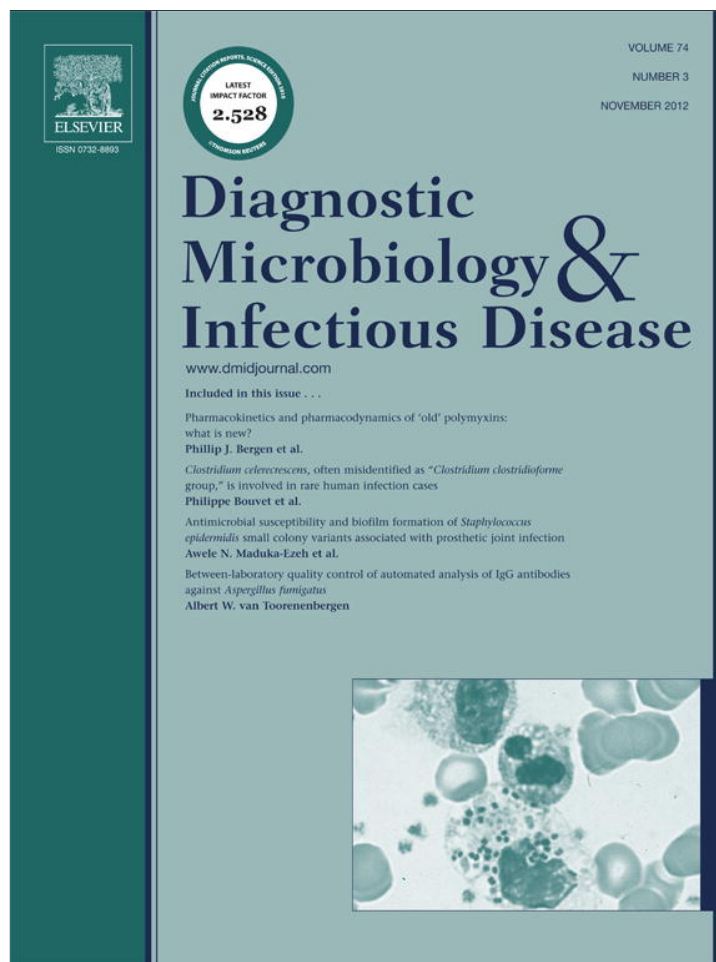


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Between-laboratory quality control of automated analysis of IgG antibodies against *Aspergillus fumigatus*[☆]

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ABSTRACT

Measurement of IgG antibodies against *Aspergillus fumigatus* is an important criterion for the diagnosis of aspergilloma, allergic bronchopulmonary aspergillosis, and extrinsic allergic alveolitis. In the present study, we compared IgG antibody analysis against *A. fumigatus* using 2 widely used automated immunochemistry systems. In a between-laboratory quality control program, good agreement was found between the results from laboratories that used the ImmunoCAP system; however, a laboratory that used the Immulite 2000 system found approx. 2-fold higher antibody levels in the quality control samples than did the ImmunoCAP system. Measurements of IgG against *A. fumigatus* in patient sera were significantly correlated ($r_s = 0.77$, $P < 0.0001$). These results demonstrate that analysis of IgG antibodies against *A. fumigatus* with these 2 systems has reached a level of standardization that allows for direct comparison of quantitative results from different laboratories. For longitudinal analysis of IgG against *A. fumigatus*, reagents from the same manufacturer should be used.

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1. Introduction

Measurement of IgG antibodies against the mold *Aspergillus fumigatus* is an important criterion for the diagnosis of bronchial aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), and mold-induced extrinsic allergic alveolitis (Stevens et al., 2000). In nonneutropenic patients with invasive aspergillosis, the detection of IgG antibodies is thought to be the best noninvasive means of establishing the diagnosis of subacute invasive aspergillosis (Hope et al., 2005). Furthermore, IgG antibody testing in patients with hematologic malignancies prior to their becoming immunocompromised has a predictive value for development of invasive aspergillosis (Sarfati et al., 2006).

Initially, antibodies against *A. fumigatus* were determined using the double immunodiffusion (DID) technique according to Ouchterlony or with the immunoelectrophoresis technique (Barton, 2010; Longbottom and Pepys, 1964). However, these methods are time consuming, labor intensive, require relatively large amounts of *A. fumigatus* extract and patient serum, and give at best only semiquantitative results. At present, the DID technique has been largely replaced by the enzyme-linked immunosorbent assay (ELISA), which is a considerably more rapid and sensitive technique that produces quantitative results with significantly less *A. fumigatus* extract and patient serum per test, and is easily automated (Barton,

2010). These benefits stimulated many laboratories to develop their own in-house ELISA for *A. fumigatus*-specific IgG antibodies (Barton, 2010; Kauffman et al., 1983; Sepulveda et al., 1979), later followed by a number of commercial suppliers. Because each of these assays expressed its results in different, arbitrarily chosen quantitative units, and because the *A. fumigatus* preparations used were not standardized, comparison of results between laboratories was difficult.

The ImmunoCAP system (Phadia, Nieuwegein, Netherlands) is a widely used ELISA technique for automated analysis of specific IgE antibodies. This system uses standardized allergen extracts covalently coupled to a solid phase. Meier and Müller (1998) described the use of this ImmunoCAP system for the assay of venom-specific IgG antibodies during venom immunotherapy. Kränke et al. (2001) used ImmunoCAP allergens produced for analysis of specific IgE antibodies for measurement of IgG antibodies against *A. fumigatus* and several other molds. Subsequently, the manufacturer (Phadia) introduced ImmunoCAP allergens certified for analysis of specific IgG antibodies against these antigens. Since then, the results of this ELISA for IgG antibodies have been compared with clinical data and with results of the Ouchterlony test against these antigens in a number of studies (Barton et al., 2008; Makkonen et al., 2001; Van Hoeyveld et al., 2006).

Another widely used system for automated analysis of IgE antibodies is the Immulite 2000 system (Siemens, Breda, Netherlands) (Ollert et al., 2005). This analyzer also facilitates the measurement of specific IgG antibodies. In the present study, we compared the analysis of IgG antibody against *A. fumigatus* on these 2 automated immunochemistry analyzers and describe their performance in a between-laboratory quality control program.

[☆] Conflict of interest: None.

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2. Methods and sera

2.1. Methods

Routine analysis of specific IgG against *A. fumigatus* was performed every week with the ImmunoCAP 100 system or its enlarged version, the ImmunoCAP 250 system (from 2007 onwards), according to the manufacturer's instructions (Phadia). Results are expressed in milligrams of antigen-specific IgG per liter (mg_A/L). The calibration for antigen-specific IgG analysis (Yman, 2001) follows the same principle as the heterologous calibration that is used for allergen-specific IgE analysis (Plebani, 2003; Yman, 2001). Analysis of specific IgG against *A. fumigatus* with the Immulite 2000 system was performed according to the manufacturer's instructions (Siemens). The Immulite 2000 also uses the heterologous standardization principle for quantitation of IgG antibodies against *A. fumigatus*.

2.2. Sera

After routine analysis of specific IgG against *A. fumigatus* with the ImmunoCAP system, sera were frozen at -20°C . For use as between-run quality control samples, selected sera were pooled and refrozen after aliquoting. Moreover, every year new serum pools that contained various levels of IgG antibodies against *A. fumigatus* were prepared for use in an external quality control scheme. These serum pools were composed of approx. 25 individual sera. NaN_3 was added as a preservative at a final concentration of 0.01%. Samples ($n = 3$) were distributed together each year to a number of laboratories in Belgium and the Netherlands by SKML, section for humoral immunology, type III allergy (<http://www.skml.nl>). Furthermore, 70 sera with *A. fumigatus*-specific IgG levels evenly distributed over the range 0–200 mg_A/L by analysis with the ImmunoCAP system were selected for analysis on the Immulite 2000.

The Institutional Review Board of Erasmus University Medical Center approves the use of leftover samples for quality control purposes.

2.3. Statistical analysis

GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis of results.

Outliers were assessed with the Grubbs' test (<http://www.graphpad.com/quickcalcs>).

3. Results

3.1. Reference values

After analysis of 30 sera collected from healthy blood donors in 2003, a mean value of 13.16 mg_A/L was found, with a standard deviation of 14.27 mg_A/L . The median value was 8.75 mg_A/L . Statistical analysis showed that the results were not normally distributed. Therefore the upper 97.5% level was estimated by nonparametric statistical analysis at 35 mg_A/L . At a level of 62 mg_A/L of anti-*A. fumigatus* IgG antibodies, the between-run coefficient of variation (CV) was 8.1% for routine assay runs conducted during 2006 using the ImmunoCAP 100. At a level of 92 mg_A/L of anti-*A. fumigatus* IgG antibodies, the between-run CV was 12.9% for routine assay runs conducted during 2008 using the ImmunoCAP 250.

In 2005, 152 sera from healthy blood donors were tested for IgG antibodies against *A. fumigatus* using the Immulite 2000 system. A mean value of 13.71 mg_A/L was found with a standard deviation of 2.68 mg_A/L . The median value was 13.20 mg_A/L . Analysis with the EP Evaluator software (Siemens) gave an upper reference value of 19.3 mg_A/L . At a level of 9.7 mg_A/L , the between-run CV was 10.0%.

3.2. Between-laboratory results

Fig. 1 shows the results of analyses conducted with ImmunoCAP and Immulite 2000 on 12 different serum pools received during 2008, 2009, 2010, and 2011 by laboratories in Belgium and the Netherlands. All participants that used the ELISA technique correctly identified the sera with the lowest and highest level of IgG antibodies against *A. fumigatus*. In 2008, 2009, 2010, and 2011, 20, 22, 19, and 20 laboratories, respectively, used the ImmunoCAP system for analysis of anti-*A. fumigatus* IgG. After exclusion of outliers, the between-laboratory CVs for participants that used the ImmunoCAP system were 13.7%, 10.8%, and 9.8% for QC samples 2008A, 2008B, and 2008C, respectively; 16.6%, 12.9%, and 13.3% for QC samples 2009A, 2009B, and 2009C, respectively; 16.3%, 11.7%, and 18.1% for QC samples 2010A, 2010B, and 2010C, respectively; and 12.8%, 7.3%, and 11.4% for QC samples 2011A, 2011B, and 2011C, respectively.

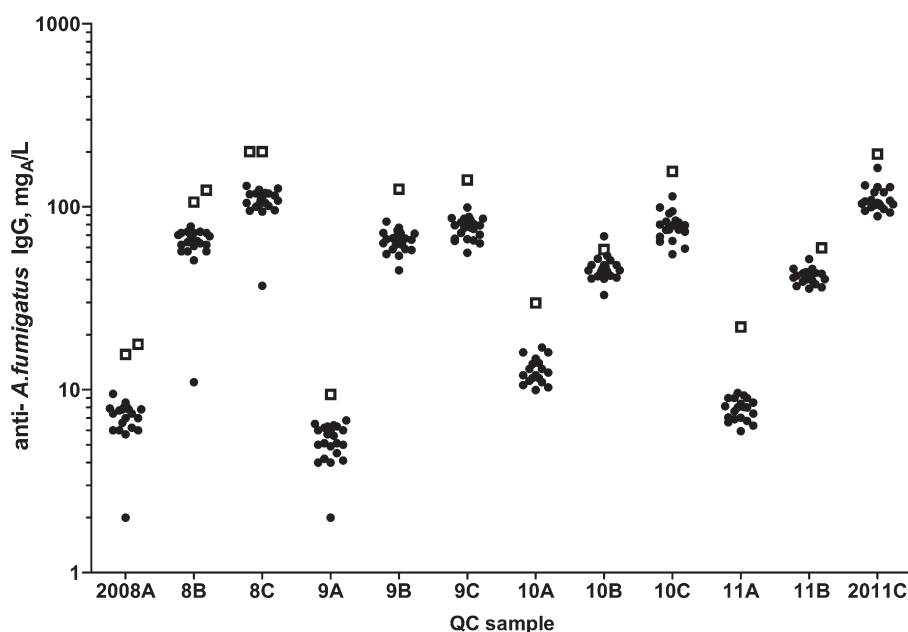


Fig. 1. Between-laboratory results for analysis of anti-*A. fumigatus* IgG in quality control samples. Closed circles: ImmunoCAP system. Open squares: Immulite 2000 system.

In 2008, 2 laboratories, and in 2009, 2010, and 2011 only 1 laboratory, used the Immulite 2000 system for analysis of anti-*A. fumigatus* IgG. Fig. 1 shows that the results obtained with the Immulite 2000 were approx. 2-fold higher than the mean values obtained with the ImmunoCAP system. The mean ratio (Immulite 2000 result divided by the corresponding mean ImmunoCAP result) was 1.78 (SD 0.33) with a significant correlation ($r_s = 0.986$, $P < 0.0001$) between these 2 variables.

In 2008, 2009, 2010, and 2011, 5, 3, 2, and 2 laboratories, respectively, used the Ouchterlony technique. Seven of 12 results with a C-sample and 2 of 12 results with a B-sample were reported as positive in the Ouchterlony test; all A-samples scored negative.

3.3. Comparison of individual sera

A total of 70 sera, which were evenly distributed over the range 0–200 mg_A/L based on analysis with the ImmunoCAP system, were analyzed in a single run for IgG antibodies against *A. fumigatus* on the Immulite 2000. Twelve sera scored >200 mg_A/L and were reanalyzed after dilution. Fig. 2 shows a comparison between the results of the ImmunoCAP system and those of the Immulite 2000 system. A significant correlation was found between both quantitative measurements (Spearman rank analysis: $r_s = 0.77$, $P < 0.0001$). The average level of specific IgG against *A. fumigatus* was 65.2 mg_A/L, when analyzed with the ImmunoCAP system, and 118.2 mg_A/L, when analyzed with the Immulite 2000. The upper reference values for the ImmunoCAP and Immulite 2000 are depicted as vertical and horizontal lines in Fig. 2. Chi-square analysis of the distribution of results over the 4 sections in Fig. 2 also demonstrated a significant correlation between the 2 assays ($P < 0.0001$). The analysis of 5 sera with strongly divergent results between the 2 assays was repeated, and, essentially, the same results were obtained in the second run (Table 1).

4. Discussion

A. fumigatus preparations have been particularly difficult to standardize. Longbottom and Pepys (1964) concluded that variations in the antigens in different cultures of *A. fumigatus* made it desirable to use a battery of several extracts for serologic testing. English and Henderson (1967) noted that the antigenic extracts that were commercially available at that time were complex and variable, and that each yielded different precipitation patterns in the Ouchterlony test. Vailes et al. (2001) showed that *A. fumigatus* extracts from different manufacturers and different lots from the same company

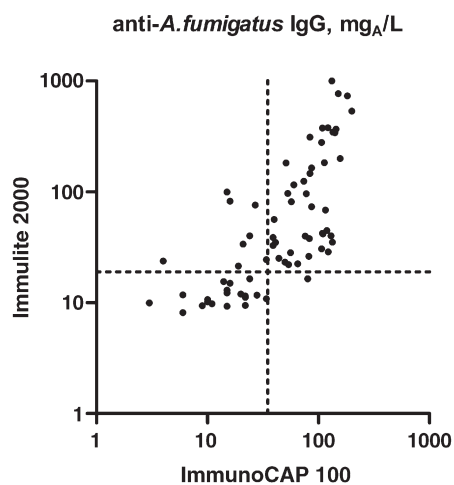


Fig. 2. Measurement of anti-*A. fumigatus* IgG in patient sera by Immulite 2000 and ImmunoCAP systems. Upper reference values are indicated by dashed lines.

Table 1

Repeated analysis of 5 sera with strongly divergent results between analysis with the ImmunoCAP 100 and the Immulite 2000 system.

Sample	ImmunoCAP 100		Immulite 2000	
	Assay run 1	Assay run 2	Assay run 1	Assay run 2
	Anti- <i>Aspergillus fumigatus</i> IgG in serum, mg _A /L			
a	15	16	100	103
b	123	108	29	30
c	16	16	82	75
d	80	70	16	16
e	27	29	76	70

showed large variations in their levels of Asp f1, which is a major allergen in *A. fumigatus*.

The use of standardized *A. fumigatus* preparations in the ImmunoCAP and Immulite 2000 systems should make it possible to compare results of IgG antibody analyzed between different laboratories. Indeed, the results depicted in Fig. 1 show good agreement for study participants who used the ImmunoCAP system. A further step toward standardization might be the use of recombinant *A. fumigatus* antigens for IgG antibody analysis on the ImmunoCAP and Immulite 2000 systems, as has already occurred for IgE antibody analysis (Delhaes et al., 2010). Technically, such an analysis is available on the ImmunoCAP system (Directions for use of the ImmunoCAP specific IgG assay; code 52-5293-EN/02, dated 2011-Dec-10, <http://www.phadia.com>). Furthermore, (<http://www.bio-rad.com>) Bio-Rad has developed the Platellia *Aspergillus* IgG ELISA, based on the use of an *A. fumigatus* recombinant antigen; the exact nature, however, of this recombinant protein was not disclosed (Guitard et al., 2012).

The ratio (Immulite 2000 result divided by the average ImmunoCAP result) remained fairly constant during the 4-year quality control period (Fig. 1). This suggests that the manufacturers of both systems have indeed developed a reproducible in-company standardization procedure for the assay of anti-*A. fumigatus* IgG.

In our hands, the between-run CV was 8.1% for the ImmunoCAP 100 system and 12.9% for the ImmunoCAP 250 system, whereas Van Hoeyveld et al. (2006) reported between-run CV values of up to 23%, and Barton et al. (2008) noted a between-run CV of <5%.

The variability of the *A. fumigatus* extracts (English and Henderson, 1967; Vailes et al., 2001) may explain the poor performance of the Ouchterlony test in the between-laboratory quality control program: no precipitins were found in 40% of the tests conducted on sera with a high level of anti-*A. fumigatus* IgG antibodies. Quality control testing of bulk mold extracts include tests for sterility, safety (toxicity in animals), pH, protein nitrogen units, and preservative (phenol or glycerine) (Esch, 2004). Additional analysis of the amount of IgG-binding antigens in the extracts may be needed for better performance in the Ouchterlony test. This issue is further complicated by the presence of nonprecipitating antibodies against *A. fumigatus* in some patients with aspergillosis (Kauffman et al., 1983). Three of the 5 laboratories that used the Ouchterlony test in 2008 had abandoned the technique in 2010.

Although a significant correlation existed, considerable scatter was observed in a comparison of individual sera (Fig. 2). Some samples with strongly divergent results were retested, but the same results were obtained upon retesting (Table 1). This indicates that despite the in-company standardization achieved by both manufacturers, a difference remains between the components of their reagents used for specific IgG antibody analysis. Diversity of the *A. fumigatus* extracts (Esch, 2004), the solid-phase matrix, and the method of immobilization involved in the preparation of the *A. fumigatus*-containing reagent used in each of the assays (Hamilton et al., 2008) may be an explanation for the interassay difference. Therefore, reagents from the same manufacturer should be used for longitudinal analysis of IgG against *A. fumigatus*.

The Immulite 2000 system reported approx. 2-fold higher antibody levels in the 12 quality control samples (which were composed of ~25 individual sera) than the ImmunoCAP system (Fig. 1), with a mean ratio of 1.78. In close agreement with this result, a ratio of 1.81 was found when the average level of specific IgG against *A. fumigatus* in 70 individual sera (Fig. 2) after analysis with the Immulite 2000 (118.2 mg_A/L) was divided by the average level (65.2 mg_A/L), when analyzed with the ImmunoCAP system. Hamilton et al. (2008) found 3.3-fold higher levels of anti-timothy grass pollen IgG with the Immulite 2000 system, compared with the ImmunoCAP system, in sera from hay-fever patients undergoing grass pollen immunotherapy.

Users of standardized ImmunoCAP reagents have reported different reference values for IgG against *A. fumigatus*. The upper reference value of 35 mg_A/L used in this laboratory was based on analysis of sera from 30 healthy blood donors. Results described by Saito et al. (2010) after testing 40 patients with pulmonary diseases other than aspergillosis or bird fanciers' lung add up to an upper reference value of 35 mg_A/L. Kränke et al. (2001) calculated an upper reference value of 39 mg_A/L based on analysis of 48 sera from healthy persons without clinical signs of lung disease. In Finland, upper reference levels of 50 and 90 mg_A/L were established after analysis of 18 and 13 sera obtained from healthy males and females, respectively (Makkonen et al., 2001). An upper reference value of 70 mg_A/L was reported in Belgium based on analysis of 42 healthy individuals (Van Hoeyveld et al., 2006). However, a cut-off point of 35 mg_A/L was found more optimal for differentiating aspergillosis and ABPA patients from healthy individuals and diseased controls (Van Hoeyveld et al., 2006). In the United Kingdom, an anti-*A. fumigatus* IgG level >40 mg_A/L is considered evidence of increased exposure to this mold (Barton et al., 2008; Fairs et al., 2010).

These different reference values may have resulted from the small number of individuals involved and from differences in local exposure; interestingly, Simmonds et al. (1994) found that patients with high titres of antibodies to *A. fumigatus* were significantly more likely to live in an area of low population density. Living in a rural environment may predispose a person to *A. fumigatus* colonization.

In close agreement with the upper reference value of 19.3 mg_A/L for IgG against *A. fumigatus* reported above for the Immulite 2000 system, an upper reference value of 21.4 mg_A/L and a median value of 11.8 mg_A/L were found in Germany after analysis of 246 sera from healthy individuals on the Immulite 2000 system (Grosse et al., 2007).

In conclusion, the results presented above demonstrate that analysis of IgG antibodies against *A. fumigatus* with these 2 systems has reached a level of standardization that allows for direct comparison of quantitative results from different laboratories. However, for longitudinal analysis of IgG against *A. fumigatus*, reagents from the same manufacturer should be used.

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