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ORIGINAL ARTICLE

Could cattle dung burning have contributed to the epidemic of COVID-19-associated mucormycosis in India? Results of an experimental aero-mycological study

Soundappan Kathirvel[1] | Valliappan Muthu[2] | Shivaprakash Mandya Rudramurthy[3] | Harsimran Kaur[3] | Arunaloke Chakrabarti[3] | Ritesh Agarwal[2]

[1]Department of Community Medicine and School of Public Health, Postgraduate Institute of Medical Education and Research, Chandigarh, India

[2]Department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India

[3]Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Correspondence
Ritesh Agarwal, Professor, Department of Pulmonary Medicine, Postgraduate institute of medical education and Research, Chandigarh, India.
Email: agarwal.ritesh@outlook.in

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Abstract

Background: Several hypotheses have been proposed for explaining the outbreak of coronavirus disease 2019 (COVID-19)-associated mucormycosis in India, including the burning of cattle dung cakes, though no study has yet been conducted to support this claim.

Methods: We conducted an aero-mycological study to evaluate whether Mucorales in the air increased during or after burning cattle dung cakes. We further compared the growth of Mucorales in the indoor air samples from houses with and without cattle. We also cultured fresh and dried cattle dung and soil samples for Mucorales.

Results: We noted no significant difference in the proportion of air samples growing Mucorales during (4/22 [18.2%]) and after (3/2 [13.6%]) cattle dung burning than that collected immediately before (4/22 [18.2%]). Mucorales were isolated in 15.4% of the indoor air samples obtained from different houses (both rural and urban); the proportion of samples growing Mucorales was not significantly different in households with and without cattle. We also observed growth of Mucorales in 6 of the 8 [75%] fresh and 3 of the 6 [50%] dried dung samples. The most common Mucorales isolated from soil and dung samples was *Lichtheimia corymbifera*, while *Rhizopus arrhizus* was the most common species isolated from indoor air samples.

Conclusions: We found no significant increase in the proportion of air samples growing Mucorales during or after burning cattle dung cake than that before. It seems unlikely that cattle dung burning contributes to the occurrence of mucormycosis.

KEYWORDS
cow dung, Mucorales, Rhizopus, SARS-CoV-2, zygomycosis

1 | INTRODUCTION

Recently, India experienced an outbreak of mucormycosis during the second wave of the coronavirus disease 2019 (COVID-19) pandemic.[1,2] The unprecedented rise in COVID-19-associated mucormycosis (CAM) cases remains largely unexplained.[3,4] Several factors were proposed for this outbreak. The COVID-19 treatment practices, including inappropriate glucocorticoids, zinc supplementation and host factors (e.g. uncontrolled diabetes mellitus and others), have been associated with CAM in case–control studies from

Soundappan Kathirvel and Valliappan Muthu contributed equally.

India.[5–9] As India accounted for most cases of CAM, it was speculated that certain practices unique to the subcontinent were responsible for the outbreak. There was a hypothesis linking CAM to the inhalation of fumes after burning cattle dung cakes.[10–12] While burning various kinds of biomass (dry grass, leaves, twigs and flood detritus) in experimental conditions has shown to disperse fungal spores,[13] it is unknown whether combustion of cattle dung cake results in the dispersion of Mucoralean spores. We hypothesised that burning cattle dung cakes will be associated with a greater Mucorales burden in the air. Our study's primary objective was to evaluate the growth of Mucorales in air samples during and after burning cattle dung cakes than before burning. The secondary objective was to compare the growth of Mucorales in the ambient indoor air of houses with and without cattle. We also cultured outdoor air (rural and urban), soil and cattle dung (fresh and dried) samples to exclude confounders.

2 | METHODS

We conducted a prospective cross-sectional aero-mycological analysis between 1 March and 30 April 2022, in selected villages (rural) from Haryana and the union territory of Chandigarh (urban), respectively. Ethical clearance was not sought as the study did not involve human participants. However, we obtained informed consent from the house inmates to collect air and other samples.

2.1 | Study setting

2.1.1 | Rural

We randomly selected 22 houses from three villages of Haryana, India (rural), where cattle rearing and related activities, including dried cattle dung cake preparation, storage and use, are routinely practiced. We performed the combustion experiment using the cattle dung cakes stored in each house. We collected air samples before, during (at 3–5 min of combustion) and after 5 min of burning the cattle dung cakes. The routinely used earthen stove was employed for burning the cattle dung. We ensured that at least two-thirds of the stove was filled with dried cattle dung cake (usually required 2–3 dried cakes of approximately 300–400 g each). The air sampler was positioned about one foot from the stove (Figure 1).

For the indoor air sampling, we randomly selected nine (two houses with and one without cattle rearing from each of the three villages) of these 22 houses. Three air samples per house were obtained from various sites, including bedrooms, kitchens and the living areas of the houses. We also collected air samples from the cattle shed in households rearing cattle.

Outdoor air samples were obtained from common gathering areas of the villages and at storage places of wet and dry cattle dung (Figure 2A,B).

2.1.2 | Urban

We selected three houses in Chandigarh urban area for indoor air (four samples per house) sampling from various indoor sites, including bedrooms, kitchens, living areas and the entrances of the houses. We obtained three outdoor air samples from different common gathering areas in Chandigarh.

2.1.3 | Soil and dung samples

We also collected soil samples from the cattle shed and common gathering areas of the rural and urban areas using a sterile spatula and transferred them in a sterile zipper bag. We also collected fresh ($n = 8$) and dry ($n = 6$) cattle dung samples in a sterile zipper bag (Figure 2C,D).

2.1.4 | Sample collection and processing

We used a sieve air sampler (BioMe'rieux AESAP1076, Sampl'air™ Pro or high-performance microbial air sampler, Bruz, France) for air sampling. The air was allowed to impact on 90 mm Petri plates containing Sabouraud dextrose agar (SDA) and dichloran rose-Bengal

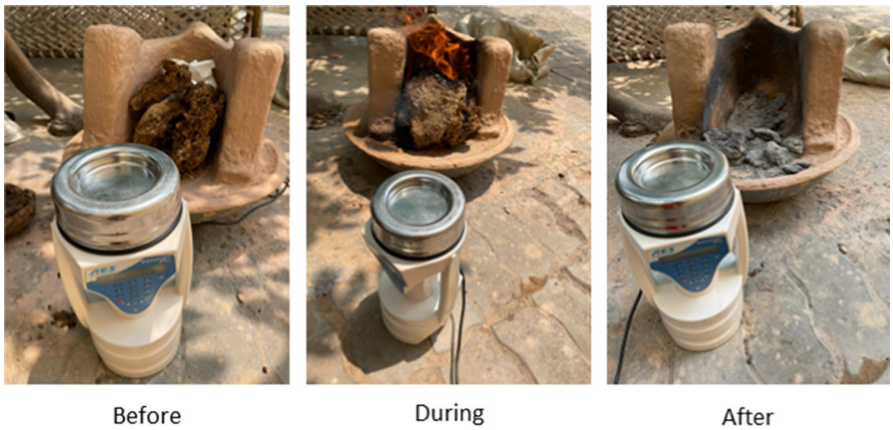

FIGURE 1 Air sample collection before, during, and after combustion of dried cattle dung cakes

FIGURE 2 Collection of air samples from storage areas of wet (A) and dried (B) cattle dung. Collection of dried (C) and fresh (D) cattle dung samples

TABLE 1 Growth of Mucorales in ambient air samples linked with the burning of cattle dung cakes

	Before burning	During burning	After burning
[a]Growth of Mucorales, *n*/*N* (%)	4/22 (18.2)	4/22 (18.2)	[b]3/22 (13.6)
Species isolated	*L. corymbifera* (*n* = 3), *R. arrhizus* and *R. microsporus* (*n* = 1)	*L. corymbifera* (*n* = 2), *R. microsporus* (*n* = 1), *L. corymbifera* and *R. arrhizus* (*n* = 1)	*L. corymbifera* (*n* = 2), *R. arrhizus* (*n* = 1)
Average spore count, CFU/m^3	0.007×10^3	0.006×10^3	0.006×10^3

Abbreviations: CFU, colony-forming units; *L. corymbifera*, *Lichtheimia corymbifera*; *R. arrhizus*, *Rhizopus arrhizus*; *R. microsporus*, *Rhizopus microsporus*.
[a]The *p*-value for the comparison was .89.
[b]In two of the three occasions where Mucorales grew, the same organism was also cultured from air obtained before or during the burning process.

chloramphenicol (DRBC) agar supplemented with 10 μg/ml of benomyl at a flow rate of 100 L/min for 1 min. We incubated the Petri plates at 37°C for 48 h and counted the colony-forming units (CFUs). Colonies suggesting Mucorales were purified and identified using standard mycological methods.

All the soil (and cattle dung) samples were processed as described by Madrid et al.[14] Briefly, 1 g of soil sample was homogenised using 10 ml of autoclaved deionised water in a sterile 50 ml falcon tube. We centrifuged the suspension at 160 rpm and incubated them at 37°C for 20 min. The tubes were kept undisturbed for 10 min to allow the soil to settle. We then spread 100 μl of the soil suspension using a Digralsky glass spreader on DRBC agar plates (Oxoid) containing benomyl (10 μg/ml). The plates were incubated at 37°C for up to 4 days. Any growth of Mucorales observed every 24 h was subcultured on potato dextrose agar (PDA) to ensure purity and identify the species using standard mycological methods.

2.2 | Statistical analysis

Data are presented descriptively as numbers with percentages. The difference between proportions was analysed using chi-square or Fisher's exact test.

3 | RESULTS

3.1 | Growth of Mucorales in the air pre- and post-cattle dung cake burning

We did not find a statistically significant difference in the growth of Mucorales in air samples obtained during (4/22 [18.2%]) and after (3/22 [13.6%]) burning of cattle dung cakes than that obtained before (4/22 [18.2%]; $p = .89$). The isolates were identified as *Lichtheimia corymbifera*, *Rhizopus arrhizus* and *Rhizopus microsporus* (Table 1).

3.2 | Mucorales in air samples from houses with and without cattle

Of the 18 indoor samples collected from six houses rearing cattle, we noted the growth of Mucorales in three samples (16.7%; each from a different house). None of the six air samples collected from the cattle sheds and dung storage areas showed Mucorales growth. Of the 21 air samples obtained from six houses not rearing cattle (three houses each from villages [$n = 9$ samples] and urban areas [$n = 12$ samples]), the growth of Mucorales was noted in three (14.3%) samples (Table 2). There was no statistically significant difference between the proportion of indoor air samples growing Mucorales from houses with and without cattle. *Rhizopus arrhizus* was isolated from all the samples except a single sample from the urban area that grew *L. corymbifera*. We did not isolate Mucorales in air samples collected from the common gathering areas of the three villages and three urban sites.

3.3 | Mucorales in the soil and cattle dung

Six of the eight (75%) fresh and three of the six (50%) dried dung samples grew Mucorales (Table 3). One of the six (16.7%) soil samples collected from the cattle shed showed Mucorales growth. All the soil samples collected from the common areas of the villages (except one in the urban area) showed the growth of Mucorales (Table 3). *L. corymbifera* was the most observed Mucorales in cattle dung and soil samples.

4 | DISCUSSION

We found no significant increase in the proportion of air samples growing Mucorales during or after burning cattle dung cakes than that before. Mucorales were isolated in only a small number of the indoor air samples obtained from the rural and urban households. There was no difference in Mucorales growth in houses with and without cattle. None of the air samples collected from cattle sheds, cattle dung storage areas, and common gathering areas grew Mucorales. While the soil obtained from the cattle shed did not grow Mucorales, almost all the other soil samples from rural or urban areas grew Mucorales. Finally, half the dried and three-fourths of the fresh cattle dung samples demonstrated Mucorales.

What do our study results imply? Few media reports of cattle dung application over the body[15] and the use of cattle dung cakes for cremation[16] led to a hypothesis that the inhalation of fumes after

TABLE 2 Growth of Mucorales in air samples collected from indoor areas of the houses in rural and urban areas

	Samples from houses with cattle	Samples from houses without cattle
[a]Growth of Mucorales, *n/N* (%)	3/18 (16.7)	3/21 (14.3)
Rural	3/18	1/9 (11.1)
Urban	0	2/12 (16.7)
Species isolated	*R. arrhizus* ($n = 1$), *R. microsporus* ($n = 1$), *R. arrhizus* and *R. microsporus* ($n = 1$)	Rural: *R. arrhizus* ($n = 1$) Urban: *L. corymbifera* ($n = 1$), *R. arrhizus* ($n = 1$)
Average spore count, CFU/m^3	0.013×10^4	0.001×10^3

Abbreviations: CFU, colony-forming units; *L. corymbifera*, *Lichtheimia corymbifera*; *R. arrhizus*, *Rhizopus arrhizus*; *R. microsporus*, *Rhizopus microsporus*.

[a]The *p*-value for the comparison was 1.0.

TABLE 3 Growth of Mucorales in soil samples and cattle dung (fresh and dry)

	Growth of Mucorales *n/N* (%)	Species isolated	Average spore count, CFU/g
Cattle dung			
Fresh	6/8 (75.0)	*L. corymbifera* ($n = 5$), *R. arrhizus* ($n = 1$)	0.38×10^4
Dry	3/6 (50)	*L. corymbifera* ($n = 2$), *R. arrhizus* ($n = 1$)	0.6×10^3
Soil			
Cattle shed	1/6 (16.7)	*L. corymbifera* ($n = 1$)	1.5×10^3
Common area, village	3/3 (100)	*L. corymbifera* ($n = 2$), unidentified Mucorales ($n = 1$)	0.6×10^3
Common area, urban	2/3 (66.7)	*L. corymbifera* ($n = 1$), *R. arrhizus* ($n = 1$)	0.1×10^4

Abbreviations: CFU, colony-forming units; *L. corymbifera*, *Lichtheimia corymbifera*; *R. arrhizus*, *Rhizopus arrhizus*.

burning cattle dung contributed to the CAM outbreak in India.[10] We found Mucorales in the ambient air even before burning the cattle dung cakes that did not change significantly during or after combustion. The growth of *Lichtheimia* from fresh dung samples indicates that fresh dung application over the body or its ingestion could potentially lead to mucormycosis.[15,17] Indeed, outbreaks of cutaneous mucormycosis with *Lichtheimia* have been reported in hospitalised patients with burns (due to contaminated bandages) or rarely following traumatic inoculation.[18–21] However, in the recent CAM outbreak in India, the rhino-orbital presentation was the most common, and *R. arrhizus* was the most frequently isolated organism, comparable to the pre-pandemic mucormycosis data (from India and elsewhere).[22,23] Also, sites other than rhino-orbital were uncommonly reported.[24] There was no increase in the cases of cutaneous mucormycosis or *Lichtheimia* infections during the CAM outbreak in India. Further, the occurrence of CAM was not exclusive to India, and several other countries reported an increase in mucormycosis following COVID-19 than the pre-pandemic data.[23,25] A high environmental burden of Mucorales in India is well recognised. It possibly explains that the country accounted for >80% of the global burden of mucormycosis before and after the COVID-19 pandemic. Thus, it seems unlikely that cattle dung burning led to the CAM outbreak in India.

The most common Mucorales isolated from soil and dung samples was *L. corymbifera*, while *R. arrhizus* was the most common species isolated from indoor air samples. In contrast, *Lichtheimia* was the predominant species in air samples after and during the combustion of cattle dung cakes. The possible explanation could be the thermotolerant nature of a few fungi (*Lichtheimia* can sustain temperatures beyond 37 degrees Celsius, while *R. microsporus* can tolerate temperatures up to 45 degrees Celsius), compared to the sub-thermotolerant nature of *R. arrhizus*.[26] *R. arrhizus* was also the most frequently isolated Mucorales (indoor and outdoor) in large aero-mycological environmental,[27,28] and clinical studies conducted prior to and following the COVID-19 pandemic.[1,29] The results of the current study and the earlier mentioned studies suggest no significant change in the environmental fungi in India that could have precipitated the CAM outbreak.

Our study has a few limitations. We did not select the houses and villages based on stratified randomisation, which is a more robust method for avoiding bias. However, the practices related to cattle rearing, handling, storage of fresh and dried cattle dung cakes, and using dung cakes as fuel are generally similar across the villages. The Mucorales growth observed in the current study could change with the season, ambient temperature and humidity. We did not measure ambient temperature and humidity in the current study. A recent study from Iran reported the seasonal variation in the growth of Mucorales in hospital soil samples.[30] We performed our study on a single occasion during the Indian summer and could have missed a potential seasonal variation in Mucoralean growth in air samples. Nevertheless, we conducted the study in March and April and thus would resemble the time of the outbreak of CAM in India following the second wave in 2021. Also, we have no clear explanation for the predominance of *Lichtheimia* in the air samples obtained before the burning of cattle dung cakes. Several other practices could influence environmental fungal spores, including major agricultural activities like harvesting, ploughing, stubble burning, and applying pesticides or other chemicals. Notably, indoor sampling in the houses of patients with CAM could have provided more insight. Future studies may be carried out to evaluate the indoor and outdoor environment of prospectively identified patients with mucormycosis. Finally, we did not perform genotypic methods for Mucorales identification. While the lack of genotyping does not influence our results' implication, it is one limitation of our manuscript.

In conclusion, we found no significant increase in the proportion of air samples growing Mucorales during or after burning cattle dung cakes than that before.

CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

Data will be made available on request with the corresponding author.

ORCID

Soundappan Kathirvel https://orcid.org/0000-0002-4839-0138
Valliappan Muthu https://orcid.org/0000-0003-0410-8468
Shivaprakash Mandya Rudramurthy https://orcid.org/0000-0002-9097-9253
Ritesh Agarwal https://orcid.org/0000-0003-2547-7668

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