Original Article

HEAT FIXED BUT UNSTAINED SLIDE SMEARS ARE INFECTIOUS TO LABORATORY STAFF

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Summary

***Background and Aim*:** In a clinical microbiology laboratory, heat fixed slide smears are commonly transported from one place to another for staining with different stains and also for onsite proficiency testing of laboratory technicians for accreditation of the laboratories. These smears are frequently handled without gloves by the staff in developing countries. Therefore, this study was conducted to check the survivability of tubercle bacilli on smears after physical and chemical treatments.

***Methods*:** A total of 196 AFB positive smears were analyzed. Of these, 116 were stained with Ziehl Neelsen (ZN), 60 with cold Kinyoun and 10 were unstained but heat fixed and 10 were neither stained nor heat fixed. The last 20 smears served as controls. The ZN and Kinyoun stained smears were 0-1.5-year-old and stored at room temperature in slide boxes, while control smears were freshly prepared. All smears were prepared from sputum samples positive for acid fast bacilli. All four sets were subjected to slide culture to see if mycobacteria could survive and grow in any. For slide culture, a new and safe device was used, which is designed for three in one purpose: cell cultivation, direct observation of the growth under microscope and cell harvesting inside the closed tube. The slide smears were directly dipped into this tube that contained liquid culture medium. The tubes were incubated at 37^0C for four weeks. The growth, if any, was confirmed by MPT-64 rapid test and subculture on LJ slants.

***Results*:** No growth was observed in ZN and Kinyoun stained slide smears. However, significant growth was observed in both control sets; the unstained non heat fixed as well as heat fixed slide smears.

***Conclusions*:** The results of our study indicate that tubercle bacilli remain viable even after heat fixation and carry risk of infection by contact. However, stained smears are safe for handling and storage. **[*Indian J Tuberc 2013; 60:* 142-146]**

Key words: Smear, Slide culture, Ziehl Neelsen, Kinyoun, *M. tuberculosis*.

INTRODUCTION

Tuberculosis (TB) is a highly contagious disease with the incidence of 8.8 million new cases every year. Microscopy remains the mainstay of any clinical microbiology laboratory, whether dedicated to the diagnosis of TB or it caters to other infectious diseases.[1] Though sputum remains the main clinical material but some patients are unable to produce sputum, such as children, immuno-compromised patients and patients with neurological impairment.[2] Also patients with chronic intractable diarrhoea are investigated for opportunistic coccidian parasites and for mycobacterial causes. The immuno-compromised patients, especially, may shed a high number of the opportunistic coccidian parasites as well as *Mycobacterium* species in their feces. These suspected fecal smears are stained with cold Kinyoun and as well with hot carbol fuchsin stains for the opportunistic coccidian parasites and *Mycobacterium* species, respectively.[2-4]

In resource limited settings such as primary health care centres and designated microscopy centres, these stained slides are usually being handled without gloves by the staff. Also often, the national and regional reference laboratory staff carries with them, during inspections, unstained smears to evaluate the proficiency of the local laboratory technicians. Hence this may be perilous to the laboratory personnel and the staff who carries such slides to the distant RNTCP laboratories. It has been documented that Laboratory Acquired Infections (LAI) of TB are three to nine times higher in laboratory workers than general

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population[5]. Hence, safety of the laboratory workers from *mycobacterial* infection should be the first consideration in mycobacteriology laboratory.

Some studies have been performed to check the viability of *Mycobacterium tuberculosis* (*M. tb*) from heat fixed smears as well as Ziehl Neelsen (ZN) and Auramine Rhodamine (AR) stained smears.[6,7] But, to the best of our knowledge, no study is published on viability of *mycobacteria* using cold Kinyoun stain. Hence, in the present study, we evaluated the viability of Mycobacteria in the heat fixed smear with and without staining with hot and cold acid fast stains.

MATERIAL AND METHODS

Slide smears used for culture

The study was conducted at the Division of Clinical Microbiology, Department of Laboratory Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi from February 2011 to February 2012. A total of 196 AFB 3+ sputum slide smears were used in this study. Of these, 70 were 12-18 months' old stored ZN stained slides and taken out from the slide storage boxes after reviewing the old records. All old slides are stored only after cleaning with xylene. Remaining 126 were freshly prepared slide smears from the sputum of known pulmonary TB patients and confirmed positive for acid fast bacilli. As a standard practice, the slide smears from sputum samples were prepared and allowed to dry in a biological safety cabinet (HR40-IIB2, Haier, China). Slide smears are then routinely heat fixed by passing the slide three times through the flame of gas burner before removing the slides from biosafety cabinet for staining.[6] Of the 126 freshly prepared slide smears, 46 were stained with ZN (total 70+46=116) and 60 with cold Kinyoun methods.[8,9] The smears were examined microscopically under oil immersion field and results were graded as per the WHO guidelines. For growth control, 10 heat fixed unstained smears and another 10 slide smears were taken which were neither heat fixed nor stained with any stain. Medium without any inoculation was considered as a negative growth control.

Decontamination of slide smears

Slide smears (heat fixed unstained as well as stained) were decontaminated to prevent the growth of other micro-organisms. In the beginning, decontamination of the slides was done by using 4%, 2% and 1% NaOH solution for different time periods of four, three, two and one minute. It was found that 4% NaOH was too harsh for the mycobacteria but 1% NaOH was not able to decontaminate properly hence decontamination with 2% for one minute was considered as optimum, and this protocol was used throughout the study. For this, two sterilized coplin jars were used. One was filled with 2% NaOH and the second with phosphate buffer solution (to neutralize effect of alkali). With the help of sterilized forceps, slides were dipped in 2% NaOH for one minute and after that neutralized in phosphate buffer solution for 30 seconds.

Smear culture by Thin Layer Agar (TLA) method

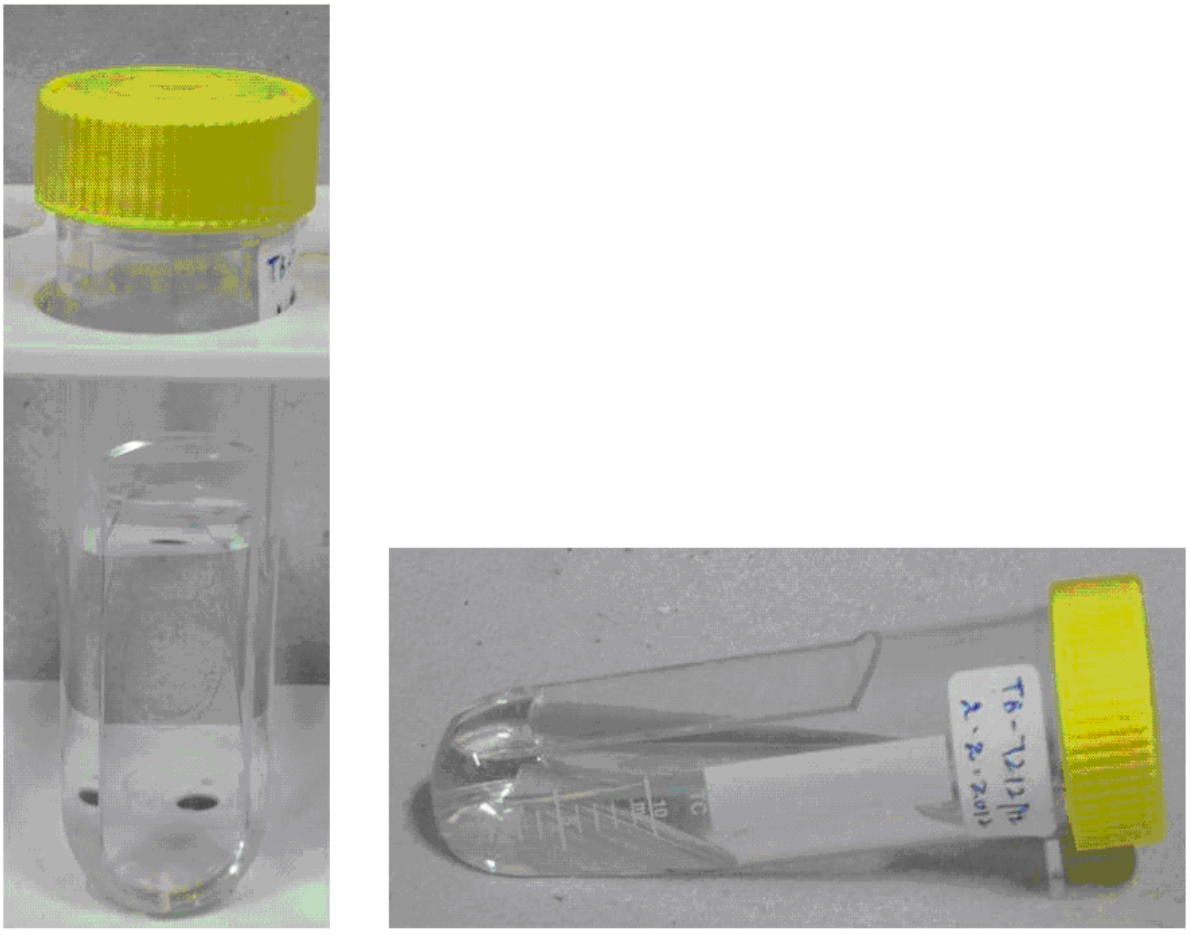
Initially, we designed our experiment with the use of TLA method to test the viability of *M. tuberculosis* in ZN stained smears. The smeared side of the decontaminated slides (20) was placed up-side-down on the agar plate containing middle brook 7H10 agar medium with PANTA (Becton, Dickinson and Company, USA). In each experiment, positive and negative controls were also used. The plates were incubated at 37°C upto six weeks. Plates were observed every 96 hours to check for contamination and cord forming micro-colonies using inverted microscope (RTC-7, Radical Instruments, India).

Smear slide culture by tissue culture tube method

Since the thin layer agar plate method was not very successful, we performed slide culture method using a special type of tissue culture tube (Figure) manufactured by Techno Plastic Products (Ref: 91253, TPP , Switzerland). This tube is designed especially for three in one purpose: cell

cultivation, direct observation under microscope and cell harvesting in a single tube. For slide culture, 10ml Middlebrook 7H9 medium with OADC-PANTA supplement was dispensed into these culture tubes. In the culture tube, the slides under study were placed. The slides were placed in such a way as the whole smear was covered by the liquid medium. Tubes were then incubated at 37°C upto four weeks and examined twice a week using the inverted microscope to detect the growth (cord forming) of *M. tuberculosis* (40x). After incubation of four weeks, the 200µl liquid medium from the tube was taken out and inoculated on LJ medium in order to confirm the further growth/viability of *M. tuberculosis*.

Fifty microliter of culture was used to confirm the growth using commercially available strips (TB Ag MPT-64 Rapid®, SD BIOLINE, India). The strip is based on the principle of immunochromatography, which detects *M. tuberculosis* MPT-64 antigen.[10]



A B

Figure: Inoculated slide smear into flat bottom tissue culture tube: (A) standing position during incubation, (B) position during microscopy

Table: Survivability of Mycobacteria in heat fixed, un-fixed and stained slide smears

Slide smears (n=176)	Number	Results		
		Mycobacterial growth observed under inverted microscope[§]	Mycobacteria grew in Middle brook 7H9 *	Mycobacteria grew on LJ slants
ZN stained old smears	70#	0/70	0/70	0/70
ZN stained fresh smears	46	0/46	0/46	0/46
Kinyoun stained fresh smears	60	0/60	0/60	0/60
Unstained heat fixed smears	10	10/10	10/10	10/10
Neither stained nor heat fixed smears	10	10/10	10/10	10/10

[§] Cord formation
[#] three to 18 months' old stored AFB 3+ smear slides were used
[*] Confirmation by TB Ag MPT-64 Rapid

RESULTS

None of the ZN stained and Kinyoun stained old slides showed growth after four weeks. However, cord formation was observed within 10 days in both the growth control slides cultured under the same culture conditions (Table).

DISCUSSION

M. tuberculosis is usually transmitted through respiratory aerosols in shared air environments and TB infection through surface contact in human-to-human transmission is low. Aerosol may be generated at any stage during laboratory processing of TB specimens, any manipulation with *Mycobacterium* cultures and working with infected animals. Therefore, incidence of TB infections in laboratory staff has been reported three to nine times higher than general population.[5] Moreover, *M. tuberculosis* infections by contacts and cutaneous injuries have also been documented.[11,12]

Even though hot carbol fuchsin stain (ZN stain) is widely perceived that it kills all mycobacteria, yet no reports are available in the literature to demonstrate, if the cold acid fast stain (such as Kinyoun stain) would also carry the same detrimental effect on the mycobacteria. This study was conducted to compare if the detrimental effect of ZN stain on mycobacteria is due to heat or it is a chemical sterilization. To compare this, we compared heat fixed unstained smears with heat fixed but stained with two types of stains: one uses hot carbol fuchsin while the other uses cold carbol fuchsnin. The study clearly showed that mycobacteria resist physical sterilization but cannot withstand chemical sterilization.[13,14]

The conclusion of the present study is that tubercle bacilli do not remain viable after the slide smears are stained whether with hot ZN or with cold Kinyoun method and are safe for handling and storage. However, the unstained slide smears prepared from samples positive for mycobacteria remain infectious and should be handled as potentially infectious.

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