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Isolation, Sporulation and Characterization of Fungi from Bihazardous Hospital Waste of Hazaribag

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A B S T R A C T

The fungus in hospital garbage has spread because of the bio-hazardous clinical waste specimens utilized in the diagnostic procedure. The current investigation's goal was to use the phenotypic approach to identify the fungal isolates collected from clinical wastes. On Potato Dextrose Agar (PDA) medium, the fungal isolates were obtained by the direct plate technique. They were purified by single-spore separation by streaking after growing for 7–14 days at 28°C. A light microscope was used to evaluate the morphology of the fungal isolates, and various mediums were used to record the culture properties. More than sixty different fungus species were discovered, ranging from numerous genera, such as *Aspergillus, Trichoderma, Rhizopus, Cladosporium, Candida, Oidiodendron, Fusarium, Curvularia, Oidiodendron, Aspergillus, Trichothecium, Mucor* and *Acinetobacter* from several genera. They were also characterized at physico-chemical as well as at biochemical levels. According to these findings, a variety of fungus found in clinical wastes might pose a health danger to people if they haven't been rendered inactive before being released into the environment. These studies revealed that there may be a health concern associated with clinical wastes, and it was urged that they be managed carefully to limit the spread of infectious germs.

Introduction

"Healthcare wastes" is a general term used to characterize the waste generated by healthcare facilities (WHO, 2005). Some terms used to describe these wastes in different sources include hazards, biohazard wastes, biological wastes, clinical wastes, and hospital wastes. These waste materials include blood and other human body fluids in addition to highly infectious loads (Saini *et al.,* 2004). The fast expansion of the population generally and the number of healthcare facilities results in the production of large amounts of medical waste (Ambali *et al.,* 2013). India is the seventh-largest country in the world, India generates a massive volume of biological waste as a result of its well-developed medical infrastructure spread over several regions and towns.

Although actual output may not match official statistics, the Government of India reports that 484 tonnes of BMWs are generated daily in the country, with 10%–

15% of them being either untreated or not treated in compliance with regulations. This issue must be addressed at the national and healthcare facility levels and can be done so by implementing policies that have been decided upon by the authorities and increasing awareness and knowledge of them. The biological waste generated by various hospitals, labs, health camps, and research institutes varies and weighs between 1.5 and 2 kg per bed each day.

In 2013, 18,055 tonnes of medical waste were generated by hospitals and clinics in India; by 2023, that number is expected to increase to 33,000 tonnes yearly. The wastes generated by healthcare facilities are referred to as "healthcare wastes" in general. In some textbooks, these wastes are referred to as biohazard, hazardous, clinical, medical, and biological wastes from hospitals. The presence of fungus in clinical wastes is associated with high pH and organic matter levels that promote the growth of fungi.

Fungi are a diverse collection of eukaryotic organisms that are known to be a chlorophyllous, which means that they are parasitic, symbiotic, saprophytic, and heterotrophic. Their cell walls are composed of both chitin and β-glucans. Known to have about 5 million species, they are thought to be the second largest kingdom after Animalia.

As their primary source of carbon, fungi may use a variety of substrates, such as proteins, polysaccharides, lipids, aromatic hydrocarbons, and other chemicals. Fungi are saprophytic in origin; they produce external spores which are airborne. Among the many functions they perform in wastewater systems that sustain them are detoxification, biodegradation, and decolorization of pollutants.

A wide range of fungal species have been identified in the clinical wastes, including Aspergillus species, Fusarium species, Mucor species, Scopulariopsis species, Paecilomyces species, Cladosporium species, Penicillium species, Basipetospora species, Curvularia species, Aureobasidium sp., Scytalidium sp., Alternaria sp., and Acremonium spp. (Noman *et al.,* 2016). Fungi are linked to biomedical wastes that have a high content of organic components and a pH that encourages fungal growth. Fungi are recognised to be extremely important, yet despite this, the taxonomy of these species is still difficult since there aren't enough sophisticated methods for identifying them or doing systematic research.

There aren't many studies that have looked into Malaysian clinical waste fungi. Noman *et al.,* (2016) identified the most common species as *T. harzianum*, *P. chrysosporium, A. fumigatus*, and *A. niger*. Testing hospital textiles and plastics for the survival of Aspergillus, Fusarium, Mucor, and Paecilomyces species revealed a varying vitality, with most fungus lasting at least a day and several lasting weeks. These findings reinforce the need for appropriate disinfection and conscientious contact control precautions (Neely and Orloff, 2001).

Previous research on the fungal content of wastewater was either limited to the use of one molecular marker gene to identify the fungi to species level or relied on a conventional identification approach based on growth, morphology, metabolism, and enzymatic activity.

Nevertheless, not much research has been done on the use of many marker genes to identify fungal populations in wastewater. Thus, the goal of the current investigation was to determine if fungi were present in the clinical wastes in order to determine the fungal load in these wastes.

Materials and Methods

The samples were collected in the biohazards clinical waste bags. The medical waste samples included tissue papers, gloves, cotton, gauze, pasture pipette, needles, urine strips, kits, serum containers, blood wastes, ACCU-CHEK Safe-T-Pro Plus lancets, strips of glucose test lancets, microscopic slides, yellow tips, HB cuvettes and wood sticks. They were then brought to University Department of Biotechnology, Vinoba Bhave University, Hazaribag. The fungi were recovered on Potato Dextrose Agar (PDA) medium using direct plate technique and purified based on the single spore technique. Further, studies were done to characterize & identify different fungus from the above samples.

Study area

The study was conducted in Hazaribag district of Jharkhand. Few local hospitals and nursing homes were selected for the study. Different BMWs were collected in sterile polythene bags and brought to University Department of Biotechnology, Vinoba Bhave University, Hazaribag, Jharkhand for further studies.

Sampling sites

Following sampling sites were selected to complete the objectives of this work. These were:

1) Sheikh Bhikhari Medical College & Hospital, Hazaribag.
2) Sadar Hospital, Nawabganj, Hazaribag.
3) Arogyam Multispeciality Hopital, Hazaribag.
4) Kshitij Hospital, Hazaribag and
5) Kunal Nursing Hospital-Research-Centre Hazaribag.

Different BMWs were collected in sterile polythene bags carefully and brought to University Department of Biotechnology, Vinoba Bhave University, Hazaribag, Jharkhand for further studies to isolate, characterize and identify the fungus from the above samples collected from these sampling sites.

Isolation and purification of fungi from BMW

About 60-80 different isolates of fungi were undertaken for study. These were then streaked on Potato Dextrose Agar (PDA) media aseptically using a sterilized inoculating loop and then incubated at 28°C for 3-5 days (Vieira *et al.,* 2010). The pure fungus was stored on slants of PDA in a refrigerator at 4℃ for identification process.

Morphological characterization

Colony morphology and microscopic characterization of fungal Isolates

By sub-culturing each of the many colonies that formed onto the PDA plates and incubating at 28°C for five days, a pure culture was established and maintained. The surface features of media, including as texture, colour, zonation, sporulation, and diameters, were used to identify the colonies. To achieve pure isolates, the distinct colonies were sub-cultured on PDA slant and incubated at 28°C for 7 days. To investigate the microscopic features, a tiny section of the fungal growth zone was mounted on a grease-free, clean slide, along with a drop of lactophenol cotton blue. The slide was then covered with a cover slip and studied using an electron microscope with a × 40 objective lens. The isolates were characterized and identified using taxonomic guide. The pure isolates were maintained in PDA slants and stored in refrigerator for further identification.

Agar Disc Diffusion Method

This method is commonly used to estimate the antimicrobial activity and efficacy of potential drug candidate before the clinical trial. In this method, we commonly use the commercially prepared discs or prepare a stock of an antibiotic of suitable strength using sterile water.

Potato Dextrose Broth was used for the test. 5 selected fungal colonies, cultured on PDA by streaking were taken for the study. These isolates were then inoculated in freshly prepared PDA broth. Using a sterile inoculating loop, each isolate was inoculated separately in 100 ml of PDA broth. These were then incubated at 28℃ for 24 hours. Turbidity of the broth was noted next day. Then, 10 µl of this broth was spreaded in sterilized plates containing PDA media. Different antibiotic discs were then placed in the petriplates at euqidistant position uisng a sterile forecep. Upon incubation of the plates at 28℃ for 48-72 hours and even for more duration. The zone of inhibition around the antibiotic discs/area where antibiotic was used was observed. The diameter of the inhibition zone was measured and reported as susceptible, intermediate, and resistant. The antibiotics used were Fluconazole, Posconazole, Voriconazole, Ketoconazole and Grisefulvin.

Identification of isolated fungi

A later identification was made of the isolated fungus. Cultural and morphological characteristics, including as pigmentation, conidial morphology, and colony development pattern, were then used to identify the isolates (Tafinta, 2013). The Oyeleke and Manga approach (Oyeleke and Manga, 2008) involved the use of lactophenol and cotton blue stain to detect the fungus. The process of identifying the fungus involved removing a little culture and inserting it in a drop of lactophenol after applying a drop of the stain on a clean slide using a mounting needle. The needle was then used to distribute the mycelium across the slide. It was carefully given a cover slip. Air bubbles were removed with caution by avoiding pressure. Subsequently, the slide was mounted and examined using a ×10 and ×40 objective lens light microscope. As per Adebayo-Tayo *et al.,* (2012); Onuorah *et al.,* (2015); Klich (2002); Samson and Varga (2007). The morphological traits and appearance of the observed fungal organisms were recognised.

Results and Discussion

The task involved isolating and characterizing various fungi from a variety of materials, including tissue paper, gloves, cotton, gauze, pasture pipette, needles, urine strips, kits, serum containers, blood wastes, microscopic slides, yellow tips, HB cuvettes, wood sticks, and ACCU-CHEK Safe-T-Pro Plus lancets. The table below shows the frequency of occurrence of fungi from the above mentioned samples collected.

Fungal colonies

An attempt was made to observe the zone of Inhibition for selected 5 fungal isolates, which were earlier characterized at morphological and physico-chemical levels. Few antifungal antibiotics were taken for the study.

Following 5 test isolates were further selected for biochemical characterization in near future.

Physico-chemical characterization

Physico-chemical characterization of selected test isolates/strains was then performed. Their growth was checked at varying temperature as well as pH.

Most of the test isolates showed least growth at 20℃, moderate growth at 24℃ & 28℃, optimum growth at 32℃ and dense growth at 36℃.

Similarly, most of test isolates showed no growth at pH 4.8, moderate growth at pH 5.2 & 5.6 and dense growth at pH 6.0 while no growth at pH 6.4.

Biochemical characterization

At biochemical level a number of important biochemical tests such as starch hydrolysis, casein hydrolysis, gelatin hydrolysis, IMVIc test, hydrogen sulphide production test, nitrate test, catalase test, oxidase test, urea hydrolysis, phosphatase test, cellulase test and acid production from some sugar discs such as of arabinose, fructose, galactose, glucose, mannitol, meso-inositol, raffinose, rhamnose, sucrose, salicin and xylose will be performed later.

An assortment of fungus that are harmful to humans and animals was discovered by the isolation and dissemination of fungi from BMW of Hazaribag. In the cultured media that was incubated for four to seven days at 28˚C, the colony size (diameter, mm), texture, and surface of the fungal growth were noted. Depending on the spores' presence in the culture, the sporulation was also documented. With an inverted microscope, the dimensions of the fungus spores were measured. *Aspergillus* spp, *Candida albicans*, *F. solani*, *F. avenaceum*, *P. digitatum*, *R. stolonifer*, yeast (Saccharomyces species), and a few other fungal organisms have been linked to BMW in the study area.

These results suggest that it may be possible to safely regulate these fungal species to prevent the spread of infectious pathogens. In this investigation, various biomedical wastes were used to isolate and characterize fungal organisms. It has to mention that the fungal species obtained here are those have the ability to grow in the culture medium, while non-culturable fungi are not investigated in the study. Further, 5 selected test isolate(s) will only be identified and catalogued from a reputable laboratory and assigned an MTCC no. (Accession number). Also, their phylogenetic characterization will be done.

Regulations for the treatment of biological waste must be scrupulously adhered to. Healthcare wastes contain different species of the fungi and thus represent a biohazards wastes with adverse effects on the human and environmental health. The health risk concerns related clinical wastes lie in the potential of the pathogens for the regrowth or persistence and then their transmission into the food chain (Rose *et al.,* 1991). It should be mandatory for healthcare institutions to mandate that their employees undergo training from accredited training institutes; this should not be a one-time event but rather a continuous procedure.

The application of suitable waste management techniques and their inclusion in the curriculum improves their understanding of best practices in BMW management. We also need fairly priced and ecologically friendly technology. More training for administrators and healthcare staff is also required, as is more coordination between the pollution control agencies' activities. Numerous harmful bacteria and fungi have been found to be present in hospital solid waste, as well as in different hospital units and dumpsites. This has been nearly validated by the studies mentioned above.

Table.1 Frequency of occurrence of fungal species

Media used	Dilution used	No. of colonies/plate	Colony characteristics	Colony colour
Potato Dextrose Agar	10^{-3}	22	Circular	Black
	10^{-3}	20	Irregular	Dark green
	10^{-3}	18	Filamentous	Brown
	10^{-4}	18	Raised	Light green
	10^{-4}	16	Umbonate	Dark green
	10^{-4}	14	Circular	Black
	10^{-5}	14	Irregular	Light green
	10^{-5}	12	Filamentous	Dark green
	10^{-5}	10	Circular	White

Table.2

Growth at Temperature		1.	2.	3.	4.	5.
	20℃	+	+	+	+	+
	24℃	++	++	++	++	++
	28℃	++	++	++	++	++
	32℃	+++	+++	+++	+++	+++
	36℃	+++	+++	+++	+++	+++
Growth at pH						
	4.8	-	-	-	-	-
	5.2	+	+	+	+	+
	5.6	+	+	+	+	+
	6.0	++	++	++	++	++
	6.4	-	-	-	-	-

Figure.1 Biomedical Wastes samples collected from different sampling sites.

Figure.2 Photographs of fungal colonies isolated on PDA media from different BMWs.

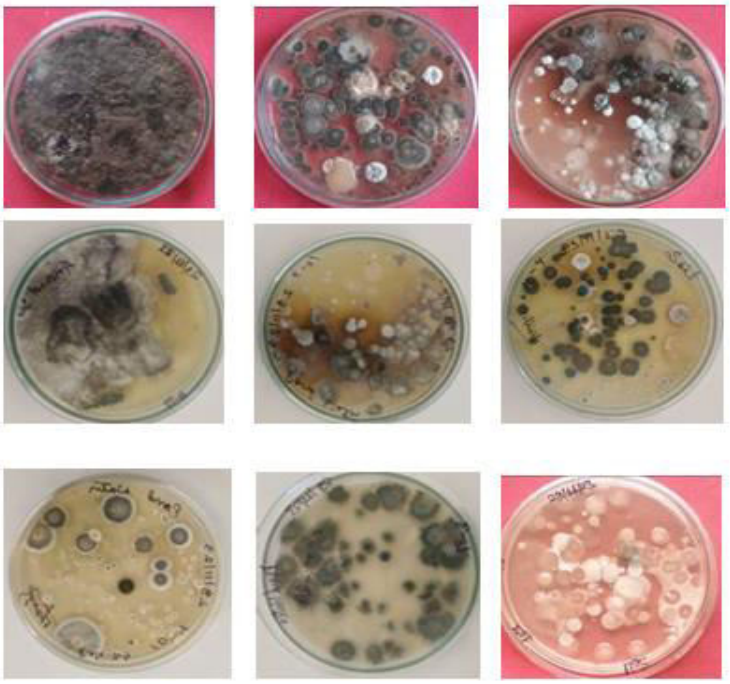

Figure.3 Streaking of different fungal isolates grown on PDA media**.**

Colour: Dark Green | Colour: Bluish Green | Colour: Bluish Green

Colour: Light Green | Colour: Dark Green | Colour: Dark Green

Figure.4 Microphotographs of fungi stained with Lactophenol cotton blue grown on PDA media.

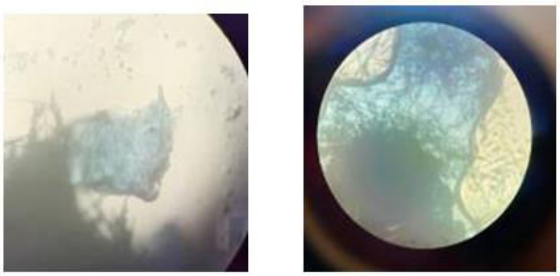

Figure.5 Photographs of PDA broth and selected 5 test isolates used for antibiotic sensitivity test.

Figure.6 Photographs of 5 selected fungal isolates for further work.

The type of clinical waste, where it comes from, and how it is used all affected the amount of fungi present in samples of biomedical waste. The solid waste from hospitals contains a variety of non-commercial and opportunistic pathogenic bacteria and fungi. These microorganisms have been shown to exhibit site variation and seasonal variation, indicating that seasonal and geographical factors influence the prevalence of different types of microorganisms.

The fungus in hospital garbage has spread because of the biohazardous clinical waste specimens utilized in the diagnostic procedure. The current investigation's goal was to use the morphological, physico-chemical as well as biochemical approach to identify the fungal isolates collected from clinical wastes.

On Potato Dextrose Agar (PDA) medium, the fungal isolates were obtained by the direct plate technique. They were purified by single-spore separation by streaking after growing for 7–14 days at 28°C. A light microscope was used to evaluate the morphology of the fungal isolates, and various mediums were used to record the culture properties. Over sixty fungal species, including species of *Rhizopus, Trichoderma,* and *Curvularia,* were discovered, spanning many genera. *Cladosporium werneckii*, *Candida, oidiodendron, Fusarium, Curvularia, Aspergillus, Trichothecium, Mucor, Acinetobacter*, and *Rhizopus*. Moreover, they underwent biochemical and physico-chemical characterization.

The aforementioned evidence indicates that a variety of fungus found in clinical wastes may provide a health concern to persons in the event that they have not been rendered inactive prior to their release into the environment. Given these facts, which indicated a potential health danger, it was recommended that clinical wastes be handled properly to stop the spread of pathogenic germs.

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Author Contribution

Aditya Kumar Singh & Shreya Kumari has performed the agar disc diffusion test. Mukul Kumar Das & Priyanshu Kumar Soni have done the morphological characterization of isolates. Raghujeet Kumar & Vidya Kumari have done the physico-chemiccal characterization. Athar Hussain Ansari & Piyush Indra Guru have written the abstract and introduction. Kumar Anand has overall edited of the paper. This work was completed under his supervision only. I would also like to acknowledge Kajal Gupta also for her sincere co-operation.

Data Availability

The datasets generated during and/or analyzed during the

current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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