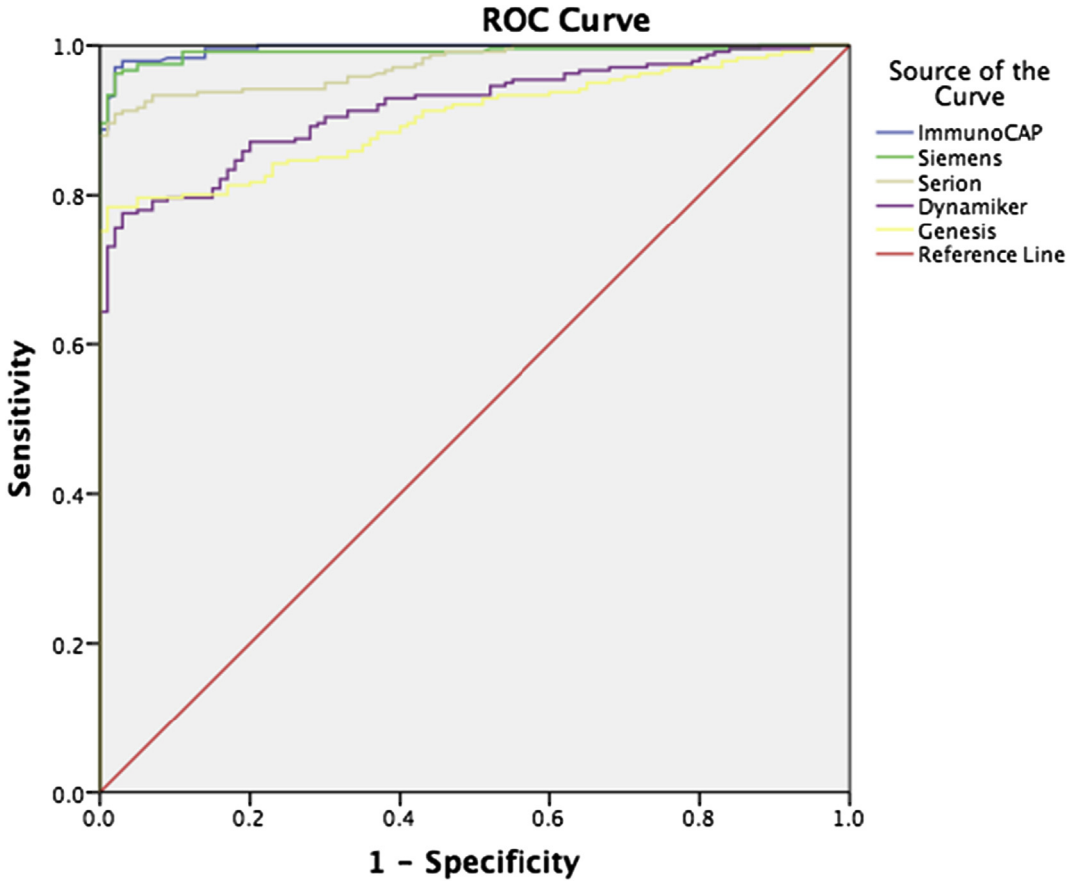

Figure 6 ROC curve for CPA cases vs. healthy controls.

Table 4 Performance of potential diagnostic cut offs.

Assay (Unit)	Diagnostic cut-off	Sensitivity	Specificity	Youden's J statistic
ImmunoCAP	10	100%	86%	0.86
(mg/L)	**20**	**96%**	**98%**	**0.94**
	30	91%	99%	0.9
	40	88%	100%	0.87
Immulite	5	99%	78%	0.77
(mg/L)	**10**	**96%**	**98%**	**0.94**
	15	94%	98%	0.92
	20	93%	99%	0.92
	30	91%	99%	0.9
Serion	30	91%	95%	0.86
(U/ml)	**35**	**90%**	**98%**	**0.88**
	40	88%	99%	0.87
	45	85%	100%	0.85
Dynamiker	60	78%	94%	0.72
(AU/ml)	**65**	**77%**	**97%**	**0.74**
	70	75%	98%	0.73
	75	73%	99%	0.72
Genesis	10	86%	65%	0.51
(U/ml)	15	79%	95%	0.74
	20	**75%**	**99%**	**0.74**
	25	71%	100%	0.71
Precipitins	—	59%	100%	0.59

Optimal diagnostic cut-offs are highlighted in bold.

therapy were slightly higher than those who received no antifungal therapy, indicating that this relatively short course of antifungal treatment did not introduce bias by reducing *Aspergillus*-specific IgG levels.

Our data suggests that existing cut-offs are sub-optimal for the diagnosis of CPA and probably require revision. We do not recommend reporting intermediate results or re-testing, as antibody levels are likely to be stable in CPA. We have therefore suggested single diagnostic cut-offs for CPA.

For ImmunoCAP our analysis suggests a cut-off of 20 mg/L is optimal. This is a reduction on the currently accepted cut-off of 40 mg/L, which was derived from a single study involving 10 patients with CPA.[14] We have not measured levels in European healthy controls, however our proposed cut-off is higher than the median ImmunoCAP levels found in Dutch blood donors (8.75 mg/L) and female Belgian laboratory workers (13.75 mg/L) in previous studies.[14,17] For Serion we suggest a diagnostic cut-off of 35 AU/ml, which is above the median level in pregnant French women (20 AU/ml).[15] There are no previous published studies for Genesis or Dynamiker. Our proposed cut-off for Genesis (20 U/ml) is higher than the manufacturer's suggested cut-off (12 U/ml). For Dynamiker our suggested cut-off (65 U/ml) is also slightly higher than the manufacturer's suggested cut-off (60 U/ml).

Our analysis produces a diagnostic cut off of 10 mg/L for Siemens Immulite. This is the first proposed cut-off for this assay as the manufacturer does not provide a cut-off and

there are no prior published studies on which to base a cut-off. The median Immulite value in our healthy Ugandan controls was 4 mg/L, but the level in Dutch blood donors in a prior study was higher than our cut-off at 13.2 mg/L.[17] This significant difference might be explained by different levels of *Aspergillus* exposure in these different environments or differences in the median age of the healthy control groups.

We chose to use healthy Ugandan blood donors as a control group. A key goal of this study was to select an assay for use in a CPA prevalence survey to be conducted in Uganda and our proposed cut-offs are appropriate for use in this context. It is reassuring that the median levels of ImmunoCAP and Serion assays in European healthy control cohorts are below our suggested cut-offs, implying that our cut-offs for these assays are also appropriate for use in a European population.

However, direct comparisons to well-matched European controls are probably required before the Immulite assay can be used with confidence in a European population. Further comparisons for all assays to locally appropriate control groups remain desirable as levels of *Aspergillus* exposure might vary from one environment to another.

The comparison to healthy controls is appropriate given that the role of serological testing in the diagnosis of CPA is to fulfil the 'microbiological evidence' aspect of a composite diagnostic criteria.[21] In this context the *Aspergillus*-specific IgG test is acting as a proxy marker for abnormal *Aspergillus* growth in the airways and any residual cavities. The control group that best represents 'normal' levels of immune response to ubiquitous *Aspergillus* exposure is healthy controls. This is especially true in the context of diagnosing CPA in patients fully treated for pulmonary tuberculosis, who should not have any ongoing active lung disease.

CPA also occurs in patients with ongoing active chronic lung diseases, such as COPD or sarcoidosis.[21,27] Excess *Aspergillus* growth may be common in the diseased airways of these patients and this may be accurately reflected in frequently raised levels of *Aspergillus*-specific IgG. Higher levels of *Aspergillus*-specific IgG have been found in cystic fibrosis and treated tuberculosis than in healthy controls.[24,28,29] It is likely that some patients with active chronic lung disease will have raised *Aspergillus*-specific IgG levels in the absence of CPA. Raised levels of *Aspergillus*-specific IgG are also found in a variety of other conditions including invasive aspergillosis, *Aspergillus* bronchitis, *Aspergillus* rhinosinusitis, allergic bronchopulmonary aspergillosis and current or past airways colonization.[15,30–32] This is why the diagnosis of CPA requires clinical and radiological evidence in addition to serological evidence.

Further studies to define the frequency of different forms of *Aspergillus*-associated disease and associated levels of *Aspergillus*-specific IgG in patients with these conditions and other chronic lung diseases are now required. These will complete our understanding of the interpretation of this test in these populations. However the cut-off produced by comparison of diseased cases to healthy controls will continue to provide the basic definition of 'abnormal' antibody levels for use in case definitions.

We have utilised a large group of sera from CPA cases who were not on long-term antifungal therapy at the time of sampling. These were compiled over a 10 year period at a national referral centre. This allows us to produce the first statistically meaningful assessment of diagnostic cut-offs and comparative efficacy of the assays. However a drawback of accessing sera banked over such a long period is that the understanding of CPA has evolved over this time. As a result case definition for CPA we have used does not perfectly match the prior criteria by Denning et al.[12] or the more recent ESCMID guidelines.[21] However the differences involved are minor and should not alter our basic conclusions.

The most common 'microbiological evidence' used to diagnose CPA in our clinical cohort was raised *Aspergillus*-specific IgG. As a result it is not surprising that all *Aspergillus*-specific IgG assays demonstrated good sensitivity. Unfortunately there is no gold standard test for CPA to compare assay performance against and comparison of test results in clinically defined cohorts remains the best option available and as such has been used in all CPA existing diagnostic studies.[14,16,17]

The ImmunoCAP and precipitins assays were routinely used for clinical testing in our reference laboratory throughout the study period. The Immulite, Serion, Genesis and Dynamiker assays are not used routinely at any UK diagnostic laboratory. This may result in some selection bias in favour of ImmunoCAP and precipitins. This bias might be removed by conducting a prospective study identifying new cases of CPA in at risk groups, but the uncommon nature of CPA makes such a study extremely challenging to perform. We note that this potential bias did not prevent the Siemens assay from demonstrating equal ROC AUC performance to the ImmunoCAP assay.

We have described the diagnostic performance of six *Aspergillus*-specific IgG assays for the diagnosis of CPA, probably the most common form of pulmonary aspergillosis, a poorly researched global public health problem. These validated assays can now be used with greater confidence in studies to measure the prevalence of CPA.

The Immulite and ImmunoCAP assays performed so well that screening for the earliest signs of CPA after tuberculosis becomes possible.[29] This is likely to yield a major health benefit with early diagnosis and treatment of CPA. If the predicted prevalence of CPA is confirmed, significant effort will be required to expand access to *Aspergillus*-specific IgG testing, which may require the development of new point-of-care test formats.

Ethics

Control samples were acquired as part the 'Pulmonary aspergillosis in association with tuberculosis' study. Ethical approval was granted by Gulu University IRB (ref GU/IRC/04/07/12), the Ugandan National Council for Science and Technology (ref HS1253) and the University of Manchester (ref 11424). Stored serum was taken from samples provided by CPA patients for the purpose of *Aspergillus*-specific IgG testing as part of routine care of CPA and ABPA at the National Aspergillosis Centre, Manchester, UK. Further stored

serum samples were acquired from the ManRAB biobank. Ethical approval was granted by the ManRAB REC committee (ref 10/H1010/7).

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The funders played no role in the design of the study or any aspect of the interpretation of results or write up of the paper.

Conflicts of interest

Dr. Page has received grant support from Astellas in addition to the funding for this study described above.

Prof. Malcolm Richardson lectures on behalf of, and provides educational material and advice for Gilead Sciences Europe, Astellas Pharma. MSD and Pfizer.

Prof. Denning holds Founder shares in F2G Ltd a University of Manchester spin-out antifungal discovery company, in Novocyt which markets the Myconostica real-time molecular assays and has current grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health Research, North West Lung Centre Charity, Medical Research Council, Astellas and the Fungal Infection Trust. He acts as a consultant to Trinity group, T2 Biosystems, GSK, Sigma Tau, Oxon Epidemiology and Pulmicort. In the last 3 years, he has been paid for talks on behalf of Astellas, Gilead, Merck and Pfizer. He is also a member of the Infectious Disease Society of America Aspergillosis Guidelines and European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines groups.

Preliminary versions of these results were presented as abstracts at the following conferences:

1 — "Comparative efficacy of five *Aspergillus*-specific IgG ELISAs for the diagnosis of chronic pulmonary aspergillosis (CPA)" poster presentation at the 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2015), 25—28 April 2015, Copenhagen, Denmark.

2 — "Comparative efficacy of five *Aspergillus*-specific IgG ELISAs for the diagnosis of chronic pulmonary aspergillosis (CPA)" poster presentation at the 19th Congress of the International Society for Human and Animal Mycology (ISHAM 2015), 4—8 May 2015, Melbourne, Australia.

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We would like to thank the staff at Manchester Royal Infirmary immunology laboratory for their assistance in performing ImmunoCAP *Aspergillus*-specific IgG testing on control samples.

We would like to thank the staff at Christie Hospital pathology laboratory, Manchester for permitting access to their Siemens Immulite 2000 system to perform this study.

We would like to thank Siemens, Serion, Genesis and Dynamiker for kindly donating test kits to perform this study and for their practical assistance in installing the test kits and relevant software prior to undertaking the study.

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Appendix 1. *Aspergillus*-specific IgG levels in CPA patients with and without antifungal therapy

Test	Median *Aspergillus*-specific IgG level in those with <3 months antifungals n = 42	Median *Aspergillus*-specific IgG level in those not on antifungals n = 199	p-value
ImmunoCAP	130 mg/L	125 mg/L	0.375
Immulite	533 mg/L	250 mg/L	0.051
Serion	143 U/ml	125 U/ml	0.372
Genesis	90 U/ml	47 U/ml	0.006
Dynamiker	141 AU/ml	119 AU/ml	0.230

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