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Evaluation of hospital environment for presence of Mucorales during COVID-19-associated mucormycosis outbreak in India – a multi-centre study

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S U M M A R Y

Background: An unprecedented rise in the number of COVID-19-associated mucormycosis (CAM) cases has been reported in India. Myriad hypotheses are proposed for the outbreak. We recently reported uncontrolled diabetes and inappropriate steroid therapy as significant risk factors for the outbreak. However, Mucorales contamination of hospital environment was not studied.
Aim: To perform a multi-centre study across India to determine possible Mucorales contamination of hospital environment during the outbreak.
Methods: Eleven hospitals from four zones of India representing high to low incidence for mucormycosis cases were included in the study. Samples from a variety of equipment used by the patients and ambient air were collected during May 19th, 2021 through August 25th, 2021.
Findings: None of the hospital equipment sampled was contaminated with Mucorales. However, Mucorales were isolated from 11.1% air-conditioning vents and 1.7% of patients'

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used masks. Other fungi were isolated from 18% of hospital equipment and surfaces, and 8.1% of used masks. Mucorales grew from 21.7% indoor and 53.8% outdoor air samples. Spore counts of Mucorales in air were significantly higher in the hospitals of North and South zones compared to West and East zones ($P < 0.0001$). Among Mucorales isolated from the environment, *Rhizopus* spp. were the most frequent genus.

Conclusion: Contamination of air-conditioning vents and hospital air by Mucorales was found. Presence of Mucorales in these areas demands regular surveillance and improvement of hospital environment, as contamination may contribute to healthcare-associated mucormycosis outbreaks, especially among immunocompromised patients.

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Introduction

During the COVID-19 pandemic an unprecedented rise in number of mucormycosis cases has been reported in India [1]. Government of India portal recorded nearly 50,000 mucormycosis cases during May 5th, 2021 through August 3rd, 2021. The same portal mentioned that 'It is very likely that the actual figures are considerably higher than this' (https://governmentstats.com/mucormycosis/index.html). The outbreak was not uniform across the country, with high incidence in West India followed by South, North, and East India [1]. Though poor glycaemic control and inappropriate steroid therapy were found to be important risk factors for development of mucormycosis, myriad hypothesis such as high Mucorales spores in air, contamination of oxygen supplies, respiratory equipment, humidifier water, reused face masks, and zinc supplementation are proposed for the causation of COVID-19-associated mucormycosis (CAM) outbreak [2–5]. Recently, our study does not conclusively support the hypothesis that zinc supplementation contributed to the pathogenesis of CAM [6]. However, considering earlier reported iatrogenic transmissions of Mucorales in susceptible patients through hospital linen, contaminated catheters, arm rest, tongue depressors, and construction activities, we planned the present multi-centre study to evaluate possible environmental contamination by Mucorales at Indian hospitals during the outbreak period [7,8]. Participating centres from both high prevalent to low prevalent zone of mucormycosis were included in the study [1].

Methods

Hospitals of study

Eleven hospitals from four zones of India representing high and low incidence for mucormycosis were included in the study (Figure 1; Table I). Samples from a variety of equipment and ambient air were collected in those hospitals during May 19th, 2021 through August 25th, 2021.

Collection of samples

The methodologies adapted to collect samples from the equipment, surfaces, and ambient air were as follows:

Oxygen ports of humidifiers

Ready-to-use humidifiers attached to hospital piped oxygen supply were screened by three methods.

(a) Maintaining a flow rate of oxygen at 12 L/min, Sabouraud Dextrose Agar (SDA) plates (Hi Media, Mumbai, India) were placed ∼10 cm in front of the outlet of a ready-to-use oxygen port for a period of 5 min (Supplementary Figure S1).
(b) Outlet of ∼15 cm oxygen tubing, attached to the outlet port of the humidifier, was placed inside 15 mL Sabouraud Dextrose broth (SD broth; HiMedia, Mumbai, India) in 50 mL test tubes. The flow rate was set to 6 L/min to allow bubbling of gas into the broth (Supplementary Figure S2).
(c) Maintaining oxygen flow rate at 12 L/min, ready-to-use oxygen tubing with Ventimask (used for the patients) was placed directly on the SDA plates for duration of 5 min.

Samples from oxygen cylinders

The above three methods of sampling from piped oxygen supply were repeated for sample collection directly from portable oxygen cylinders, maintaining the flow rate of oxygen at 12 L/min. While collecting samples in SD broth, the flow rate was maintained at 6 L/min.

Samples from humidifiers

(a) Pre-moistened commercially available swabs (HiMedia, Mumbai, India) were used to collect samples from hubs of the humidifier ports. The swabs were inoculated on site on the SDA plate.
(b) Samples were also collected from the reservoir of humidifiers using pre-moistened swabs and inoculated on site on the SDA plate.
(c) A volume of 100 mL water from the reservoirs was collected in sterile McCartney bottles. The water samples were then passed through a membrane (0.45 μm) filtration assembly by applying a vacuum of 500 mmHg. The filter paper was thereafter removed using a sterile forceps and inoculated on to the SDA plate.

Samples from masks

Masks being used by patients in hospital were collected randomly in sterile zip lock pouches. Inside a biosafety hood,

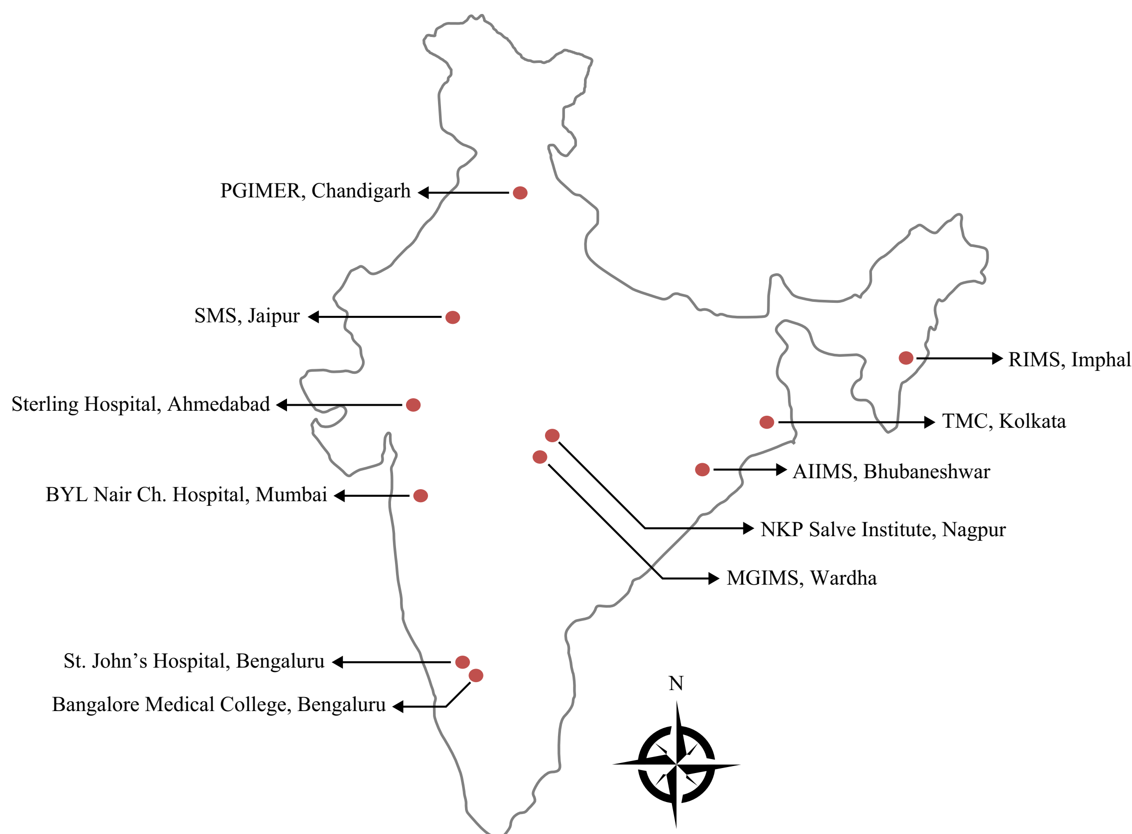

Figure 1. Locations of 11 healthcare centres participating in study. AIIMS, All India Institute of Medical Sciences; SMS, Sawai Min Singh; RIMS, Regional Institute of Medical Sciences; Mahatma Gandhi Institute of Medical Sciences; TMC, Tata Medical Center; PGIMER, Post Graduate Institute of Medical Education & Research.

the inner surface of the mask was scraped off with the bent wire over Dichloran Rose Bengal Chloramphenicol (DRBC) agar with benomyl (10 μg/mL) (Sigma–Aldrich, Bengaluru, India).

Ambient air sampling

Air samples were collected from indoors (hospital wards including intensive care units (ICUs)) and outdoors (parking lots and outside environment of the hospital) using the active air samplers. Inside hospital, both wards/ICUs with heating, ventilation, and air conditioning (HVAC; with and without high-efficiency particulate air (HEPA) filters) as well as conventionally ventilated (rooms with fans or window AC units) areas were sampled. Sieve samplers (bioMérieux, Sampl'air™ or HiMedia air sampler) were used for active air sampling. The spores were allowed to impact on 90 mm Petri plates of SDA and DRBC agar with a flow rate of 100 L/min for 10 min (total volume of air impacted on each plate was 1000 L over 10 min) [9]. Colony-forming units (cfu) were counted.

Air-conditioning (AC) vents

Pre-moistened cotton swabs were used for sampling the front, i.e. hospital side of the AC vent, and the swabs were inoculated on site on to SDA plates.

All plates and broths were incubated at 25 °C for seven days and were checked every day for any growth. Participating centres identified the fungi phenotypically. Unidentified isolates were transferred to the reference centre at Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, for molecular identification. At the reference centre, genomic DNA was extracted from the culture isolate using the phenol–chloroform–isoamyl extraction method and semi-nested PCR was performed for amplification of the 18S region of rDNA with Mucorales-specific primers ZM1 (5′-ATTACCATGAGCAAATCAGA-3′), ZM2 (5′-TCCGTCAATTCCTTTAAGTTTC-3′) and ZM3 (5′-CAATCCAAGAATTTCACCTCTAG-3′ [10]. Subsequent sequencing of the product was performed by Sanger's method (ABI 3500 Dx genetic analyzer) and the obtained sequences were compared with NCBI database for accurate identification.

Statistical analysis

Data analysis using SPSS Statistics 25.0 (IBM, Inc., https://www.ibm.com) was performed. Descriptive statistics of spore counts were expressed as mean ± standard deviation with ranges. The comparisons of contamination in samples from various areas and between centres were done using independent *t*-test and analysis of variance. Comparative analysis and correlation were conducted by the Pearson χ^2-test. GraphPad Prism Version 9 was used for graphical representation of the data. All tests were two-tailed and $P < 0.05$ was considered significant.

Table I
Weather conditions on sampling sites at study centres

Hospital		Dates of sampling	Temperature (°C)	Humidity (%)	No. of patients with mucormycosis treated during the study
North India					
1	Postgraduate Institute of Medical Education and Research, Chandigarh	May 20th to Jun 14th	32–42	35.4 ± 5	538
2	Sawai Man Singh Medical College, Jaipur, Rajasthan	Jul 4th	38–41	73.8 ± 16.8	1340
East India					
3	Regional Institute of Medical Sciences, Imphal, Manipur	Jul 3rd to 17th, 2021	32–36	91.1 ± 7.6	4
4	All India Institute of Medical Sciences, Bhubaneswar, Odisha	Jul 2nd to 9th, 2021	31–36	88 ± 5.7	60
5	Tata Medical Centre, Kolkata, West Bengal	Jul 19th to Aug 10th	29–34	87.7 ± 5.8	0
South India					
6	Bangalore Medical College and Research Institute, Bengaluru, Karnataka	Jul 1st to 7th	22–27	81.7 ± 9.2	207
7	St John's Medical College, Bengaluru, Karnataka	Jul 13th to 20th	24–28	81.8 ± 9.6	100
West India					
8	Sterling Hospital, Ahmedabad, Gujarat	Jul 3 to 27th	33–38	78.5 ± 12.4	101
9	Mahatma Gandhi Institute of Medical Sciences, Wardha, Maharashtra	Jul 15th to Aug 20th	27–33	81.8 ± 9.2	21
10	NKP Salve Institute of Medical Science & Research centre and LMH, Nagpur, Maharashtra	Jul 12th to 17th	32–35	84.2 ± 8.3	55
11	BYL Nair Ch. Hospital, Mumbai Maharashtra	Jul 20th to Aug 25th	29–32	89.3 ± 3.5	99

Results

A total of 622 environmental samples were collected from equipment and surfaces, and 295 from ambient air. The centres in the North India had the highest temperatures while those in the South had the lowest temperatures during the period of sampling. Apart from Chandigarh, where the humidity level was in the thirties, all the remaining centres had higher humidity ranging between 73.8 ± 16.8 and 91.1 ± 7.6 g/kg (Table I). Mucorales and other fungi were detected in 101 (11.1%) and 257 (28%) environmental samples, respectively. Mucorales were not isolated from any hospital equipment and surfaces sampled, whereas other fungal species were isolated in 18% of samples. Mucorales were isolated from 10 (11.1%) out of 90 AC vents sampled, and three (1.7%) out of 172 patients' masks. Fungi other than Mucorales were isolated from 8.1% of those masks.

The proportion of air samples positive for Mucorales was 21.2% of indoor air and 51.8% of outdoor air of the hospitals ($P < 0.00001$). The spore counts of Mucorales were significantly higher in hospitals of North and South zones compared to West and East zones ($P < 0.001$) (Table II). The spore counts varied among hospitals, and the mean spore counts were 28.3 ± 56.4, 15.7 ± 25.0, 7.1 ± 4.85, and 2.9 ± 7.3 cfu/m^3 in the North, South, East and West zones respectively ($P = 0.0026$).

Mucorales spore counts in the indoor air varied depending on the type of ventilation of the rooms (Table II). Rooms with HEPA-filtered air were minimally contaminated (2.1%) compared to rooms (20.5%) without HEPA filters (mean Mucorales spore counts: 0.14 ± 10.95 vs 3.53 ± 11.4 cfu/m^3, respectively) ($P = 0.01$). Air of rooms with an individualized air-conditioning (AC) facility was significantly more contaminated with Mucorales than air of those with a central AC with attached micro-filters (mean Mucorales spore counts: 7.7 ± 13.6 vs 2.5 ± 10.9 cfu/m^3, respectively, $P = 0.0388$). At one centre (PGIMER, Chandigarh), the effect of cleaning of AC filters of five window ACs was determined by Mucorales spore count. Pre-cleaning, all five filters (100% positivity) grew Mucorales with an average spore count of 24.8 ± 10.5 (range: 10–35) cfu/m^3. The spore counts decreased after cleaning with soap and water (mean: 1.7 ± 1.2; range: 0–3 cfu/m^3) (Supplementary Table S1).

Among Mucorales *Rhizopus* spp. were common isolates (67% from air-conditioning vents and masks, and 78% from air) (Tables III–V). Some of the *Rhizopus* spp. could not be speciated, as transferred isolates could not be revived at the reference centre (PGIMER, Chandigarh). Environmental Mucorales, *Spinellus fusiger*, and *Choanephora cucurbitarum*

Table II
Spore counts of Mucorales in ambient air study centres

Hospital	No. of samples positive for Mucorales[a]			P value
	HVAC with HEPA	Non-HEPA filter AC (microfilters, window, split)	Natural ventilation and outdoor air	
PGIMER, Chandigarh	0/5	13/22 15 ± 19.2 (0–71)	12/24 45.6 ± 66.6 (0–237)	0.016
SMS, Jaipur	0/5	0/15	3/15 0.5 ± 1.1 (2–3)	0.1573
North zone	0/10	10/32 7.96 ± 15.8 (0–71)	15/39 28.3 ± 56.4 (0–237)	0.0359
AIIMS, Bhubaneswar	0/5	4/15 2.4 ± 1.6 (0–3)	6/10 2.4 ± 2.3 (0–6)	0.0562
TMC, Kolkata	0/2	0/4	0/3	–
RIMS, Imphal	Not sampled	3/5 18.8 ± 18.6 (0–43)	6/10 7.9 ± 9.9 (0–24)	0.15
East zone	0/7	7/24 10.6 ± 13.6 (0–43)	12/23 7.1 ± 4.9 (0–24)	0.2291
Sterling Hospital, Ahmedabad	0/5	0/15	5/10 6.1 ± 8.0 (0–25)	0.0001
MGIMS, Wardha	0/5	0/15	8/10 5.2 ± 3.8 (0–10)	0.0001
NKLP Salve, Nagpur	1/5	0/10	5/15 0.86 ± 1.3 (2–3)	0.1207
BYL Nair Ch. Hospital, Mumbai	0/5	0/15	0/5	–
West zone	1/20	0/55	17/40 2.9 ± 7.3 (0–25)	0.0001
BMCRI, Bengaluru	Not sampled	5/5 22.6 ± 11.1 (9–36)	5/5 60 ± 24.0 (36–100)	0.0135
St John's Medical College, Bengaluru	0/10	Not sampled	11/201 15 ± 1.2 (0–3)	0.05
South zone	0/10	5/5 22.6 ± 11.1 (9–36)	21/30 15.7 ± 25.0 (0–100)	0.1263
Total	1/4 7 (2.1%)	25/122 (20.5%) 3.5 ± 11.4 (0–71)	60/127 (47.2%) 13.1 ± 34.9 (0–237)	0.0019

HVAC, heating, ventilation, and air conditioner; HEPA, high-efficiency particulate air filter.
[a] Mean ± SD (range) in cfu/m^3.

constituted 17% of all Mucorales isolated from air. Among non-mucorales, *Aspergillus spp*. were the most frequently isolated from environmental samples and ambient air. Other fungi included *Cladophialophora* spp., *Penicillium* spp., *Candida* spp., *Fusarium* spp., *Alternaria* spp., *Bipolaris* spp., as well as rare hyalohyphomycetes and dematiaceous fungi.

Table III
Proportion of Mucorales species isolated from air

Species	%
Rhizopus arrhizus	40%
Rhizopus spp.	23%
Rhizopus microsporus	3%
Rhizopus stolonifera	1%
Rhizopus homothallicus	2%
Spinellus fusiger	5%
Cunninghamella bertholletiae	1%
Syncephalastrum racemosum	8%
Mucor spp.	4%
Choanephora cucurbitarum	12%
Lichtheimia corymbifera	1%

Discussion

The present multi-centre study was conducted to evaluate fungal contamination of the hospital environment by Mucorales and other fungi in 11 hospitals across India during the CAM outbreak. No Mucoraceous fungi was isolated from hospital equipment and surfaces. However, Mucorales were isolated

Table IV
Proportion of Mucorales species isolated from equipment

Species	%
Rhizopus arrhizus	42%
Rhizopus microsporus	8%
Rhizopus spp.	17%
Mucor circinelloideae	17%
Syncephalastrum racemosum	8%
Lichtheimia corymbifera	8%

Table V
Hospital environment contamination by various Mucorales species

Location/equipment	*Rhizopus arrhizus*	*Rhizopus* spp.	*R. microsporus*	*R. stolonifera*	*R. homothallicus*	*R. oryzae*	*Spinellus fusiger*	*Cunninghamella bertholletiae*	*Syncephalastrum racemosum*	*Mucor* spp.	*Choanephora cucurbitarum*	*Lichtheimia corymbifera*
Outdoor air	24	9	1	1		1	4			2	10	
Rooms without AC	4	5	1		1			1	3	2		
Room with AC (non-HEPA)	14	1	1		1		1		6		1	
Rooms with HEPA												1
AC vents	8	1								2		1
Patients' masks		1	1									
Humidifier water	1											
Humidifier reservoirs												
Oxygen cylinders	1											

AC, air conditioning; HEPA, high-efficiency particulate air filter.

from 11.1% of AC vents and 1.7% of masks used by the patients. Ambient air contamination levels varied across centres, with the centres in North and South India exhibiting higher Mucorales spore counts than the West and East India centres. However, the spore counts of all centres in a particular zone were not similar and variation had been noted. Mucorales spore counts also varied depending on the type of ventilation of hospital wards or ICUs. HEPA-filtered room air had minimal Mucorales spore count, while 19% samples from rooms with other ventilation systems grew Mucorales. *Rhizopus* spp. were the Mucorales most frequently isolated.

In previous studies, nosocomially acquired mucormycosis in patients with uncontrolled diabetes, immunosuppression, or trauma had been linked to environmental contamination of tongue depressor, wooden arm rest, linen, or hospital air, especially after construction-related activities [8,11–14]. Building construction was implicated in five cases of pulmonary mucormycosis [15]. In the present study, environmental contamination was restricted to AC vents and hospital air. AC vents, which draw fresh air from the outdoor environment, could easily be contaminated with Mucorales spores from outside air unless the filter vents are cleaned regularly. The contamination of AC vent was markedly reduced after cleaning of the filter at one hospital. Hospital water may also be a reservoir for fungi [16]. However, we found no Mucoraceous fungi in the water used in humidifiers for oxygen supply to the patients. No Mucorales contamination was also noted in piped oxygen port, oxygen cylinder, or humidifier reservoir to support those hypotheses of environmental contamination of respiratory equipment as the source of the outbreak.

Masks can become contaminated if worn repeatedly and not washed regularly, though we found that only 1.7% of 172 such cloth masks worn by patients were contaminated with Mucorales. This finding indicates that repeated use of masks was unlikely to be a major source for mucormycosis outbreak, though the patients should be advised to wear clean cloth masks only, as 8.1% of masks were contaminated with fungi other than Mucorales.

The presence of fungi such as *Aspergillus* spp., *Cladophialophora* spp., *Penicillium* spp., *Fusarium* spp., and other hyalohyphomycetes and dematiaceous fungi at oxygen sources, humidifier water, respiratory equipment, and hospital air even in HEPA-filtered rooms is a matter of concern. It emphasizes the need for improvement in overall house-keeping activities in hospitals. Regular replacement of HEPA filter along with regular scraping, painting, humidity, and temperature control within HVAC premises and anti-fungal paint may minimize hospital environment contamination by fungi.

The presence of Mucorales in the hospital air is a matter of concern. *Rhizopus* spp. were commonly isolated Mucorales in both pre and post-COVID-19 period [8,11–15,17]. *Rhizopus* spp. were also common isolates from air in the present study. We noted a significantly different Mucorales spore count in air between air-conditioned and non-air-conditioned wards, similar to previous studies [9]. The limitations of the present study are that all centres could not collect all samples as planned in the study, and we did not perform molecular strain typing to correlate clinical isolates of Mucorales and those found from the environment. It is, therefore, difficult to pinpoint the source of Mucorales during the CAM outbreak. However, the study does not support the hypothesis of Mucorales contamination of the hospital equipment as the source of CAM

outbreak. In any case, practices for management of COVID-19 patients in hospitals have been shown to have a bearing on causation of mucormcyosis. In our earlier multi-centre study, we noted that poor glycaemic control and inappropriate steroid therapy were important risk factors for development of mucormycosis [2]. This was further demonstrated in a cohort of 1027 patients in a Mumbai ICU where a protocol of appropriate steroid doses and strict glycaemic control was maintained, and no case of mucormycosis was reported during their stay in the hospital and during immediate outpatient follow-up [4]. The authors concluded that elimination of those risk factors eliminated the risk of acquisition of mucormycosis. Mucorales contamination of the hospital environment may be an additional risk factor, though the susceptible patients may acquire Mucorales from the home environment before reaching the hospital. A detailed molecular study correlating environment and patient isolates is required to resolve the issue of the source of Mucorales during the outbreak.

In conclusion, this study did not support the hypothesis of hospital equipment contamination by Mucorales as the source of the CAM outbreak. The presence of Mucorales in hospital air and the air conditioning system is a matter of concern and demands regular surveillance and improvement of the hospital environment, as susceptible patients may acquire the life-threatening mucormycosis while admitted in the hospital. Universal clean mask use is also important for the susceptible patients in hospital and home environment to minimize exposure from Mucorales supplementary table S1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2022.01.016.

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