

Studying the use of a proposed three-level grossing protocol in the histopathological diagnosis of COVID-19-associated rhinomaxillary mucormycosis

Krishna Sireesha Sundaragiri¹, Shikha Saxena¹, Bharat Sankhla¹, Akshay Bhargava¹, Geeta Sharma², Isha Gaurav³

Departments of ¹Oral Pathology and ³Oral Medicine and Radiology, RUHS College of Dental Sciences (Govt. Dental College), Jaipur, Rajasthan, ²Department of Oral Pathology, Patna Dental College and Hospital, Patna, Bihar, India

Abstract

Introduction: Mucormycosis is an acute and rapidly progressing opportunistic fungal infection. COVID-19-associated mucormycosis (CAM) had re-emerged as a complication of COVID-19 infection during the second wave of the pandemic in 2021. The rhinomaxillary form is a variant of the rhino-cerebral mucormycosis that presents a diagnostic challenge to the dentist and the oral and maxillofacial pathologist. Gross examination of pathological specimens is the most undermined step even though it plays a vital role in the final diagnosis. No studies have described this post-clinical step for the maxillofacial soft and hard tissue submitted for examination.

Material and Methods: A prospective comparative study was carried out on 52 COVID-19-associated rhinomaxillary mucormycosis (CARM) cases to achieve complete, representative, and informative sampling of the submitted tissue and establish a three-level gross macroscopic examination protocol. Complete clinical and radiological histories were recorded after informed, written consent from every patient was received. Details of the number and type of samples received were recorded, grossing procedure was done as per the proposed three-level grossing protocol and were then compared to the presence of fungal hyphae in the soft tissue or decalcified hard tissue.

Result: All 100% of the samples consisted of soft tissue (maxillary sinus lining), while 90.4% of the samples contained different hard tissue specimens. Seventy percent of the grossing workload was carried out by first-year oral pathology residents. Sixty-seven point three percent of the total soft tissue samples submitted showed no presence of fungal hyphae, while 69.2% of total decalcified sections of hard tissue were positive for fungal hyphae with a positive correlation. Out of the 29 cases grossed via the three-level grossing protocol, 89.6% of the cases were histopathologically positive for fungal hyphae. Thus a positive association ($P < 0.05$) between histopathological diagnosis and the proposed three-level grossing protocol was found.

Conclusion: It is imperative to recognise that no mucormycosis report is to be signed out without multi-site (three-level grossed) bone decalcified reports. There is an immediate need to realise how vital documentation, correct laboratory practices, and grossing are for accurate histopathological diagnosis.

Keywords: COVID-19, decalcification technique, mucormycosis, pathologists

Address for correspondence: Dr. Krishna Sireesha Sundaragiri, B-209, Anshu Hospital, Rajendra Marg, Bapunagar, Jaipur - 302 015, Rajasthan, India.
E-mail: sksireesha@gmail.com

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INTRODUCTION

A total of 51,775 cases of mucormycosis have been reported as of 29 November 2021 in India.^[1] This led to a diversion of massive efforts in its diagnosis, treatment, and prevention. COVID-19-associated mucormycosis (CAM) is an opportunistic fungal infection with high mortality. Its treatment requires an invasive surgical approach, so early diagnosis is vital to save patients' lives and reduce morbidity.^[2] Early diagnosis of CAM is challenging for inexperienced pathologists and personnel. Observations of soft tissue invasion (mucosal penetration), destruction of cartilage or bone (maxillary bone, nasal turbinates), necrosis, and inflammation help confirm clinical suspicion at an early stage of the disease, but histopathological examination of the biopsy specimen is still the gold standard.^[3]

Histopathological examination complements the microbiological examination in diagnosing mucormycosis. However, the fungal hyphae are fragile, and in most cases microbiological culture may remain negative. For histopathological examinations, it is recommended that the specimen be routinely processed, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) stain and examined for the presence of fungal elements,^[4] but grossing pathological specimens is the most disparaged and underrated step.

Grossing is the process by which pathology specimens are inspected with bare eyes to obtain diagnostic information by trained personnel as only a small portion from a large specimen is subjected to microscopic examination. During this procedure, identification of the specimen, its anatomical biopsy site, and clinical details are noted along with a written record of the physical appearance of the specimen. During gross examinations, all the pathological specimens should be completely, representatively, and informatively sampled. It is the most important step to obtain the most diagnostically important tissue onto the slide. Theresa *et al.*,^[5] in their review, listed the location from which specific sections of tissue are taken for microscopic evaluation as one of the seven important points for grossing. The three main conditions that determine successful grossing for bone sections are specimen immobilisation, the appropriate cutting instrument, and correct specimen orientation.^[6] The rhinomaxillary variant of CAM primarily affects the maxillofacial skeleton; thus, maxillary bone decalcification process with the use of acids has become an essential prerequisite for tissue processing before its examination and diagnosis. The acids affect the soft tissue components, but double wall fungal hyphae are

accentuated. We propose a three-level grossing protocol for the resected maxillary specimens for faster definitive detection of fungal hyphae. This protocol was applied on our CAM cases submitted during the second wave of COVID-19 in a government-approved mucormycosis treating centre in a government-run dental college and hospital.

MATERIAL AND METHODS

This prospective comparative study was carried out on 52 clinically and radiologically diagnosed rhinomaxillary mucormycosis (RMM) maxillectomy specimens submitted to the department of oral pathology during the second wave of COVID-19 in the month of May 2020 to September 2020. Complete clinical and radiological COVID-19 histories were recorded after informed, written consent from every patient was received. The institutional ethics committee approval was obtained in RUHS-CDS/EC/2021/Proposal/002. Intraoperative KOH diagnosis was provided to determine fungal-free margins. Based on the grossing protocol carried out for all the hard tissue maxillectomy specimens, three groups were defined as

- Group 1: Only soft tissue (no hard tissue received)
- Group 2: Soft and hard tissue received but hard tissue not grossed as per three-level grossing protocol
- Group 3: Soft and hard tissue received and hard tissue grossed as per three-level grossing protocol.

Procedure: Each pathological soft and hard tissue was received in 10% formalin in separate labelled sample bottles and marked accordingly. Gross examination was carried out 24 hours after specimen submission as per COVID-19 specimen handling protocols. The gross macroscopic examinations were performed by oral pathology residents under the supervision of a faculty member. The assessment of hard tissue samples was categorised into no hard tissue received, only necrotic bony bits, or total/hemi/partial maxillectomy specimens with several teeth present. After the photographic documentation, the grossing and sectioning of hard tissue specimens was done with a hand chisel and mallet as deemed necessary. A three-level grossing for the submitted maxillectomy specimens was developed and adopted [Figure 1]. That site was selected where adequate interdental bone, the maxillary bone, and the sinus floor were available. In case only bone fragments were available, they were kept for decalcification. In cases where no hard tissue/bone was submitted, the same was recorded. Based on the College of American Pathologists (CAP) guidelines for each block's unique identifier, the samples were labelled as HP number DE A/B/C number^[6] as 1 for interdental

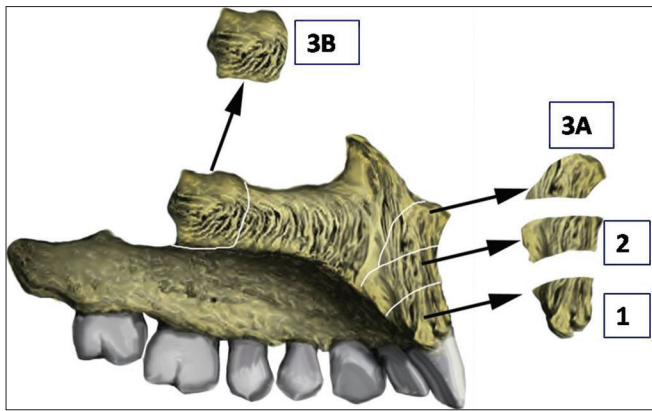


Figure 1: Schematic diagram of a hemimaxillectomy specimen with three-level grossing protocol. (1) Inter-dental area of teeth, (2) peri-apical area, and (3A) nasal floor (anterior) or (3B) sinus floor (posterior)

bone between the teeth, 2 for periapical tissue between the teeth, and 3 for the floor of sinus/nasal floor [Figure 2].

Decalcification technique: All the samples were decalcified with 5% hydrochloric acid, and the time required for decalcification was standardised. Their decalcification endpoint was noted by testing for softness using a sharp pin.^[7] The ready samples were then thoroughly drained of acid by washing them in running water and were routinely processed, sectioned, and stained by H&E and periodic acid–Schiff (PAS) stains. All the sections for each case were scanned thoroughly by a minimum of two qualified oral pathologists for the presence of fungal elements, and all the findings were tabulated in a Microsoft Excel sheet designed for the study. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) version 20 software and frequency distribution charts for type of sample received, grossing conducted by, the three-level grossing in the resected maxillary specimen, and presence of fungal hyphae in soft and hard tissue were tabulated. Pearson's Chi-squared test with a *P* value of less than 0.05 was considered for a significant association between the final histopathological diagnosis and the parameters.

RESULTS

Only 72.5% of patients were COVID-19 positive while the rest of them reported symptoms of fever or cold prior to developing any signs of CAM. In our study, 24 patients (47.1%) had underlying diabetes mellitus while 9 patients (12.8%) had hypertension and 24 patients (47.1%) had received steroids.

All the 52 specimens (100%) submitted included soft tissue (maxillary sinus lining), while no decalcified sections were possible for 9.6% of all samples with no hard tissue/bone submitted. The three-level grossing

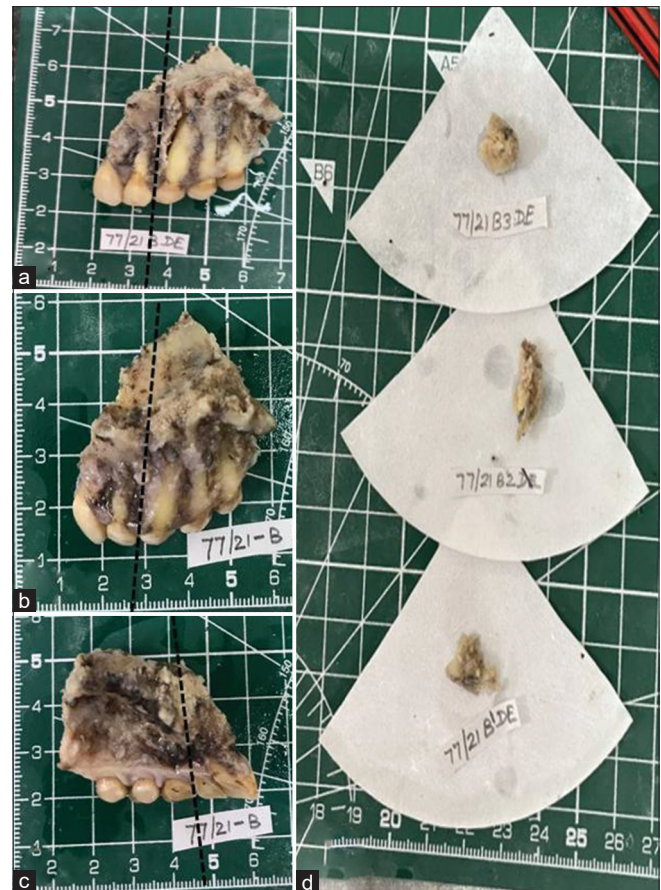


Figure 2: Left hemimaxillectomy specimen of a 33-year-old male patient suspected of mucormycosis. (a) Buccal view, (b) superior view, and (c) palatal view with site for three-level grossing of the specimen (dotted black line). (d) Three hard tissue specimens grossed at inter-dental area of teeth 22, 23 (1), peri-apical area, (2) and nasal/sinus floor (3) shown

protocol was carried out in 29 out of 52 cases (55.8%) that had hard tissue (resected maxillectomy) in the CAM samples [Table 1]. Forty-four point two percent of hard tissue was from the anterior maxillary bone area including the area between the canine and the first premolar where an adequate mass of bone was available for the three-level grossing protocol.

A total of 69.2% of the gross examination was carried out by first-year residents while only 21.1% and 9.6% of the total samples were grossed by second- and third-year residents [Table 2]. There was no positive association between histopathological diagnosis and residency year of the grossing resident doctor.

Sixty-seven point three percent of the total soft tissue samples submitted showed no presence of fungal hyphae, while 69.2% of total decalcified sections of hard tissue were positive for fungal hyphae [Table 3] [Figure 3]. Thus, there was a positive correlation ($P < 0.05$) between

histopathological diagnosis and fungal hyphal presence in the decalcified sections.

Six (11.2%) cases were diagnosed as simple maxillary sinusitis with no evidence of fungal hyphae, out of which 83.3% of cases had no hard tissue submitted among the samples [Table 4]. Out of the 29 cases grossed via the three-level grossing protocol, 89.6% were histopathologically positive for fungal hyphae; thus, a positive association ($P < 0.05$) between histopathological diagnosis and the three-level grossing protocol for maxillectomy CAM specimens was found.

DISCUSSION

Grossing of bone is considered cumbersome, dirty, and physically demanding. Dimenstein stated that an effectively performed bone gross section helped in accurate diagnosis.^[6] Gross macroscopic examination of

the resected maxilla and maxillofacial soft tissue is highly demanding and challenging. Owing to bone fragility, often the complete maxillary bone specimens have been kept for decalcification. Grossing of oral maxillofacial skeleton for suspected mucormycosis has been neither described in detail nor the problems encountered discussed.

Rajasekaran and Thangavelu^[8] discussed a combination of procedures such as partial/total maxillectomy, functional endoscopic sinus surgery, and curettage for surgical management for RMM; thus, surgical specimens include both sinus lining and maxillectomy specimens. Similarly, 100% of our surgical specimens consisted of debrided sinus lining (soft tissue) and necrotic maxillectomy/bone bits (hard tissue). A 24-hour fixation time minimised tissue fragmentation, and distortion due to ease of bone

Table 1: Frequency distribution of the type of hard tissue received for RMM and status of maxillectomy specimens grossed at three-levels

Grossing status	Hard tissue	
	Frequency	Percentage (%)
Three-level grossing done	29	55.8
Hard tissue not kept as per protocol	18	34.6
Only soft tissue (no hard tissue received)	5	9.6
Total	52	100

Table 2: Frequency distribution of the residency year of the grossing resident doctor and soft and hard tissue grossing conducted

Residency year	Frequency	Percentage (%)
First	36	69.2
Second	10	21.1
Third	5	9.6
Total	52	100

Table 3: Frequency distribution of fungal hyphal presence in soft and hard tissue samples of RMM

Fungal Hyphal Presence	Fungal Hyphae in Soft Tissue		Fungal Hyphae in Decalcified Sections	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Absent	35	67.3	8	15.4
Present	17	32.6	36	69.2
Hard tissue not available/lost	-	-	8	15.4
Total	52	100	52	100

Table 4: Frequency distribution and correlation of histopathological diagnosis and status of three-level grossing of RMM maxillectomy specimens

Histopathological Diagnosis (in percent)	Three- Level Grossing of Maxillectomy Specimens Status			
	Not Done	Done	No Hard Tissue Received	Total
Maxillary sinusitis	1	-	5	6 (11.5)
Maxillary sinusitis with maxillary osteomyelitis	4	3	-	7 (13.5)
Maxillary sinusitis with invasive fungal infection	3	-	-	3 (5.8)
Maxillary sinusitis and maxillary osteomyelitis with invasive fungal infection	10	26	-	36 (69.2)
Total	18 (34.6)	29 (55.8)	5 (9.6)	52 (100)

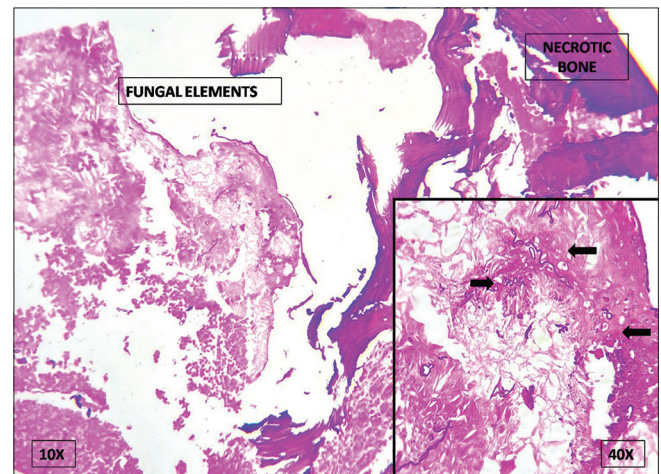


Figure 3: Photomicrograph of decalcified section of CAM-affected maxillary bone shows necrotic bone without osteocytes and fungal elements. A high-power view shows aseptate oval to long cylindrical fungal hyphae adjacent to bone (arrows represent fungal hyphae) (H&E stain)

sectioning facilitated easy soft tissue stripping of mucosal margins and aided in avoiding processing delays.

All gross macroscopic tissue examinations should be performed by a pathologist or pathology resident, physician assistant, histotechnologist, or a biomedical scientist under the supervision of a qualified pathologist. Activities and the nature of supervision should be defined in a written protocol.^[9] In our study, 69.2% of the grossing of CAM maxillary bone specimens was done by first-year residents. Shashidara *et al.*,^[10] in their review of grossing in oral pathologies, felt that the junior trainees were the ones entrusted with the important work of grossing.

Skill in bone cutting depends very much on the choice of the right cutting instrument. Due to the nature of the disease, the CAM maxillary bone was much softer and fragile to section and gross. Thus, a chisel and mallet was preferred over a saw. The standardised decalcification time for CARM maxillary bone specimens was comparatively less than that for normal bone. Simple methods for determining the end-point of decalcification, such as using a sharp pin, can completely satisfy the goal.^[6] After decalcification, the tissues have to be thoroughly washed in running water. This stops the continuation of the acid decalcification process and staining properties.

Statistically significant fungal hyphae or their cross-sectional forms were frequently seen in the decalcified sections (69.2%) of the floor of the sinus or the nasal floor in maxillary teeth region, which could have been missed if only the bone fragments or random sampling was done. The final diagnosis of invasive fungal infection is highly dependent on the type of tissue submitted.

We attempted to establish a gross examination protocol that could be easily understood and repeated anywhere to achieve maximum success in the detection of fungal hyphae in routine H&E sections in a basic pathological laboratory setting. Thus, this would help in reducing interobserver variability during site selection for gross examination. The protocol was adopted to incorporate as much tissue as possible and to study the invasive characteristics of the hard and soft tissue of the suspected fungal species.

The advantages of a three-level grossing technique included the following: (i) It considered the pathologist's diagnostic requirements and expectations.^[6] (ii) It helped in assessing maximum possible transverse, mid-specimen tissue; the protocol helped assess remote inter radicular, interdental bone, as well as both cortical and cancellous bone of the

maxilla. (iii) Adequate tissue size could be accessed via a faster decalcification process, processing protocols and diagnosis. (iv) It helped to follow basic bone grossing details of utilising the anatomical structure, making sections in one direction as much as possible and maintaining as much as possible the organ/specimen integrity after the section has been taken.^[6]

Various limitations for implementing this protocol could be possible: human misjudgement or human errors in sampling of diverse oral and maxillofacial tissues; multiple random samples add to laboratory workload and increase the number of sections and slides for the pathologist to screen. More laboratory studies will be required to assess the efficacy of this three-level grossing protocol.

CONCLUSION

Grossing is a skill that is mastered over a period of time with the right attitude, knowledge, and efforts. There exists a lacuna of print material on the exact grossing protocol standards for CARM maxillary bone maxillectomy resection specimens especially for a beginner. Thus by establishing a set protocol, faster and accurate diagnosis of CAM can be achieved. The three-grossing techniques help in complete, representative, and informative sampling of the submitted tissue suspected for mucormycosis. Adjunct staining techniques such as PAS can be used and they definitely enhance the visualisation of fungal elements. Thus, there is an urgent need to realise how vital documentation and correct laboratory practices are for a perfect diagnosis.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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