[12/08/2022]

Dear Editors and Reviewers,

We wish to re-submit the attached manuscript as a Case Report. The manuscript ID is 78320.

Thank you very much for reviewing our manuscript and for your valuable comments.

We have addressed these comments in our point-by-point responses and revised the manuscript accordingly.

We hope that the revised manuscript is now suitable for publication in your journal.

The responses to all comments have been prepared and given below.

We attach a revised manuscript at the end of this document for your reference.

Responses to the Comments by Reviewer #1:

1. "Introduction" This is the first case report detailing the autopsy results of disseminated mucormycosis in a patient affected with COVID-19" However, recently another 58-years old Japanese male with disseminated mucormycosis was reported. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8841239/). In addition, another case has been reported in https://pubmed.ncbi.nlm.nih.gov/34075329/. Therefore, it is recommended the authors revise the corresponding sentence and cite these two another similar articles.

Reply:

Thank you for your helpful comments. We have revised the text in the abstract, core tips, and introduction, and conclusion, and added the references.

Change:

Abstract (page 2, line 6-7)

Before

This is the first documented autopsy report of disseminated COVID-19-associated mucormycosis.

After

This is a rare pathological autopsy report on COVID-19-associated mucormycosis.

Core tips (page 3, line 6-7)

Before

This is the first documented autopsy report of disseminated COVID-19-associated mucormycosis.

After

Our paper is a rare pathological autopsy report on COVID-19-associated mucormycosis.

Introduction (page 4, line 15-18)

Before

We present an autopsy case of systemic Mucorales infection after COVID-19 treatment. This is the first case report detailing the autopsy results of disseminated mucormycosis in a patient affected with COVID-19.

After

COVID-19 is a known risk factor for disseminated mucormycosis, but there are very few reports detailing related autopsy results [7, 8].

We present an autopsy case of systemic Mucorales infection after COVID-19 treatment. Our paper is a rare pathological autopsy report on COVID-19-associated mucormycosis.

Conclusion (page 10, line 16-17)

Before

our case report is the first report detailing an autopsy of disseminated COVID-19-associated mucormycosis.

After

our paper is a rare report detailing an autopsy for disseminated COVID-19-associated mucormycosis.

References (page 14)

7 Krishna V, Morjaria J, Jalandari R, Omar F, Kaul S. Autoptic identification of disseminated mucormycosis in a young male presenting with cerebrovascular event, multi-organ dysfunction and COVID-19 infection. *IDCases* 2021; 25: e01172 [PMID: 34075329 PMCID: PMC8161734 DOI: 10.1016/j.idcr.2021.e01172]

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2. Case presentation: "A nasopharyngeal swab specimen tested positive for COVID-19". COVID-19 is a disease. Nasopharyngeal swab test can be positive for SARS-CoV-2. Please correct the sentence.

Reply:

We agree with you and have corrected the text in the case presentation.

Change:

Case presentation (page 5, line 3)

Before

A nasopharyngeal swab specimen tested positive for COVID-19.

After

A nasopharyngeal swab specimen tested positive for SARS-CoV-2.

3. Case presentation: ". He sought treatment at an emergency hospital for COVID-19 and was managed with oxygen and glucocorticoids". Considering glucocorticoids are risk factor for mucormycosis development, it is necessary to outline the indications to start glucocorticoids for the patient.

Reply:

We corrected the text in the case presentation and added a reference to clarify the indication for glucocorticoids.

Change:

Case presentation (page 5, line 4-5)

Before

He sought treatment at an emergency hospital for COVID-19 and was managed with oxygen and glucocorticoids.

After

He was diagnosed with moderate pneumonia at an emergency hospital for COVID-19 and was managed with oxygen and glucocorticoids [9].

References

9 Recovery Collaborative Group, Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, Staplin N, Brightling C, Ustianowski A, Elmahi E, Prudon B, Green C, Felton T, Chadwick D, Rege K, Fegan C, Chappell LC, Faust SN, Jaki T, Jeffery K, Montgomery A, Rowan K, Juszczak E, Baillie JK, Haynes R, Landray MJ. Dexamethasone in Hospitalized Patients with Covid-19. N Engl J Med 2021; 384(8): 693-704 [PMID: 32678530 PMCID: PMC7383595 DOI: 10.1056/NEJMoa2021436]

4. Case presentation: "Seventeen days later, …" was the history taking significant during that 17 days? What happened during that period? Was patients status stable or

deteriorative? Was the respiratory symptoms developed in acute-phase or chronically? These questions need to be answered.

Reply:

We appreciate your important question. Despite the treatment with oxygen and glucocorticoid, his oxygen requirements increased and his pneumonia gradually worsened.

We have added relevant text in the case presentation.

Change:

Case presentation (page 5, line 5)

Before

Seventeen days later,

After

However, his oxygen requirements increased and his pneumonia gradually worsened. Seventeen days later, …

5. Please cite the following article for this sentence in the Introduction "The presentations of COVID-19 can range from symptomless to multi-organ failure and death". - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7779976/.

Reply:

We have added the reference in the introduction.

Change:

Introduction (page 4, line 5-6)

Before

The presentations of COVID-19 can range from symptomless to multi-organ failure and death [2].

After

The presentations of COVID-19 can range from symptomless to multi-organ failure and death [2, 3].

References

3 Rakhsha A, Azghandi S, Taghizadeh-Hesary F. Decision on Chemotherapy Amidst COVID-19 Pandemic: a Review and a Practical Approach from Iran. Infect Chemother 2020; 52(4): 496-502 [PMID: 33263246 PMCID: PMC7779976 DOI: 10.3947/ic.2020.52.4.496]

6. Figure 3F: Please indicate the CMV particles.

Reply:

As noted, we modified Figure 3F and its legend.

Change:

Figure 3F

We added the black arrows for the CMV-infected cells.

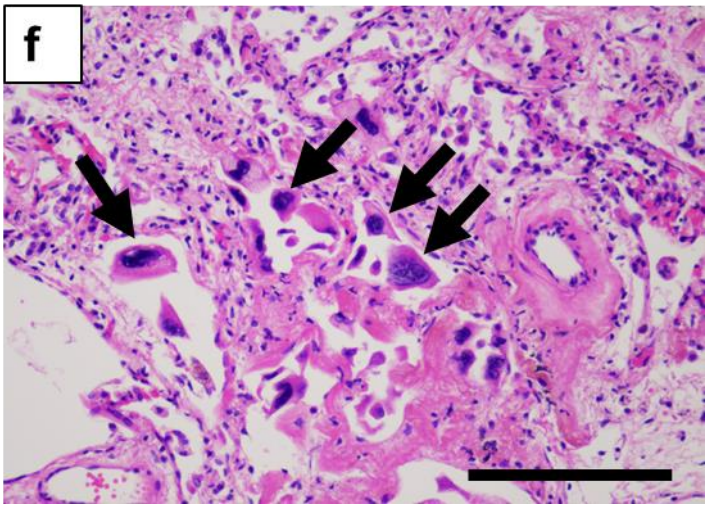

Figure legends (page 19, line 4-5)

Before

(F) Cytomegalovirus infection.

After

(F) Cytomegalovirus infection. Cytomegalovirus-infected cells are indicated by black arrows.

Responses to the Comments by Reviewer #2:

Manuscript is well written and is of scientific interest. May be accepted. However, minor grammar correction required prior to publication.

Reply:

Thank you for your kind comment. We re-sent our revised manuscript to a professional English language editing company to polish the language.

Change:

The manuscript has been revised by the company and received a new language certificate.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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COVID-19-associated disseminated mucormycosis: An autopsy case report

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Keywords: COVID-19, mucormycosis, Mucorales infection, *Rhizopus oryzae*, SARS-CoV-2, case report

Running title: COVID-19-associated mucormycosis

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Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; IL, interleukin; ECMO, extracorporeal membrane oxygenation; ARDS, acute respiratory distress syndrome, CT, computed tomography

Abstract

Background: Reports of mucormycosis, an infectious disease that commonly affects immunocompromised individuals, have increased during the ongoing coronavirus disease 2019 (COVID-19) pandemic. Disseminated mucormycosis associated with COVID-19 is rare but fatal and is characterized by an aggressive clinical course and delayed diagnosis. Our report documents a case of disseminated mucormycosis after COVID-19 infection. This is a rare pathological autopsy report on COVID-19-associated mucormycosis.

Case summary: A 58-year-old man was transferred to our hospital with severe COVID-19 pneumonia. During treatment for acute respiratory distress syndrome, he developed intra-abdominal bleeding that required a right hemicolectomy and ileostomy for hemostasis. The ileostoma and surgical wound developed necrosis followed by sepsis and multi-organ failure, which led to death. An autopsy revealed multiple thrombi associated with *Rhizopus oryzae* infection, which led to the necrosis of multiple infected organs.

Conclusion: Early suspicion and diagnosis followed by treatment are keys to better outcomes of mucormycosis in patients with severe COVID-19.

Core tips

We document a case of disseminated mucormycosis post-COVID-19 infection. A 58-year-old man underwent a right hemicolectomy during COVID-19 pneumonia. The surgical wound developed necrosis, which was followed by multi-organ failure, leading to death. An autopsy revealed multiple thrombi with *Rhizopus oryzae* infection, which led to the necrosis of multiple infected organs. Our paper is a rare pathological autopsy report on COVID-19-associated mucormycosis. It is important that treating physicians be aware of the increased risk of mucormycosis in patients with severe COVID-19. Early suspicion and diagnosis followed by treatment are keys to better outcomes.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has greatly impacted the global population. As of 1 February 2022, there were more than 38,000,000 confirmed cases and 5,500,000 deaths according to the World Health Organization [1]. The presentations of COVID-19 can range from symptomless to multi-organ failure and death [2, 3]. Current interventions include glucocorticoids and anti-interleukin (IL)-6 drugs to suppress inflammation, extracorporeal membrane oxygenation (ECMO) to address fatal lung injury caused by COVID-19, and mRNA-based vaccines [4]. There have been several reports about concomitant bacterial and fungal infections [2]. An increase in SARS-CoV-2 infections increases the risk of opportunistic fungal infections, including candidiasis, pulmonary aspergillosis, and mucormycosis, resulting in multi-organ failure and death [2, 5]. Among opportunistic fungal infections, Mucorales, the order of fungi that causes mucormycosis, causes extensive angioinvasion, resulting in vessel thrombosis and tissue necrosis. The mortality rate for mucormycosis is 30%−50%, which increases to 90% once dissemination occurs [6]. COVID-19 is a known risk factor for disseminated mucormycosis, but there are very few reports detailing related autopsy results [7, 8].

We present an autopsy case of systemic Mucorales infection after COVID-19 treatment. Our paper is a rare pathological autopsy report on COVID-19-associated mucormycosis.

Case presentation

A 58-year-old man with a history of hypertension visited a local hospital with a primary complaint of a sore throat. A nasopharyngeal swab specimen tested positive for SARS-CoV-2. He was diagnosed with moderate pneumonia at an emergency hospital for COVID-19 and was managed with oxygen and glucocorticoids [9]. However, his oxygen requirements increased and his pneumonia gradually worsened. Seventeen days later, he developed severe respiratory fatigue and was diagnosed with acute respiratory distress syndrome (ARDS). The patient was intubated and started on mechanical ventilation and tocilizumab (an anti-IL-6 receptor drug). Subsequently, he was transferred to the intensive care unit of our hospital for venovenous ECMO in the prone position to improve dorsal atelectasis. Computed tomography (CT) revealed bilateral and peripheral ground-glass and consolidative pulmonary opacities (Fig. 1a).

Nine days after his admission to our facility, he developed hypotension and had an intra-abdominal bleed from the mesentery of the ascending colon. He underwent a right hemicolectomy to remove the bleeding mesenteric vessels. After confirming hemostasis in the abdominal cavity, an ileostoma was created. Histopathological examination revealed the absence of morphological abnormalities, aneurysms, or stenosis in the blood vessels that caused the abdominal bleed. Further, thrombi, bacteria, or fungi were not observed in the mesenteric vessels or tissue.

Eighteen days later, the ileostoma exhibited ischemic changes, and the patient underwent stomal reconstruction (Fig. 1b and c). According to enhanced CT imaging and surgical findings, ischemic changes occurred only in the part of the intestines adjacent to the stoma. However, the surgical wound gradually exhibited necrotic changes that required repeated debridement. No fungi were present on culture-based examination of venous blood and necrotic tissues, including the skin, muscular tissue, and adjacent intestine. However, histopathological examination revealed that the necrotic stoma contained many thrombi and Mucorales. Grocott staining demonstrated that Mucorales had invaded the vessel walls and fat tissue in the mesentery of the necrotic intestine (Fig. 1d and e). The patient's respiratory status showed mild improvements, and his SARS-CoV-2 antigen test result was negative at 29 and 31 days after transfer. However, the patient eventually developed multiple organ failure and died 46 days after admission to our hospital.

An autopsy was performed with permission from the patient's family. Macroscopic examination revealed necrosis of the reconstructed stoma, skin, and abdominal wall muscles (Fig. 2a and b). A large thrombus was found in the common iliac vein (Fig. 2c) along with the necrosis of multiple abdominal organs, including the small intestine, liver, gall bladder, right kidney, and spleen. Histopathological examination revealed Mucorales in the thrombi and necrotic organs except for the spleen (Fig. 3a-d). The stomach and urinary bladder showed Mucorales but no

necrosis. We extracted the fungal gene from formalin-fixed paraffin-embedded tissue of the thrombus using NucleoSpin DNA FFPE XS (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Using DNA sequencing, we identified the Mucorales as *Rhizopus arrhizus*, also known as *Rhizopus oryzae* (The Basic Local Alignment Search Tool [BLAST]; results are shown in Table 1). Mucorales were undetectable in the nasal cavity, pharyngeal mucosa, trachea, or lungs. Immunohistochemical analysis of both lungs did not reveal SARS-CoV-2 S proteins. There was partial proliferation of myofibroblasts and lymphocytes in the interstitial and intra-alveolar spaces (Fig. 3e). These findings were consistent with the proliferative/organizing phase of diffuse alveolar damage caused by ARDS in COVID-19. In addition, swollen cells with intranuclear inclusion bodies, positive for cytomegalovirus antigen, were observed in the left lung (Fig. 3f).

Significant inflammation was absent in the heart. Sepsis and multi-organ failure secondary to mucormycosis due to COVID-19 were determined as the causes of death.

Discussion

This report describes a case of unexplained necrosis in multiple organs after COVID-19 treatment. Mucorales are highly angioinvasive and are associated with thrombus formation,

infarction, and hemorrhage [2]. *R. oryzae* is the most common organism isolated from patients with mucormycosis, accounting for 70% of cases [10]. In the current case, pathological examination during autopsy and DNA sequencing revealed that *R. oryzae* had created a large thrombus and invaded the necrotic tissues.

Although COVID-19-associated pneumonia and ARDS were appropriately treated, fungal thrombosis, necrosis, and multi-organ failure due to mucormycosis led to a fatal outcome in this patient. Mucorales were detected in multiple ventral organs, the skin, and surgical wounds (Fig. 4a). The predominant site of infection for mucormycosis is the nasal cavity or respiratory tract [2, 5]. According to a systematic review of COVID-19-associated mucormycosis, 86% of infections were peri-nasal, 10% were pulmonary, and only 1% were disseminated [2, 11]. As Mucorales were not found in the pharyngeal mucosa, trachea, or lungs, it was concluded that the ileostoma and surgical wound were the points of entry of Mucorales in this case. Mucorales are known to invade the gastrointestinal tract, skin, and the upper airway [12, 13]. Uncontrolled diabetes mellitus, steroids, numerous cytokines during ARDS, and drugs such as lopinavir, ritonavir, and remdesivir used in COVID-19 treatment increase the risk of mucormycosis in COVID-19 patients [2]. This patient had an increased risk of mucormycosis due to glucocorticoid use, surgical stress, and ARDS.

Histopathological examination of the right hemicolectomy specimen did not reveal the existence of Mucorales or anatomical abnormalities, and stomal necrosis caused by mucormycosis was observed 21 days after the right hemicolectomy procedure. Since mucormycotic symptoms were not found during the intra-abdominal hemorrhage, we concluded that the mucormycosis was unrelated to the hemorrhage and occurred after surgery. The cause of intra-abdominal hemorrhage remained unclear from the histopathological diagnosis, but we speculate that several factors such as venovenous ECMO, anticoagulation, prone position, and sepsis might have contributed to the abdominal bleeding [14].

Culture-based examination of blood and necrotic tissue could not identify the causative organism, but histopathological examination revealed that mucormycosis was the cause of necrosis. As the patient's condition rapidly deteriorated due to multi-organ failure, the clinicians lacked sufficient time to manage the mucormycosis. Thus, early diagnosis before the systemic dissemination of mucormycosis is essential for better outcomes. Although routine serological tests do not help diagnose Mucorales infections, a histopathological examination can play a significant role in confirmation. Antigen-antibody reactions or the beta-D-glucan test used to diagnose other fungal infections, such as *Candida* and *Aspergillus*, cannot be used to diagnose mucormycosis [12]. Thus, the diagnosis of mucormycosis before the formation of an infected or necrotic site is quite challenging [15]. A recent case report demonstrated that cell-free DNA next-

generation sequencing effectively detected disseminated *Rhizomucor pusillus* in a patient with Philadelphia-like acute lymphoblastic leukemia [16]. After receiving sufficient treatment with posaconazole and liposomal amphotericin B, the patient survived the infection. Moreover, a prospective multicenter study demonstrated that quantitative polymerase chain reaction examination using the patient's serum could help diagnose mucormycosis with 85.2% sensitivity and 89.8% specificity [17]. It is important to note that this method detected the presence of Mucorales four days prior to mycological or histopathological evaluation. Recent articles have reviewed other detection methods [15, 18]. These novel serum-based techniques may increase the chances of better outcomes in patients with systemic mucormycosis by facilitating the initiation of treatment before tissue necrosis.

Conclusion

Our report documents a case of disseminated mucormycosis associated with COVID-19. Autopsy revealed that Mucorales could infect stomas and surgical wounds, apart from the nasal cavity in high-risk patients, and rapidly spread to various organs. Disseminated mucormycosis is rare but lethal in COVID-19 patients, and our paper is a rare report detailing an autopsy for disseminated COVID-19-associated mucormycosis. The flowchart of autopsy diagnosis should

be helpful for fellow clinicians (Fig. 4b). Although ARDS can be resolved in intensive care, opportunistic infections triggered by COVID-19 are a critical problem, and a diagnosis of mucormycosis is often delayed. Physicians should be aware of the increased risk of mucormycosis among COVID-19 patients. Early suspicion and timely diagnosis followed by treatment are crucial for better outcomes among affected individuals.

Disclosure of potential conflicts of interest

None declared.

Author contributions

DK, TK, and MH performed the autopsy. DK, TK, MT, SS, YO, and KM performed the histopathological examination. AT, YK, and KT performed the DNA analysis. HI, KH, and EN provided treatment for this case and performed visualization of the work. TH and MO supervised this project and helped with writing the manuscript. DK drafted the manuscript. All authors reviewed and revised the manuscript.

Declarations

This project was approved by our institutional ethics committee. This investigation was conducted in accordance with the Declaration of Helsinki (1975).

Consent for publication

Appropriate written informed consent was obtained from the patient's family to publish this case report and the project.

CARE Checklist (2016)

The authors have read the CARE Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

Acknowledgements

Thanks to ondoku3.com for creating the audio core tip.

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Figure legends

Fig. 1: Imaging and histological findings during treatment. (A) Computed tomography (CT) images of acute respiratory distress syndrome (ARDS). Left image: CT image at the time of ARDS diagnosis. Right image: CT image after 38 days. (B) The necrotic ileostoma caused by the mucormycosis can be seen in this image. (C) Surgical findings at the stomal reconstruction. (D and E) Presence of thrombus with Mucorales in the mesenteric vessels of the necrotic ileostoma. H&E staining; magnification ×200 (D), Grocott staining; magnification ×200 (E). Bar, 200 μm.

Fig. 2: Autopsy findings of the Mucorales infection. (A) The image shows the necrotic stoma, skin, and abdominal wall. (B) The necrotic abdominal organs are seen in this image. A pathologist held the intestine on the oral side of the stoma. (C) Dorsal view of the incised common iliac vein. The thrombus can be seen in the common iliac vein (indicated by arrows).

Fig. 3: Histopathological findings of the Mucorales infection in the organs and thrombus. (A and B) Presence of Mucorales in the thrombus; H&E staining; magnification ×100 (A), ×200 (B). (C) Macroscopic image showing partial necrosis of the liver. (D) Hepatic infarction caused

by the thrombus including Mucorales; H&E staining; magnification ×200. (E) The proliferative/organizing phase of diffuse alveolar damage of the left lung. Restoration of type II pneumocytes and proliferation of myofibroblasts are partially shown. H&E staining; magnification ×40. (F) Cytomegalovirus infection. Cytomegalovirus-infected cells are indicated by black arrows. H&E staining; magnification ×200. Bar in the autopsy images, 2 cm; Bar in the microscopic images, 200 μm.

Fig. **4**: The autopsy diagnosis. (A) The image shows the organs and part of the Mucorales infection. The gray areas indicate areas infected with Mucorales. The resected ascending colon is transparent in the drawing. (B) A flowchart of the autopsy diagnosis.