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Performance of the Euroimmun *Aspergillus* Antigen ELISA for the Diagnosis of Invasive Pulmonary Aspergillosis in Bronchoalveolar Lavage Fluid

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ABSTRACT Invasive pulmonary aspergillosis (IPA) is a life-threatening disease that affects mainly immunocompromised hosts. Galactomannan testing from serum and bronchoalveolar lavage fluid (BALF) represents a cornerstone in diagnosing the disease. Here, we evaluated the diagnostic performance of the novel *Aspergillus*-specific galactomannoprotein (GP) enzyme-linked immunosorbent assay (ELISA; Euroimmun Medizinische Labordiagnostika) compared with the established Platelia *Aspergillus* GM ELISA (GM; Bio-Rad Laboratories) for the detection of *Aspergillus* antigen in BALF. Using the GP ELISA, we retrospectively tested 115 BALF samples from 115 patients with clinical suspicion of IPA and GM analysis ordered in clinical routine. Spearman's correlation statistics and receiver operating characteristics (ROC) curve analysis were performed. Optimal cutoff values were determined using Youden's index. Of 115 patients, 1 patient fulfilled criteria for proven IPA, 42 for probable IPA, 15 for putative IPA, 10 for possible IPA, and 47 did not meet criteria for IPA. Sensitivities and specificities for differentiating proven/probable/putative versus no IPA (possible excluded) were 74% and 96% for BALF GP and 90% and 96% for BALF GM at the manufacturer-recommended cutoffs. Using the calculated optimal cutoff value of 12 pg/mL, sensitivity and specificity of the BALF GP were 90% and 96%, respectively. ROC curve analysis showed an area under the curve (AUC) of 0.959 (95% confidence interval [CI] of 0.923 to 0.995) for the GP ELISA and an AUC of 0.960 (95% CI of 0.921 to 0.999) for the GM ELISA for differentiating proven/probable/putative IPA versus no IPA. Spearman's correlation analysis showed a strong correlation between the ELISAs (rho = 0.809, $P < 0.0001$). The GP ELISA demonstrated strong correlation and test performance similar to that of the GM ELISA and could serve as an alternative test for BALF from patients at risk for IPA.

KEYWORDS *Aspergillus* antigen, galactomannan, hematologic malignancy, intensive care unit, invasive aspergillosis, respiratory disease

*A*spergillus is an ubiquitous fungus that can cause a spectrum of disease in humans ranging from allergy to severe invasive infection (1). Invasive pulmonary aspergillosis (IPA) occurs when inhaled conidia germinate in the lower airways and invade tissue, leading to potential hematogenous dissemination, and is associated with high morbidity and mortality (2). While IPA tends to occur in patients with established

immune deficiencies, such as those with prolonged neutropenia in the setting of hematopoietic stem cell transplantation (HSCT), solid organ transplantation (SOT), or aggressive chemotherapy (3), it is now a well-recognized complication in nonneutropenic patients suffering from influenza or COVID-19-associated acute respiratory failure (4), as well as in patients with significant corticosteroid exposure (2).

The diagnosis of IPA is challenging. Proven diagnosis requires either culturing the fungus from a sterile site or identifying *Aspergillus* hyphae and associated tissue damage on histopathologic examination (5, 6). Since this is rarely feasible *in vivo*, diagnosis relies mainly on the detection of *Aspergillus* antigens or DNA fragments in serum or bronchoalveolar fluid (BALF) (7). Pathogenesis of IPA differs in nonneutropenic patients (primarily airway invasive growth) compared to neutropenic patients (primarily angioinvasive growth) (2). Hence, diagnostic testing in BALF is superior to testing serum in nonneutropenic patients (8, 9).

One of the most widely employed methods for BALF testing is analysis for the galactomannan (GM) antigen, a polysaccharide component of the *Aspergillus* cell wall, using the Platelia GM enzyme-linked immunosorbent assay (ELISA; Bio-Rad, Marnes-la-Cocquette, France) (10). Data on the sensitivity and specificity of this test vary. A recent Cochrane review found that at an optical density index (ODI) cutoff of 1.0, the sensitivity and specificity of this test in BALF were 78% and 93%, respectively (11). However, sensitivity has been shown to decrease in patients on mold-active prophylaxis (12–14). Positive results may also occur in other fungal diseases, including fusariosis (15) and histoplasmosis (16).

Importantly, many mycology laboratories globally do not have access to GM testing, with only 23% of laboratories surveyed in Asia able to offer this test (17). Therefore, alternative and more broadly available assays are needed to assist with the diagnosis of IPA. The purpose of this study was to evaluate a novel *Aspergillus* antigen assay, the *Aspergillus*-specific galactomannoprotein ELISA (GP ELISA; Euroimmun Medizinische Labordiagnostika, Lübeck, Germany), which has recently been validated for use in serum samples (18), for the diagnosis of IPA in BALF.

MATERIALS AND METHODS

A total of 115 BALF samples from 115 patients with various underlying diseases and clinical suspicion of IPA and GM testing ordered between 2015 and 2019 at the University of California San Diego (UCSD) were analyzed retrospectively. IPA was classified according to the revised European Organization for Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) criteria and slightly modified AspICU criteria (i.e., including positive BAL fluid GM of >1.0 ODI as entry criterion) for patients in the intensive care unit (ICU) who did not fulfill EORTC/MSG host criteria (6, 19, 20).

GM testing with the Platelia ELISA was routinely and prospectively performed in all BALF samples before samples were stored at $-70°C$ for up to 6 years. In December 2021 and January 2022, stored BALF samples were selected based on IPA classification. Samples were then thawed, vortexed, and tested with the GP ELISA according to the manufacturer's recommendations, comprising mixing of 300 μL of BALF with 100 μL of sample buffer, heating in a boiling water bath for 3 min, and centrifuging at 10,000 × *g* for 10 min, followed by incubation of 100 μL supernatant for (i) 1 h without reagent, (ii) 1 h with biotin solution, (iii) 30 min with enzyme conjugate, (iv) 15 min substrate incubation, and (v) adding stop solution into each of the microplate wells. Photometric measurement of the color intensity was made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 min of adding the stop solution. Prior to measuring, the micro plate was carefully shaken to ensure homogenous solution distribution.

For additional comparative purposes, 94 BALF samples were tested using the IMMY sona *Aspergillus* galactomannan lateral-flow assay (LFA; IMMY, Norman, USA) (21, 22). Automated readout of test line intensities was performed applying the sona LFA cube reader and displayed in ODI. LFA testing was performed according to the manufacturer's instructions. All testing procedures were performed by personnel blinded to IPA classification and GM ELISA results.

Statistical analyses were performed using SPSS 25 (SPSS Inc., Chicago, IL, USA). Sensitivity and specificity for IPA, i.e., fulfilling criteria of probable/proven/putative, (i) versus possible and no IPA and (ii) versus no IPA with possible IPA excluded were calculated for the manufacturer-recommended GP cutoff (25 pg/mL) and GM cutoff (ODI 1.0). For GM and GP, receiver operating characteristic (ROC) curve analyses were performed and area under the curve (AUC) values were calculated, including 95% confidence intervals (CI) for the outcomes proven/probable/putative aspergillosis (versus no aspergillosis) in the overall study cohort. In addition, performances of tests were calculated for various subgroups, including those samples that yielded positive results in BAL culture (i.e., to help overcome incorporation bias associated with GM testing). Correlation between (i) GP and GM, (ii) GP and LFA, and (iii) GM and LFA was calculated using Spearman's rho correlation analysis due to the nonnormal distributions as well as Cohen's

TABLE 1 Demographic characteristics of overall study population and subgroups[a]

Characteristic	Proven/probable IPA (n = 43)	Putative IPA (n = 15)	Possible IPA (N = 10)	No IPA (n = 47)	Overall (n = 115)
Age (median, IQR)	62.5 (54–76)	64 (51–76)	59.5 (53–67)	54 (39–73)	62 (46–86)
Female sex (n, %)	14 (30%)	6 (13%)	6 (13%)	20 (43%)	46 (40%)
Underlying disease					
Hematologic malignancy (n, %)	19 (58%)	1 (3%)	5 (15%)	8 (24%)	33 (28%)
SOT recipient (n, %)	4 (13%)	5 (17%)	4 (3%)	17 (57%)	30 (26%)
ICU patient (n, %)	20 (38%)	9 (17%)	1 (2%)	22 (42%)	52 (45%)
Mycological evidence					
GM ELISA > 1.0 ODI (n, %)	37 (88%)	14 (93%)	0 (0%)	2 (4%)	
GP ELISA > 25 pg/mL (n, %)	31 (72%)	12 (80%)	1 (10%)	2 (4%)	
GP ELISA > 12 pg/mL (n, %)	39 (90%)	13 (87%)	1 (10%)	2 (4%)	
LFA > 1.0 ODI (n, %)	41 (95%)	13 (87%)	2 (20%)	23 (48%)	
Culture (n, %)[b]	15 (34%)	1 (6%)	0 (0%)	0 (0%)	
Antifungal prophylaxis at time of BAL fluid sampling	11 (26%)	1 (7%)	2 (20%)	6 (13%)	20 (17%)
Overall mortality (n, %)	18 (42%)	5 (33%)	1 (10%)	9 (19%)	32 (28%)
30-day mortality (n, %)	12 (28%)	5 (33%)	1 (10%)	8 (17%)	24 (21%)
90-day mortality (n, %)	16 (37%)	5 (33%)	1 (10%)	9 (19%)	29 (25%)

[a]BALF, bronchoalveolar lavage fluid; GM, galactomannan; GP, galactomannoprotein; ICU, intensive care unit; IPA, invasive pulmonary aspergillosis; IQR, interquartile range; LFA, lateral-flow assay; ODI, optical density index; SOT, solid organ transplantation.
[b]Results of culture missing in two patients.

Kappa statistics. Optimal cutoff values were determined using Youden's index. A two-sided P value of <0.05 was taken as the cutoff for statistical significance.

The study protocol and all study-related procedures were approved by the Human Research Protections Program at UCSD.

RESULTS

Demographic characteristics of overall study population and subgroups are displayed in Table 1. The majority of patients were male (67/113, 58%, missing data on sex in 2 patients), and median age was 59 years (range 42 to 86). Underlying hematological malignancy was present in 33/115 patients (29%), 30/115 (26%) patients were solid organ transplant (SOT) recipients, and 52/115 (45%) patients were in the ICU for various diseases. BALF culture grew *Aspergillus* spp. in 16/115 patients (all patients with proven/probable/putative IPA). One patient met the criteria of proven IPA, 42 patients met the criteria of probable IPA, 15 met the putative IPA, 10 met possible IPA, and 47 did not fulfill criteria for IPA (34 patients classified according to AspICU criteria; 81 patients classified according to EORTC/MSG criteria; of the 47 patients with no IPA, 22 were in the ICU setting, of which 19 patients did not fulfill classical host risk factors). Thirty-eight percent (22/58) of those with proven/probable/putative IPA and 17% (10/57) of patients with possible IPA or no IPA had a fatal outcome; 30- and 90-day mortality for patients with proven/probable/putative IPA were 26% and 33%, respectively.

The GP assay produced results in all 115 samples. Test performances are displayed in Tables 2 and 3. For differentiating proven/probable/putative versus no IPA (possible excluded), sensitivities and specificities were 74% (43/58) and 96% (45/47) for BALF GP and 90% (52/58) and 96% (45/47) for BALF GM, respectively (P = 0.081 comparing sensitivities). In patients with BALF cultures positive for *Aspergillus*, sensitivity of GP (median level 141.5 pg/mL) and GM (median 5.2 ODI) was 75% (12/16) and 94% (15/16), respectively. The GP assay tested positive in 1 of 10 patients with possible IPA (361.6 pg/mL), while the GM assay showed no positive results in this group. Thus, specificity for proven/probable/putative versus possible/no IPA was 95% (54/57) for the GP ELISA and 97% (55/57) for the GM ELISA.

Calculated Youden's index for GP ELISA yielded a cutoff value of 11.955 pg/mL for optimal diagnostic discriminatory power. Using the alternative cutoff value, sensitivity and specificity were 90% and 96% for differentiating proven/probable/putative versus no IPA (possible excluded), and sensitivity was 88% (14/16) in patients with BALF

TABLE 2 Sensitivity and specificity for the GP ELISA, the GM ELISA, the LFA, and culture for the diagnosis of probable/proven/putative versus no IPA (possible excluded)

Diagnostic test (cutoff, n)/patient group	Sensitivity (n)	Specificity (n)
Euroimmun GP (25 pg/mL, n = 115)	74% (43/58)	96% (45/47)
Euroimmun GP (12 pg/mL, n = 115)	90% (52/58)	96% (45/47)
Bio-Rad GM (ODI > 1.0, n = 115)	90% (52/58)	96% (45/47)
IMMY LFA (ODI >1.0, n = 90)	98% (51/52)	52% (20/38)
Culture (n = 109)	30% (16/54)	100% (45/45)

cultures positive for *Aspergillus*, respectively. Above a threshold of 58.8 pg/mL, the GP reached a specificity of 100%.

ROC curve analysis yielded an AUC of 0.959 (95% CI 0.923 to 0.995) for the GP assay compared to an AUC of 0.960 (95% CI 0.921 to 0.999) for the GM test when differentiating proven/probable/putative IPA versus no IPA (Fig. 1). For the LFA GM assay, ROC curve analysis yielded an AUC of 0.910 (95% CI 0.844 to 0.976) when differentiating proven/probable/putative IPA versus no IPA. When using culture positivity as the reference test, the AUC for the GP, GM, and LFA was 0.959 (95% CI 0.905 to 1.00), 0.956 (95% CI 0.882 to 1.00), and 0.933 (95% CI 0.857 to 1.00), respectively.

Spearman's correlation analysis showed a strong correlation between the two ELISAs (rho = 0.809, $P < 0.0001$; Fig. 2). Cohen's Kappa was calculated to be 0.715, suggesting substantial agreement ($P < 0.001$). Spearman's correlation analysis also showed a substantial correlation between the GP ELISA and the LFA (rho = 0.701, $P < 0.0001$) and the GM ELISA and LFA (rho = 0.746, $P < 0.0001$).

Samples of 12 patients with proven/probable/putative IPA were obtained while mold-active antifungal prophylaxis or treatment was administered. Sensitivity of GP and GM was 67% (8/12) and 100% (12/12), respectively, in these patients. When using the optimized cutoff for the GP ELISA, sensitivity increased to 75% (9/12) in this setting.

In patients with proven/probable/putative IPA, ROC curve analysis of GP results showed an AUC of 0.684 (95% CI 0.568 to 0.800) for identifying those with a fatal outcome within 30 days and an AUC of 0.706 (95% CI 0.603 to 8.10) for identifying those with a fatal outcome within 90 days. The respective results for GM testing were 0.625 (95% CI 0.495 to 0.754) and 0.659 (95% CI 0.542 to 0.776), respectively. Calculated Youden's index for GP ELISA yielded a cutoff value of 7.84 pg/mL for optimal diagnostic discriminatory power. Using the alternative cutoff value, sensitivity and specificity were 79% and 50% (proven/probable/putative versus no IPA), respectively, for identifying patients with a fatal outcome withing 90 days.

DISCUSSION

We retrospectively evaluated the GP ELISA in BALF in a mixed patient cohort, for which BALF GM testing had been performed as part of the routine diagnostics. Our data suggest that the two assays performed comparably in diagnosing IPA, particularly when considering that GM results were included in classification of the disease, which puts the GP test at a considerable disadvantage.

TABLE 3 Diagnostic performance of the GP ELISA using the alternative cutoff 12 pg/mL for different diagnostic criteria and underlying disease groups[a]

Diagnostic criteria, underlying disease group/patient group	Sensitivity (n)	Specificity (n)	AUC (95% CI)
EORTC/MSG (n = 71)[b]	91% (39/43)	93% (26/28)	0.962 (0.920–1.00)
AspICU (n = 34)[c]	87% (13/15)	100% (19/19)	0.942 (0.842–1.00)
Intensive care unit/others (n = 51)	97% (28/29)	100% (22/22)	0.9997 (0.988–1.00)
Hematological malignancy (n = 33)	90% (18/20)	88% (7/8)	0.913 (0.792–1.00)
Solid organ transplant (n = 30)	67% (6/9)	95% (16/17)	0.895 (0.746–1.00)

[a]EORTC/MSG, European Organization for Research and Treatment of Cancer/Mycoses Study Group; AspICU, Asp intensive care unit.
[b]Proven/probable versus no IPA (possible excluded).
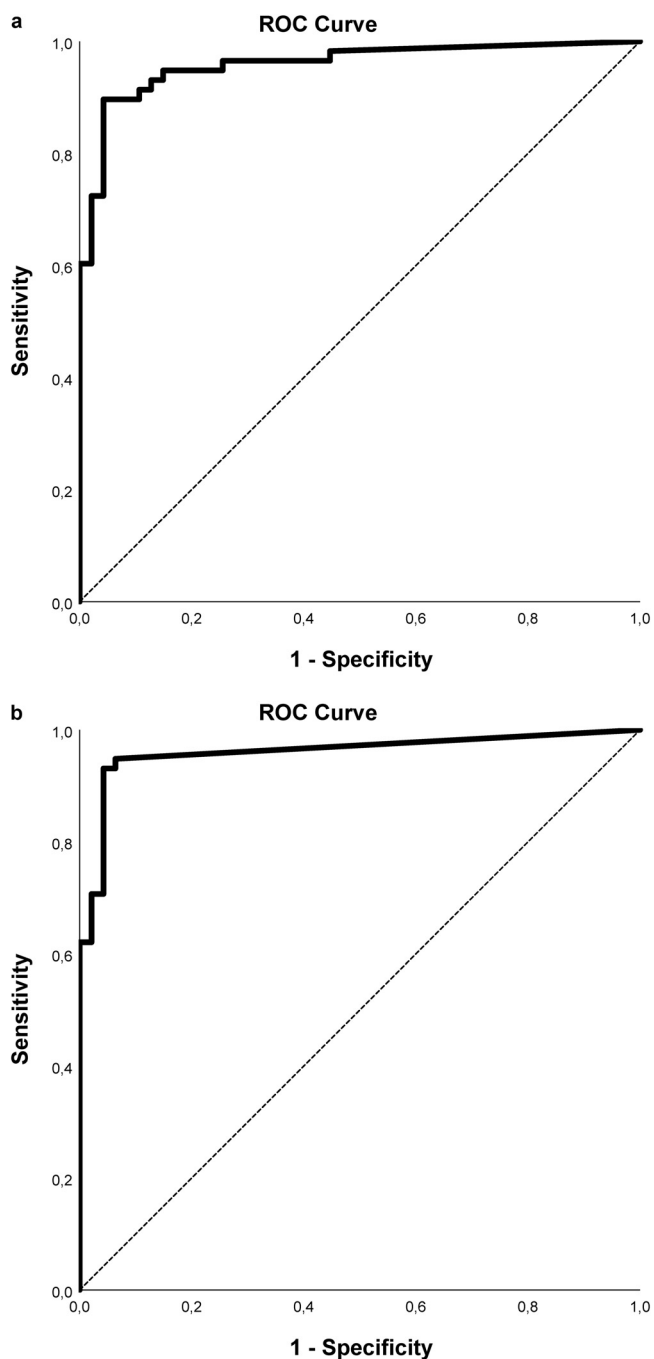[c]Putative versus no IPA.

FIG 1 Receiver operating characteristics (ROC) analysis curve for proven/probable/putative IPA versus no IPA (possible excluded) for the GP (a) and GM (b) ELISA.

Both assays are ELISAs that bind and identify *Aspergillus* antigens. The GP test relies on the monoclonal antibody JF5 detecting a glycoprotein in the hyphal cell wall and septa, whereas the GM assay uses the monoclonal antibody EB-A2 directed against a cell wall polysaccharide (23). It is thought that tests using the JF5 antibody may avoid cross-reactivity with antibiotics or bacterial lipoteichoic acids (23), and work has shown that lateral-flow devices using JF5 antibodies have shown high specificity, although lower sensitivity in comparison to the GM ELISA (22, 24, 25). To date, there have been only two direct comparisons of the two ELISAs. The first showed that they performed similarly on serum samples with sensitivities of 40% (18). The second study compared
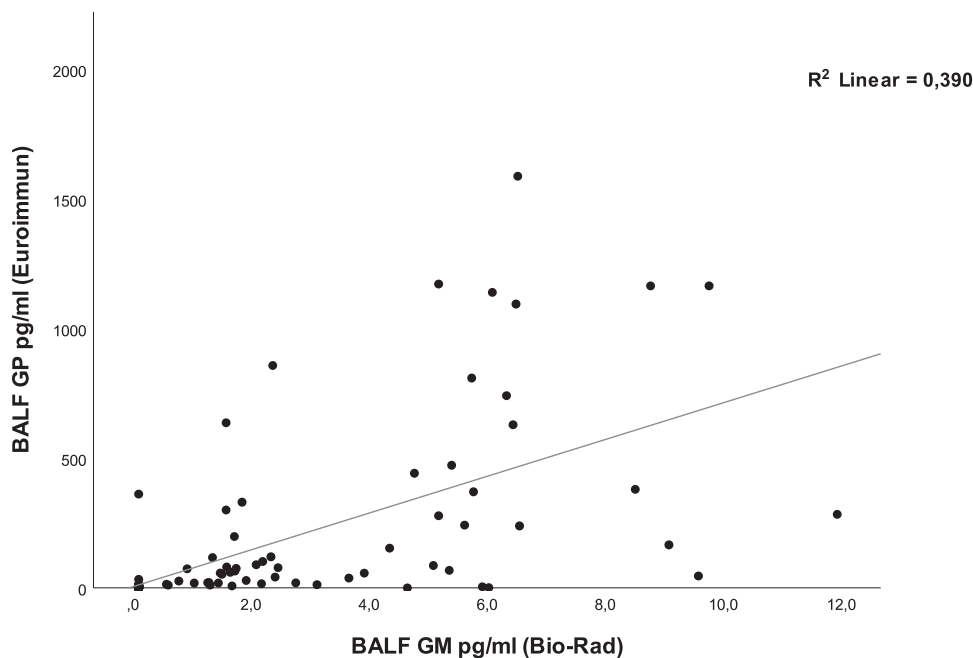
FIG 2 Scatterplot of galactomannan (GM) and galactomannoprotein (GP) ELISA results from BALF (n = 115). ODI, optical density index.

how the two tests performed in a smaller set of serum and BALF specimens (26). Sensitivity of GP and GM in serum was 43% and 89%, respectively, and specificity was 92% for both. In 28 BALF samples evaluated (including 9 from IPA patients), sensitivity was 89% and 100%, respectively, and specificity was 84% and 68% (26).

In our study of 115 BALF samples (including 58 from IPA patients), the ELISAs showed similar specificities (94% versus 96%), while GP testing had lower sensitivity than GM testing (74% versus 90%) at the current manufacturer-recommended cutoffs for differentiating proven/probable/putative versus no IPA. However, ROC curves were nearly identical: GP exhibited an AUC of 0.959 (95% CI 0.923 to 0.995), whereas GM had an AUC of 0.960 (95% CI 0.921 to 0.999). This finding indicates that modifying the GP BALF cutoff might have the potential to further increase the test performance and to mirror performance of the GM ELISA. This suggestion is reinforced by the observation that the two assays correlated strongly according to both the Spearman coefficient (rho = 0.809) and Cohen's Kappa coefficient (0.715). When using a cutoff 11.995 pg/mL (calculated by using Youden's index), sensitivity and specificity were 90% and 96% (same performance with a 12 pg/mL cutoff), respectively, for differentiating proven/probable/putative IA versus no IA. This cutoff renders the GP ELISA an equivalent alternative to the GM ELISA in terms of diagnostic discriminatory power.

Our study has several limitations, including the low number of proven IPA cases. In addition, this study was performed with banked BALF samples that were tested after they were stored and frozen, although it is unlikely that this had a relevant impact on test performance (27). Finally, considering that the GM ELISA was used for classification of the majority of cases, performance of that test was likely overestimated. Considering the significant correlation between the two tests, this may have also led to an overestimation of GP performance.

In conclusion, our study indicates that GP BALF testing might be a valuable alternative for settings without rapid access to GM BALF testing. Further data comparing the two assays and potentially some optimization of the officially recommended GP cutoff for BALF may help to further establish this test as a viable alternative. Globally, additional broadly available and reliable antigen-based assays for the diagnosis of IPA are needed in order to increase our ability in diagnosing these infections.

ACKNOWLEDGMENTS

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