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RESEARCH ARTICLE

Comparison of diagnostic techniques in patients with COVID-19-associated pulmonary aspergillosis (CAPA) in a Venezuelan population

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ABSTRACT

Background: Invasive aspergillosis related to COVID-19 or CAPA is defined by the association of a group of histological, mycological, radiological and clinical criteria that establish this disease as a secondary infection of high morbidity without timely diagnosis. Clinical and histological methods, due to the non-specific characterization of the disease, have been supported by techniques based on microbiological methods, such as culture and direct examination, with a sensitivity close to 50%, allowing the identification of biomarkers such as the GM of the pathogen, being the immunological techniques of greater sensitivity and specificity, the use of radial immunodiffusion and ELISA with antigens secreted by Aspergillus spp. are recommended. The objective of this article was to compare three different serological diagnostic methods used in the mycology laboratory to diagnose CAPA in a Venezuelan population.
Methods: Based on a longitudinal study, 77 patient samples diagnosed as positive for SARS-CoV-2 by RT-PCR were analyzed, of which 24 CAPA BAL samples were subsequently evaluated by conventional mycological diagnostic methods (direct examination and culture) and corresponding CAPA serum samples by three serological methods: DID, ELISA with *Aspergillus* pool antigen, and the Galactomannan detection with Lateral flow (GM- LFA) essay.
Results: By microscopy, fungal structures were observed in 18/24 patients and 16/24 were positive for GM-LFA. Regarding the DID assays, precipitating bands were obtained in 21/24 patients with 86% sensitivity; and 97% specificity. To use ELISA technique, shows that 16/24 sera from patients with aspergillosis were positive, which represents 66% sensitivity and 97% specificity. As for GM- LFA, all sera were tested and 16/24 positive sera were obtained with the commercial kit, representing 66% sensitivity and 97% specificity.
Conclusions: Finally, when comparing the three tests studied, all techniques were found to be highly sensitive and have excellent specificity, recommending that the diagnosis of CAPA and IPA be based on a combination of diagnostic assays.
Keywords: Aspergillosis, COVID-19 Associated Pulmonary Aspergillosis, diagnostics, lateral flow assay, ELISA, serology.

Introduction

COVID-19 associated Pulmonary Aspergillosis (CAPA) is defined as a secondary or concurrent fungal infection due to *Aspergillus sp.* along with SARS-COV-2, according to histological, mycological, radiological and clinical criteria[1]. CAPA can be a life-threatening comorbidity with nearly 38% mortality if diagnosis and treatment are not in time [2]. The genus *Aspergillus* spp belongs to the phylum *Ascomycetes, order Eurotiales*, and family *Trichocomaceae*, with cosmopolitan distribution in soil, air, water and present in the human oral and pulmonary mycobiome. Frequent species related to Aspergillosis are *A. fumigatus, A. flavus* and *A. niger*; followed by *A. terreus, A. nidulans, A. glaucus, A. tubingensis* and *A. versicolor* [3,4]. Clinical and radiological diagnosis can be challenging due to its clinical resemblance to other infections; approximately, 5% of patients will develop severe symptoms such as dyspnea, complex immune deregulation, intravascular disseminated coagulation and diffuse alveolar damage, leading to a multiorgan failure [5]. Latin America has been one of the region's most severely affected by the COVID-19 pandemic, accounting for 25% of global infections. Eight of the ten countries with the highest mortality rates in the world are now known to be in the Region, including Colombia and Brazil. Deaths from COVID-19 in the region amount to nearly 1.3 million people, but the pandemic continues to cause deaths due to the failure of health services, economic instability, and deepening inequalities in Latin America[6,7]. Studies in different countries of the Americas have shown the convergence of COVID-19 and co-infections of three fungal groups: *Aspegillus*, Mucorales and C*andida*, with a higher number of records for CAPA in patients with invasive mechanical ventilation registered since the beginning of 2020 using the combination of different diagnostic tools[8].

On the other hand, various studies have shown an overall incidence of CAPA of 13.5%, with most patients acquiring the infection while requiring invasive mechanical ventilation (IMV). The time from onset of illness to diagnosis of CAPA ranged from 8.0 to 16.0 days. However, the time from intensive care unit (ICU) admission and initiation of IMV ranged from 4.0 to 15.0 days and 3.0 to 8.0 days [9]

In Venezuela, there is scarce information about Mycoses post COVID-19, including aspergillosis; reports before the SARS-CoV-2 pandemic, depicted 4.5% cases of aspergillosis included in the group of Invasive Fungal Infections and 1.6% of superficial mycoses, Monagas state was the most prevalent state with the highest reports of Invasive Pulmonary Aspergillosis (IPA) cases, probably related to greater agricultural and farming activities, followed by Caracas and Zulia States[10]. However, COVID-19 has changed the epidemiology of mycoses throughout the world including our country [11].

Mycological diagnosis is characterized by positive cultures from sterile anatomical sites and visualization of fungal structures in studied samples with a sensitivity of 50%. The discrimination between invasive infection and colonization is difficult; this is because the available diagnosis tests excepting histopathological examination do not yield absolute evidence of infection [12]. In addition, some laboratories have developed the detection of the biomarker galactomannan. This is a complex polysaccharide present in the fungal cell wall. Diagnostic strategies specifically focus on the detection of an extracellular mannoprotein antigen secreted exclusively during active growth of *Aspergillus* species by a monoclonal antibody using enzyme-linked immunoassay (ELISA) and immunochromatography (LFA, lateral flow assay) [13,14]. The LFA is worldwide distributed and each year more applied to human samples, obtaining high sensibility and specificity of 80% an 94% respectively, to achieve rapid diagnosis of CAPA. The LFA can qualitatively and quantitatively detect galactomannan antigen concentrations in several samples: bronchoalveolar lavage (BAL) and serum according fabricant indications [15,16]. Regarding the serum detection of specific antibodies by double agar gel immunodiffusion test (DID) and ELISA, it shows good sensitivity and specificity for the diagnosis of pulmonary aspergillosis and is characterized as a simple method with low cross-reactivity. Moreover, the serological progression of patients after the establishment of antifungal treatment [17,18]. The aim of this article is to compare three different diagnostic methods used in the medical mycology laboratory to diagnose CAPA in a Venezuelan population.

Methods

STUDIED GROUP

We transversely studied a cohort of 77 patients with moderate to severe diagnosis of pneumonia associated with SARS-CoV-2 proven by RT-PCR technique, hospitalized at the Hospital Vargas de Caracas – Venezuela. Patients included in the study were adults over 18 years old, admitted from May 15th 2020 to May 15th 2021. In all cases, demographic, clinical and epidemiological data were considered: age, gender, comorbidities, date of Intensive Care Unit (ICU) admission (if required), date of discharge and/or date of disease. Also, other information was gathered such as laboratory findings, microbiological results (cultures, hemocultures, bacilloscopies, medication received during hospitalization and radiological information. The Bioethical Committee of the Instituto Autónomo de Biomedicina "Dr. Jacinto Convit" granted ethical approval for this study (06/2022).

DIAGNOSTIC CRITERIA

For COVID-19 associated Pulmonary Aspergillosis (CAPA), we applied the modified criteria based on the EORTC and MSGERC and Asp ICU and expert case definitions [1].

- Proven CAPA: Patient with COVID-19 needing intensive care and at least one of the following: Invasive Pulmonary Aspergillosis (IPA) confirmed by positive biopsy/histology and/or autopsy from sterile site (detection of growing fungal hyphae with peripheral tissue damage), Aspergillus sp., recovered from sterile site or PCR obtained by sterile aspiration/biopsy from pulmonary site.
- Probable CAPA: Patient with COVID-19 needing intensive care with radiological findings such as Pulmonary infiltrate documented by chest CT or cavitating infiltrate (not attributed to another cause) with at least one of the following:

microscopic detection of fungal elements in bronchoalveolar lavage, indicating a mould; positive bronchoalveolar lavage culture, serum galactomannan index >0·5 or serum LFA index >0·5; bronchoalveolar lavage galactomannan index ≥1·0 or bronchoalveolar lavage LFA index ≥1·0; two or more positive aspergillus PCR tests in plasma, serum, or whole blood; a single positive aspergillus PCR in bronchoalveolar lavage fluid (<36 cycles); or a single positive aspergillus PCR in plasma, serum, or whole blood, and a single positive in bronchoalveolar lavage fluid (any threshold cycle permitted).

- Possible CAPA: Patient with COVID-19 needing intensive care with Pulmonary infiltrate, preferably documented by chest CT, or cavitating infiltrate (not attributed to another cause) and at least one of the following: microscopic detection of fungal elements in non-bronchoscopic lavage indicating a mould; positive non-bronchoscopic lavage culture; single non-bronchoscopic lavage galactomannan index >4·5; non-bronchoscopic lavage galactomannan index >1·2 twice or more; or non-bronchoscopic lavage galactomannan index >1·2 plus another non-bronchoscopic lavage mycology test positive (non-bronchoscopic lavage PCR or LFA)

SAMPLES OBTAINED

For each patient, two serum samples during the first 14 days of hospitalization were received, to perform immunoassays. Additionally, 24 bronchoalveolar lavage (BAL) were performed by physicians and sent for mycological study (direct examination, conventional culture in selective agar).

SAMPLES MANIPULATION

The serum samples were analyzed using three methods: DID, ELISA with *Aspergillus* pool antigen manufactured at the Mycology Laboratory of the Institute of Biomedicine "Dr. Jacinto Convit" and additionally the Galactomannan detection kit provided by IMMY Diagnostic was used.

DIAGNOSTIC ANTIGENS PREPARATION

We developed antigens with three Aspergillus reference strains: *Aspergillus niger* ATCC 0613, *Aspergillus fumigatus* ATCC 0607 and *Aspergillus flavus* ATCC 0605.

These strains were maintained by successive subcultures on Sabouraud Dextrose Agar with chloramphenicol at 25°C. Subsequently, 0.5 cm fragments of each culture on Sabouraud agar were transferred to 2 L flasks containing 500 mL of liquid Sabouraud medium. The cultures were maintained at 31°C in the dark for 5 weeks, to reach stationary phase, where the excretion-secretion antigenic components are produced. Subsequently, the cultures were filtered first through Whatman N°1 paper (Whatman, Springfield Mill, UK), centrifuged at 800 g for 30 min. and filtered again through Millipore 0.45 µm nitrocellulose paper (Millipore Corporation, Bedford, MA, USA). The sterilized filtrate was dialyzed overnight, at 4 °C, against distilled water, using dialysis tubing with a 10 kDa cut-off, and the sample was lyophilized and resuspended in sterile distilled water [19,20].

Subsequently, protein concentration was determined by the BCA Protein assay Kit (Pierce), the filtrate was analyzed by DID and ELISA.

To assay cross-reactivity antigen produced was tested with heterologous serums diagnosed with other diseases: histoplasmosis (n=16), paracoccidioidomycosis (n=16), coccidioidomycosis (n=16) and sporotrichosis (n=16). Also, 25 sera from healthy individuals were included as negative controls to determine specificity of the antigen and the determination of the cut-off value. (The healthy individual's serums were donated by the Municipal Blood Bank of Capital District, Caracas).

DOUBLE IMMUNODIFFUSION METHOD (DID)

The Ouchterlony technique with some modifications was used to evaluate the sera [21].

DID was performed on agarose slides. Each slide was punched with seven holes, the main central hole of 1 mm Ø was filled with 5 µL of the antigenic component, and 20 µL of the sera in 4 mm diameter were placed in the other six holes alternatively. The slides were placed in a wet chamber, and left for 24 h at 24 °C, then at 4 °C for 24 h. Afterwards, the slides were washed with water and in 5% tri-sodium citrate solution for 90 min. Subsequently, they were rinsed several times with saline solution. The agarose was dehydrated by placing the slides on filter paper moistened with distilled water in a 37° C oven for 24 h. Ultimately, the slides were colored with a protein dye solution (Amidoschwarz) for 15 minutes and bleached with 4% acetic acid solution [22].

ELISA IMMUNOASSAY

The protocol used was that of Guimaraes et al. (2004)[23] with a few minor modifications. 96-well polystyrene plates were used, dispensing 100 µl per well of the antigenic compound (1 µg/ml) prepared in a 10 mM of saline phosphate buffer (PBS). The plate was incubated for 1 h at 37 °C and then left at 4 °C overnight. Then the plate was washed 3 times with PBST wash buffer (10 mM, 0.1% v/v Tween 20, pH 7.3), for 5 min, each wash, 200 µl of blocking buffer (PBST with 5% w/v nonfat milk) was added for 2 h at 37 °C. The plates were then plated according to the previously described conditions and patient sera samples were added in duplicate to each well (100 µl) at 1:1000 dilution in wash buffer (PBST) followed by incubation for 1 h at 37 °C. Subsequently, the plates were washed and 100 µL per well of secondary antibody (Dako Po214, Denmark) was added at a 1:8000 titer diluted in blocking buffer, incubated at 37 °C for 1hr and the plates were washed again. Finally, the reaction was developed with 0.4 mg/ml of O-phenylenediamine dihydrochloride (OPD) per mL in 0.01 M sodium citrate buffer pH 5.5 and added 0.04% v/v Hydrogen Peroxide (30% H_2O2) at the time of use (100 µl), plates were incubated for 10 min in dark at 24 °C. The reaction was stopped with 50 µl 3 M HCl. The absorbance was measured in an ELISA reader (BT 2000 Microkinetics Reader, Biotek Instruments, Inc., USA) at a wavelength of 490 nm. The cut-off point was established as the mean of the absorbances plus three standard deviations [20].

GALACTOMANNAN DETECTION WITH LATERAL FLOW (GM- LFA) ESSAY

For the evaluation of titers of GM in serum, we used the

GM Aspergillus Lateral Flow Assay kit (GM-LFA, IMMY). The galactomannan lateral flow assay test was performed in 300µl of each patient sera, following the manufacturer instructions. The assay was performed by a single operator, using a GM Index (GMI) >0.5 and ≥1.0 in serum as a positivity threshold. To eliminate subjectivity, validity was confirmed and the Sona LFA cube reader (IMMY Diagnostics) [24].

To evaluate cross-reactivity, the antigen produced was tested with heterologous sera diagnosed with other diseases: histoplasmosis (n=16), paracoccidioidomycosis (n= 16), coccidioidomycosis (n=16) and sporotrichosis (n=16). Also, 25 samples of healthy patients were assessed as negative controls to determine specificity of the antigen (Healthy patients' serums were donated by the Municipal Blood Bank of Capital District, Caracas).

STATISTICAL ANALYSIS

The determination of the sensitivity and specificity variables considering that they are proportions was performed using confidence intervals with standard methods based on the binomial proportion and the Central Limit Theorem ($p \pm 1.96^{*}\sqrt{(p(1-p)/N)}$), taking into consideration that the sample size was less than 30, which infers that the estimated value is close to 1 (or 100%) or 0 (0%), the standard method based on the binomial proportion was used. The Z value was obtained from the normal function (inverse function of a standard normal), with 1.96 being the corresponding value for a two-sided 95% confidence interval. In this context, "p" represents the proportion for which the confidence interval is to be calculated, while N corresponds to the denominator of this proportion. In the case of sensitivity, N is the sum of true positives and false negatives [25].

Results

During the 2020-2021 study period, we analyzed 77 patients previously diagnosed with SARS-CoV-2 infection, of which 24/77 (31.16%) were positive for CAPA. Of these patients that fulfilled the CAPA criteria, 9/24 (37.5%) have probable CAPA and 15/24 (62.5%) possible CAPA. Regarding the mycological cultures, *Aspergillus fumigatus* was the main fungi recovered from BAL samples in 3/9 (33.33%) male and 6/9 (66.66%) female patients. About microscopy, fungal structures were observed in 18/24 (75%) patients and 16/24 (66.66%) were positive for GM-LFA (Table 1).

Table 1. Demographic and clinical characteristics of the CAPA patients

Patient	Age	sex	State	Comorbidities	clinical manifestations	Coinfections	CAPA	Microscopy/ Fungal Culture/ GM-LFA	Cortisteroid Treatment
1	56	M	Distrito capital	diabetes	cough Hemoptysis Fever Dyspnea Fatigue Chest pain		Probable	positive microscopy *Aspergillus fumigatus*/GM+	Yes
2	47	F	Miranda		cough Hemoptysis Fever Dyspnea, weight loss Chest pain	TBC	Probable	positive microscopy *Aspergillus fumigatus*/ GM+	Yes
3	33	M	Distrito capital		cough Hemoptysis Fever Dyspnea	HIV	Possible	positive microscopy	Yes
4	45	M	Distrito capital		cough Hemoptysis Fever Dyspnea		Possible	positive microscopy	Yes
5	56	M	Distrito capital		cough Hemoptysis Fever Dyspnea Fatigue Chest pain	HIV	Probable	positive microscopy *Aspergillus fumigatus*/GM+	Yes
6	19	M	Distrito capital		cough Hemoptysis Fever Dyspnea		Possible	positive microscopy	Yes
7	45	M	Distrito capital		cough Hemoptysis Dyspnea		Possible	positive microscopy/ GM+	Yes
8	46	M	Miranda		cough Hemoptysis Dyspnea		Possible	positive microscopy	Yes
9	36	F	Distrito capital		cough Hemoptysis Fever Dyspnea		Possible	positive microscopy/ GM+	Yes

Patient	Age	sex	State	Comorbidities	clinical manifestations	Coinfections	CAPA	Microscopy/ Fungal Culture/ GM-LFA	Cortisteroid Treatment
10	33	M	Distrito capital		cough Hemoptysis Dyspnea		Possible	positive microscopy	Yes
11	31	F	Distrito capital		cough Hemoptysis Fever Dyspnea		Possible	GM+	Yes
12	66	F	Distrito capital	diabetes	cough Hemoptysis Fever Dyspnea, weight loss		Probable	positive microscopy *Aspergillus fumigatus*/ GM+	Yes
13	40	F	Distrito capital		cough Hemoptysis Dyspnea		Possible	GM+	Yes
14	40	F	Distrito capital		cough Hemoptysis Fever Dyspnea		Probable	positive microscopy *Aspergillus fumigatus*/ GM+	Yes
15	29	F	Distrito capital		cough Hemoptysis Fever Dyspnea	TBC	Possible	positive microscopy	Yes
16	30	M	Distrito capital		cough Hemoptysis Dyspnea		Possible	GM+	Yes
17	44	F	Miranda		cough Hemoptysis Fever Dyspnea		Probable	positive microscopy *Aspergillus fumigatus*	Yes
18	20	M	Distrito capital		cough Hemoptysis Fever Dyspnea		Possible	GM +	Yes
19	65	M	Miranda	hypertension	cough Hemoptysis Fever Dyspnea		Possible	positive microscopy	Yes
20	53	M	Miranda	diabetes	cough Hemoptysis Fever Dyspnea		Possible	GM +	Yes
21	41	M	Distrito capital		cough Hemoptysis Fever Dyspnea		Probable	positive microscopy *Aspergillus fumigatus*/GM+	Yes
22	36	M	Distrito capital		cough Hemoptysis Dyspnea		Possible	GM +	Yes
23	46	F	Miranda		cough Hemoptysis Fever Dyspnea	TBC	Probable	positive microscopy *Aspergillus fumigatus*/GM+	Yes
24	34	F	Distrito capital		cough Hemoptysis Fever Dyspnea		Probable	positive microscopy *Aspergillus fumigatus*/GM+	Yes

Table 2. Sensitivity and specificity of the tested techniques

	IDD	ELISA	GM-LFA
Sensitivity	86%	66%	66%
Specificity	97%	97%	97%

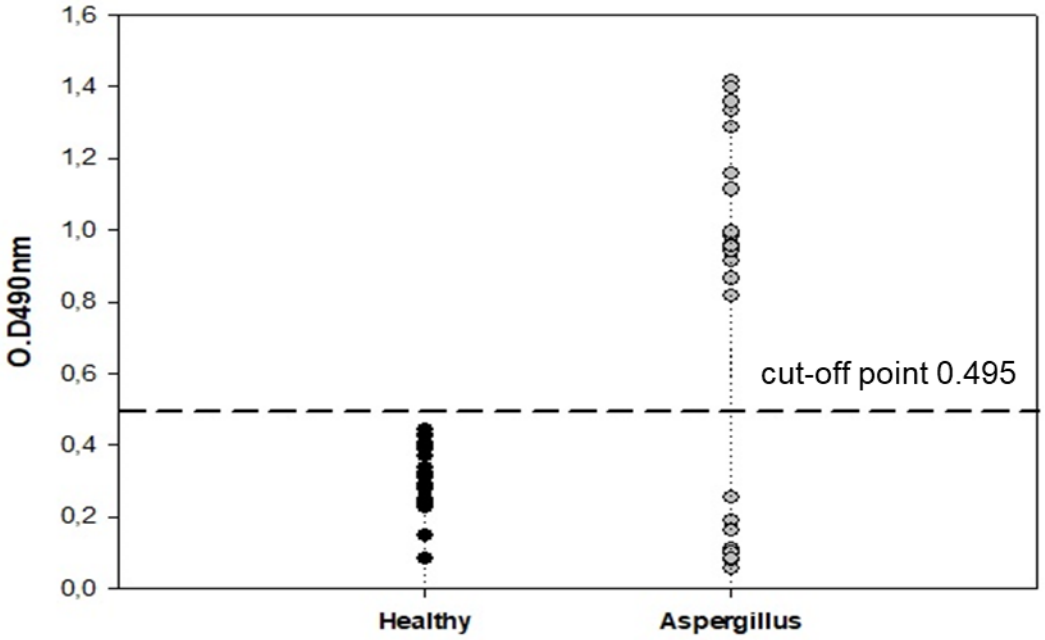

Figure 2. Detection of antibodies by indirect ELISA in serum of patients with aspergillosis, using *Aspergillus* spp. antigen Pool.

This study included 14/24 (58.33%) male patients and 10/24 (41.66%) female patients. The age range was between 19 and 65 years, with a mean of 41.29 years. The patients resided in the Capital District and Miranda state. Regarding clinical manifestations, patients presented cough, hemoptysis, fever, dyspnea, fatigue and chest pain. The proportion of comorbidities and co-infections were: 3/24 (12.5%) diabetes mellitus, 3/24 (12.5%) tuberculosis, 2/24 (8.33%) HIV/AIDS, 1/24 (4.16%) hypertension (Table 1). All patients received broad-spectrum antibiotics and corticosteroids. Radiographically, patients had bilateral interstitial pneumonitis, and nodular infiltrates were widely distributed with predominance of bilateral bases.

Regarding the DID assays, precipitating bands were obtained in 21/24 (87.5%) patients with 86% sensitivity; and 97 % specificity (Figure 1). The positive predictive value was 97.73% and the negative predictive value was 82.50%. (Table 2). As for cross-reactivity, the prevalence is 1/16 (6.25%) for coccidioidomycosis, paracoccidioidomycosis and sporotrichosis, and no serum sample for histoplasmosis.

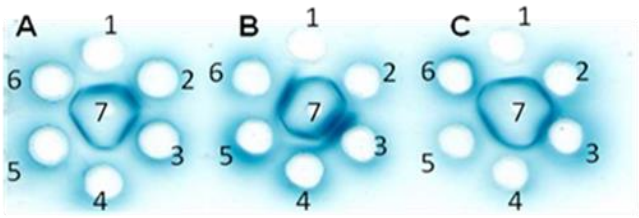

Figure 1. Double immunodiffusion (DID) using Aspergillus spp. antigen
Aspergillus spp. antigen, (central well 7), patient sera (wells 1-6)

In order to use ELISA technique, considering the measures obtained, we established the cut-off point in 0.495, so higher absorbances to this value were stated as positive (Figure 2), shows that 16/24 (67%) sera from patients with aspergillosis were positive, which represents 66% sensitivity and 97 % specificity. The positive predictive value was 97.06% and the negative predictive value was 66%. Concerning cross-reactivity, we obtained 1/16 for coccidioidomycosis, 3/16 paracoccidioidomycosis, 0/16 sporotrichosis, and 5/16 for histoplasmosis (Table 2).

As for GM-LFA, all sera were tested and 16/24 (67%) positive sera were obtained with the commercial kit, which represents 66% sensitivity and 97 % specificity; the positive predictive value was 97.06% and the negative predictive value was 66%, with regard to cross-reactions, 3/10 (30%) cross-reactivity was obtained with positive sera for histoplasmosis (Table 2).

Discussion

SARS-CoV-2 increases the risk of patients developing bacterial and fungal superinfections, including invasive pulmonary aspergillosis (IPA). Influenza-associated pulmonary aspergillosis (IAPA) has complicated the clinical course of many critically ill patients with acute respiratory distress syndrome (ARDS)[26]. COVID-19 causes direct damage to the airway epithelium, facilitating *Aspergillus* spp. invasion. In addition, viral infection reduces ciliary clearance and leads to local or systemic immune dysfunction or dysregulation. The extent of dysregulation associated with ARDS is not fully understood, but some patients develop marked immunosuppression that facilitates bacterial and fungal superinfection, such as patients with CAPA[26].

In Venezuela, the first wave of COVID-19 showed significant geographic heterogeneity, with most cases reported in the north-central region. Despite the lack of official casuistry, as of October 28, 2021, 401 259 cases and 4822 deaths from COVID-19 had been reported in the country; the total number of cases is

closely related to access to diagnosis (RT-PCR, rapid tests) and the estimated tested population was 3,359,014 people, 1/9 of the total population of the country [27]. The first cases were initially reported on March 13, 2020, almost three weeks after the detection of the first case in Brazil and one week after the first report from Colombia[28].

There are few data on the impact of systemic or invasive mycoses in the Venezuelan population due to lack of suspicion by clinicians and lack of laboratory tests or understanding of the clinical evolution of mycoses in hosts; cases of aspergillosis have been previously reported in our country[10], so it is reasonable to hypothesize that a population previously affected by SARS-CoV-2 may develop or reactivate some systemic or invasive mycoses due to the immunological dysregulation observed in hosts with SARS-CoV-2 [26].

A previous study conducted in Venezuela analyzed 576 patients with COVID-19, most of whom presented comorbidities such as: arterial hypertension and diabetes mellitus, consistent with publications from other countries. However, this study, Forero-Peña et al. 2022, did not address the co-infections observed in the cohort of patients, reporting only 44 deaths (7.6%), apparently unrelated to other infections [28]. Early case series studies of patients with presumed CAPA have shown that establishing an accurate diagnosis of CAPA can be challenging. Although host factors, clinical factors (including radiology), and mycologic evidence are often used to diagnose and classify patients with IFD, patients with CAPA may lack host factors and typical radiologic features [29-32].

Invasive aspergillosis (IA) is one of the many known complications of intensive immunosuppressive therapy. In addition, the population at risk for developing aspergillosis has now been significantly expanded by malnutrition, HIV/AIDS, cancer treatment, and other newly categorized patients, including those admitted to intensive care units (ICUs) with severe influenza, ARDS, and COVID-19 [33]. Global estimates indicate that more than 1.8 million cases of IFI were reported in 2017, including 250,000 cases of IPA[34]. Subsequently, after the COVID-19 pandemic, several complications associated with an increased predisposition and/or risk factors to develop fungal diseases, have been described, and many international organizations and literature have developed efforts to classify, diagnose, and treat algorithms for CAPA [1,33].

Recent studies suggest that individuals with severe COVID-19, especially those with compromised immune systems, are at higher risk of developing secondary infections, including those associated with *Aspergillus* spp. On the other hand, immunosuppression induced by the use of IL-6 inhibitors and corticosteroids [35] contributes to the development of CAPA through various downstream pathways: altering the maturation of phagolysosomes involved in the degradation of *Aspergillus fumigatus*; meanwhile, IL-6 inhibitors contribute to the development of aspergillosis by inhibiting the development of Th17 cells, which together with Treg and TCD4 represent the main immune response against *Aspergillus fumigatus* [36].

Many studies have shown that patients with comorbidities are prone to develop severe COVID-19; comorbidities such as cardiovascular disease, hypertension, diabetes, chronic pulmonary obstructive disease, cancer, cerebrovascular disease and others have been described [1, 31, 36]. In this study, we found 3 patients with diabetes, 2/3 died, and 1 patient with hypertension survived. In relation to comorbidities/coinfections, the probable CAPA group (7/16) provided the higher number of samples; however, we could not be correlated as a predictive condition; only the relationship between aspergillosis and treatment with corticosteroids for the development of invasive aspergillosis is confirmed with respect to other similar studies with small number of samples that describe the association between comorbidities and treatment with corticosteroids in CAPA patients [35,36].

As for conventional mycological diagnosis, sensitivity is low, as referred to in a large number of cases of CAPA. Several studies have shown that only a quarter of autopsies have demonstrated that IPA were diagnosed *in vivo* by culture-based methods, which has led to serological studies and molecular methods becoming the first choice for diagnosis [37]

Obtaining mycological evidence of airway-invasive aspergillosis in patients with COVID-19 is complicated [1,31,37], and the low sensitivity of detection of circulating galactomannan in serum. Furthermore, detection of *Aspergillus* in specimens of the upper respiratory tract, such as sputum or tracheal aspirate, often does not distinguish between aspergillus colonization and invasive disease[1]. Given the challenges that are associated with diagnosis and management of patients with CAPA, there is an urgent need to study the epidemiology and characteristics of this secondary infection.

The present study was aimed to compare the accuracy parameters of serologic tests (DID and ELISA), using antigen preparations (*Aspergillus* spp, antigen pool). In addition, serum detection of galactomannan antigen (GM-LFA, IMMY), which can be used to BAL and serum, this GM-LFA is widely used for diagnosis and point of care of IPA treatment [38].

Serological methods such as DID reactions, complement fixation, latex particle agglutination, electrophoretic tests and enzyme immunoassays have been previously evaluated for the diagnosis of aspergillosis[12]. The agar gel immunodiffusion reaction is the most preferred method, because of its simplicity of execution, reproducibility, low cross-reactivity, and high positive likelihood ratio, which facilitates the standardization for the detection of anti-*Aspergillus* antibodies in most clinical laboratories [13,39].

However, this technique has a variable sensitivity dependent-operator, remaining as the main drawback of this method. On the other hand, antigen preparation, and the concentration of antigen needed to detect precipitins in DID, is a critical factor of this reaction, and it can only be read 96 hours after preparation [39].

In this study, the double immunodiffusion (DID) technique obtained a high sensitivity of 86% and specificity of

97%, comparable to the values described in similar studies, where the sensitivity ranges between 92% and 100% and the specificity between 90% and 100% [18,39,40]. Azevedo et al. in 2015,[18] studied 55 patients with chronic pulmonary aspergillosis and 30 healthy individuals; for this purpose, they produced antigenic components of *A. fumigatus*, *A. flavus*, and *A. niger* mixed in an *Aspergillus* pool. They found the sensitivity of DID using the *Aspergillus* pool to be 92.7% and the specificity to be 90%, and when evaluating pure *A. fumigatus* antigenic compounds, they found an increase in sensitivity inversely proportional to specificity. In our present study, samples were evaluated using our own manufactured *Aspergillus* pool and we found that the sensitivity was 4% lower compared to Azevedo et al; however, the specificity was 10% higher than the aforementioned study.

Stopiglia et al. 2017 characterized and standardized the antigens of *Aspergillus* spp. required for serological diagnosis[41]. The authors obtained exoantigens from *A. fumigatus*, *A. flavus* and *A. niger*. The authors evaluated the antigens of each species separately and obtained slight variations between species, with sensitivities ranging from 93% to 100% and specificities from 97% to 100%. Therefore, although our sensitivity is slightly lower and our specificity is 97%, we strongly recommend DID assays as an effective method for diagnosis and follow-up of patients with aspergillosis, regardless of the association with SARS-CoV-2.

Regarding the presence of cross-reactions observed in this study, the DID test showed reactivity with coccidioidomycosis, paracoccidioidomycosis and sporotrichosis sera and no reactivity with any histoplasmosis sera. The presence of cross-reactivity with histoplasmosis sera and different *Aspergillus* species has been described in other studies [42]. This phenomenon is due to the fact that the species share antigenic epitopes, which may be present in 2%-10%. These include mannan antigens, galactomannan, chitin, protein antigens with similar amino acid sequences and glycoprotein antigens, which are also found in the cell wall of these fungi. It is important to emphasize that the cross-reactions obtained in our study do not reduce the diagnostic value of the test [43].

With respect to ELISA assays, variations in sensitivity and specificity have been reported, ranging from 59 to 83% and specificity between 96 and 100%. These differences are closely related to the type of ELISA, its cut-off point and the sample used, in addition to the variability factors of the serological tests previously described [42,43].

In the present work, patient sera were used as the sample for the ELISA test because of the low cost and accessibility of the samples. When using sera, the sensitivity may decrease significantly, but the observed specificity values are reliable and even increased compared to other techniques. In this study, the sensitivity was 66% and the specificity was 97%. These percentages suggest good predictive behavior as a diagnostic test. Cross-reactions are also a common phenomenon; in this assay, one reaction was obtained with 1 coccidioidomycosis serum, 3 paracoccidioidomycosis sera, and 5 histoplasmosis sera. However, other authors report predominantly histoplasmosis and paracoccidioidomycosis, ranging from 9 to 52% [39].

In addition, GM-LFA detection is widely used in the diagnosis of aspergillosis. Chang et al. 2020 [41] performed a comparison of data published up to September 2020 and observed sensitivity values between 47% and 79% and specificity values between 57% and 95%. More recent studies on the use of GM-LFA in the diagnosis of aspergillosis report a sensitivity of 90.9% and a specificity of 90.8%, demonstrating a better diagnostic outcome than ELISA assays [42]. In our work, we obtained a specificity of 97% and a sensitivity of 66%, within the expected range, so that its use is recommended for the diagnosis of all forms of aspergillosis. It is important to mention that it has been reported that the sensitivity of the test decreases when serum samples are used compared to BAL samples [43]. On the other hand, although the patient cohort is small and it is a retrospective study from a single center, the results showed high sensitivity and specificity for the three techniques used. GM-LFA may be a valuable tool for early therapeutic decisions and can be used in laboratories that do not have access to GM-EIA.

Conclusions

Finally, when comparing the three tests studied, all techniques were found to be highly sensitive and have excellent specificity. It is recommended that the diagnosis of CAPA and IPA be based on a combination of diagnostic imaging, mycological examination of respiratory specimens, immunodiagnostic assays and serum biomarkers.

Conflicts of Interest: None declared.

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