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Brief Report

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Acid and heat fastness in microsporidia: How acid fast are acid fast microsporidium?

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Abstract

Microsporidia are obligate spore-forming microorganisms with strong resemblance to fungi and can affect almost every organ system in immunocompetent or immunocompromised individuals. Mixed infections are also reported in immunocompromised hosts. Microsporidial spores show marked morphological variations and the small and slender forms can resemble bacilli. Modified Zeihl Neelsen (ZN) stain, cold method demonstrates them as bright red in color, leaving several spores blue or incompletely stained; thus, they are reported as weakly or variably acid fast. Variability in staining results with ZN stain and considering the fact that *Mycobacterium tuberculosis*, the commoner bug in developing countries is identified by its resistance to stronger acids on ZN staining, authors wished to demonstrate acid and heat fastness in microsporidium using corneal tissue specimens. Microsporidial spores stained bright red in color with conventional ZN stain, demonstrated strong acid fastness, and interestingly the staining results improved on heating. Thus, the authors conclude that they are strongly acid and heat fast and care must be warranted so that they are not misdiagnosed as *Mycobacterium* or other acid-fast organisms. Careful observation of morphology, battery of special stains, and molecular diagnostics should be advocated for diagnostic confirmation. To the best of the authors' knowledge, this is the first explicit report on acid and heat fastness on microsporidial spores.

Key words: Microsporidia, *Mycobacterium*, Zeihl Neelsen stain, acid fast bacilli, histopathology.

Microsporidia are highly specialized spore forming obligate intracellular microorganisms, strongly related to fungi. They produce infective spores with marked morphological variations. Spores may be as small as 0.8–1.5 μm in length, with a width of 1 μm, appearing like round to oval dots, cell debris, or like bacteria on light microscopy or measure up to 5 μm in largest dimensions, resembling yeast.[1] They can affect almost every organ system including gastrointestinal, genitourinary, pulmonary, rhino cerebral, and eye, with predilection for both immunocompetent and immunocompromised hosts.[2,3] Several stains like Gram's chromotrope stain, modified Ziehl Neelsen (ZN) stain(1% Sulphuric acid or 1% acid alcohol; cold method), potassium hydroxide-calcofluor white stain(KOH + CFW), Gomori's methenamine silver stain, Giemsa and toluidine blue stain have been described as sensitive and ideal for detection of microsporidial spores.[1,2,4–6] Modified ZN stain shows discrete bright red staining of spores with a characteristic horizontal polar band against a bluish background that helps in their easy identification; however, lack of visualization of polar band in slender forms and background debris may lead to false negatives and/or cause diagnostic dilemma with other acid fast microorganisms. Further literature describes variable sensitivity of staining with modified ZN stain,[4,6] leaving some spores as partially stained or unstained, that is, blue in color.

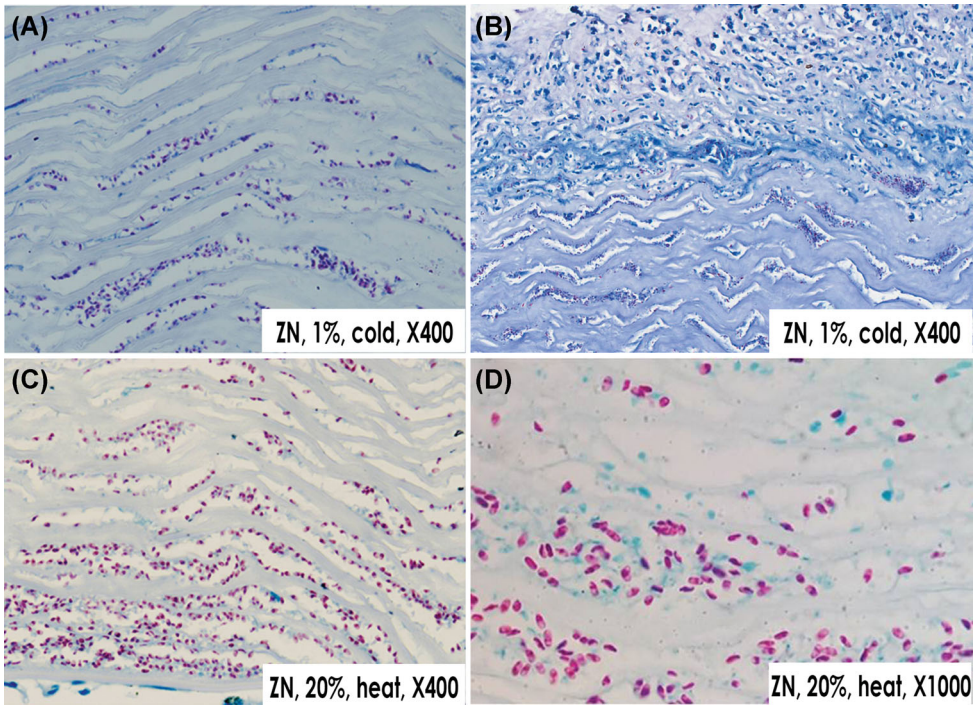

Figure 1. Demonstration of heat and acid fastness in microsporidium. (A) Corneal stromal tissue shows oval yeast like forms and (B) slender, small forms of microsporidial spores stained red with modified Ziehl Neelsen (ZN) stain (1% sulphuric acid-cold method [×400]). Also noted are admixed unstained and purple colored spores. (C) An increase in the number of spores and intensity of staining can be appreciated; when the same tissue as in panel A is subjected to conventional ZN stain (20% sulphuric acid-heat [×400]). (D) Morphology can be beautifully discerned using conventional ZN stain at oil immersion objective.

Light microscopy and ZN stain have been recommended by World Health Organization (WHO) as a method of screening, case finding, referral and treatment of tuberculosis at peripheral and intermediate laboratories, advocated to be supplemented by other tests.[7] In spite of being gradually phased out by molecular diagnostics, ZN stain is a common diagnostic armamentarium on sputum samples for suspected *Mycobacterium tuberculosis* in developing countries, and also recommended for treatment monitoring.[8] It is also popularly used in histopathology labs on biopsy samples and cytology smears that display granulomatous inflammation with or without caseation, especially in cases with suspected extra pulmonary tuberculosis and instances when appropriate tissue samples are not available for molecular diagnostics. It is also performed with modifications for screening/diagnosis of other acid fast microbes like *Mycobacterium leprae*, *Nocardia* spp., and intestinal *Coccidia*. Considering that microsporidia is also acid fast and can infect immunocompromised individuals with multiple infections,[2,3] the authors wished to explicitly study acid fastness in microsporidium.

In a retrospective observational study, approved by our Institutional Review Board, all of the patients undergoing therapeutic penetrating keratoplasty (TPK) during the year 2011–2015 were analyzed and nine of 387 patients were diagnosed as microsporidial stromal keratitis on histopathology; clinicolaboratory details of seven of these nine cases has been previously described by us.[6] Six of these nine cases had demonstrated microsporidial spores on corneal scrapings stained with KOH + CFW stain, Gram stain and/or modified ZN stain. No growth on culture of corneal scrapings prior to TPK was noted in these cases, when inoculated on 5% sheep blood agar, chocolate agar, brain heart infusion agar, thioglycollate broth, Robertson's cooked-meat medium, Sabouraud's dextrose agar, potato dextrose agar, and nonnutrient agar with *Escherichia coli* overlay. Pan-microsporidial PCR targeting small subunit rRNA was found to be positive in all the cases.[9] Biopsy sections of these nine cases of corneal stromal microsporidiosis were evaluated for acid fastness, using five different concentrations of sulphuric acid as decolorizer (1%, 5%, 20%, 30%, 40%) with and without heating the slide bearing three sections each, overlaid by carbol fuschin stain. In addition, sections were also studied by staining with ZN stain with 3% hydrochloric acid alcohol (kit method) as decolorizer with and without heating. All staining experiments were performed in duplicates and stained slides were evaluated by light microscopy. All the cases were clinically immunocompetent.

Colonoscopic biopsy of intestinal tuberculosis and sputum sample of confirmed pulmonary tuberculosis (Xpert MTB/RIF assay) were included as positive controls in the staining experiments. Negative controls of sputum sample and corneal biopsy were also run with each batch of staining.

Modified ZN stain (1% acid alcohol or 1% sulphuric acid; Fig. 1A, B), without heating demonstrated spores varying from

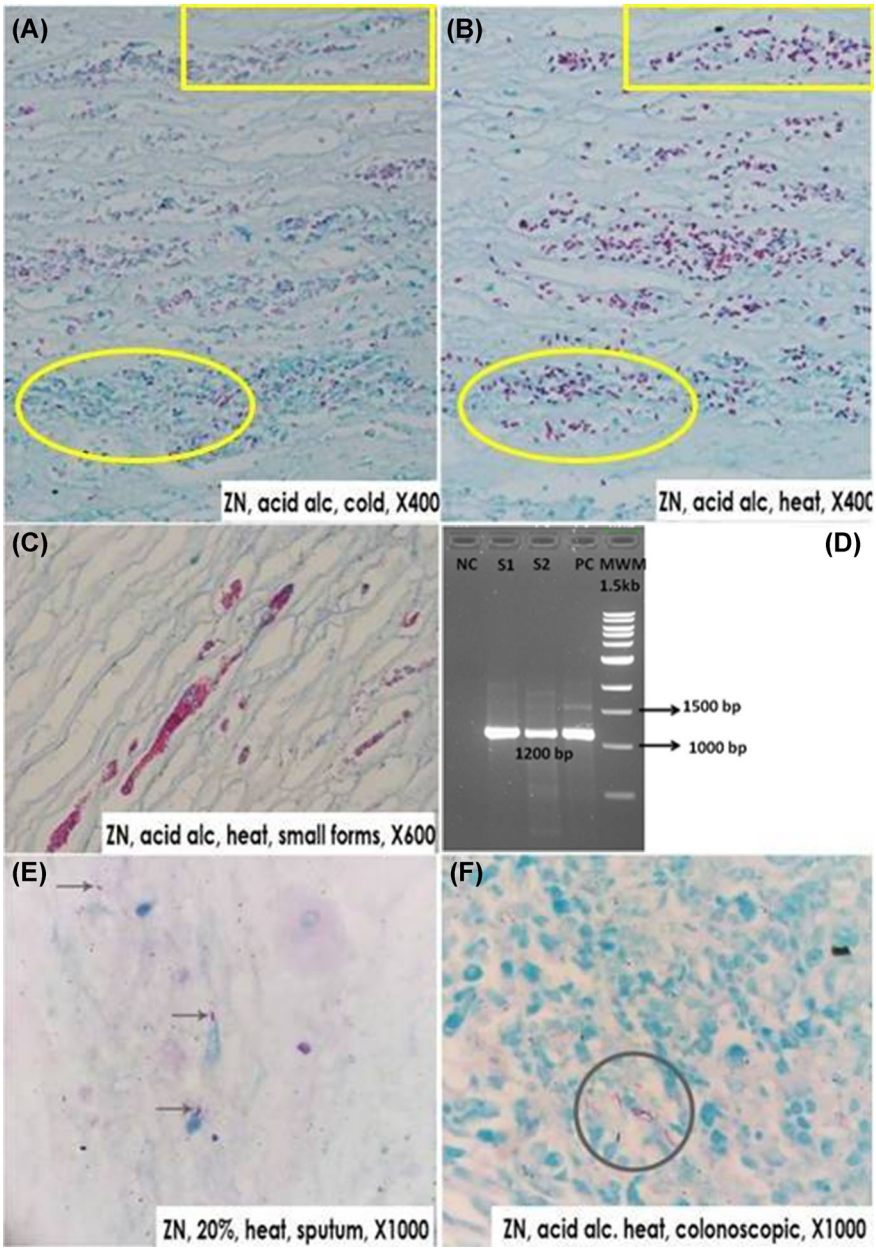

Figure 2. Demonstration of heat and acid fastness. (A) Corneal stromal tissue demonstrates spores stained with Ziehl Neelsen (ZN) stain using 3% hydrochloric acid alcohol, without heating. Several spores are left unstained, when the tissue is not heated. (B) An increase in the number of spores stained with better contrast, can be observed in the same tissue at similar magnification using heat (ZN stain; 3% acid alcohol ×400; highlighted in yellow oval and rectangular inserts). (C) Smaller forms of microsporidia are beautifully discerned using ZN stain, 3%hydrochloric acid alcohol with heating (×600). (D) Representative agarose gel picture of pan-microsporidial polymerase chain reaction targeting small subunit r-RNA. (E, F) Photomicrographs of positive controls of *Mycobacterium tuberculosis* using ZN stain, 20% sulphuric acid-heat (×1000) in a sputum sample (arrow marked) and colonoscopic biopsy (circle marked) are presented.

bright red, bluish red, or pale red, leaving spores unstained, giving them debris like appearance. Microsporidial spores stained bright red using conventional ZN stain. There was an increase in the number of spores that got stained red as compared to results with modified ZN stain, which was distinctly obvious by minimal bluish debris like material. (20% H_2SO_4, heat; Fig. 1C, D). Significant increase in intensity of staining and number was also observed on staining with ZN stain using 3% hydrochloric acid alcohol(kit method) with heating(Fig. 2A-without heating, 2B, 2C-with heating). Further modifications in sulphuric acid concentrations (5%, and even 30%; with heating) demonstrated the spores brightly. Tissue sections crumbled, got folded, or lifted with sulphuric acid concentration of 40%.

In addition, panel of special stains like Gomori's methenamine silver stain and periodic acid Schiff stain was performed on

all the sections to confirm the presence of microsporidia and molecular confirmation of all the cases of microsporidial keratitis was also obtained on pan microsporidial PCR (Fig. 2D) using appropriate positive and negative controls. Appropriate positive control samples of confirmed *Mycobacterium tuberculosis* and negative samples were run with each batch of staining of ZN stain (Fig. 2E, F)

We conclude that microsporidial spores are strongly acid fast and could resist an acid concentration of even 30%, beyond which it could not be elicited as tissue sections crumbled. Heating allowed better penetration of carbol fuschin stain, and it was interesting to observe, that the sections that were heated overlaid with carbol fuschin stain showed an increase in the number of microsporidial spores that got discretely stained red, as compared to staining without heating.

The slender forms of microsporidia mimics bacteria on morphology can be misinterpreted as *Mycobacterium* or other acid fast organisms, specifically in cases with low microbial load or in patients with multiple infections.[3,10] It is therefore important to be aware of the fact that microsporidia are also acid and heat fast and are therefore likely to be misdiagnosed as *Mycobacterium* in biopsy samples. Careful study of morphology, alternative stains for microsporidium spp. and/or molecular diagnostics needs to be performed for diagnostic confirmation. In addition to morphology at oil immersion(×1000), confirmatory tests for *Mycobacterium* should therefore be warranted across all health centers, on all biological samples like sputum, urine, stool, bronchoalveolar lavage, lymph node aspirates, intestinal biopsy, and so forth, if they demonstrate acid fast microorganisms. More species specific studies on different biological samples would further contribute to our observations.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Ramanan P, Pritt BS. Extraintestinal microsporidiosis. *J Clin Microbiol*. 2014; 52: 3839–3844.
2. Fedorko DP, Hijazi YM. Application of molecular techniques to the diagnosis of microsporidial infection. *Emerg Infect Dis*. 1996; 2: 183–191.
3. Saigal K, Sharma A, Sehgal R, Sharma P, Malla N, Khurana S. Intestinal microsporidiosis in India: a two year study. *Parasitol Int*. 2013; 62: 53–56.
4. Joseph J, Vemuganti GK, Garg P, Sharma S. Histopathological evaluation of ocular microsporidiosis by different stains. *BMC Clin Pathol*. 2006; 23: 6.
5. Weber R, Bryan RT, Owen RL, Wilcox CM, Gorelkin L, Visvesvara GS. Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. The Enteric Opportunistic Infections Working Group. *N Engl J Med*. 1992; 326: 161–166.
6. Mittal R, Balne PK, Sahu S, Das S, Sharma S. Coexistence of herpes simplex virus infection in microsporidial stromal keratitis associated with granulomatous inflammation. *Indian J Ophthalmol*. 2017; 65: 276–281.
7. WHO. *Implementing Tuberculosis Diagnostics: A Policy Framework*. Geneva: World Health Organization, 2015. https://www.who.int/tb/publications/implementing_TB_diagnostics/en/. Accessed: February 2, 2019.
8. WHO. *Global Tuberculosis Report*. Geneva: World Health Organization, 2018. https://www.who.int/tb/publications/global_report/en/. Accessed: March 23, 2019.
9. Reddy AK, Balne PK, Gaje K, Garg P. PCR for the diagnosis and species identification of microsporidia in patients with keratitis. *Clin Microbiol Infect*. 2011; 17: 476–478.
10. Weber R, Deplazes P, Flepp M, Mathis A, Baumann R, Sauer B et al. Cerebral microsporidiosis due to Encephalitozoon cuniculi in a patient with human immunodeficiency virus infection. *N Engl J Med*. 1997; 336: 474–478.