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Receiver operating characteristic curve analysis of four *Aspergillus*-specific IgG assays for the diagnosis of chronic pulmonary aspergillosis

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ABSTRACT

Measurement of *Aspergillus*-specific IgG is central to the diagnosis of chronic pulmonary aspergillosis (CPA), but manufacturers' guidance on test interpretation is based on unpublished data. We performed the first receiver operating characteristic (ROC) area under the curve (AUC) analysis to identify optimal cut-offs for this test in relation to European controls. *Aspergillus*-specific IgG levels were measured in sera from British adults with CPA and European healthy controls by ImmunoCAP, Immulite, Serion and Bio-Rad assays. ROC AUC analysis was performed to identify optimal cut-offs. ROC AUC results were; Bio-Rad 0.955, Immulite 0.948, ImmunoCAP 0.956 and Serion 0.944. Optimal diagnostic cut-offs were 1.5 AU/mL for Bio-Rad (93% sensitive, 98% specific), 25 mg/L for Immulite (93% sensitive, 99% specific), 50 mg/L for ImmunoCAP (84% sensitive, 96% specific) and 50 U/mL for Serion (84% sensitive, 91% specific). These cut-offs differ from manufacturers' guidance and from those previously calculated in relation to Ugandan controls.

1. Introduction

Chronic pulmonary aspergillosis (CPA) is a serious condition that can complicate many pulmonary disorders. It is estimated to afflict 1–3 million persons worldwide (Denning et al., 2011, 2013a, 2013b). *Aspergillus*-specific IgG is the most common source of evidence of infection in CPA and is recommended for use in recently published international guidelines(Denning et al., 2016; Jhun et al., 2013; Ohba et al., 2012; Patterson et al., 2016).

Published data regarding the optimal interpretation of *Aspergillus*-specific IgG in CPA diagnosis are scarce (Richardson and Page, 2017). One study demonstrated that the Bio-Rad (USA) and ImmunoCAP (USA) assays both have markedly superior sensitivity to precipitins (Baxter et al., 2013). Another showed similar performance for Bio-Rad and Serion assays(Guitard et al., 2012). A third study suggested that the Bio-Rad and Bordier (Switzerland) assays both had superior performance than the Serion assay(Dumollard et al., 2016).

These studies reported results in line with manufacturers' instructions or conventional practice, based on unpublished data. Proposed diagnostic cut-offs for the ImmunoCAP and Immulite assays have been published based on analysis of healthy controls alone (van Hoeyveld et al., 2006; van Toorenenbergen, 2012; Watkins et al., 2012), but the performance of these proposed cut-offs in CPA diagnosis has not been described.

A previous study by our group reported ROC curve analysis results comparing 241 CPA patients and 100 Ugandan healthy controls (Page et al., 2016). Optimal diagnostic cut-offs were identified for ImmunoCAP, Immulite, Serion, Dynamiker (China) and Genesis (UK) assays. The performance of the ImmunoCAP and Immulite assays were both significantly superior to all other assays assessed. All ELISAs demonstrated markedly superior sensitivity to precipitins.

These analyses identified optimal cut-offs for use in Uganda. It is not clear whether they are also optimal for use in other settings. To

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Table 1
Patient characteristics

Characteristic	Immulite, ImmunoCAP and Serion CPA patients (n = 241)	ImmunoCAP controls (n = 114)	Serion and Bio-Rad controls (n = 222)
Female gender	101 (42%)	84 (74%)	222 (100%)
Mean age (years)	65	-	-
Age range (years)	23–92	20–63	-
Chronic cavitary pulmonary aspergillosis	238 (99%)	-	-
Aspergillus nodule disease	3 (1%)	-	-
ImmunoCAP *Aspergillus*-specific IgG >40mg/L	211 (88%)	-	-
Positive precipitins	138 (57%)	-	-
Aspergillus fumigatus growth in sputum culture	89 (37%)	-	-
Aspergillus niger growth in sputum culture	3 (0.5%)	-	-
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%)	-	-

our knowledge, no study has assessed the optimal cut-off for an *Aspergillus*-specific IgG ELISA for the diagnosis of CPA by ROC AUC analysis in relation to European controls. One study combined European healthy controls with a small mixed population of pulmonary aspergillosis, but did not specifically report on CPA(van Hoeyveld et al., 2006). Another performed ROC AUC analysis with European controls, but did not assess the optimal cut-offs for the assays involved (Dumollard et al., 2016).

We conducted a collaborative analysis involving teams in Manchester, Leuven, Rotterdam and Paris (Guitard et al., 2012; van Hoeyveld et al., 2006; van Toorenenbergen, 2012). We shared pre-existing data to perform a series of novel ROC AUC analyses comparing *Aspergillus*-specific IgG levels in British CPA cases to various European healthy controls cohorts, to identify optimal cut-offs for the Bio-Rad, Immulite, ImmunoCAP, and Serion assays for use in this setting.

2. Materials and methods

Aspergillus-specific IgG levels were measured by a variety of methods. Sera from 241 CPA patients, who were not on long term antifungal therapy, were stored at the UK National Aspergillosis Centre in Manchester between 2004 and 2014 and retrospectively tested by ImmunoCAP, Immulite 2000 and Serion *Aspergillus fumigatus*-specific IgG assays in 2014 as previously described (Page et al., 2016). Sera from 118 patients with CPA were tested in the same lab by the Bio-Rad Platelia *Aspergillus* IgG kit, which uses a combination of thus far unspecified recombinant *Aspergillus* antigens, in 2010 as previously described (Baxter et al., 2013). However, in this analysis when ImmunoCAP or Immulite analysis produced a result of >200 mg/L a result of 200 mg/L was recorded. When Bio-Rad produced a result of >80 AU/mL a result of 80 AU/mL was recorded.

Sera from 152 healthy Dutch blood donors were tested by the Immulite 2000 assay in 2005 as previously described (van Toorenenbergen, 2012). Sera from 114 healthy laboratory technicians were tested by ImmunoCAP assay in 2007 in line with manufacturer's instructions at University Hospitals, Leuven, Belgium. Sera from 222 healthy French pregnant women were tested in 2010 by Serion and Bio-Rad assays at Hopital St. Antoine, Paris, France as previously described (Guitard et al., 2012).

Results from CPA cases were compared to healthy controls using ROC AUC analysis. Sensitivity and specificity for a variety of points on the ROC curve were described. Optimal diagnostic cut-offs for each assay were calculated using Youden's J statistic (sensitivity + specificity − 1). 95% confidence intervals for sensitivity and specificity were calculated by Wilson's method. Statistical analyses were performed using SPSS version 20 (IBM, USA).

3. Results

Patient characteristics for the CPA patients are shown in Table 1, together with characteristics for the controls, where available. Specific characteristics of 118 CPA patients tested by Bio-Rad were not recorded, but these patients were recruited from the same clinical cohort as the 241 CPA patients tested by other methods.

ROC curves for the assays are shown in Fig. 1. ROC AUC results and optimal diagnostic cut-offs are shown in Table 2, together with the sensitivity and specificity of the assay at the optimal cut-off and 95% confidence intervals for these figures. The sensitivity and specificity for Bio-Rad, Immulite, ImmunoCAP and Serion assays at various points on the ROC curve are shown in Table 3, with optimal results highlighted.

4. Discussion

We have performed the first ROC AUC analysis comparing *Aspergillus*-specific IgG levels in a large group of patients with CPA to healthy European controls. We have identified optimal cut-offs for CPA diagnosis for each assay based on the ROC AUC results. This represents the most robust attempt to date to calculate cut-offs for use in the diagnosis of CPA in a developed region with a temperate climate and includes the first published analysis of the optimal cut-off for the Bio-Rad assay.

The cut-off identified for Bio-Rad (1.5 AU/mL) is lower than the manufacturer's recommended cut-offs of 5-10 AU/mL for intermediate and >10AU/mL for positive results. This change is associated with a significant improvement in sensitivity, while maintaining excellent specificity (Table 3).

The cut-off identified for ImmunoCAP (50 mg/L) differs from those previously calculated in relation to mixed aspergillosis populations or from healthy controls alone (35–70 mg/L) (van Hoeyveld et al., 2006; van Toorenenbergen, 2012; Watkins et al., 2012). These differences are also likely to be clinically significant (Table 3).

The cut-off we identified for Immulite (25 mg/L) is similar to the upper reference values reported by the manufacturer (21.4 mg/L) and in our previous analysis of healthy controls alone (19.3 mg/L) (van Toorenenbergen, 2012). Our analysis suggests that these cut-offs all have similar diagnostic performance (Table 3).

Our analysis produced higher cut-offs than our previous paper comparing the same 241 CPA cases to Ugandan controls (Page et al., 2016). The ImmunoCAP cut-off increased from 20 mg/L to 50 mg/L, the Serion cut-off from 35 U/mL to 50 U/mL and the Immulite cut-off increased from 10 mg/L to 25 mg/L. The previously calculated cut-offs performed poorly in relation to European controls. For Immulite a 10 mg/L cut-off was associated with 5.3% specificity in this context.

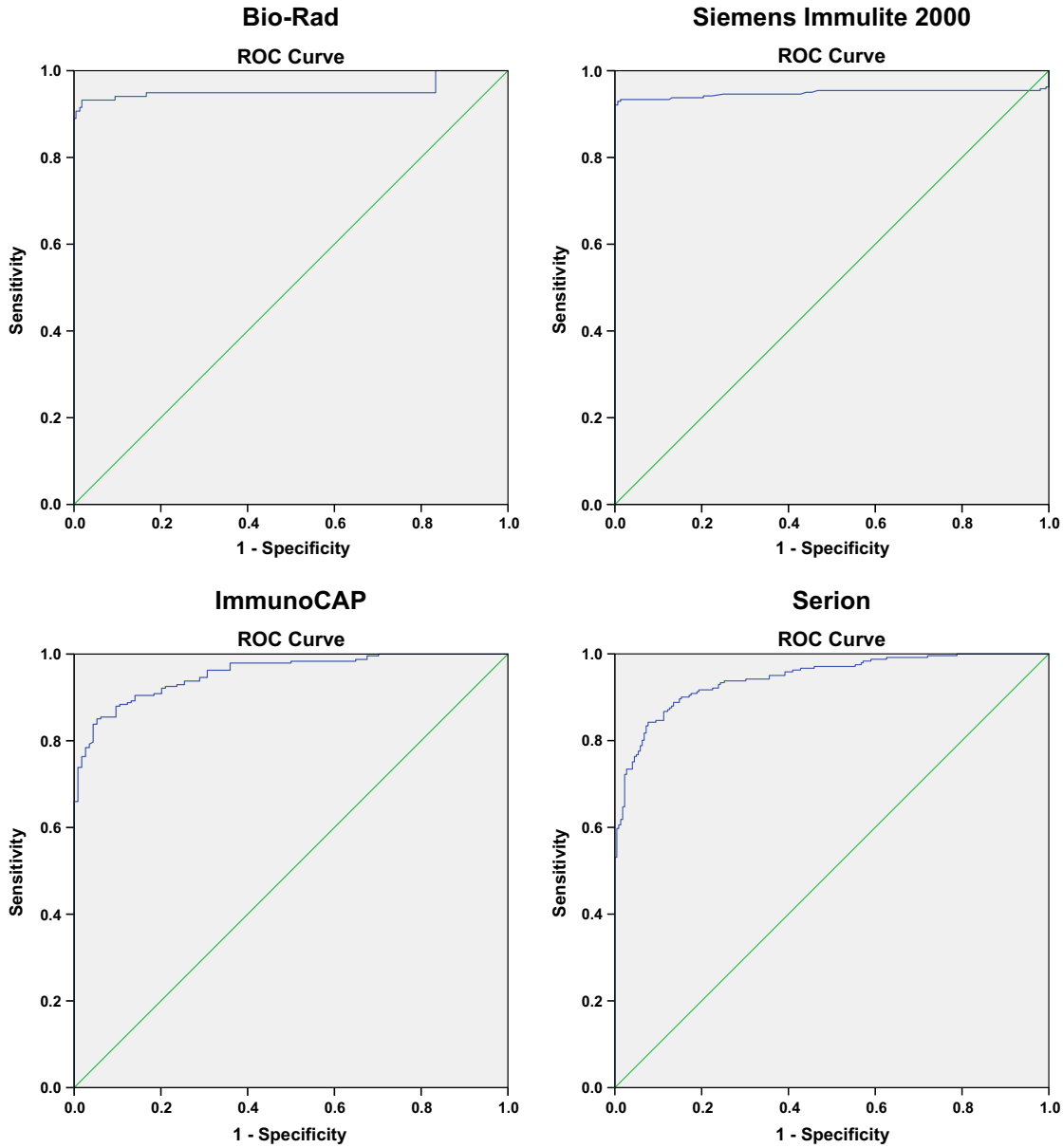

Fig. 1. ROC curves for different assays

It is not clear why the analyses with Ugandan and European controls produced different cut-offs. Batch-to-batch variation is not described for these assays, but would be unlikely to result in the consistent pattern of higher antibody levels in European controls identified in each of the three assays across three laboratories.

Ugandan controls had a mean age of 19 years, which is likely to be younger than the laboratory workers, pregnant women and blood donors used as healthy controls in this study. The relationship between *Aspergillus*-specific IgG and age is not well described. *Aspergillus*-specific IgG levels are lower in healthy children than healthy adults (Bardana, 1974). It is not known at what age adult antibody levels are attained. The inclusion of many adolescents in the Ugandan healthy control cohort might have affected results.

Table 2
ROC AUC results and optimal diagnostic cut-offs for CPA

Assay	ROC AUC	95% CI	Optimal diagnostic cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Bio-Rad	0.955	0.922–0.988	1.5 AU/mL	93.2	87.2–96.5	98.2	95.5–99.3
Immulite	0.948	0.921–0.975	25 mg/L	92.9	89.0–95.6	99.3	96.4–99.9
ImmunoCAP	0.956	0.937–0.974	50 mg/L	83.8	78.6–87.9	95.6	90.1–98.1
Serion	0.944	0.925–0.964	50 U/mL	84.2	79.1–88.3	91	86.5–94.1

Table 3
Assay performances at various points on the ROC AUC curve

Cut-off	Sensitivity (%)	Specificity (%)	Youden's J statistic
Bio-Rad (AU/mL)			
0.5	94.1	83.3	0.774
0.75	93.2	91	0.842
1	93.2	93.7	0.869
1.5	**93.2**	**98.2**	**0.914**
2	91.5	98.6	0.901
5	89.8	99.5	0.893
10	85.6	1	0.856
Immulite (mg/L)			
10	95.4	5.3	0.007
15	94.6	73	0.676
20	93.4	98.7	0.921
25	**92.9**	**99.3**	**0.922**
30	90.9	100	0.909
35	89.6	100	0.896
40	87.6	100	0.876
ImmunoCAP (mg/L)			
10	98.8	32.5	0.313
20	96.3	66.7	0.63
30	91.3	79.8	0.711
40	86.7	90.4	0.771
50	**83.8**	**95.6**	**0.794**
60	78.4	96.5	0.749
70	73.9	98.2	0.721
Serion (U/mL)			
30	91.3	80.6	0.719
35	90.5	82.4	0.729
40	88	86.5	0.745
45	84.6	89.6	0.742
50	**84.2**	**91**	**0.752**
55	81.7	92.8	0.745
60	78	94.1	0.721

Environmental exposure to *Aspergillus* might vary between rural Uganda and Europe. The species epidemiology of aspergillosis in Uganda has not been described, but *A. niger* is common elsewhere in Africa (Meawed et al., 2012; Oladele et al., 2017; Osman et al., 2013). Our study includes *Aspergillus fumigatus*-specific IgG assays, but serological cross-reactivity between species does not always occur (Chaparas et al., 1980). Helminth and schistosomiasis infections are common in northern Uganda (Ashton et al., 2011; Kabatereine et al., 2007) and are known to impact on immune function (Haseeb and Craig, 1997; Kamal and El Sayed Khalifa, 2006; Muniz-Junqueira et al., 1996). The impact on *Aspergillus*-specific IgG production has not been described.

Our study is not designed to provide head-to-head comparison of assay performance. We describe results of a novel retrospective analysis of existing data from studies performed separately at a variety of centers to identify optimal cut-offs for individual assays for use in Europe. Head-to-head comparisons of assay performance in single cohorts are described elsewhere (Baxter et al., 2013; Dumollard et al., 2016; Guitard et al., 2012; Page et al., 2016).

Ninety-nine percent of patients in our CPA cohort had chronic cavitary pulmonary aspergillosis (CCPA). It is not clear whether cut-offs derived from this study are also optimal for uncommon related conditions such as *Aspergillus* nodules or sub-acute invasive pulmonary asperillosis (Muldoon et al., 2016; Nam et al., 2010). It is, however, probably reasonable to use them until individual studies can be performed for each condition.

We report cut-offs calculated in relation to healthy controls, but over 85% of CPA occurs in patients with underlying disease (Smith and Denning, 2011). An ideal study would therefore describe cut-offs in relation to diseased controls. Recent studies describe attempts to achieve this for CPA and allergic bronchopulmonary aspergillosis (ABPA) (Agarwal et al., 2017; Dumollard et al., 2016). Interpretation of these studies is challenging as they do not describe whether or by what method control cohorts were screened for cases of CPA/ABPA. The optimal study would involve diseased controls systematically and effectively screened to remove cases of CPA. However, we believe analysis involving healthy controls remains the best available option until this is achieved.

CPA is predicted to affect up to 3 million people worldwide. Accurate interpretation of *Aspergillus*-specific IgG levels is likely to be a prerequisite to identifying and treating these predicted cases. Our studies represent the most robust attempts to date to identify optimal cut-offs for the assays for use in Europe and Africa. Further work is now needed to explain the discrepancies between these studies and to determine whether different cut-offs are needed in different geographical regions or in relation to different underlying conditions or manifestations of aspergillosis.

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Conflict of interest statements

Iain Page received kit donations from Siemens and kit donations a grant from Serion in relation to this study. He has also received kit donations from Genesis and Dynamiker and grant funding from Astellas and Dynamiker for related studies.

Caroline Baxter has received travel grants from Merck and Pfizer and been paid for talks on behalf of Astellas. She has acted as an advisor to Gilead and Basilea.

Christophe Hennequin has been paid for talks by MSD, Basilea and Astellas. During the last 5 years he received grants from Basilea and Pfizer for research purposes.

Malcolm Richardson is a Consultant for Astellas, Gilead Sciences, Dynamiker, MSD, Pfizer and Basilea. He is a member of the European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines group.

Erna van Hoeyveld reports no conflicts of interest.

Albert van Toorenenbergen reports no conflicts of interest.

David Denning and family hold Founder shares in F2G Ltd, a University of Manchester spin-out antifungal discovery company, in Novacyt which markets the Myconostica real-time molecular assays. He acts or has recently acted as a consultant to Astellas, Sigma Tau, Basilea, Scynexis, Cidara, Biosergen, Quintiles, Pulmatrix and Pulmocide. In the last 3 years, he has been paid for talks on behalf of Astellas, Dynamiker, Gilead, Merck and Pfizer. He is a longstanding member of the Infectious Disease Society of America Aspergillosis Guidelines group, the European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines group and the British Society for Medical Mycology Standards of Care committee

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Siemens and Serion donated test kits for testing of CPA cases in Manchester. Serion and Dynamiker provided funding for laboratory consumables in Manchester. Financial support was given by Bio-Rad for testing performed in France. No funders had any role in the study design, data collection, analysis or decision to submit for publication.

Ethical approval

South Manchester Research Ethics Committee approved testing of stored serum from CPA patients by Bio-Rad assay in June 2007 (REC reference number 07/Q1403/70). Stored sera from CPA patients tested by ImmunoCAP, Immulite and Serion assays were acquired from the ManRAB biobank and from samples provided for the purpose of *Aspergillus*-specific IgG testing as part of routine care at the National Aspergillosis Centre, Manchester, UK. The South Manchester Research

Ethics Committee granted ethical approval in December 2013 (REC reference number 10/H1010/7). Testing of healthy control samples was performed on leftover samples and analysed in an anonymous fashion in line with local regulations in Belgium, the Netherlands and France.

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