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Original Article

Pathology Section

Rapid Diagnosis of Rhino-Orbito-cerebral Mucormycosis by Double-concentration Method using Cytospin: A Cross-sectional Study

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ABSTRACT

Introduction: Rhino-Orbito-Cerebral Mucormycosis (ROCM) is an opportunistic, aggressive and rapidly progressive infection requiring early diagnosis and treatment. Potassium Hydroxide (KOH)-mount preparations aid in this, however, sometimes diagnosis is elusive. Biopsy and culture are gold-standards but time consuming. Cytology may, thus, be an important alternative for rapid and definitive diagnosis. The present study proposes a double concentration technique for the same.

Aim: The study was aimed to assess diagnostic accuracy of cytopathology, compare it with KOH mount and characterise tissue reactions in ROCM.

Materials and Methods: The current cross-sectional study was done in the Cytopathology section of Department of Pathology, UCMS and GTB Hospital, a tertiary care and dedicated Coronavirus Disease 2019 (COVID-19) hospital in New Delhi, India from May to July 2021. Samples (nasal crusts/ scrapings, orbital tissue aspirates and drained pus) from 47 cases of suspected ROCM sent for cytology were included. Double-concentration method was used to prepare the smears from all samples; however, an additional step of crushing was used for nasal crust/scrapings prior to concentration. Smears were stained with May-Grünwald Giemsa (MGG) and Papanicolaou (PAP) stains. Periodic Acid-Schiff (PAS) stain was used for confirmation of mucormycosis. The parameters studied included identification of fungal hyphae and associated tissue reaction patterns. A comparison with KOH mount and histopathology was done. Histopathology was taken as gold standard for the calculations diagnostic accuracy parameters.

Results: Among 47 suspected ROCM cases, the double-concentration cytospin technique yielded cytological evidence of mucormycosis in 11/47 (23.4%) cases (broad, pauciseptate hyphae with right-angle branching). Two of 11 (18.2%) cytology-positive cases were inconclusive on KOH mount. Tissue reaction patterns such as acute inflammatory exudate, foreign-body giant-cell reaction and fat necrosis were noted. With histopathology as the gold standard, the double-concentration cytology technique showed sensitivity of 50%.

Conclusion: Double-concentration method can be used as a fast effective method for detection of mucormycosis on cytology especially in cases of doubt where cross confirmation is required. It is a rapid and diagnostically method, providing same day results with high specificity and optimal sample utilisation for identifying fungal hyphae and the type of tissue reactions associated with ROCM.

Keywords: Concentration technique, Cytomorphology, Fungal elements, Same-day reporting, Smear preparation

INTRODUCTION

Mucormycosis is an opportunistic fungal infection that is rare and may be associated with angioinvasion. It is caused by fungi of the order Mucorales (previously called Zygomycetes), which include *Mucor, Rhizopus, Lichtheimia* and *Rhizomucor* [1]. These fungi are predominantly found in the soil, decayed wood and manure and mostly affect immunocompromised patients [2]. ROCM is the most common presentation of mucormycosis and is a rapidly progressing condition [1].

During the COVID-19 pandemic, a surge in the cases of this aggressive fungal infection was seen, particularly in immunocompromised patients who underwent steroid therapy as a treatment for the COVID-19 infection [3]. Due to the fast-progressing nature of this condition and already immunosuppressed status of the patients infected, there was a need for urgent, rapid diagnosis in those suspected to have mucormycosis. Biopsy and culture, though gold-standard, are time consuming [4]. KOH mount preparations are invaluable in rapid diagnosis of fungal infection but sometimes the diagnosis may be elusive and identification of the type of fungus may not be possible, which may cause delay in timely initiation of the correct type of therapy (e.g. posaconazole versus voriconazole) [5]. Thus, early diagnosis of mucormycosis is a genuine challenge in routine clinical practice [5,6].

Fine Needle Aspiration (FNA) and exfoliative cytology are minimally invasive procedures with lesser processing time and less turn-around time that is preferred for rapid screening and diagnosis of conditions requiring urgent therapeutic decisions [7]. Further, there is evidence in existing literature that cytology plays an important role in the early diagnosis of mucormycosis [6]. Current challenges in cytological diagnosis, however, remain improper sample utilisation, which may require repeated processing and hence, may delay the diagnosis [7]. In the present study, authors propose a double-concentration cytospin method to generate highly concentrated cytology smears from specimens obtained from patients with suspected ROCM, with the aim of enhancing diagnostic yield for rapid evaluation. Specifically, the authors sought to evaluate the diagnostic utility and accuracy of this method for cytological diagnosis of ROCM, compare its performance with KOH preparation, and characterise the cytomorphological features of mucormycosis.

MATERIALS AND METHODS

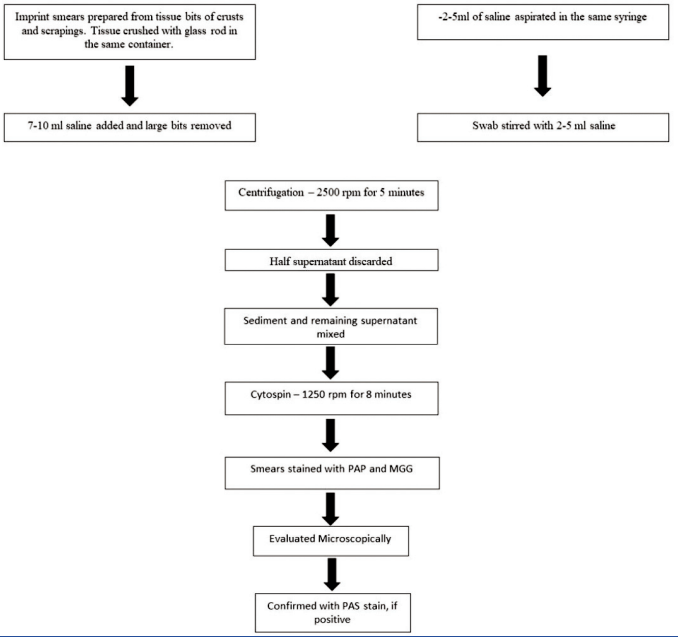
The present cross-sectional diagnostic accuracy study was conducted over three months (May-July 2021) in the Cytopathology Section of the Department of Pathology, UCMS and GTB Hospital, New Delhi, India, a tertiary healthcare center that functioned as a

dedicated COVID-19 hospital during the study period. Institutional Ethical Committee clearance was obtained (IEC No. GTBHEC 2022/P-175), and patient identifiers were anonymised for analysis.

Inclusion and Exclusion criteria: A total of 47 consecutive patients with clinical and/or radiological suspicion of ROCM, from whom specimens were submitted for cytological evaluation, were included. The specimens comprised nasal crusts/scrapings, orbital tissue/scraping/aspirates and drained pus. Samples considered inadequate after processing or yielding non-interpretable smears were excluded; further, histopathology was used as the reference standard for diagnostic accuracy calculations, and cases without histopathology correlation were not included in sensitivity, specificity, PPV and NPV computations.

Study Procedure

For all specimens, liquid suspensions were prepared from the received material using sterile normal saline. A double-concentration method was employed to enhance the yield of fungal elements and tissue reaction patterns on cytology; the exact processing steps varied slightly according to specimen type and are illustrated in [Table/Fig-1]. For nasal crusts/scrapings, an additional crushing/mechanical dissociation step was performed prior to concentration to disperse thick particulate material and improve recovery of fungal hyphae. The final concentrated deposit was used to prepare cytospin smears, which were stained with MGG and PAP stains. PAS stain was used as a special stain for confirmation of fungal elements on cytology, and Grocott-Gomori Methenamine Silver (GMS) staining was not performed on cytology smears as PAS was available in the routine cytology workflow with rapid turnaround during the surge period; where biopsy was performed, histopathology served as the confirmatory reference. Smears were evaluated for the presence of fungal hyphae and associated tissue reaction patterns, including acute inflammatory exudate, foreign-body giant-cell reaction and fat necrosis. Cytology was considered positive for mucormycosis when broad, ribbon-like aseptate to pauciseptate hyphae with right-angle branching were identified, supported by PAS positivity. Cytology findings were compared with KOH mount results (where available) and with histopathology, which was taken as the gold standard for diagnostic accuracy assessment. As this was a time-bound observational study conducted during a defined three-month period, no prior sample size calculation was performed; all consecutive suspected ROCM cases received for cytology during the study window were included.



[Table/Fig-1]: Steps followed for processing of samples using double concentration technique.

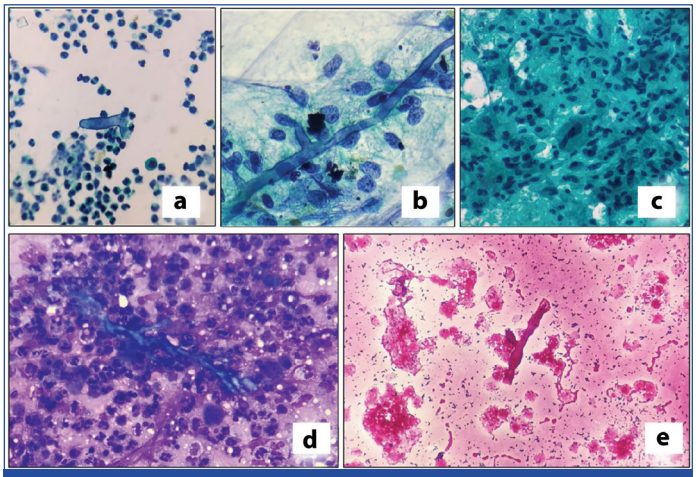
STATISTICAL ANALYSIS

Data were analysed using Statistical Package for the Social Sciences (SPSS) version 24.0 (IBM Corp.), with categorical variables summarised as frequencies and percentages, and diagnostic accuracy parameters (sensitivity and PPV) calculated using a 2×2 contingency table with histopathology as the reference standard.

RESULTS

Samples were received from 47 cases of suspected mucormycosis, including 30 males (63.8%) and 17 females (36.2%). Age ranged from 18 to 84 years (mean 50.5 years). At the time of sample collection, 26/47 (55.3%) patients had recently recovered from COVID-19, while 21/47 (44.7%) were COVID-19 positive but asymptomatic. Cytology smears prepared using the double-concentration cytospin technique were considered positive for mucormycosis when broad, ribbon-like aseptate to pauciseptate hyphae with right-angle branching were identified and confirmed on PAS stain [Table/Fig-2]. In addition to fungal hyphae, tissue reaction patterns such as acute inflammatory exudate, foreign-body giant cell reaction and varying degrees of fat necrosis were observed in positive cases [Table/Fig-2].

Overall, cytology detected mucor hyphae in 11/47 cases (23.4%). Among these 11 cytology-positive cases, KOH mount was positive in 9/11 (81.8%), while 2/11 (18.2%) were reported as equivocal on KOH. Histopathology correlation was available in 22/47 cases (46.8%); all 22 correlated cases were diagnosed as mucormycosis on histopathology. Within this histopathology-correlated subset, cytology was positive in 11/22 cases, giving a sensitivity of 50.0% for the double-concentration technique (11 true positives; 11 false negatives). Because no histopathology-negative cases were available



[Table/Fig-2]: a)& b) Broad, aseptate, fungal hyphae with right angle branching with acute inflammatory infiltrate, (PAP, a) 200x; b) 400x). c) Foreign body giant cell reaction along with plenty of histiocytes (PAP, 200x); d) Fungal hyphae amidst the acute inflammatory infiltrate (MGG, 200x); e) Fungal hyphae, consistent with Mucor PAS, 200x).

in the correlated set, specificity and NPV could not be estimated from this dataset [Table/Fig-3]. The sensitivity of KOH preparation was 40.9%. Using histopathology as the reference standard and considering only clearly positive KOH results as test-positive, the PPV of KOH was 100% (9/9); however, this estimate should be interpreted cautiously because no histopathology-negative cases were present in the correlated subset.

DISCUSSION

The ROCM is the most common presentation of mucormycosis and is associated with high morbidity and mortality. In view of its rapidly progressive and angio-invasive nature, it is treated as a surgical emergency [1,8].

Patients usually present with thick, dark nasal discharge and black necrotic areas in the turbinates, nasal septum, paranasal sinuses and orbit that may ultimately slough off completely. The fungus

KOH result	Cytology positive	Cytology negative	Histopathology positive	Histopathology negative	Total cases
Positive	9	0	9	0	9
Equivocal	2	0	2	0	2
Negative	0	11	11	0	11
Total	11	11	22	0	22

[Table/Fig-3]: Combined comparison of KOH, double-concentration cytology and histopathology results (n=22).

has a tendency to spread along the nerves leading to necrosis and infarction of paranasal sinuses, orbit and the brain [9,10].

Early and quick diagnosis of ROCM is imperative as it can improve the clinical outcome and also obviate the need of debilitating and distorting surgeries [10]. Treatment for ROCM includes immediate treatment of underlying systemic illness, systemic antifungal therapy, surgical debridement and where indicated, orbital exenteration. Intravenous amphotericin B (lipid formulation) is the antifungal drug of choice. Posaconazole and isavuconazole have been also been used for treatment [11]. Thus, treatment cannot be initiated based on mere suspicion in view of wide-reaching deleterious effects of these drugs and disfigurement and other complications related to surgeries. A confirmatory diagnosis is, therefore, mandatory for initiating the treatment [5]. The gold standard diagnostic tests used are culture and biopsy, both of which are time-consuming. KOH mount preparations may help hasten the diagnosis, however, these may at times be inconclusive and ambiguous [4,5]. FNA and exfoliative cytology is widely used for rapid diagnosis of both infectious and neoplastic conditions.Although cytology can be used as one of the rapid diagnostic tests for mucor but it is usually not included in the diagnostic work up of mucormycosis. The various samples which can be used for diagnosis of mucormycosis on cytology include aspirates, drained pus, tissue scrapings and crusts from the involved tissue [6,7,12].

Centrifugation is a technique to concentrate liquid suspensions/fluid samples in order to yield satisfactory smears. However, sometimes simple centrifugation may not be sufficient to yield satisfactory preparations. In such cases, cytospin technique proves to be of utility as it, concentrates the liquid suspensions/fluid samples of low cellularity to the maximum [13-15]. Combining both these methods, i.e., centrifugation followed by cytospin, in samples from ROCM patients may, thus, help in attaining high concentration smears and a higher yield of mucor hyphae.

Following the second wave of COVID-19, a surge of mucormycosis cases was witnessed in India [3]. Our hospital was a dedicated COVID-19 centre and hence, treated a number of patients with signs and symptoms of ROCM. Routinely, owing to the heavy case load and in order to get a rapid diagnosis, samples were mainly sent for KOH evaluation and some were sent to cytology laboratory. In view of limited exposure of sample preparation for diagnosis of mucor in cytology and the massive load of cases requiring urgent and rapid diagnosis, we performed the double concentration technique described above in all cases with the aim to get a better concentration of the tissue sample and higher yield of mucor hyphae.

In the present study, liquid suspensions were prepared by adding saline to the samples received in the laboratory so that maximum particles could be shed into the solution. This suspension was then subjected to concentration twice by using centrifugation followed by cytospin. This was done to maximise the yield and improve detection of fungal hyphae. Scant material could also be concentrated maximally by this double concentration technique. Since centrifugation followed by cytospin were carried out for five minutes and eight minutes respectively, this method was not time consuming. It, thus, had advantages of being rapid and yielding high concentration smears at the same time. This technique may, thus, have utility in other infectious and neoplastic conditions with low yield on routine cytology methods.

Therefore, the efficiency of the double-concentration technique in the present study is explained on to its ability to provide rapid diagnosis with high positive predictive yield (100%), added diagnostic yield over KOH mount and proper and complete utilisation of limited samples using routine cytology instruments.

Previous studies have shown that the sensitivity of KOH mount preparations in detecting fungi varies from 64% to 84.9% [16-19]. Gangadhara KS et al., reported a lower KOH sensitivity of 41.7%, which was similar to the KOH sensitivity in the current study (40.9%). The sensitivity of KOH in the present study was slightly lower than the sensitivity of double concentration technique. This, further, underscores the mutability of KOH mount technique in rapid and effective detection of mucormycosis as compared to the technique described in the present study [20].

Cytology and histopathology correlation was found in 22 cases. All 11 cases positive in cytology were diagnosed as mucormycosis in histopathology. However, the type and quantity of sample sent for cytology varied from that sent for histopathology, which could explain the lack of hyphae in the remaining 11 cases. Also, majority of the samples received in cytology were sent from orbital tissue/aspirates of the same patients for whom nasal crusts/tissues/scrapings were sent for histopathology. Not all the cases of ROCM had involvement of the orbit. Sinonasal mucormycosis was more common form of ROCM seen at our centre, which could explain this correlation.

Despite the utility of the present study, the small sample size and lack of comparison with a single concentration technique, i.e., double concentration versus cytospin only or double concentration versus centrifugation alone, are the major limitations of the present study.

CONCLUSION(S)

The ROCM is a rapidly progressing, aggressive condition requiring rapid diagnosis and treatment. Double concentration technique of sample preparation helped in maximising the concentration of the sample for cytological evaluation and hence increased detection of fungal hyphae. Additionally, the type of tissue reactions associated with mucormycosis could also be studied in cytology smears. Thus, a routine adoption of the double concentration technique and addition of cytology in the diagnostic protocol of mucormycosis may aid in early diagnosis and treatment of these cases.

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