Original Article

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Microbiology Section

Identification and Speciation of Various Fungal Co-infections in COVID-19 Patients by Different Laboratory Diagnostic Methods: A Retrospective Observational Study

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ABSTRACT

Introduction: Respiratory illness caused by a novel coronavirus was first noted in December 2019 in Wuhan, Hubei Province, China. Later, the novel coronavirus was referred to as Severe and critical Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses (ICTV). However, the types of co-infected pathogens and the proportion of co-infection in SARS-CoV-2-positive patients were unclear. Co-infection with certain pathogens may also hinder accurate disease diagnosis.

Aim: To determine the most suitable sample type for a better culture outcome and to identify the various opportunistic fungal infections related to Coronavirus Disease 2019 (COVID-19) patients.

Materials and Methods: This retrospective observational study was conducted between November 2021 to January 2022 at Department of Microbiology, Government Medical College, Surat, Gujarat, India. The data collection was done in the month of November 2021 followed by data analysis in December 2021 to January 2022. A total of 252 suspected cases of fungal co-infection were sampled (including swabs, tissues, skin scrapings, Cerebrospinal fluid (CSF), sputum, pus, and drain) from different wards for fungal wet mount Potassium Hydroxide (KOH) and culture. The data was entered into Microsoft Excel (Windows 10), and analysis was performed using Microsoft Excel, including frequency distribution and percentage.

Results: A total of 252 samples were received. Demographic details showed that individuals aged 41-50 years were more commonly affected, and males were affected more than females. Among the sample types, tissue samples showed the highest positivity 43 (17%), followed by scraping materials 14 (6%). Out of the total 252 cultures performed, 70 were positive. In terms of fungal speciation, *Mucor* species accounted for 24 (34.28%) isolates, while *Aspergillus flavus* accounted for 26 (37.14%), followed by other isolated fungal species.

Conclusion: Fungal co-infection is observed in COVID-19 positive patients, which not only contributes to morbidity but also increases mortality. Tissue samples demonstrated the highest culture positivity. The culture results showed a higher frequency of *Mucor* species isolates and *Aspergillus flavus* compared to other isolates.

Keywords: Fungal diagnostics, Lactophenol cotton blue mount, Pandemic

INTRODUCTION

History was changed by the 1918 influenza epidemic, which killed one-third of the world's population. Fifty million individuals died as a result of the illness. Only 80 years after the 1918 influenza pandemic, Morens and colleagues from the National Institute of Allergy and Infectious Diseases examined lung tissue samples preserved in paraffin blocks and found that the majority of deaths occurred from secondary bacterial pneumonia caused by common upper respiratory tract bacteria, not viral pneumonia. This suggests that subsequent infections significantly affect patients' outcomes [1]. COVID-19, caused by SARS-CoV-2, is a global pandemic. Fungal co-infections associated with COVID-19 patients, especially those who are severely ill or immunocompromised, have a higher probability of suffering from invasive mycoses. The frequency and effects of co-infections, among other causes of morbidity and mortality in COVID-19 patients, are still poorly understood [2-5].

The aggressive nature of the SARS-CoV-2 virus against lung tissue and the presence of extensive bilateral alveolo-interstitial lesions suggest that the establishment of an invasive fungal infection is a plausible explanation for these phenomenon. The novel pathophysiology of COVID-19 may contribute to the previously unrecognised high rate of co-morbidity with invasive pulmonary aspergillosis, pneumocystosis, and mucormycosis [6,7]. However, due to the focus on controlling SARS-CoV-2 transmission, secondary illnesses caused by bacteria or fungi are not receiving the attention they deserve in infection control efforts [8]. In fact, secondary infection was found in 94.2% of patients receiving COVID-19 treatment. Clinical information on secondary bacterial and fungal infections is crucial for developing an evidence-based COVID-19 treatment plan [9].

Early detection of fungal infections in COVID-19 patients will require a comprehensive diagnostic intervention, including histopathology, direct microscopic examination, culture, (1,3)-β-D-glucan, galactomannan, and Polymerase Chain Reaction (PCR)-based assays, to ensure early and effective treatments. Additionally, appropriate sample type and transportation of samples are mandatory for better yield of results. The investigation and reporting of bacterial and fungal infections in COVID-19 patients have been insufficient so far. Only few articles with clinical data that have been published have reported secondary infection [10-12]. The aim of the present study was to determine the most suitable sample type for better culture outcome and identify the various opportunistic fungal infections related to COVID-19 patients.

MATERIALS AND METHODS

This laboratory-based retrospective observational study was conducted at Department of Microbiology, Government Medical

College, Surat, Gujarat, India, between November 2021 to January 2022. The data collection was done in the month of November 2021 followed by data analysis in December 2021 to January 2022. The study was conducted after obtaining ethical clearance from the institutional ethics committee, with HREC No. GMCS/STU/ETHICS/Approval/25565/21.

Inclusion criteria: The study included suspected fungal co-infection samples from COVID-19 positive patients, irrespective of age and sex.

Exclusion criteria: Samples for bacterial culture were excluded from the study.

Procedure

A total of 252 samples were received for suspected fungal co-infection COVID-19 patients and included in the study. Samples such as swabs, tissues, skin scrapings, CSF, sputum, pus, and drain samples from different wards, were received for fungal wet mount KOH and culture at the Microbiology Department of New Civil Hospital, Surat. These samples were subjected to culture on Sabouraud's Dextrose Agar (SDA) at 37°C and 25°C, as well as KOH wet mount. The culture on SDA was kept for 21 days before finally declaring it as negative. After obtaining a positive culture, fungal speciation was performed through macroscopic observation of colony morphology (front and reverse plate) and microscopic examination using Lactophenol Cotton Blue (LPCB) mount. The results of microscopy and culture were recorded.

STATISTICAL ANALYSIS

The data was entered into Microsoft Excel (Windows 10), and analysis was performed using Microsoft Excel, including frequency distribution and percentage.

RESULTS

A total of 252 samples were received. Samples from all age groups and both sexes were included in the study. In terms of age-wise distribution, all age groups were affected, but the highest number of cases 81 (32.14%) were observed in the 41-50 years age group. The number of male cases were higher than female cases [Table/Fig-1].

Age (in years)	n (%)
20-30	11 (4.37)
31-40	34 (13.49)
41-50	81 (32.14)
51-60	73 (28.97)
61-70	46 (18.25)
>70	7 (2.78)
Gender-wise distribution	
Male	179 (71.03)
Female	73 (28.97)

[Table/Fig-1]: Age and Gender wise distribution

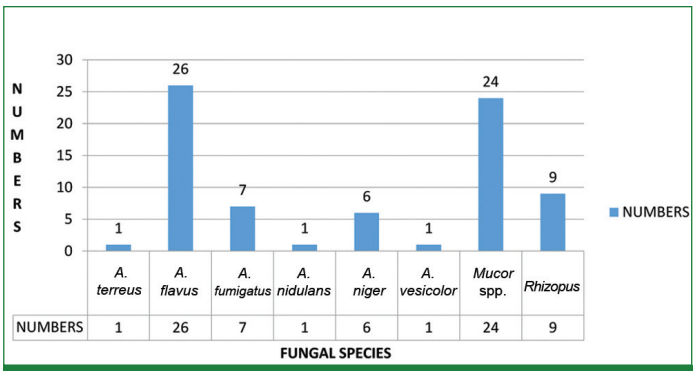
[Table/Fig-2] displays the different sample types received and their corresponding culture positive data. The samples included CSF, drain, pus, scraping material, sputum, swab, and tissue samples. Among these sample types, tissue samples demonstrated the highest culture positivity, with 43 (17%) samples yielding positive results. Swabs accounted for 13 (5%) positive cultures, while scraping material accounted for 14 (6%). The type of sample directly influences the outcome of the culture result, highlighting the importance of selecting the appropriate sample type. Out of the total 252 samples, 70 cultures showed positive results for fungal isolation. The percentage of samples with positive cultures is shown in [Table/Fig-2].

Comparing the positivity of KOH and culture, KOH showed a positivity rate of 69 (27.3%), while culture had a positivity rate of 70 (27.7%). The positive cultures revealed various fungal species, which were identified through LPCB mount and observation of culture characteristics

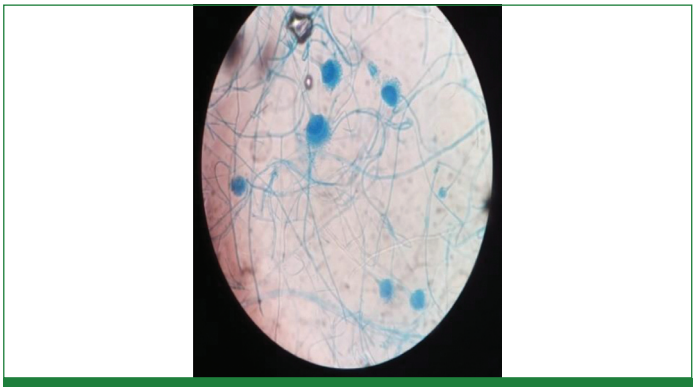
Sample type	Total sample (total N=252)	Frequency of positivity n (%)
Tissue	142	43 (17%)
Scrapping Material	25	14 (6%)
Swab	76	13 (5%)
Pus	3	0
Drain	1	0
Sputum	2	0
CSF	3	0

[Table/Fig-2]: Sample types and culture positive.

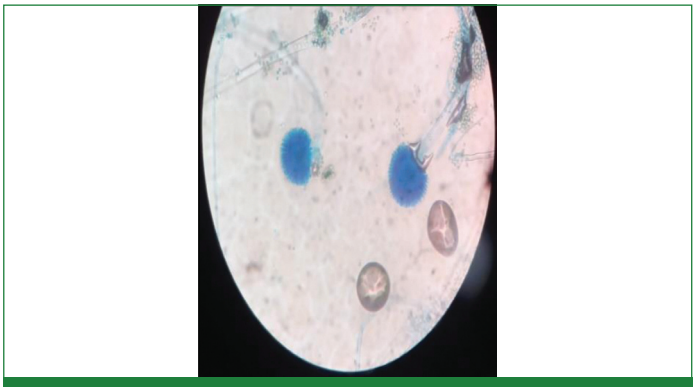
on the front and reverse plates. Among the total positive cultures, *Mucor* species accounted for 24 (34.28%), followed by *Aspergillus flavus* with 26 (37.14%) isolates. *Rhizopus* accounted for 9 (12.85%) isolates, *Aspergillus fumigatus* for 7 (10%) isolates, *Aspergillus niger* for 6 (8.57%) isolates, *Aspergillus terreus*, *Aspergillus vesicolor*, and *Aspergillus nidulans* were 1 (1.42%) isolate each. So 75 fungal species isolated as in five patients showed 2 fungal species viz *Mucor* plus *Aspergillus flavus* were isolated [Table/Fig-3].



[Table/Fig-3]: Fungal speciation.

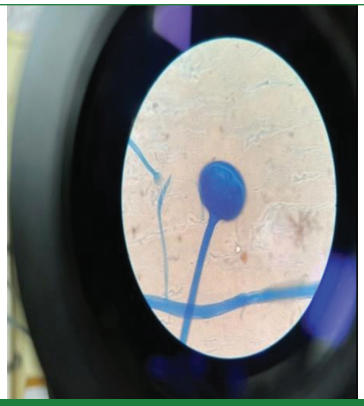


[Table/Fig-4]: *Aspergillus flavus.*
(Lactophenol cotton blue stain) at 40x magnification



[Table/Fig-5]: *Aspergillus fumigatus.*
(Lactophenol cotton blue stain) at 40x magnification

And various pictures (original images from the study) of LPCB mounts of *Aspergillus flavus* can be seen in [Table/Fig-4], *Aspergillus fumigatus* in [Table/Fig-5], and *Mucor* species in [Table/Fig-6], showcasing the fungal morphology.



[Table/Fig-6]: *Mucor* species.
(Lactophenol cotton blue stain) at 40x magnification

DISCUSSION

Mucormycosis, although uncommon in healthy individuals, is more prevalent in individuals with immune system disorders. Examples of conditions that fall under this category include haematological disorders, uncontrolled Diabetes Mellitus (DM) with or without diabetic ketoacidosis (DKA), other cancers, organ transplantation, prolonged neutropenia, immunosuppressive and corticosteroid therapy, iron overload or hemochromatosis, deferoxamine or desferrioxamine therapy, voriconazole prophylaxis for transplant recipients, severe burns, Acquired Immunodeficiency Syndrome (AIDS), intravenous drug users, malnutrition, and open wounds following trauma [7,13-15].

Co-infection makes the diagnosis and outcome of mucormycosis difficult. Mucormycosis is a rare but potentially fatal fungal infection caused by a group of moulds collectively known as Mucormycosis [16]. Mucorales, the fungi responsible for mucormycosis, are widespread in nature, including in soil, decaying plants, bread, and dust. Infection occurs through inhalation of Mucorales spores, ingestion of contaminated food, or through damaged skin or wounds. Microbiological identification of the hyphae based on their diameter, presence or absence of septa, branching angle (right or acute branching), and pigmentation helps distinguish mucormycosis from other fungal infections [17].

Comparing the components of present study with different studies published in 2021, variations was observed in the age range and gender distribution. For example, Singh AK et al., reported an age range of 22-86 years, while Ravani SA et al., reported a mean age of 56.3 years [7,14]. Sen M et al., suggested a mean age of 60.5±12 (range 46.2 to 73.9) years [5], and Rabagliati R et al., reported a median age of 65 (range 30-89) years [18]. In present study, the predominant age group was 41-50 years, with 81 cases (32.14%).

Regarding gender distribution, male patients were found to be predominant in various studies. Singh AK, et al., reported 78.9% male patients [7], while Zhu X et al., found that 138 (53.7%) were male [9]. Comparing present study with the analysis by Heard KL et al., *Aspergillus* species, *Candida glabrata* and *Aspergillus fumigatus* were the fungal isolates [11]. J Gangneux et al. reported *Aspergillus* species infections in COVID-19 patients hospitalised in the Intensive Care Unit (ICU) for Acute Respiratory Distress Syndrome (ARDS) [6]. Yang X et al., isolated *Aspergillus flavus* and *A. fumigatus* from Respiratory Tract Secretions [19]. Lansbury L et al., identified *Aspergillus flavus*, *Aspergillus fumigatus*, and *Candida glabrata* from respiratory samples [20].

One more study showed *Aspergillus flavus, Candida glabrata, Candida albicans* as isolates [21]. Another study by Santos HM et al., reported an upsurge of mucormycosis cases, reaching 17 in a span of four months (May 5-September 6, 2021) during the COVID-19 period [22].

[Table/Fig-7] shows comparison between different studies. The present study demonstrated a higher culture yield with tissue samples, followed by scraping material and swab samples, indicating that tissue samples are superior to other sample types for isolating fungi. Proper sample collection and selection of the appropriate sample type directly impact the outcome. Furthermore, more studies are needed to investigate fungal co-infections in COVID-19 patients.

	Heard KL et al., study [11]	Gangneux JP et al., study [6]	Present study (in numbers)
Fungal species isolated	*Aspergillus* species, *Candida glabrata*, *Aspergillus fumigatus*	*Aspergillus* species	*Mucor* speices (24), *Aspergillus flavus* (26) predominant

[Table/Fig-7]: Comparison of isolated fungal species.

Limitation(s)

Although traditional culture tests are still considered the gold standard for identifying fungal infections, there are several challenges associated with their use. These challenges include the often low sensitivity of the tests, lengthy turnaround times, arduous process, and the invasive nature of the specimens required.

CONCLUSION(S)

The second wave of COVID-19 had a significant impact worldwide, and the presence of fungal co-infections further complicated the situation. Fungal co-infections in COVID-19 positive patients contribute to increased morbidity and mortality. Tissue samples exhibited the highest culture positivity, with *Mucor* species and *Aspergillus flavus* being the most frequently isolated species.

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