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Original Article

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Investigation of the value of precipitins in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) patients with a positive marker for *Aspergillus* species

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Abstract

Although a high prevalence of COVID-19-associated pulmonary aspergillosis has been reported, it is still difficult to distinguish between colonization with *Aspergillus fumigatus* and infection. Concomitantly, similarities between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and hypersensitivity pneumonitis were suggested. The objective of this study was to investigate retrospectively if precipitin assays targeting *A. fumigatus* could have been useful in the management of SARS-CoV-2 patients hospitalized in an Intensive Care Unit (ICU) in 2020. SARS-CoV-2 ICU patients were screened for Aspergillus co-infection using biomarkers (galactomannan antigen, qPCR) and culture of respiratory samples (tracheal aspirates and bronchoalveolar lavage). For all these patients, clinical data, ICU characteristics and microbial results were collected. Electrosyneresis assays were performed using commercial *A. fumigatus* somatic and metabolic antigens. ELISA were performed using in-house *A. fumigatus* purified antigen and recombinant antigens.

Our study population consisted of 65 predominantly male patients, with a median ICU stay of 22 days, and a global survival rate of 62%. Thirty-five patients had at least one positive marker for *Aspergillus* species detection. The number of arcs obtained by electrosyneresis using the somatic *A. fumigatus* antigen was significantly higher for these 35 SARS-CoV-2 ICU patients (P 0.01, Welch's t-test). Our study showed that SARS-CoV-2 ICU patients with a positive marker for *Aspergillus* species detection more often presented precipitins towards *A. fumigatus*. Serology assays could be an additional tool to assess the clinical relevance of the *Aspergillus* species in respiratory samples of SARS-CoV-2 ICU patients.

Lay Summary

This study showed retrospectively that precipitin assays, such as electrosyneresis, could be helpful to distinguish between colonization and infection with *Aspergillus fumigatus* during the management of severe acute respiratory syndrome Coronavirus-2 (SARS CoV-2) patients in an intensive care unit.

Key words: precipitins, SARS-CoV-2, tracheal aspirates, *Aspergillus fumigatus*, electrosyneresis, somatic antigen.

Introduction

During the early stages of the COVID-19 pandemic, similarities between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and hypersensitivity pneumonitis (HP) were hypothesized.[1,2] HP are interstitial lung diseases characterized by a type III hypersensitivity reaction to microbial antigens, which is mediated by the formation of antigen-antibody aggregates called immune complexes.[3] The papers reporting a relationship between SARS-CoV-2 pneumonia and HP highlighted the potential role of immune complexes in the occurrence of the hyper-inflammation response, characterized by the release of proinflammatory cytokines and the influx of polynuclear cells (also called "cytokine storm").[1,2]

At the same time, several papers reported clinical cases of fungal superinfections of SARS-CoV-2 pneumonia, called COVID - 19-associated pulmonary aspergillosis (CAPA).[4,5] Guidelines to better define and manage CAPA were published in 2021.[6]

Several precipitation reactions that allow a macroscopic visualization of the antigen-antibody reaction by generating precipitation arcs, are applied for the serological diagnosis of HP.[7,8] Among them, electrosyneresis is performed on cellulose acetate sheets while other methods such as double diffusion and immune-electrophoresis are performed on agar gel; electrosyneresis was previously shown more performant for the differential serodiagnosis of HP.[9]

The first objective of this study was to investigate retrospectively if precipitin assays targeting *A. fumigatus* could have been useful in the management of SARS-CoV-2 patients hospitalized in an Intensive Care Unit (ICU) in 2020. The second objective of this study was also to assess retrospectively the contribution of ELISA assays using in-house purified and recombinant antigens from *A. fumigatus* (Glucose-6-isomerase and Glu/Leu/Phen/Val dehydrogenase).[10–11]

Methods

Fungal screening of SARS-CoV-2 ICU patients

In total, 225 SARS-CoV-2 ICU patients were managed between March 2020 and December 2020 at the ICU of the University Hospital of Besancon. The fungal screening was performed prospectively on a weekly basis in all patients. Respiratory samples (tracheal aspirates, broncho-alveolar lavage (BAL)) were cultivated on Sabouraud media. Identification of the fungal species was performed macroscopically, microscopically and verified using Bruker MALDI-TOF techniques. Serum samples were used to measure the galactomannan antigen using an ELISA assay (Platelia Aspergillus EIA; Bio-Rad, Marnes la Coquette, France) and to detect *A. fumigatus* DNA by qPCRs using in-house techniques.[12]

During both the first COVID-19 wave in France (March 2020-May 2020), and the second one (October 2020-December 2020), 16/135 and 19/90 SARS-CoV-2 ICU patients presented a positive marker for *Aspergillus* species detection during their ICU stay (either a positive culture in tracheal aspirate, a positive culture in BAL, a positive galactomannan in serum, or a positive *A. fumigatus* qPCR in serum). Overall, 35 SARS-CoV-2 ICU patients (35/225; 15.5%) were included retrospectively in the "*A. fumigatus* group".

Thirty SARS-CoV-2 ICU patients were randomly chosen among the 185 patients with no positive marker for *Aspergillus* species detection during their ICU stay (30/225; 13.3%) to be included in the "control group".

For all patients included in the study, clinical data, residence details, ICU characteristics, and microbial results were collected retrospectively.

During the COVID-19 waves of 2020, the galactomannan antigen was locally measured only in the serum of SARS-CoV-2 patients; Respiratory samples were considered as a risk of exposure for the technicians.

Electrosyneresis on cellulose acetate

Electrosyneresis assays were performed retrospectively for all SARS-CoV-2 ICU patients included. Electrosyneresis was performed on residual sera that were sampled initially to measure the galactomannan antigen, in median 6 days [1-26] after the ICU admission.

Electrosyneresis assays were performed using commercial *A. fumigatus* somatic and metabolic antigens (R-Biopharm®, St Didier au Mont d'Or, France) and commercial cellulose acetate sheets (Cellogel electrophoresis®, Milano, Italy). Briefly, samples of 15 μl of each serum were placed on three spots on the anode side and a 15-μl line of somatic antigen was placed on the cathode side 3.5 cm from the first deposit (Figure 1). The samples were washed and stained after 125 min of migration in a 90 mV current, as recommended by the supplier. The number of arcs was determined for each serum by two independent readers using a magnifying glass.

ELISA tests

ELISA were performed using an in-house proteic purified antigen, which depart from in-house somatic antigen that is further submitted to an enzymatic lysis of cell wall polyosids, protein acidic precipitation and acetone purification[8] and ii) recombinant antigens, Glucose-6-phosphate isomerase (G6PI) and Glu/Leu/Phen/Val dehydrogenase (GLPV), selected among immunogenic proteins from *Aspergillus fumigatus* in a previous study.[10,11]

Briefly, the wells of 96-well plates (PolySorp Immunomodule®, Nalge Nunc, Rochester, NY) were coated by incubation with 200 μl of 1 μg/ml recombinant antigen or purified antigen solution in 50 mmol/l K2HPO4 buffer,

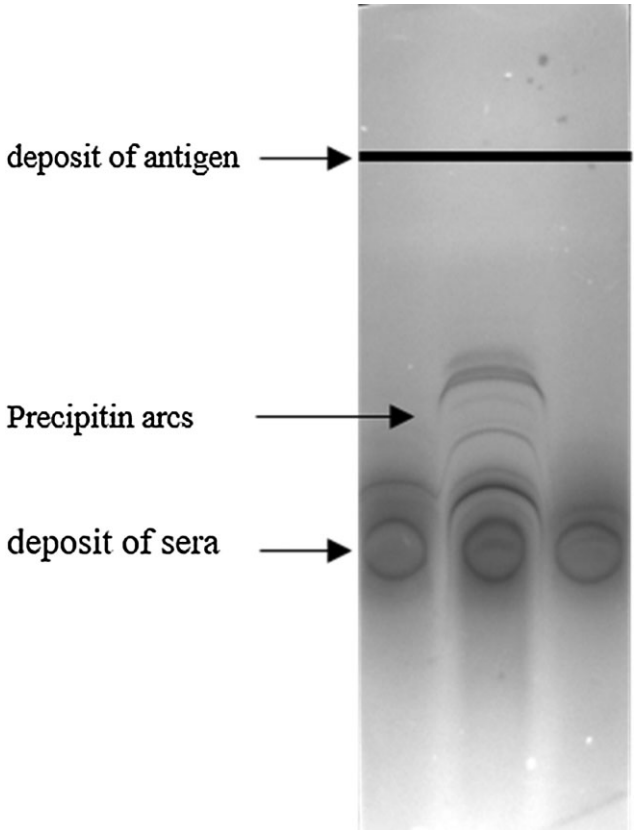

Figure 1. Illustration of precipitation arcs obtained by electrosyneresis.

pH 8.5, at 4°C for 48 h. ELISA tests for specific IgG were then conducted, as described previously.[10,11] Optical densities at 450 nm were read with a spectrophotometer (VictorTM 2 Multilabel Counter, PerkinElmer, Courtaboeuf, France).

ELISA was performed on residual sera sampled in median 6 days [1-26] after the ICU admission.

Statistical analysis

Data were analyzed using Jamovi, version 2.2. A *P*-value < 0.05 was considered significant. In order to compare frequencies between the two defined groups, Pearson's chi-squared test or Fisher's exact test (when the theoretical numbers were too small) were used. Student's or Welch *t*-tests were used to compare means based on the equality of variances.

Results

Characteristics of the studied population

The study population consisted of 65 predominantly (80%) male SARS-CoV-2 ICU patients, with a median age of 72 years, a median ICU stay of 22 days and a global survival rate of 62%.

No significant difference was observed between the two studied groups in terms of mean age at admission, sex ratio or residence type (urban or rural) (Table 1). Risk factors comparison showed that patients from the "*A. fumigatus* group" had significantly more frequently chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), than patients from the "control group" (in mean ± SD, respectively, 0.34 ± 0.6 *vs* 0.07 ± 0.25, *P* 0.016 Welch's *t*-test; Table 1). Indeed, eight patients from the "*A. fumigatus* group" had asthma or COPD and two patients from the "*A. fumigatus* group" had both asthma and COPD (10/35 patients with chronic respiratory diseases) while only two patients from the "control group" had COPD (2/30 patients with chronic respiratory diseases).

No significant difference was observed for cardiovascular risk and the fact to be immunocompromised (Table 1). Cardiovascular risks listed were diabetes, obesity, hypertension and cardiopathy, 29 patients from the "*A. fumigatus* group" had cardiovascular risks (1 to 4 among the risks listed) and 23 patients from the "control group" had cardiovascular risks (1 to 3 among the risks listed). Immunosuppression risks listed were corticotherapy long course, solid organ transplantation, hemopathy, allograft, solid cancer. Five patients from the "*A. fumigatus* group" had immunosuppression risk factors (1 to 3 among the risks listed) and ten patients of the 'control group È had immunosuppression risk factors (1 to 2 among the risks listed).

Table 1. Description of sex, age, residence details and risk factors for both groups of patients.

	A. fumigatus group N = 35	Control group N = 30	*P* value
Male	28 (80%)	24 (80%)	1
Age at admission mean	67	72	0.06
Median [range]	70 [23–82]	73 [51–82]	
Residential area			0.21
– Urban	18 (51%)	20 (67%)	
– Rural	17 (49%)	10 (33%)	
Chronic respiratory diseases (mean ± SD)	0.3 ± 0.6	0.07 ± 0.2	*0.*016
Cardiovascular risk (mean ± SD)	1.4 ± 1	1.5 ± 1.1	0.70
Immuno-suppression (mean ± SD)	0.2 ± 0.7	0.4 ± 0.6	0.28

Table 2. ICU characteristics and fungal screening.

Clinical data	*A. fumigatus* Group N = 35	Control Group N = 30	*P* value
Median ICU stay (in days) [range]	21 [3–44]	25 [3–70]	0.17
Median Apache II Score [range]	19 [8–38]	22 [13–33]	0.14
qSOFA Score ≥ 2	15/27 (43%)	13 (48%)	0.68
Survival at D90	21 (60%)	19 (63%)	0.78
Frequency of voriconazole treatment	14 (40%)	1 (3%)	**< 0.001**
Median number of tracheal aspirates analyzed during the ICU stay per patient [range]	5 [0–11]	4 [1–17]	0.83
Median number of broncho-alveolar lavage (BAL) analyzed during the ICU stay per patient [range]	1 [0–4]	0 [0–5]	0.45
Median number of antigen galactomanan measurements in serum [range]	2 [0–7]	3 [1–10]	**0.04**
Number of *A. fumigatus* qPCR on serum [range]	2 [0–7]	3 [1–10]	**0.04**

ICU characteristics of the two studied groups did not differ significantly neither on the mean duration of the ICU stay, nor on their gravity scores (Apache II and qSOFA) (Table 2).

No difference in survival was observed neither between both groups of patients (Table 2).

Patients from the "*A. fumigatus* group" were significantly more often treated with voriconazole (40%) than patients included in "the control group" (3%) ($P < 0.001$, Fisher's exact test, Table 2).

Fungal screening

Both groups of patients were equally frequently screened by respiratory samples (tracheal aspirates and broncho-alveolar lavage) during their ICU stay (Table 2). Fungal biomarkers galactomannan and *A. fumigatus* qPCR on serum were performed on the same sample. During their ICU stay, patients from the"control group" were screened for fungal biomarkers in serum more frequently than patients from the "*A. fumigatus* group" (in mean ± SD, respectively, 3.6 ± 2.5 *vs* 2.5 ± 1.8, *P* 0.04, Student's *t*-test; Table 2).

Most patients of the "*A. fumigatus* group" had positive *A. fumigatus* cultures (N = 34, 97%). Only two had positive *A. fumigatus* qPCR in serum, and only one had a positive galactomannan antigen in serum.

Based on the criteria proposed by Koelher *et al.*,[6] 12 patients from the "*A. fumigatus* group" were retrospectively classified as probable CAPA and 23 patients from the "*A. fumigatus* group" were classified as colonized (Table 3). To be classified as probable CAPA, patients had either a positive culture in BAL, a positive galactomannan on serum or a positive *A. fumigatus* qPCR in serum (Table 3). Patients with only tracheal aspirates positive for A. *fumigatus* culture (but not BAL) were considered as colonized with *A. fumigatus* and not as having probable CAPA (Table 3).

Seven patients classified as probable CAPA died (7/12, 58%), seven patients classified as colonized died (7/23, 30%) and eleven non-colonized patients died (11/30, 37%). No relationship was found between the Koelher's classification and the outcome (*P* 0.15, Fisher's exact test). No difference in terms of residential area (rural *vs* urban) was observed between probable CAPA patients (5/12, 42%) and colonized patients (12/23, 52%) (*P* 0.55, Fisher's exact test).

Serology results

Serology results are presented in Figure 2. Based on electrosyneresis using *A. fumigatus* somatic antigen, the number of arcs was significantly higher in the "*A. fumigatus* group" (*P* 0.01, Welch's *t*-test) (Figure 2A). In contrast, no significant difference was observed between the "*A. fumigatus* group" and the "control group" using *A. fumigatus* metabolic antigen (*P* 0.17, Welch's *t*-test) (Figure 2B). No significant difference was found between probable CAPA patients and colonized patients by electrosyneresis, using either the somatic antigen (*P* 0.4, Welch's *t*-test) or the metabolic antigen (*P* 0.8, Welch's *t*-test).

ELISA tests were performed using either a purified *A. fumigatus* antigen or using recombinant antigens (G6PI and GLPV).

No significant difference was observed between the "*A. fumigatus* group" and the "control group" in ELISA using the purified *A. fumigatus* antigen (*P* 0.08, Welch's *t*-test) (Figure 2C).

However, significant higher indices for colonized patients compared to probable CAPA patients were observed (1.3 ± 0.9 *vs* 0.68 ± 0.4, *P* 0.02, Welch's t test) (Figure 3).

No significant difference was observed between the "*A. fumigatus* group" and the "control group" in ELISA using the recombinant antigen G6PI (*p* 0.65, Welch's t test) (Figure 2D).

However, significant higher indices for colonized patients compared to probable CAPA patients were observed (0.8 ± 0.4 *vs* 0.6 ± 0.2, *P* 0.008, Welch's *t*-test) (Figure 3).

No significant difference was observed between the "*A. fumigatus* group" and the "control group" in ELISA using the recombinant antigen GLPV (*P* 0.09, Welch's *t*-test) (Figure 2E).

Table 3. Criteria for probable CAPA/colonization classification for patients included in the "*A. fumigatus* group", based on Koelher's classification.[6]

Patients	Positive culture in tracheal aspirates	Positive culture in BAL	Positive galactomannan (serum)	Positive *A. fumigatus* qPCR (serum)	Classification
P1	Yes	Yes	No	No	Probable CAPA
P2	Yes	Yes	No	No	Probable CAPA
P3	Yes	Yes	No	No	Probable CAPA
P4	Yes	yes	no	no	Probable CAPA
P5	Yes	yes	no	no	Probable CAPA
P6	Yes	yes	no	no	Probable CAPA
P7	Yes	yes	no	no	Probable CAPA
P8	Yes	yes	no	no	Probable CAPA
P9	Yes	yes	no	no	Probable CAPA
P10	Yes	yes	no	no	Probable CAPA
P11	Yes	yes	no	yes	Probable CAPA
P12	No	No	yes	yes	Probable CAPA
P13	Yes	No	no	no	Colonized
P14	yes	No	no	no	Colonized
P15	yes	No	no	no	Colonized
P16	yes	No	no	no	Colonized
P17	yes	No	no	no	Colonized
P18	yes	No	no	no	Colonized
P19	yes	no	no	no	Colonized
P20	yes	no	no	no	Colonized
P21	yes	no	no	no	Colonized
P22	yes	no	no	no	Colonized
P23	yes	no	no	no	Colonized
P24	yes	no	no	no	Colonized
P25	yes	no	no	no	Colonized
P26	yes	no	no	no	Colonized
P27	yes	no	no	no	Colonized
P28	yes	no	no	no	Colonized
P29	yes	no	no	no	Colonized
P30	yes	no	no	no	Colonized
P31	yes	no	no	no	Colonized
P32	yes	no	no	no	Colonized
P33	yes	no	no	no	Colonized
P34	yes	no	no	no	Colonized
P35	yes	no	no	no	Colonized

No difference in indices was observed between colonized patients and probable CAPA patients (1.1 ± 0.9 *vs* 1.2 ± 0.9, *P* 0.9, Welch's *t*-test) (Figure 3).

Discussion

Our study aimed at investigating retrospectively if *A. fumigatus* precipitin detection could have been useful in deciding whether or not to use antifungal treatment for SARS-Cov-2 ICU patients who present positive respiratory samples in culture for *A. fumigatus*. Indeed, since the prevalence of COVID Associated Pulmonary Aspergillosis (CAPA) may increase with the deterioration of respiratory conditions, authors have recently emphasized the importance of acting quickly: the sooner the antifungal prescription based on the first positive culture/test, the better.[4–6]

Precipitin assays targeting *A. fumigatus* allow the detection of serum-specific immunoglobulin which is merely indicative of previous exposure and immunologic sensitization, and does not prove infection.[7] We observed a difference in positivity according to the nature of the *A. fumigatus* antigen used for electrosyneresis, which can be explained by the protein composition of each antigen: somatic antigen is obtained from the mycelial culture of *A. fumigatus* while metabolic antigen is obtained by filtration of *A. fumigatus* liquid culture media. So, the metabolic antigen contains proteins produced in the culture media by *A. fumigatus* and not directly the proteins composing *A. fumigatus* mycelium and spores. Precipitation reactions remain the "gold standard" to

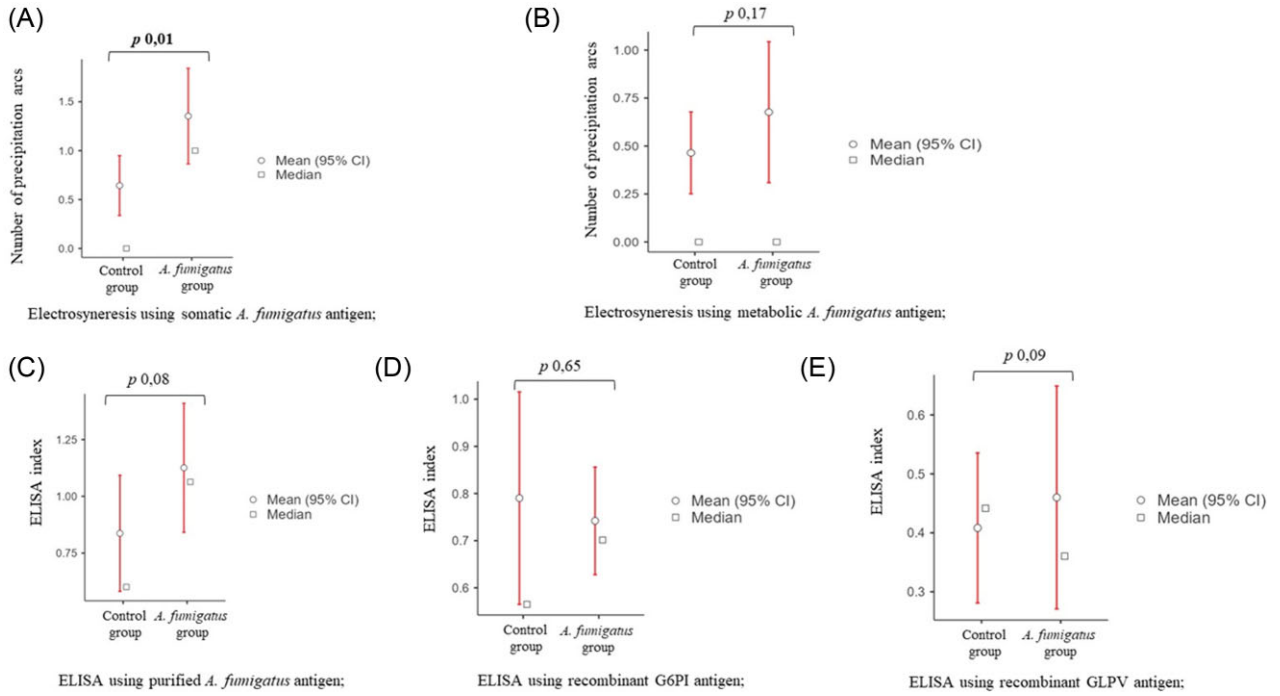

Figure 2. Serology results. (A) Significant difference by electrosyneresis between the two groups studied using the somatic *A. fumigatus* antigen (*P* 0.01, Welch's *t*-test). (B) No significant difference by electrosyneresis between the two groups studied using the metabolic *A. fumigatus* antigen (Welch's *t*-test). (C) No significant difference by ELISA using the purified *A. fumigatus* antigen (Welch's *t*-test). (D) No significant difference by ELISA using the recombinant G6PI antigen (Welch's t test). (E) No significant difference by ELISA using the recombinant GLPV antigen (Welch's *t*-test).

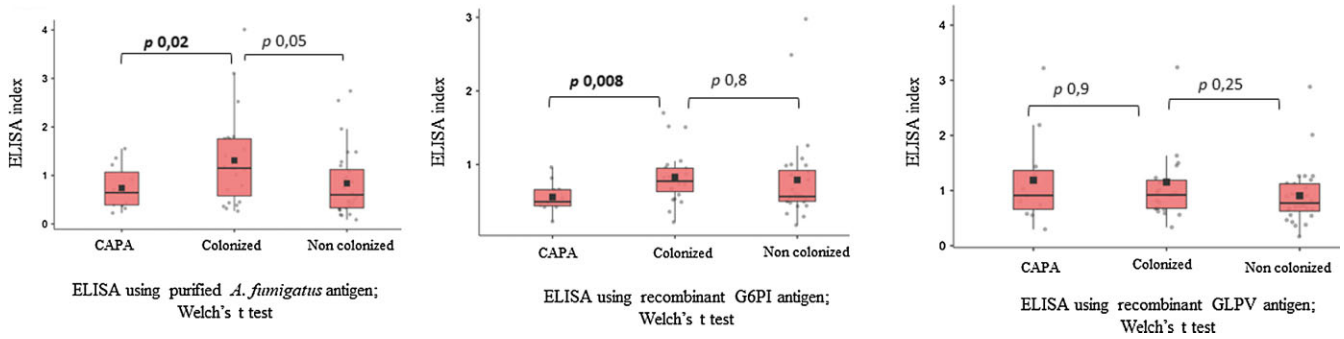

Figure 3. Serology results using in-house ELISA, when considering the groups probable CAPA (n = 12), colonized (n = 23) and non-colonized (n = 30).

highlight macroscopically the precipitation arcs that result from the antigen-antibody reaction.[8] Most probably, these precipitating antibodies are not as well detected by ELISA method than by precipitation techniques. ELISA tests did not highlight significant differences between the two main groups of patients ("*A. fumigatus* group" *vs* "control group"), however, ELISA tests using *A. fumigatus* purified antigen and the recombinant G6PI antigen showed significantly higher indices for patients classified retrospectively as colonized compared to those classified retrospectively as probable CAPA. This result was not expected and may be related to the small size of the subgroup of patients classified retrospectively as probable CAPA (n = 12).

Before the study, we hypothesized that the rural/urban habitation of SARS-Cov-2 ICU patients could have played a role on their exposure to molds at home and their probability to arrive in the ICU colonized with *A. fumigatus*. The environmental exposure plays indeed a key role in HP investigations[7,8] and also in the risk to develop invasive aspergillosis.[13] However, no significant difference was observed between residential area between the two groups of patients, and also between probable CAPA patients and colonized patients.

One could consider that the fact that the patients from the"control group" were randomly chosen and not matched to the patients from the "*A. fumigatus* group" represents a bias. However, the descriptive analysis showed that both groups were similar in terms of age and sex (Table 1) and in terms of ICU characteristics (Table 2).

The management of SARS-CoV-2 pneumonia has evolved over time, and a regimen of dexamethasone for 10 days is now recommended in severe cases.[14] A SARS-CoV-2 ICU patient receiving dexamethasone while showing evidence of *A. fumigatus* colonization (i.e. positive culture in tracheal aspirate) and of *A. fumigatus* sensitization (i.e. positive precipitin assay) could be a possible candidate for CAPA, as the steroids may create a favorable local environment for *A. fumigatus* growth *in situ*. When a patient had a positive result in culture for *A. fumigatus* in BAL, physicians rapidly treated the patient with voriconazole, which was illustrated by the high frequency of treated patients in the "*A. fumigatus* group" (40%, Table 2) compared to the "control group"(3%). However, when SARS-CoV-2 ICU patients had no positive result for *A. fumigatus* detection, the screening in serum remained sustained, as illustrated by the high rate of galactomannan and *A. fumigatus* qPCR measurements in serum performed in the "control group" (Table 2). Fungal biomarkers were rarely positive, <10% in our series, which was also the case in other reports.[15] There is thus a need for additional tools, and serology assays could be appropriate candidates.

Conclusion

Our study has demonstrated that SARS-CoV-2 ICU patients with a positive marker for *Aspergillus* species detection more often presented precipitins towards the somatic *A. fumigatus* antigen. Serology assays could thus be an additional tool to manage SARS-CoV-2 ICU patients.

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Declaration of interest

The authors have declared no conflict of interest.

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