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Article

Evaluation of the *Aspergillus* Lateral Flow Assay in Pretreated Sputum and Serum for the Diagnosis of Invasive Aspergillosis

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Abstract

Invasive aspergillosis (IA) is a disease with a complex diagnosis due to its non-specific clinical manifestations, which delays treatment and consequently leads to a worsening of the disease. The *Aspergillus* galactomannan (GM) lateral flow assay was evaluated in pretreated sputum (PS) and serum samples for the diagnosis of IA. Clinic epidemiological data were collected from 76 patients at the Instituto Médico La Floresta between January 1, 2022, and April 30, 2024. The GM lateral flow assay was performed on serum, BAL (bronchoalveolar lavage), and PS samples for subsequent classification according to the criteria established by the EORTC/MSG into possible, probable, proven IA, and no IA. Only 28 patients were presented with possible IA, 28 with probable IA, and 20 did not present with IA. A descriptive analysis was applied to 32 patients with galactomannan results ≥ 0.7 in serum and ≥ 0.8 in sputum or BAL, yielding statistically significant values for sputum and the associated serum ($p=0.00$), and for BAL ($p=0.041$) and its accompanying serum ($p=0.05$). Furthermore, the means obtained were within the 95% confidence intervals, being consistent and reliable. A correlated t-test was performed on 3 patients who provided all three sample types, revealing $p(0.453) > 0.05$, which indicates no significant difference between pretreated sputum and BAL. PS is an alternative sample to BAL for high-risk patients. The use of respiratory samples (BAL/PS) increases the probability of galactomannan detection in non-neutropenic patients compared to the use of serum and can be used for IA classification; however, it is considered that EORTC/MSG criteria have limited applicability in this population.

Keywords: serum; pretreated sputum; galactomannan lateral flow; aspergillosis

1. Introduction

Invasive fungal diseases (IFD) have shown an increased incidence in recent years, with invasive aspergillosis (IA) ranking among the top positions. This disease causes more than 300,000 cases annually, with mortality potentially exceeding 90% according to research by Goyeneche-García *et al.* For this reason, prompt diagnosis and adequate treatment are of vital importance for individuals who acquire this disease [1].

The development of assays to detect biomarkers such as galactomannan (GM) antigen for *Aspergillus* in clinical samples from neutropenic patients at risk of IA has significantly improved the

management of this mycotic infection [2]. Likewise, tests for GM detection exist via enzyme immunoassay techniques (GM-EIA), the FungiXpert Aspergillus galactomannan enzyme-linked immunosorbent assay (ELISA) kit, the AGMAg ELISA kit, the Aspergillus galactomannan Ag Virclia Monotest, and the Dynamiker Aspergillus galactomannan assay [3].

However, to expedite clinical diagnosis, a kit is currently available that provides faster results: the Aspergillus Galactomannan Lateral Flow Assay (GM-LFA-IMMY), an immunochromatographic method that detects the presence of galactomannan antigen located in the fungal cell wall, either qualitatively or quantitatively [3]. Studies to date have focused on the performance of GM-LFA-IMMY in analyzing bronchoalveolar lavage (BAL) samples, yielding a sensitivity of 83% to 92% and a specificity of 91% to 92% [4,5].

The techniques are standardized primarily for serum and BAL samples. Taking into consideration the implications of BAL sampling for the patient and their hemodynamic stability, this research proposed pretreated sputum as an alternative to BAL due to its ease of collection. A pretreatment with phosphate-buffered saline (PBS) at pH 7.2 was applied through three successive washes [6]. After this preparation, the sputum was processed using the GM-LFA-IMMY test in the same manner established for BAL [7].

The pretreated sputum option will benefit patients in the ICU or those considered at high risk for BAL collection with suspected IA. Pretreatment of the sample eliminates interferents from the respiratory tract microbiota that could cause false positives in the galactomannan test [6]. Consequently, this study allowed for the evaluation of the Aspergillus Galactomannan Lateral Flow assay in pretreated sputum and serum samples for the diagnosis of invasive aspergillosis, in addition to classifying the study patients into IFD categories using EORTC/MSG definitions [8,9].

2. Materials and Methods

2.1. Study Patients

Seventy-six patients with signs and symptoms of invasive aspergillosis were evaluated between January 2022 and April 2024. These patients visited the microbiology service of the Instituto Médico La Floresta for the detection of Galactomannan antigen in serum, spontaneous sputum (SS), induced sputum (IS), endotracheal or bronchial secretion (ETBS), and bronchoalveolar lavage (BAL). The study was approved by the institutional review board of the Instituto Médico La Floresta and the Bioethics Committee of the School of Bioanalysis at the Central University of Venezuela. Written informed consent was obtained from all participating patients. Ethical standards established by the Declaration of Helsinki and the World Health Organization (WHO) for research in humans and animals were followed. As inclusion criteria, respiratory and serum samples from patients with lower respiratory infection and suspected aspergillosis had to be collected on the same day.

2.2. Clinic-Epidemiological Data Collection

A data sheet was used to collect epidemiological, clinical, and microbiological information, including: name, age, sex, underlying condition or disease, presumptive diagnosis, origin (outpatient, inpatient, or external centers), type of biological sample (SS, IS, ETBS, BAL, and serum), use of antifungals, antimicrobials, steroids, imaging studies, presumptive diagnosis of IA, COVID-19-associated invasive aspergillosis (CAPA), mechanical ventilation, status (alive/deceased), association with other microorganisms, and mycological culture. To enter the study, the patient had to have both types of samples (respiratory sample and serum). All data were entered into a Microsoft Excel 2016 spreadsheet for Windows.

2.3. Processing of Biological Samples

SS, IS, and ETBS samples underwent pretreatment with phosphate-buffered saline (PBS) at pH 7.2 following the standardized technique at the Mycology Department of the National Institute of Hygiene (INHRR) in Caracas, Venezuela, to obtain a sample with conditions like BAL [6]. The Galactomannan test for Aspergillus was performed using 1 mL of the final pretreated wash

suspension from SS, IS, and ETBS, along with the serum sample, following the manufacturer's instructions for BAL and serum samples [7]

2.4. *Aspergillus Galactomannan Lateral Flow Assay*

To evaluate antigenemia, the sōna *Aspergillus Galactomannan* LFA kit (IMMY Diagnostics, Oklahoma, USA) was used, following the manufacturer's instructions with modifications in incubation time. Briefly, 300 μ L of the pretreated respiratory samples (SS, IS, ETBS), BAL, and serum were mixed with 100 μ L of pretreatment buffer and vortexed. They were then placed in a heat block at 120°C for 8 min and subsequently centrifuged at 14,000 g for 10 min. 80 μ L of the supernatant was mixed with 40 μ L of the *Aspergillus* GM-LFA running buffer in a sterile tube. An *Aspergillus* GM lateral flow strip was then placed in each sample and allowed to diffuse for 10 min to visualize the run; quantitative readings were taken 20 min later using the sōna LFA Cube Reader [7]. Samples from each patient were processed and analyzed upon receipt. Each run was appropriately controlled with positive and negative controls provided by the kit for each lot. The presence of two pink lines (test and control), regardless of intensity, indicates a positive result. A single control line (top line) indicates a negative result. If the control line does not appear, results are invalid and the test was repeated; however, negative results did not rule out the diagnosis.

2.5. *Mycological Culture of Respiratory Samples*

An aliquot of the pretreated respiratory samples and BAL sediment was inoculated onto Sabouraud Dextrose Agar (SDA-Oxoid)[®] plus gentamicin, Mycosel Agar[®], and Potato Dextrose Agar (PDA)[®], then incubated at temperatures between 20-30°C and 35 $\pm$ 2°C. Cultures were reviewed weekly for one month to check for colonies suggestive of *Aspergillus* sp. In the case of growth of any *Aspergillus* species, a direct examination with lactophenol blue was performed for microscopic recognition of structures, considering the criteria described by De Hoog *et al.* [10] and Klich *et al.* [11].

2.6. *Interpretation of Results*

Reference values provided by the commercial kit manufacturer were used. For pretreated sputum (extrapolated from BAL) and serum samples, these are: Positive: ≥ 0.5 and Negative: < 0.5 . These were compared with the EORTC/MSG cut-off points for dual samples (serum and BAL): Serum or plasma: ≥ 0.7 and BAL: ≥ 0.8 . Regarding patient classification, clinical and epidemiological data were initially considered without mycological evidence. Following GM investigation and mycological culture, they were reclassified into possible, probable, or proven IA.

2.7. *Statistical Analysis*

Statistical analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Discrete or continuous quantitative variables were described using measures of central tendency and dispersion. For nominal qualitative variables, absolute and relative frequencies (percentages) were used. For processing the inferential statistical data for comparing means between paired samples, the Student's t-test was applied. All tests were two-tailed and the significance level was 5%, meaning that all p-values < 0.05 were considered statistically significant.

3. Results

The 76 patients were preliminarily categorized based on signs and symptoms suggestive of IA according to EORTC/MSG criteria: 56 (74%) met criteria for possible IA and 20 (26%) met most criteria but lacked radiological evidence (classified as suspected IA without imaging). Clinical and epidemiological characteristics are shown in Table 1. Regarding underlying diseases, 20 (26%) patients had solid tumors (lung being most frequent); 25 (33%) had unknown underlying conditions; and 14 (18%) had other pathologies like asthma, diabetes, hypertension, and SARS-CoV-2. A total of 53 (70%) had a presumptive diagnosis of pneumonia. Fifty-nine patients (70% [sic - likely 78% based on discussion]) received steroid treatment. Regarding imaging, 39 (51%) presented pulmonary

infiltrates. Seven (9%) presented SARS-CoV-2; 7 (9%) were on mechanical ventilation, and 13 (17%) died. Of the 76 patients, 32 were selected whose GM values matched EORTC/MSG criteria (serum ≥ 0.7 and PS or BAL ≥ 0.8). For three of these, GM was performed on serum, PS, and BAL simultaneously. Descriptive statistics for these values are in Table 2. The remaining 41 samples were negative. Student's t-test (Table 3) indicated the mean estimation is reliable ($p=0.00$ for PS and serum). In the 3 patients with simultaneous PS and BAL, a correlated t-test yielded a mean of 3.87 for sputum and 2.14 for BAL. Correlation showed $p(0.736) > 0.05$ between both samples, suggesting pretreated sputum behaves similarly to BAL. Mycological culture was positive for *Aspergillus* in 21 (28%) samples. *Aspergillus fumigatus* was most isolated (57%), followed by *A. flavus* (29%) and *A. niger* (14%). Table 4 shows the final classification: 20 patients (26%) originally suspected were finally classified as "No IA" due to lack of imaging (despite 4 having positive GM). Of the 56 initially possible IA, 28 remained possible (negative GM) and 28 became probable (positive GM).

3.1. Figures, Tables and Schemes

Table 1. Clinical and epidemiological characteristics of patients with suspected IA.

Characteristic	Samples (n=76)	Percentage (%)
Gender		
Female	38	50
Male	38	50
Age $\bar{X}$: 58		
< 20 years	6	8
20-60 years	26	34
60-80 years	39	51
>80 years	4	5
Underlying Condition/Pathology		
Hematological Malignancies	6	8
Hodgkin's and Non-Hodgkin's Lymphoma	4	
Leukemia	1	
Myeloma	1	
Solid Tumors	20	26
Lung	12	
Others	8	
COPD	5	7
Cystic Fibrosis	4	5
Transplants	2	3
Others	14	18
Unknown (records not available)	25	33
Presumptive Diagnosis		
Pneumonia	54	70
Others	22	30
Treatment		
Steroids	59	78
Non-steroids	11	14
No treatment	6	8

CT Scan Findings		
Infiltrates	39	51
Cavitations	5	7
Nodules	2	3
Halo Sign or Ground-glass Opacity	3	5
Others	7	8
Without Imaging	20	26
Associated Diseases		
SARS-CoV-2	7	9
Influenza	2	3
Mechanical Ventilation		
Yes	7	9
Morbidity/Mortality		
Deceased patients	13	17
Living patients	63	83

IA: Invasive Aspergillosis; COPD: Chronic Obstructive Pulmonary Disease; CT: Computed Tomography.

Table 2. Descriptive statistics for average galactomannan values obtained in pretreated sputum, serum, and BAL in evaluated patients.

Sample	n	Minimum	Maximum	Mean	Standard Error	Standard Deviation	Variance
Pretreated sputum	29	0.80	13.63	3.36	0.55	3.00	9.01
Serum	29	0.7	18.58	3.67	0.86	4.68	21.94
BAL	6	0.83	6.74	2.55	0.93	2.28	5.20
Serum	6	1.1	9.15	3.19	1.28	3.15	9.93

BAL: Bronchoalveolar Lavage; n: sample size.

Table 3. Student's t-test of single samples for galactomannan values in pretreated sputum, BAL, and their respective serum samples.

Sample	t	df	Mean	95% CI (Lower - Upper)	p
Pretreated Sputum	6.04	28	3.36	2.22 – 4.51	0.00
Serum	4.22	28	3.67	1.89 – 5.45	0.00
BAL	2.74	5	2.55	0.15 – 4.94	0.041
Serum	2.47	5	3.19	-0.11 – 6.49	0.05

BAL: Bronchoalveolar Lavage; df: degrees of freedom; CI: confidence intervals; p: statistical significance.

Table 4. Clinical and epidemiological characteristics of patients evaluated via EORTC/MSG criteria.

Characteristic	Possible IA n= 28	Probable IA n=28	No IA n=20
Gender			
Female	10	15	13

Male	18	13	7
Age			
< 20 years	3	3	0
20-60 years	11	8	7
60-80 years	13	15	11
>80 years	1	2	1
Underlying Condition/Pathology			
Hematological Malignancies	0	4	2
Solid Tumors	9	9	2
COPD	2	1	2
Cystic Fibrosis	3	1	0
Transplants	1	1	0
Others	4	5	5
Unknown	9	7	9
Presumptive Diagnosis			
Pneumonia	21	20	13
Others	7	8	7
Treatment			
Steroids	25	26	15
Non-steroids	2	2	1
No treatment	1	0	4
CT Scan Findings			
Infiltrates	19	20	0
Cavitations	2	3	0
Nodules	1	1	0
Halo Sign or Ground-glass Opacity	1	2	0
Others	5	2	0
Without Imaging	0	0	20
Associated Diseases			
SARS-CoV-2	3	3	1
Influenza	1	1	0
Mechanical Ventilation			
Yes	2	5	
Morbidity/Mortality			
Deceased patients	4	7	2
Living patients	25	20	18

IA: Invasive Aspergillosis; COPD: Chronic Obstructive Pulmonary Disease; CT: Computed Tomography.

4. Discussion

Currently in Venezuela, no studies have evaluated pretreated sputum as an alternative method for detecting GM antigen via LFA as a tool for diagnosing IA.

The patients evaluated in this study had a mean age of 58 years, with the highest percentage (51%) falling within the 61–80 age range. Gender distribution was equal, with 50% male and 50% female participants. Although the literature does not establish a specific mean age or gender as a predisposing factor for IA, the trends observed here are consistent with those of Jenks *et al.*, where the mean age was 60 years, though males predominated (55%) [12].

While IA is common in patients with hematologic malignancies, those with solid tumors are also at risk due to chemotherapy- and radiotherapy-induced neutropenia [13]. In the present study, the most frequent underlying pathologies were solid tumors (26%), followed by hematological malignancies (8%). These results differ from the findings of Cornillet *et al.*, where hematological malignancies were more frequent (57%) than solid tumors (3%) [14]. This discrepancy is likely relative to the sample size and the specific clinical focus of the health center. Notably, among solid tumors in this study, lung tumors were the most frequent, matching data obtained by Jani *et al.* [15].

The presence of pneumonia is often confused with ventilator-associated pneumonia (VAP), particularly if patients have previously received mechanical ventilation. This was described by Loughlin *et al.*, where 24 out of 194 patients with presumed VAP were confirmed to have the condition [16]. In the present study, 70% of patients had a presumptive diagnosis of pneumonia, and 7% required mechanical ventilation.

Prior steroid treatment negatively impacts the host's immune response, and prolonged use promotes the growth of *Aspergillus* spp. by reducing defense cell lines such as macrophages and neutrophils [17]. Palmer *et al.* demonstrated that even low concentrations of steroids can predispose individuals to IA [18]. In this study, 78% of patients had previously received steroids—a figure nearly identical to the 78.6% reported by Kimura *et al.*, who noted that such treatments create an environment conducive to fungal development [19].

Radiological findings are essential for diagnosing IA, though patterns vary based on host factors and disease progression. The "halo sign" is an early indicator of IA, primarily in neutropenic patients [20,21]. However, in the present study, it was only the third most frequent pattern (5%). The most frequent findings were infiltrates (51%), followed by cavitations (7%), like the findings of Zhuang *et al.* [22]. Infiltrates are the most common pattern in non-neutropenic patients, whereas cavitations likely occur due to delayed CT scans performed later in the course of the disease [20,23].

Patients with severe COVID-19 pneumonia have a higher probability of developing IA due to prolonged ICU stays and the use of steroids and immunomodulators [24]. Wang *et al.* reported a 7.7% incidence of SARS-CoV-2 as an associated disease [25]; similar figures were obtained in this study, where 9% of patients presented with both SARS-CoV-2 and IA.

The mortality rate for IA can exceed 90% [2]; however, this study observed a survival rate of 83%, like the 84.8% reported by Goyeneche *et al.* [1]. This survival rate may be influenced by the specific types of immunocompromised states or predisposing factors present upon admission.

Descriptive statistics were applied to the LFA data for serum and pretreated sputum. Sputum samples showed a mean of 3.36 (range: 0.80–13.63), while serum samples showed a mean of 3.67 (range: 0.7–18.58). A t-test confirmed the reliability of these results. The 95% confidence interval (CI) for sputum was 2.22 to 4.51 ($p < 0.05$). For serum, the CI was 1.89 to 5.45 ($p < 0.05$). These analyses confirm that the results are statistically significant.

The standard deviation for serum associated with sputum and BAL samples was 4.68 and 3.15, respectively. This suggests why serum is not the ideal sample for galactomannan determination; it is highly influenced by underlying diseases, the extent of aspergillosis, prophylactic treatments, and the site of infection. In non-neutropenic patients, there is often less vascular invasion by *Aspergillus* hyphae, and active neutrophils can eliminate galactomannan antigens, further reducing serum levels. Therefore, serum is not recommended as the sole sample for suspected IA [26].

The study by Kimura *et al.* demonstrates that serum has lower sensitivity for galactomannan compared to respiratory samples like sputum and BAL. This correlates with our results, where the largest standard deviations were observed in serum samples, indicating greater data dispersion [19].

A correlated t-test between pretreated sputum and BAL samples yielded a p-value of 0.453 ($p > 0.05$), indicating no significant difference between the two. This confirms the utility of pretreated sputum as an alternative sample for galactomannan LFA. Similarly, Fu *et al.* found a p-value of 0.655 when comparing the two, concluding that sputum galactomannan performance is comparable to BAL [27].

The three patients with positive samples in this study presented risk factors, radiological findings, and positive cultures for *Aspergillus fumigatus*. These results support the potential for a "proven IA" diagnosis if BAL and sputum cultures were considered equivalent. Currently, BAL culture is excluded from EORTC/MSG criteria because it is not from a sterile region and carries a high risk of contamination. However, Bassetti *et al.* have proposed new criteria that include positive BAL cultures as a mycological criterion for non-neutropenic patients [28]. Using pretreated sputum would benefit neutropenic and hematological patients because the collection risk is low, unlike BAL, which can lead to complications in unstable patients [29,30]. Furthermore, these samples come directly from the site of infection (the lungs), where antigen concentration is highest [26].

In this study, 28% of respiratory samples yielded positive cultures, comparable to the 25.4% positivity rate reported by Goyeneche *et al.* Both studies agree that *A. fumigatus* is the most frequently isolated species, followed by *A. flavus* and *A. niger* [1].

Finally, a new classification of IA was performed using EORTC/MSG criteria. Results showed that 56 patients had possible or probable IA, while 20 did not have aspergillosis (though they lacked radiological images for definitive classification). These results differ from Zhuang *et al.*, who observed a higher proportion of non-IA patients and a higher frequency of diabetes and solid tumors [22]. In the present study, four patients with IA had hematological malignancies, whereas Zhuang *et al.* focused on non-neutropenic patients.

Regarding treatment, steroids were more frequently used in patients with IA. Severe exogenous immunosuppression leads to prolonged neutropenia, the primary risk factor for invasive *Aspergillus* infections [1,31]. Additionally, antibiotics influence the stability of the gut microbiome, creating an environment conducive to fungal growth when the immune system is compromised [32].

Radiological findings for patients classified with possible IA were primarily infiltrates, followed by segmental atelectasis, bronchiectasis, and consolidations. These results mirror those of Zhuang *et al.* [22]. Notably, imaging was unavailable for 26% of patients, likely due to socioeconomic factors; had these images been available, these patients might have been formally classified under IA criteria.

According to EORTC/MSG criteria, 37% of patients were classified as "possible IA," 37% as "probable IA," and 26% as "non-IA." If the kit's standard cutoff point (≥ 0.5) had been used for the galactomannan test, six additional "possible" cases would have been upgraded to "probable." Furthermore, 12 patients had probable IA plus 7 with possible IA and positive cultures. This highlights the limitations of excluding non-sterile respiratory cultures from EORTC/MSG criteria. If these were included, 19 additional patients would have received a confirmed diagnosis and early treatment. New criteria should be included for non-neutropenic patients with atypical findings of IA, given that, after the COVID-19 pandemic, patients who were previously immunocompetent have been predisposed to acquiring IA.

5. Conclusions

In conclusion, EORTC/MSG criteria have limited applicability in non-neutropenic patients, who often do not meet standard radiological or host criteria. Pretreated sputum performs as well as BAL for galactomannan LFA and provides a safer alternative for patients unable to undergo invasive procedures. Respiratory samples increase the probability of detection in non-neutropenic patients compared to serum. Ultimately, pretreated sputum is a valuable tool for defining possible and probable IA cases within clinical practice.

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Investigation, Formal analysis & Writing – original draft; A.V.: Writing – original draft, data curation & Review; D.O.: Investigation, data curation, Writing – review & editing; J.C.: Investigation & Review; M.L.: Investigation & Review; N.G.: Investigation & Review. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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