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ORIGINAL PAPER

COVID-19-associated pulmonary aspergillosis (CAPA) in Iranian patients admitted with severe COVID-19 pneumonia

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Abstract

Purpose Bacterial or virus co-infections with SARS-CoV-2 have been reported in many studies; however, the knowledge on *Aspergillus* co-infection among patients with COVID-19 was limited. This study was conducted to identify and isolate fungal agents and to evaluate the prevalence of pulmonary aspergillosis (CAPA) as well as antifungal susceptibility patterns of *Aspergillus* species in patients with COVID-19 admitted to Shahid Beheshti Hospital, Kashan, Iran.

Methods The study involved 119 patients with severe COVID-19 pneumonia referred to the Shahid Beheshti Hospital, Kashan, Iran. A total of 17 *Aspergillus* spp. that were isolated from COVID-19 patients suspected of CAPA were enrolled in the study. CAPA was defined using ECMM/ISHAM consensus criteria. The PCR amplification of the β-tubulin gene was used to identify the species. The antifungal activities of fluconazole, itraconazole, voriconazole, amphotericin B against *Aspergillus* spp. were evaluated according to the Clinical and Laboratory Standards Institute manual (M38-A3).

Results From the 119 patients with severe COVID-19 pneumonia, CAPA was confirmed in 17 cases (14.3%). Of these, 12 (70.6%) were males and 5 (29.4%) were females; the mean age at presentation was 73.8 years (range: 45–88 years; median = 77; IQR = 18). *Aspergillus fumigatus* (9/17; 52.9%), *Aspergillus flavus* (5/17; 29.4%), *Aspergillus oryzae* (3/17, 17.6%), were identified as etiologic agents of CAPA, using the molecular techniques. Voriconazole and amphotericin B showed more activity against all isolates. Moreover, the MIC of fluconazole, itraconazole varied with the tested isolates. For 3 clinical isolates of *A. fumigatus,* 2 isolate of *A. flavus* and 3 *A. oryzae*, the MIC of fluconazole and itraconazole were ≥ 16 µg/mL.

Conclusions We observed a high incidence (14.3%) of probable aspergillosis in 119 patients with COVID-19, which might indicate the risk for developing IPA in COVID-19 patients. When comparing patients with and without CAPA regarding baseline characteristics, CAPA patients were older ($p = 0.024$), had received more frequent systemic corticosteroids ($p = 0.024$), and had a higher mortality rate ($p = 0.018$). The outcome of CAPA is usually poor, thus emphasis shall be given to screening and/or prophylaxis in COVID-19 patients with any risk of developing CAPA.

Keywords Pulmonary aspergillosis · COVID-19 · Azole · Antifungal drug resistance

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Abbreviations

CAPA	COVID-19-associated pulmonary aspergillosis
MIC	Minimum inhibitory concentration
IPA	Invasive pulmonary aspergillosis
CPA	Chronic pulmonary aspergillosis
ABPA	Allergic bronchopulmonary aspergillosis

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) started in December 2019 in Wuhan, China [1]. Coronavirus disease 2019 (COVID-19) is a new and emerging disease of late 2019 caused by the new coronavirus called SARS-CoV-2. Acute respiratory distress syndrome (ARDS) is a common immunopathological phenomenon in SARS-CoV-2 infections, the main mechanism of which is the cytokine storm. Cytokine storm is an uncontrolled and lethal systemic inflammatory response due to the copious release of inflammatory cytokines and chemokines by cells of the immune system against SARS-CoV-2 infection [2]. To curtail this immunopathological phenomenon, the treating physicians need to prescribe corticosteroids, which may increase the patient's susceptibility to superinfection by a variety of microorganisms, including bacteria and fungi. This can complicate clinical manifestations, create treatment problems, and even increase mortality [3, 4]. According to the results of studies conducted worldwide, few months into the emergence of COVID-19 disease, there were several reports on the isolation of fungi in affected patients. This issue might have raised the question: "Can fungi underlie to aggravate COVID-19?" Or, conversely, "Can COVID-19 affect the susceptibility to fungal infections, such as the influenza virus that has been investigated and proven by numerous studies?".

Aspergillus speciescause various pulmonary infections including invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis (CPA), allergic bronchopulmonary aspergillosis (ABPA), chronic rhinosinusitis, fungal asthma, and *Aspergillus* bronchitis [5–10]. Usually, insufficient attention to timely diagnosis and treatment of this disease leads to the patient's death. Opportunistic fungi, which are usually harmless, are among the major causes of pulmonary fungal infections that become pathogenic in abnormal and susceptible hosts. As reported in Salmanton-Garcia et al.; cases of pulmonary aspergillosis associated with COVID-19 (CAPA) have been documented by researchers since August 2020 [11]. Since then, varying reports on cumulative incidences of CAPA, including rates of 0.7–7.7% among COVID-19 cases [12, 13], 1–39.1% among COVID-19 patients admitted in ICU [12, 14, 15], and 3.2–29.6% among COVID-19 patients who received mechanical ventilation [12, 16]. Similar to influenza-associated pulmonary aspergillosis, CAPA develops few days following ICU admission. The establishment of pulmonary aspergillosis superinfection in COVID-19 and influenza patients follows exposure to common risk factors [17].

Thus, it is important to investigate the occurrence of such infections among patients with severe COVID-19 disease, in terms of nosocomial infections, especially those admitted to intensive care units and might require a long hospital stay. Therefore, this study was conducted to identify and isolate fungal agents and evaluate the prevalence of systemic fungal infections among patients with COVID-19 disease admitted to Shahid Beheshti Hospital, Kashan, Iran.

Materials and methods

Study design

This descriptive study was performed on COVID-19 patients, diagnosed using clinical, radiological, and molecular tests, and admitted to *Shahid Beheshti Hospital,* Kashan, Iran within a period of 11 months; August 2020–June 2021. Shahid Beheshti hospital (latitude 33°00′46″ N, longitude 51°24′24″ E) with an area of about 40,000 m^2 and 400 beds is located at 5 km of Kashan–Ravand road as the only general hospital in Kashan, a city in the center of Iran, that provides services to about 300,000 population. This hospital became one of the most important referral centers for the management of COVID-19 patients during the COVID-19 pandemic. The samples for mycological examination including the broncho-alveolar lavage, and sputum were collected and processed based on clinical symptoms. The study was approved by the joint Ethical Committees of *Tehran University of Medical Sciences*, with ethic number IR.TUMS.SPH.REC.1399.329. The study included adult immunosuppressed patients with COVID-19 pneumonia admitted to ICU who used invasive MV for more than 4 days. We defined a "Probable CAPA" in patients with at least one of the following conditions: The presence of new cavitary lung lesion(s) at chest computed tomography without alternative explanation, positive serum GM EIA index ≥ 0.5, positive BAL GM index ≥ 1.0, or a positive culture/PCR for *Aspergillus* species in BAL sample [18].

Diagnosis of probable CAPA tracheobronchitis requires observation of tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar, alone or in combination, on bronchoscopic analysis and mycological evidence. Definitions for 'probable' CAPA have been proposed by ECMM/ISHAM consensus criteria for research and clinical guidelines in which a positive galactomannan (GM) in serum or bronchoalveolar lavage (BAL), recovery of *Aspergillus* species in BAL culture, positive polymerase chain reaction (PCR) for *Aspergillus* species in BAL or blood, or chest

imaging consistent with a fungal infection suffices the diagnosis [18, 19]. Refer to Table 1

Sample collection and preparation

The study was conducted on 119 specimens. We collected 114 broncho-alveolar lavage (BAL) samples and 5 sputum samples from hospitalized patients.

Initial examination

The collected specimens were initially examined under the microscope using 10% KOH solution for the detection of fungal hyphae. Parts of specimens were subcultured on Sabouraud Dextrose Agar (SDA) 2% (Merck, Denmark) and incubated at 35°C for 7 days. A few of the colonies grown on SDA were also mixed with sterile saline and 3% glycerol in a 0.5 ml microtube and stored at -70°C.

DNA extraction and PCR amplification of ITS region

The genomic DNA was directly isolated from BAL specimens using the high pure PCR template purification kit (Roche, Germany) based on the manufacture's guide. The PCR amplification was performed using the 3 µL of test sample as a template, in a total volume of 25 µL (1 µL of each of forward and reverse primers, 10 µL of PCR Master Mix (Amplicon, Denmark), and 9 µL of deionized distilled water. The amplification was achieved using the β-tubulin primers (BT-forward (5′-GGTAACCAAATCGGTGCT GCTTTC-3′), and reverse (BT-reverse: 5′-ACCCTCAGT GTAGTGACC CTTGGC-3′) [20, 21] using the following program: Initial denaturation of DNA at 95°C for 2 min, 35 cycles consisted of a denaturation step at 94°C for 45 s, an annealing step at 58°C for 60s, and an extension step at 72°C for 60 s, with a final extension at 72°C for 15 min following the last cycle. The PCR products were examined by staining with a DNA-safe stain and electrophoresis on 1.5% agarose gel. The PCR products were subjected to sequencing and analyzed using the MEGA7.0.21 software.

Antifungal susceptibility test

The minimum inhibitory concentrations (MIC) of fluconazole, itraconazole, voriconazole, amphotericin B were assessed according to the Clinical and Laboratory Standards Institute (CLSI) M38-A3 guidelines [22]. After preparation of antifungal serial dilutions, the 96-well microtitre plates were inoculated with the spore suspensions to obtain 5×10^5 cells/mL in every well. The microplates were incubated at 35°C for 48 h. All tests were carried out in duplicate. The standard strain of *Candida parapsilosis* ATCC 22,019 and *Aspergillus fumigatus* ATCC®MYA-3627™ were used for quality control. The MIC cutoff values for antifungals were concluded according to the CLSI M38-A3 guideline [22].

Statistical analysis

Statistical analysis was performed using SPSS software (version 16.0). The MICs range of all antifungals were calculated. We used the Mann–Whitney *U* test or Fisher's exact test to compare differences between patients with and without CAPA when appropriate.

Results

From a total of 119 patients with severe COVID-19 pneumonia, CAPA was confirmed in 17 cases (14.3%). Of these, 12 (70.6%) patients were males and 5 (29.4%) were females; the mean age at presentation was 73.8 years (range: 45–88 years; median = 77; IQR = 18). *Aspergillus fumigatus* (9/17; 52.9%), *Aspergillus flavus* (5/17; 29.4%), *Aspergillus oryzae* (3/17, 17.6%), were identified as etiologic agents of CAPA, using the molecular techniques.

The predominant underlying diseases among patients with CAPA included diabetes (12 cases), kidney disorder (6

Table 1 Defining and diagnosing CAPA according to the 2020 ECMM/ISHAM consensus criteria

Proven CAPA	Probable CAPA (Microbiology)	Probable CAPA BAL (Clinical factors)
SARS-CoV-2 + ARDS + ICU patients	SARS-CoV-2 + ARDS + ICU patients	SARS-CoV-2 + ARDS + ICU patients
Tracheal biopsy (Histology)	BAL + Microscopy /*Aspergillus* (positive)	Tracheobronchial ulceration
Invasive growth (Microscopy) +	BAL + Culture/PCR *Aspergillus* (positive)	Nodule
Culture/*Aspergillus* (positive) +	Serum + GM / Lateral flow assay (index > 0·5)	Pseudomembrane
PCR/*Aspergillus* (positive) +	BAL + GM / Lateral flow assay (index ≥ 1·0)	Plaque
Or a combination	Or a combination	Eschar or a combination

CAPA COVID-19-associated aspergillosis, *SARS-CoV-2* severe acute respiratory syndrome coronavirus 2, *ARDS* acute respiratory distress syndrome, *ICU* intensive care unit, *BAL* bronchoalveolar lavage, *GM* enzyme immunoassay for galactomannan

cases), heart failure (5 cases), liver transplantation (3 cases), AML (2 cases), ALL (1 case), and CML (1 case). Whereas patients with CAPA had diabetes (70.6%) and kidney disorder (35.3%) as their main underlying diseases, diabetes and malignancy were seen in patients with non-CAPA at the rate of 21.6 and 13.7%, respectively.

Table 2 Demography and major presenting symptoms of COVID-19 patients with CAPA

Number of patients Characteristic, no (%)	17
Age at the time of diagnosis-years*	73.8 (median=77; IQR=18)
Sex	No
Male	12 (70.6%)
Female	5 (29.4%)
Fungal isolates	
Aspergillus fumigatus	9 (52.9%)
Aspergillus flavus	5 (29.4%)
Aspergillus oryzae	3 (17.6%)
Underlying cause of immunosuppression	
Acute lymphoblastic leukemia	1 (5.9%)
Acute myeloblastic leukemia	2 (11.7%)
Chronic myeloblastic leukemia	1 (5.9%)
Diabetes Mellitus	12 (70.6%)
Liver transplantation	3 (17.6%)
Kideny disorder	6 (35.3%)
Heart failer	5 (29.4%)
Signs and symptoms	
Headache	15 (88.2%)
Fever	13 (76.5%)
Myalgia	17 (100%)
Arthralgia	12 (70.6%)
Gastrointestinal	9 (53%)
Dyspnea	17 (100%)
Extension	
BAL	17 (100%)

Dyspnea (100%), myalgia (100%), headache (88.2%), fever (76.5%), arthralgia (70.6%), and gastrointestinal symptoms (53%) were the most frequent symptoms at presentation of the patients (Table 2).

We compared baseline characteristics of patients with and without CAPA (Table 3); we found that CAPA patients were older ($p=0.024$). On management and development of complication among the studied patients, we observed that CAPA patients had received more frequent systemic corticosteroid ($p=0.024$), and had a higher mortality rate ($p=0.018$).

The clinical course and disease outcome of patients with and without CAPA is being been demonstrated in Table 3.

MICs range of all antifungals are shown in Table 4. Voriconazole and amphotericin B showed more activity against all isolates. The MIC of fluconazole, itraconazole varied with the tested isolates. For the clinical isolates of *A. fumigatus* (three isolates), *A. flavus* (two isolates), and *A. oryzae* (three isolates), the MIC of fluconazole and itraconazole were ≥ 16 µg/mL. The remaining isolates showed sensitivity to antifungal drugs used.

The antifungal treatment, length of stay, and outcome in presumed CAPA cases is shown in Table 5

Discussion

Since COVID-19 is similar to influenza regarding clinical symptoms and characteristic host's immune response to the viral agent, it is expected that COVID-19 can favor the development of opportunistic fungal infections. The available reports in this regard can be good evidence of the importance of fungal diseases in these patients. On the other hand, in trying to control bacterial superinfection, physicians dealing with patients with COVID-19 tend to be non-restrictive and tend to over-prescribe broad-spectrum antibiotics, which may increase patients' susceptibility to fungal infections. Systemic fungal superinfections can negatively impact the prognosis and subsequently increase the mortality rate among patients with

Table 3 Characteristics of patients, clinical course, and outcome in CAPA and non-CAPA cases

Parameter	Presumed CAPA ($n=17$)	Non-CAPA ($n=102$)	*p*Value
Age, year, median (range)	73.8 (45–88)	61.2 (19–73)	0.024
Sex, M, *n* (%)	12/17 (70.6)	65/102 (63.7)	0.145
Interval from symptom onset to ICU admission, median (range), d	6 (3–12)	8 (4–16)	0.260
Interval from ICU admission to ICU discharge, median (range), d	10.5 (5–42)	11.2 (3–40)	0.425
Interval from symptom onset to death, median (range), d	16.3 (7–30)	17.7 (10–39)	0.371
Systemic corticosteroid use, *n* (%)	6/17 (35.3)	14/102 (13.7)	0.031
Mortality, *n* (%)	13/17 (76.5)	52/102 (50.1)	0.018

CAPA COVID-19-associated pulmonary aspergillosis, *COVID-19* coronavirus disease

Table 4 MICs range and MICs 90 of four antifungals agent evaluated against *Aspergillus* species

Fungi Species	MICs	Amphotericin B µg/mL	Voriconazole µg/mL	Itraconazole µg/mL	Fluconazole µg/mL
Aspergillus fumigatus (9)	Range	0.125–1	0.03–1	0.03–16	0.125–16
	MIC90	ND	ND	ND	ND
Aspergillus flavus (5)	Range	0.25–1	0.125–1	0.25–16	0.125–16
	MIC90	ND	ND	ND	ND
Aspergillus oryzae (3)	Range	0.5–2	0.25–2	≥ 16	≥ 16
	MIC90	ND	ND	ND	ND

ND not determined

Table 5 Antifungal treatment, length of stay, and outcome in presumed CAPA cases

Sex/Age	Length of stay	Fungal species	Antifungal treatment	Outcome
M/83	1 week	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Caspofungin	Died
M/79	5 days	*Aspergillus flavus*	Amphotericin B 50 mg/day, Posaconazole 300 mg/day, Itraconazole	Died
M/64	9 days	*Aspergillus flavus*	Amphotericin B 250 mg/day, Voriconazole 200 mg/day	Survived
F/56	10 days	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Caspofungin	Died
M/77	12 days	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Posaconazole 300 mg/day, Itraconazole	Died
M/86	2 weeks	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Caspofungin	Died
M/59	2 weeks	*Aspergillus flavus*	Amphotericin B 300 mg/day, Voriconazole 200 mg/day	Survived
M/73	9 days	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Posaconazole 300 mg/day, Nystatin	Survived
M/87	2 weeks	*Aspergillus oryzae*	Amphotericin B 50 mg/day, Caspofungin	Died
F/76	6 weeks	*Aspergillus flavus*	Caspofungin, Itraconazole	Died
M/78	6 days	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Caspofungin	Died
F/69	1 week	*Aspergillus oryzae*	Voriconazole 200 mg/day, Caspofungin	Died
M/78	1 week	*Aspergillus fumigatus*	Voriconazole 200 mg/day, Caspofungin	Died
M/88	6 days	*Aspergillus flavus*	Amphotericin B 50 mg/day, Posaconazole 300 mg/day	Survived
F/45	5 days	*Aspergillus fumigatus*	Amphotericin B 50 mg/day, Caspofungin	Died
F/86	1 week	*Aspergillus oryzae*	Amphotericin B 50 mg/day, Caspofungin	Died
M/70	5 days	*Aspergillus fumigatus*	Caspofungin	Died

F female, *M* male

COVID-19. Certain risk factors ascribed to the development of CAPA, include age, prior respiratory diseases, chronic renal failure, long-term use of corticosteroid, neutropenia, COVID-19 severity, and treatment of COVID-19 patient with corticosteroid or tocilizumab [12, 13, 23, 24]. In the present study, 17 isolates were recovered from cases considered to be CAPA. These isolates were identified using molecular methods to be *Aspergillus fumigatus* (9/17; 52.9%), *A. flavus* (5/17; 29.4%), and *Aspergillus oryzae* (3/17, 17.6%). In another study from China, 60/257 COVID-19 (23.3%) patients had Aspergillosis [25]. In addition, Koehler et al. showed that 5 (26.3%) out of 19 patients with COVID-19 ARDS were co-infected with *Aspergillus* [26]. Importantly, three out of the five patients were on steroid therapy but the other two cases had no comorbidity. Moreover, the results of the serum galactomannan antigen test, bronchoalveolar lavage fluid fungal culture, and polymerase chain reaction were positive in two, three, and four of the five patients, respectively. Although the patients received antifungal medication, only two survived [26]. In a study carried out by Rello et al., symptoms of bacterial/fungal infections were compared in COVID-19 patients and H1N1 influenza patients (a 2009 European study performed on influenza patients). The results showed that COVID-19 was associated with *Aspergillus flavus* (2%), *Aspergillus fumigatus* (2%), and invasive candidiasis (2%). However, *A. flavus* was reported in 3.1 of patients with H1N1 [27]. Chen et al. (2020) examined the symptoms and co-infections of the COVID-19 virus in 99 patients. In the mentioned study, among all patients, one patient tested positive for *A. flavus* [28]. In a study conducted in China, one out of five COVID-19 cases was reported to have co-infection with *A. flavus* [14]. Furthermore, several studies from France, Germany, Belgium, and the Netherlands have reported high rates of CAPA among COVID-19 cases with ARDS, ranging from 20% to 35%. The development and progression of CAPA were relatively fast, ranging from 3 to 28 days after ICU admission [26, 29–31]. Arastehfar et al. claimed the necessity to investigate patients with COVID-19 for aspergillosis. In the mentioned study, the risk

of transmission of bronchial fluid samples in patients admitted to the ICU for mycological diagnostic and galactomannan test was mentioned as a major diagnostic problem. In addition, the side effects of antifungal drugs in patients with probable diseases were discussed and the use of new antifungal drugs with more promising pharmacokinetic and pharmacodynamic properties was recommended [17]. In the present study, voriconazole and amphotericin B showed more activity against all isolates. For the 3 clinical isolates of *A. fumigatus,* 2 isolates of *A. flavus,* and *3 A. oryzae* (from the present study), the MIC of fluconazole and itraconazole were ≥ 16 µg/mL. Similarly, azole-resistant *A. fumigatus* isolates (resistance to itraconazole, voriconazole, and posaconazole with MICs 16, 2, and 0.5 µg/mL, respectively) were reported in a CAPA case from the Netherlands [32]. Although the survival benefit of antifungal treatment of CAPA is not currently being confirmed, early diagnosis shall trigger the commencement of antifungal treatment. Voriconazole remains the recommended first-line treatment for IPA, except in cases of hematologic malignancies [33, 34]. However, its tendency for drug–drug interactions, serious undesirable effects, as well as its narrow therapeutic window that may require therapeutic drug monitoring limit its use in the ICU settings [35–37]. In addition, voriconazole may interfere with experimental COVID-19 therapies, including hydroxychloroquine, atazanavir, lopinavir/ritonavir, and remdesivir [38]. The major alternative for treatment of IPA in the ICU include the isavuconazole and liposomal amphotericin B [33]. Isavuconazole shows generally a better pharmacokinetic profile, fewer undesirable effects, and a lesser drug–drug compared to voriconazole [39]. Liposomal amphotericin B is an effective alternative with broader antifungal activity; however, renal insufficiency complicates initiation and often causes withdrawal of this agent particularly in cases of severe COVID-19 infection that shows tendency for renal tropism and frequently causes kidney insult [40]. Although itraconazole is not recommended for treatment of invasive aspergillosis, it has been shown to exhibit some antiviral activity in a feline coronavirus model via cholesterol transport inhibition [41]. Thus, it may be an alternative therapy for treating COVID-19-associated IPA, albeit its problem of drug–drug interactions with other triazoles [41, 42]. Clinical trials shall compare efficacy and safety profiles of new and established antifungals, especially in cases of COVID-19-associated fungal infections [17, 41].

Conclusions

There are increasing reports of secondary fungal infections in patients with COVID-19. Based on the definition of CAPA in our study, we observed a high incidence (14.3%) of probable aspergillosis in our cohort of 119 patients with severe COVID-19 admitted to ICU, which might highlight their risk for developing IPA. On comparison, patients with CAPA were older ($p = 0.024$), received more frequent systemic corticosteroids ($p = 0.024$) and had a higher mortality rate ($p = 0.018$) than those without CAPA. The outcome of CAPA is usually poor, thus emphasis shall be given to screening and/or prophylaxis in COVID-19 patients with any risk of developing CAPA.

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Declarations

Conflicts of interest The author(s) declare that there are no conflicts of interest.

Ethical approval The Ethical Clearance Committee of the Iran University of Medical Sciences approved the study under reference no. IR.TUMS.SPH.REC.1399.329.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent to publication All co-authors agreed to the submission of the current form of the manuscript.

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