Technical note

Identification of *M. mycetomatis* fungus in pleural fluid and sputum of a patient with aggressive gluteal eumycetoma with pulmonary spread.

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Abstract

Herein, we report on the isolation of *Madurella mycetomatis* from the pleural effusion and sputum of a 38-year-old man with a long standing aggressive gluteal eumycetoma that had spread to the abdominal and pleural cavities. The disease failed to respond to several antifungal agents and extensive surgical excisions including disarticulation of the right lower limb.The diagnosis was established by grain culture, cytological smears examination and PCR and LAMP molecular techniques. It is believed that, this is the first report on the isolation of this fungus from the sputum and pleural effusion in an eumycetoma patient.

Materials &Methods

Clinical specimens

Sputum sample and pleural fluid (200 mL) were collected from a patient seen at the Mycetoma Research Centre, University of Khartoum, Sudan after a written consent was obtained.

Sputum Sample Processing:

Early morning specimen, produced by deep cough, was collected and brought immediately to the laboratory without fixation. Macroscopically it was purulent sputum with small clump of mucoid substances and black grains. It was examined grossly for tissue and blood-tinged fragments. Smears from these fragments and other randomly sampled areas were prepared and fixed immediately in 95% ethyl alcohol. Other smears were allowed to air dry.

The 95% ethyl alcohol fixed smears were stained with the traditional Papanicolaou method and the air-dried ones were stained using Diff Quick Staining Protocol, Periodic Acid-Schiff's (PAS), Grocott's Silver, Gram's stain and Ziehl–Neelsen for acid fast Bacilli (AFB).

Culture for Bacterial Isolation

Sputum samples were inoculated on blood and MacConkey agar (Hi Media Laboratories, Mumbai, India) for secondary bacterial isolates detection.

Pleural Effusion Processing

The fluid was collected into a clean, dry container, and sent to the laboratory. No anticoagulant or fixative was added to the fluid. The effusion was blood-stained and contained numerous black grains macroscopically. The fluid was centrifuged for 5 mins at 2000 rpm. Then using wire loop, two drops of the top most layer of the sediment were collected and placed onto the center of a glass slide. Using the loop, the material quickly and immediately spread on the slide in a longitudinal and criss-cross manner. The slides with smeared material were quickly immersed in 95% ethanol while other smears were allowed to air dry.

Pleural fluid culture for bacteria isolates

Pleural fluid samples were inoculated on blood and MacConkey agars (Hi Media Laboratories, Mumbai, India) for secondary bacterial isolates detection.

Preparation and culture of grains:

Grains collected from pleural fluid were washed five times in normal saline under aseptic conditions, then they were inoculated on Sabouraud dextrose agar with penicillin and streptomycin,(SDA,Hi Media Laboratories, Mumbai, India), to inhibit the growth of contaminant bacteria, at 35^0C for 15– 21 days.

DNA extraction:

Grains collected from the sputum and pleural fluid were washed twice in physiological saline and then DNA was extracted using a protocol adapted from Möller and associates, (1992). [1] In summary, grains were added to a screw-capped tube containing 6−10 acid-washed glass beads. Tubes were filled with 490 µL cetyl-trimethyl ammonium bromide (CTAB) and 10 µL proteinase K (10 mg/mL, Sigma-Aldrich, St Louis, MO, USA), and were incubated for 1 h at 60^0C mixed every 20 minutes. After incubation, 500 µL of a 24:1 chloroform: isoamylalcohol solution were added and shaken for 2 minutes followed by centrifugation for 10 minutes at 14,000 r.p.m. The supernatant was collected and 400 µL supernatant 270 µL of ice-cold iso-propanol were added followed by another centrifugation. In the final step the pellets were washed with 70% ethanol, air-dried and re-suspended in 100 µL TE buffer (Tris 0.12% w/v, Na-EDTA 0.04%, w/v; pH 8.0).

Identification of Madurella mycetomatis by PCR:

In order to detect fungal DNA, PCR technique was performed using *M. mycetomatis* species primers described previously by Ahmed and colleagues (2004)[2]. In addition, LAMP technique as described previously by Sarah Ahmed and associates(2015) was used. [3] PCR results were performed by preparing a PCR mixture (50 µL) containing 50 ng DNA, 1 × Supertaq PCR buffer 1 (HT Biotechnology), 0.2 mM nucleotide mix, 25 pmol primer 26.1A (5′ AATGAGTTGGGCTTTAAC GG-3′) and 25 pmol primer 28.3A (5′-TCCCGGT AGTGTAGTGTCCCT-3′), and 1.2 U Super taq (HT Biotechnology). PCR amplification was performed in a thermocycler using a cycling program consisting of a denaturation step at 94^0C for 4–10 min; 30 cycles of 94^0C for 30 s, 58^0C for 30 s, and 72^0C for 30 s; and a final extension step at 72^0C for 10 min. PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

Identification of *Madurella mycetomatis* by Loop-mediated isothermal amplification (LAMP)

LAMP was carried out in 25 µL reaction volumes containing 8 U Bst DNA polymerase (New England Biolabs, Beverly, MA, USA), 1 × reaction buffer provided with enzyme (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)2SO_4$, 0.1% Triton X-100). Additional concentrations of $MgSO_4$ were tested, including 4 mM, 6 mM, 8 mM, 10 mM. Primers were used in concentrations of 40 pmol of each forward inner primer and backward inner primer (BIP 5'-TCCGCCGGAGGATTATACAAC-CTTATTCAGTACAGAAGACTCAGA-3'and FIP 5'-GACACTACACTACCGGGAGG-CATACCCCCAAAACCGTT-3') and 5 pmol each forward and backward outer primers (F3 5-'TCCCCAAACCATTGTGAA-3'and B3 5'-AGAGATCCGTTGTTGAAAGT-3'). Alternative ratios of inner and outer primers were tested including 6:1 and 10:1. Betaine was used in a concentration of 1 M. Reaction without betaine was also tested and dNTPs 1 mM each. Sterile water was used as negative control. LAMP reactions were incubated at 60^0C, 63^0C, and 65^0C, and three incubation periods (60, 90, 120 min) were also tested. Final products were examined in 2% agarose gels and results were considered positive if ladder-like bands patterns were revealed.

Results:

Sputum smears examination showed large, round to oval elongated, budding yeast cells with pseudohyphae in line with Candida species. The smears also showed numerous pus cells, lymphocytes, histiocytes in a necrotic background. Several colonies of bacteria were noticed (Fig. 1). Fungal colonies were seen, and on higher magnification, they consisted of septate, branching fungal hyphae embedded in a cement-like matrix,

(Fig. 2A). The PAS and Grocott's Silver stains had highlighted the branching hyphae(Fig. 2B) These cytological smears findings are in line with *M. mycetomatis,* bacterial isolates and *candida spp* (Fig. 2C).The sputum and pleural fluid were negative for acid-fast bacilli (AFB) by Ziehl-Neelsen staining.

Sputum bacterial culture revealed growth of *E. coli, Pseudomonas aeruginosa*, and that was confirmed by Gram stain and biochemical tests. *E. coli* was sensitive to Imipenem and resistant to Ceftazidime, Ciprofloxacin, gentamycin, Cefepime whereas *Pseudomonas aeruginosa* was sensitive to Imipenem, Ceftazidime, Ciprofloxacin, gentamycin, Cefepime.

Pleural fluid grains cultured on Sabouraud's dextrose agar with chloramphenicol, but without cycloheximide, grew *M.mycetomatis* which was identified by observing its growth rate, colony morphology, pigment production (Fig.3) and confirmed by PCR and LAMP molecular techniques.

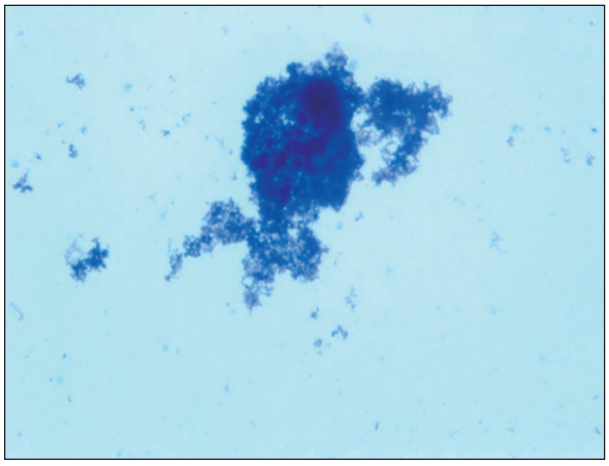

Fig. 1. Sputum cytological smear showing scattered colonies from coccobacilli organism identified by Diff Quik stain (X100).

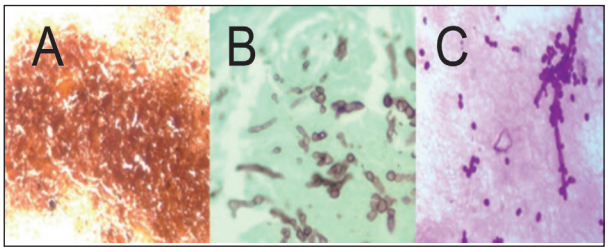

Fig. 2 A. Dark brown *M. mycetomatis* grain from sputum sample(H&E, X10), B: *Madurella mycetomatis* hyphae embedding in cement like matrix which stained pale green (Grocott's, X100), C: *Candida spp,* note the pseudohyphae and small budding yeasts (PAS, X100).

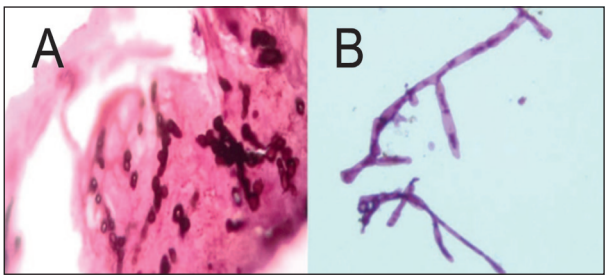

Fig. 3. Pleural effusion cytological smear showing A; *Madurella mycetomatis* hyphae within cement-like matrix (Grocott's, X100). B: Septate hyphae of *M. mycetomatis* (PAS, X100).

Comment:

In this short communication, we report on the identification of *Madurella mycetomatis* fungus in the sputum and pleural effusion of a patient with a gluteal eumycetoma which had spread wildly to the abdominal and pleural cavities. This aggressive clinical presentation is a rarity. A medical literature review revealed no previous reports on such presentation nor identification of the fungus in the sputum or pleural effusion.

The fungus identification was based on a battery of tools and techniques that included cytopathological smears, grain culture, and two molecular techniques; the PCR and LAMP.[4,] The molecular techniques were essential to confirm the diagnosis.

In mycetoma the current available diagnostic tools are invasive, expensive, and of low sensitivity and specificity. Furthermore, they are not widely available in endemic regions and are not field

friendly. However, LAMP is a potential simple, economical tool that can be a point of care test. However, clinical trials are required to validate its accuracy. Hence there is a pressing need for a new simple, non-invasive diagnostic test that can overcome these limitations.[5]

References

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