Journal of Clinical Microbiology

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J. Clin. Microbiol. **2012, 50(3):762. DOI: 10.1128/JCM.01257-11.**
Published Ahead of Print 14 December 2011.

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Evaluation of a Recombinant Antigen-Based Enzyme Immunoassay for the Diagnosis of Noninvasive Aspergillosis

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Antibody detection is a key diagnostic tool for noninvasive aspergillosis (NIA) such as allergic bronchopulmonary aspergillosis and chronic pulmonary aspergillosis. Specific immunoprecipitin detection (IPD) is considered as the reference but lacks standardization and is time-consuming. To evaluate the performance of a new anti-*Aspergillus fumigatus* IgG enzyme immunoassay (EIA) kit using a recombinant *A. fumigatus* antigen (Bio-Rad), a retrospective study was performed on 551 sera collected from patients with a definite diagnosis of NIA (group 1; $n = 64$), bronchial *Aspergillus* colonization (group 2; $n = 26$), and probable aerial *Aspergillus* contamination (group 3; $n = 44$); from patients suspected of NIA with negative serological and mycological investigations (group 4; $n = 49$); and from a group of 222 patients not suspected of NIA (group 5). The EIA exhibited excellent reproducibility with coefficients of variation below 10%. Agreement with IPD was calculated between 62.5 and 84.4% according to the group of patients with Cohen's kappa coefficient at 0.6196 ± 0.077. Taking as reference a composite status including clinical, radiological, mycological, and serological data, sensitivity (group 1) and specificity (other groups) were calculated between 90.2 and 93.8% and 54.3 and 100%, respectively. Lower specificity was observed for patients with *Aspergillus* colonization. However, Yule *Q* coefficients estimating the correlation between EIA result and the definite diagnosis of NIA were calculated between 0.97 and 0.98. The method is a highly useful screening tool for the diagnosis of NIA, reducing the need for confirmatory IPD tests.

Depending on the immune status of patients, *Aspergillus*-induced diseases are present in a wide variety of clinical forms, whose clinical manifestations are mainly pulmonary (5, 15). In the immunocompetent host, noninvasive aspergilloses (NIA) predominate and include the allergic bronchopulmonary aspergillosis (ABPA) and different forms of chronic pulmonary aspergillosis (CPA) such as aspergilloma, chronic cavitary aspergillosis, and chronic necrotizing aspergillosis. In addition, *Aspergillus* may also colonize bronchial airways of patients with altered mucociliary clearance, such as cystic fibrosis patients or heavy smokers. This colonization does not seem to impact negatively the respiratory function of these patients while it may be the *primum movens* of other clinical forms, notably allergic bronchopulmonary aspergillosis (4, 12).

Whatever is the clinical form, *Aspergillus fumigatus* predominates as the etiological agent in about 90% of cases (6, 14). While antigen detection is a reliable tool for invasive aspergillosis diagnosis, antibody detection is considered as an important criterion in the diagnosis of NIA (9). An ideal serological test should differentiate between colonization and clinical forms associated with a deleterious impact on lung function. However, it has been well documented that prolonged colonization may induce anti-*Aspergillus* antibody synthesis, leading to difficulties in interpreting serological results (12). Techniques allowing specific immunoprecipitin detection (IPD) are considered as reference methods but lack standardization and are time-consuming (7). On the other hand, enzyme immunoassay (EIA) systems are more adapted to automated systems, leading to rapid and easy routine screening.

In this study, we evaluated a new commercial EIA based on the use of an *A. fumigatus* recombinant antigen (Bio-Rad, Marnes-la-Coquette, France). Using a large panel of well-characterized sera, it is shown that its performance makes it a suitable screening assay for the detection of noninvasive aspergillosis.

MATERIALS AND METHODS

Patients and sera. A panel of 551 sera retrospectively collected from 405 patients (with a range of 1 to 10 sera per patient) was divided in 5 groups according to the clinical, radiological, mycological, and serological data. Anti-*Aspergillus* antibody detection had previously been performed using one or a combination of the following tests: indirect hemagglutination (Fumouze, France), EIA Serion\Virion (enzyme-linked immunosorbent assay [ELISA] Classic, Serion\Virion, France), and immunoelectrophoresis (home-made technique; see above for specifications). Group 1 to group 4 included patients suspected of NIA mainly based on clinical and radiological status. Group 1a ($n = 51$, 164 sera) consisted of patients with a definite diagnosis of CPA and group 1b ($n = 13$, 16 sera) of patients suffering from ABPA. In all cases but one that was due to *Aspergillus flavus* (3 sera), *A. fumigatus* was the causative agent of all the cases of NIA. Group 2 included 26 patients (35 sera) with *Aspergillus* bronchial colonization defined by at least two positive cultures for *Aspergillus* over a 6-month period. Group 3 included 44 patients (50 sera) with negative serological tests and a unique positive culture for *Aspergillus* sp. considered as aerial contamination. Group 4 included 49 patients (64 sera) with negative results for both mycological and serological investigations. Finally, group 5 consisted of 222 pregnant women (222 sera) without any history of pulmonary disease and not given tests for *Aspergillus* cultures or anti-

Received 24 June 2011 Returned for modification 24 August 2011
Accepted 1 December 2011
Published ahead of print 14 December 2011
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doi:10.1128/JCM.01257-11

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Aspergillus antibody tests. All sera were stored frozen at −35°C before testing.

Serological analyses. The EIAs were performed retrospectively in parallel according to the manufacturers' recommendations.

(i) Bio-Rad Platellia *Aspergillus* IgG. Briefly, serum samples were diluted with a final ratio of 1:400 and then were incubated 60 min at 37°C in an antigen-sensitized microplate. After a washing step, a peroxidase labeled anti-human IgG conjugate was added, and the microplate was incubated for 60 min at 37°C. After the addition of a peroxidase chromogenic substrate, incubation was done at room temperature for 30 min. The reaction was stopped by adding 1 N sulfuric acid before reading optical density (OD) measured at 450 nm. A titer in U/ml was calculated using the supplied calibration points (0 to 80 U/ml). A titer of ≥10 U/ml was considered as positive and a titer between 5 and 10 U/ml was considered as intermediate. A selection of 46 sera with the highest optical densities (overflow result) was secondarily tested after a 1/60 dilution, as recommended by the manufacturer.

(ii) Serion\Virion ELISA Classic *A. fumigatus* IgG. One hundred microliters of diluted sample (1:500) was added into the wells of microtiter plates and incubated for 1 h at 37°C in a moist chamber. Wells were then washed with 300 μl washing solution 4 times and dried. One hundred microliters of the ready-to-use IgG conjugate was added and incubated for 30 min at 37°C in a moist chamber. A washing step was performed before adding 100 μl of ready-to-use substrate solution to each well. After incubation for 30 min at 37°C in a moist chamber, the reaction was stopped by the addition of 100 μl stopping solution to each well. The optical density (OD) was measured at 405 nm against substrate blank with a reference wavelength at 650 nm. ODs were then analyzed using the Serion Evaluate software version 2.27, which calculated the antibody levels according to the values of the 4 controls simultaneously tested. Activities of >70 U/ml, <50 U/ml, and between 50 and 70 U/ml correspond to positive, negative, and borderline results, respectively.

(iii) Immunoelectrophoresis. Immunoelectrophoresis was performed using ready-to-use agarose gels (Hydragel, Sebia, France) for the migration of antigen and serum components. Briefly, 2 μl of a mix (1:1) of somatic and culture filtrate of *A. fumigatus* antigens (FKS1; Microgen Bioproducts, United Kingdom) and 80 μl of serum were deposited in precut wells, with sera being on the anodic side. Following a 30-min run (Sebia tank) under a 100-V constant current, the gels were incubated in barbital buffer, pH 9.2 (barbital 2.45%, sodium barbital 13.73%, NaN_3 0.13%) for 48 h at room temperature. Following two washes in distilled water, gels were dried for 10 min using the Paragon system (Coulter-Beckman, France). Gels were then stained for 5 min in amidoschwartz solution (amidoschwartz 4 g/liter, ethylene glycol 6.7%), destained with citric acid solution (5 g/liter), washed, and dried before reading. Positive and negative controls provided with FKS1 antigens were used for each run. The gels were then read independently by two technicians to determine the number of precipitin lines. In this study, sequential sera for a given patient were all tested in a single run, allowing accurate comparison of the precipitin line count. Sera were considered positive and equivocal when ≥2 and 1 precipitating lines were detected, respectively.

Statistical analysis. The interassay reproducibility of the EIA kits was evaluated by the calculation of coefficients of variation (CVs) (100 × standard deviation/geometric mean) of 4 sera (one negative, one slightly positive, and two positive) for the Bio-Rad kit and three sera (one negative, one slightly positive, and one positive) for the Serion\Virion kit, all tested in 15 independent runs.

Distributions of titers according to the groups of patients were compared by the mean of a dot plot graphic. For this comparison, Serion\Virion EIA titers were normalized in order that the recommended gray zones for the two tests fit each other (50 to 70 U/ml for Serion\Virion and 5 to 10 U/ml for Bio-Rad). This was achieved using the following deduced formula: normalized Serion\Virion titer = 0.25 × Serion\Virion titer − 7.5. These arbitrary units (AU/ml) are used throughout this work in order to facilitate the comparison between the kits.

Correlations between EIA results and the precipitin detection analysis were estimated by calculating global agreements (ratio between total number of sera with similar status either negative, equivocal, or positive and total number of sera tested) and Cohen's kappa coefficients. Cohen's kappa estimates the concordance of the tests beyond the agreement by chance. Landis and Koch (11), using arbitrary ranges, consider the concordance excellent, substantial, moderate, fair, slight, and null for kappa values of >0.80, between 0.80 and 0.61, between 0.6 and 0.41, between 0.4 and 0.21, between 0.2 and 0, and below 0, respectively (11).

Because some treated patients of groups 1a and 1b had their IPD turn negative during the course of their disease, the sensitivity of the EIA kits to detect the *Aspergillus*-associated disease was calculated on the first sera of these patients (64 sera, 64 patients). Specificity was calculated on groups 2 to 5 (patients without evidence of aspergillosis, including colonized patients). In addition, the Yule *Q* coefficient was calculated to measure the strength of the association between the EIA results and the *Aspergillus* disease. Again, in groups 1a and 1b only the first serum collected from each patient was considered. The Yule *Q* coefficient can be calculated as follows: $Q = (A \times D - B \times C)/(A \times D + B \times C)$, where *A*, *B*, *C*, and *D* represent true-positive, false-positive, false-negative, and true-negative results, respectively. Interpretation of the coefficient was as follows: 0, null; 0.01 to 0.09, negligible; 0.10 to 0.29, light; 0.30 to 0.49, moderate; 0.50 to 0.69, strong; and 0.70 to 1, very strong.

RESULTS

A new commercial EIA (Bio-Rad) was evaluated comparatively with a large panel of sera collected from patients either infected or suspected of a noninvasive form of *Aspergillus* disease and a group of control patients without any history of bronchopulmonary disease.

The means of the coefficients of variation calculated using the control sera were at 4.86 ± 0.79 (range, 3.86 to 5.78) and 9.82 ± 3.30 (range, 6.04 to 12.12) for the Bio-Rad and the Serion\Virion tests, respectively.

In order to compare results between the two EIAs, a box plot was drawn using normalized values (Fig. 1). The graph shows that patients with noninvasive aspergillosis (group 1a plus 1b) displayed high antibody titers for the Bio-Rad EIA (geometric means, 64.5 ± 28.6 versus 41.6 ± 28.4), with a lesser spreading of values (Fig. 1). In contrast, a higher number of patients suspected of NIA but for which an *Aspergillus*-associated disease has been ruled out (groups 3 and 4), exhibited an equivocal or a positive result with the Serion\Virion test. Indeed, 29, 8, and 27 sera of group 4 were found positive, equivocal, and negative using the Serion\Virion test (median value at 10.6 AU/ml), respectively, versus 16, 5, and 43 using the Bio-Rad test (median value 0.8 AU/ml). Similarly, among the 222 sera from the negative control group (group 5), 9 and 11 were found positive and equivocal using the Serion\Virion test, while a single one was found equivocal with the Bio-Rad test.

Overall, a better agreement between IPD and Bio-Rad results was demonstrated, except for group 1a (77.4 versus 78% for the Serion\Virion test) (Table 1). Cohen's kappa correlation coefficients with IPD calculated for groups 1 to 4 were at 0.6196 ± 0.077 and 0.4556 ± 0.085 for the Bio-Rad and Serion\Virion tests, respectively. Taking into account the equivocal results or not, the sensitivity of the EIA kits to detect *Aspergillus*-induced diseases was estimated at 90.6 or 93.8% with a specificity at 87.3 or 89.6% for the Bio-Rad test compared to a sensitivity and a specificity at 87.3 or 89.6% and 75.7 or 84.4%, respectively, for the Serion\Virion test (Table 2). Sera from a patient with non-

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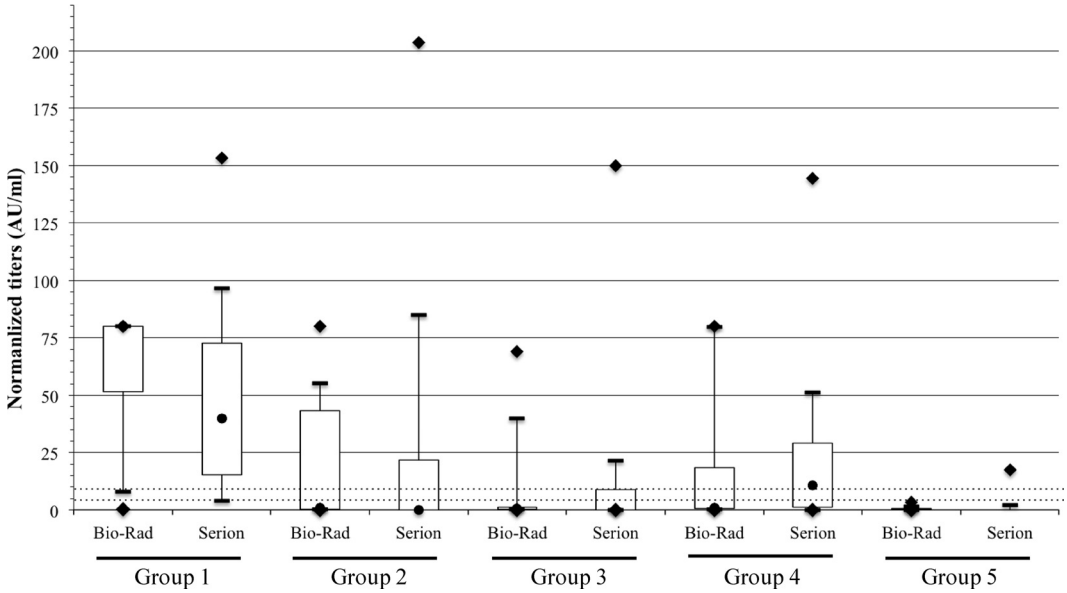

FIG 1 Box plot of normalized data from five groups of sera tested using the Bio-Rad Platellia *Aspergillus* IgG and Serion ELISA Classic *A. fumigatus* IgG. See Materials and Methods for the normalization of data and definitions of groups. Group 1 gathers sera from groups 1a and 1b. Diamonds represent minimum and maximum values, central rectangles span the first quartile to the third quartile, bars above and below the box correspond to the 10th and 90th percentiles, respectively, and circles represent medians. Dotted lines represent the cutoff values delineating the gray zone between 5 and 10 U/ml.

fumigatus infection remained negative for the 3 techniques. In contrast, a patient (4 sera) with a final diagnosis of CPA had a negative IPD in 3 sera and a single precipitin line in one, while both EIAs returned positive for the 4 sera. In 6 out of 13 patients of group 1b (ABPA), both EIAs were positive before the IPD (1 to 7.5 months) (data not shown). The Yule *Q* coefficients estimating the correlation between EIA results and the final diagnosis of NIA were calculated at 0.97 and 0.98 if the equivocal values were considered as either negative or positive for the Bio-Rad test and at 0.93 and 0.94 for the Serion\Virion test.

Quantitative correlation between the number of precipitin lines and Bio-Rad titers was low overall ($R^2 = 0.062$). However, for some patients of group 1a with a high number of precipitin lines (>5), EIA titers of diluted sera followed that of the IPD line count (data not shown).

DISCUSSION

Diagnosis of noninvasive aspergillosis may be difficult because neither clinical nor radiological presentations are specific. Direct examination and culture of bronchopulmonary samples are considered nonoptimal, because of insufficient sensitivity, and of possible misinterpretation due to aerial contamination (16). Since circulating antigens can be detected only in the course of invasive aspergillosis, the detection of anti-*Aspergillus* antibodies appears thus as a key diagnostic tool for noninvasive forms of aspergillosis. A major drawback of this approach is that some patients with a noncomplicated persistent bronchial colonization may develop an immunologic response that could interfere with the diagnosis of other clinical entities (1, 8). In our previous experience, about 28% of cystic fibrosis patients were colonized with *A. fumigatus*, among whom 37% presented with anti-*Aspergillus* precipitins as detected by immunoelectrophoresis (our unpublished data).

EIA tests are considered as sensitive methods and because of the possibility of automation of process are particularly adapted for screening surveys. In this study, we evaluated a new commercial EIA (Bio-Rad) for the detection of anti-*Aspergillus* IgG and its interest for the diagnosis of NIA. To our knowledge, this kit is the only commercial one using a recombinant *Aspergillus* antigen in

TABLE 1 General agreement between EIAs and IPD according to patient group, as defined in Materials and Methods

Group (no. of sera)	Agreement		
	Bio-Rad vs. IPD	Serion\Virion vs. IPD	Bio-Rad vs. Serion\Virion
1a ($n = 164$)	77.4%	78.0%	83.5%
1b ($n = 16$)	62.5%	56.3%	75.0%
2 ($n = 35$)	74.3%	65.7%	74.3%
3 ($n = 50$)	80.0%	62.0%	72.0%
4 ($n = 64$)	84.4%	51.6%	59.4%

TABLE 2 Sensitivity and specificity of two anti-*Aspergillus* antibody EIAs for the detection of *Aspergillus*-induced diseases

Parameter	Group[a] (no. of samples; no. of patients)	EIA[b]	
		Bio-Rad	Serion
Sensitivity	1a (51; 51)	94.1–90.2%	92.2–88.2%
	1b (13; 13)	92.3–92.3%	84.6–76.9%
	1a + 1b (64; 64)	93.8–90.6%	90.6–85.9%
Specificity	2 (35; 26)	54.3–60.0%	60.0–71.4%
	3 (50; 44)	82.0–84.0%	62.0–80.0%
	4 (64; 49)	67.2–75.0%	42.2–54.7%
	5 (222; 222)	100.0–99.5%	95.9–91.0%
	2 + 3 + 4 + 5 (371; 341)	87.3–89.6%	75.7–84.4%

[a] Groups are defined in Materials and Methods.
[b] First and second percentages were obtained when equivocal results were considered as positives or negatives, respectively.

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contrast to the crude extracts of *A. fumigatus* used in other homemade or commercial assays. This may explain, in part, the excellent reproducibility of the test with CVs below 5% for the 4 sera tested in each series. Agreement between Bio-Rad test and IPD was ≥70%, except for group 1b (chronic pulmonary aspergillosis), with a Cohen's kappa correlation coefficient at 0.6196, suggesting a good correlation between the IPD reference technique and the evaluated EIA.

Whichever the group of infected patients, either with chronic necrotizing or cavitary pulmonary aspergillosis or ABPA, the sensitivity of the Bio-Rad test was ≥90%. Our work relies on a robust testing of a wide panel of sera collected from different categories of patients suspected of NIA, and not only from healthy volunteers. Indeed, these patients have no reason to develop an immune response against *Aspergillus*, and using their sera may overestimate the specificity of the test (99.5 to 100% in group 5 of our series). Not surprisingly, specificity was lower for patients with chronic bronchial colonization (54.3 to 60.0%) while it remained higher than 75% for the other groups of patients when equivocal results were considered as negative. This suggests that the antigen used induces a humoral response not only in the various forms of NIA but also during the phase of colonization. Defining antigens specifically involved in the pathogenic processes, possibly different for the various clinical forms, and/or specific isotypes of antibodies should help in the design of more-specific tests (2, 13). Such an approach has been proposed for the diagnosis of ABPA (3, 10), aspergilloma, or invasive forms of aspergillosis (17) but, to our knowledge, none has ever been commercialized, limiting their use to reference laboratories. Despite this limitation, the Yule *Q* coefficient calculated at 0.94 and 0.95 (if the equivocal values were considered as either negative or positive) for the Bio-Rad test clearly demonstrated the value of the test in the prediction of the disease. Overall, for the majority of the evaluations performed, the Bio-Rad kit compares favorably to the Serion\Virion test.

In France, serodiagnosis of aspergillosis is usually performed in two steps: a screening test, such as EIA, indirect hemagglutination test, indirect immunofluorescence, or latex agglutination, followed, in case of a positive result, by a confirmatory technique such as Western blotting or IPD using immunoelectrophoresis. Considering its good sensitivity, the Bio-Rad assay appears to be a useful screening tool for the diagnosis of NIA. However, even when considering equivocal results as negative, thus slightly lowering the sensitivity (94.1 to 90.2) and improving the specificity, our data do not prompt the replacement of the reference method by the Bio-Rad test.

ACKNOWLEDGMENTS

We are grateful to C. Fairhead for English language revision, P. Joly and M. Tabouret for their critical advice on the paper, and D. Rocherolle, N. Imbro, and A. Beghdaoui for excellent technical assistance.

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