SHORT COMMUNICATION

Survival of Tubercle Bacilli in Heat-fixed and Stained Sputum Smears

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We used a slide culture technique to detect tubercle bacilli surviving in sputum smears (n=46) after conventional heat fixation and Ziehl-Neelsen staining. In all heat-fixed sputum smears, tubercle bacilli survived after time 0 (n=22), 24 h (n=7), 48 h (n=7), 72 h (n=4), and seven days (n=6). None of the stained sputum smears showed growth on slide cultures. Viable tubercle bacilli remaining in heat-fixed sputum smears for at least seven days may present an infection risk to laboratory staff. Thus, sputum smears should be stained immediately by the Ziehl-Neelsen method or stored in a safe container to avoid transmission of tuberculosis.

Key words: tubercle bacilli - slide culture - sputum smear

In Brazil and other developing countries the presumptive diagnosis of pulmonary tuberculosis is made by direct microscopic detection of acid fast-bacilli from sputum specimens using the Ziehl-Neelsen stain (Brasil 1992, Hijjar 1994, Campos 1996). Under Brazilian epidemiological conditions this procedure identifies 70% to 80% of the cases of pulmonary tuberculosis (Gontijo Filho & Fonseca 1979). Thus, it is an obligatory basic examination in all health units (Machado 1985, Brasil 1992, Hijjar 1994, Campos 1996).

However, health units with inadequate facilities merely collect the sputum specimens and send them "in natura" or as heat-fixed smears to a reference laboratory for microscopic examination. The latter procedure is much used in Brazil because of the ease of transport, storage, and preservation of the slide specimens (União Internacional Contra a Tuberculose 1978, Machado 1985).

Some investigators, however, have demonstrated that tubercle bacilli may survive in heat-fixed sputum smears (Blair et al. 1972, Allen 1981, Goldfogel & Sewell 1981, Peluffo & Kantor 1984). Any smear rubbed from the slide may therefore be a potential source of infection to laboratory staff (Smithwick 1976). Although tubercle bacilli can be killed in smears stained by the Ziehl-Neelsen method (Goldfogel & Sewell 1981), it is unclear how long the bacilli can survive on heat-fixed slides prior to staining.

In this study a slide culture technique was used to detect tubercle bacilli surviving in sputum smears immediately after air-drying and heat fixation (time 0), and after 24 h, 48 h, 72 h, and seven days post-heat fixation. The survival of tubercle bacilli in smears stained by the Ziehl-Neelsen method was also studied.

Sputum specimens - Forty six sputum specimens obtained from outpatients in diagnosis for pulmonary tuberculosis attended at health care unit (Centro de Referência Prof. Hélio Fraga, Rio de Janeiro, RJ) were studied. The specimens were transported by air to the city of Maringá and tested within 14 days after collection.

Preparation of smears - Each smear was made by spreading the sputum specimen over a 25 x 10 mm area of a 76 x 13 mm glass microscope slide, using a 10 ml disposable loop (Difco Laboratories, Detroit, MI, USA). The smears were allowed to dry in a safety cabinet without heating.

Slide cultures - Duplicates of controls and tests were cultured by placing each slide in a 16 x 150 mm tube containing 8 ml of a selective lysed blood (SLB) medium, and incubated at 37ºC for seven

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Received 5 July 2000
Accepted 22 August 2000

days. The SLB medium was prepared according to Stritch and Dickinson (1958) and Allen (1981). Briefly, one volume of human blood was added to one volume of sterile distilled water. The blood was obtained from out-of-date blood transfusion bags and any other blood bags unsuitable for transfusion purposes. The red blood cells were lysed by addition of a sterile solution of saponin (Inlab, São Paulo, SP, Brazil) to give a final concentration of 500 mg/l. The medium was made selective by addition of polymixin B, 200 U/ml and trimethoprim, 10 mg/l (Laboratórios Frumtost SA, Indústrias Farmacêuticas, Guarulhos, SP, Brasil); carbenicillin, 100 mg/l (Laboratórios Pfizer Ltda., Guarulhos, SP, Brasil) and amphotericin B, 10 mg/l (Bristol-Myers-Squibb Brasil SA, Santo Amaro, SP, Brasil).

Experiment 1. Heat fixation - Twelve smears were prepared from each of 46 sputum specimens. The 1st pair of slides was not heated and acted as a control. The remaining smears were heat-fixed by passing the slide, smear side up, through the flame of a gas burner two or three times. Next, the 1st and 2nd pairs of slides (control and time 0) were cultured in SLB medium. The 3rd, 4th, 5th, and 6th pairs of slides were cultured after 24 h, 48 h, 72 h, and seven days post-heat fixation, respectively. All the cultures were incubated at 37ºC for seven days.

Experiment 2. Stained smears - Four smears were prepared from each 46 sputum specimens used in experiment 1. Two smears were heat-fixed and stained using the Ziehl-Neelsen method (Smithwick 1976). The other two smears were air-dried without heating and remained unstained (controls). All smears were cultured in SLB medium at 37ºC for seven days.

Microscopic examination of slide cultures - After incubation, the slides were removed from the SLB medium, rinsed briefly in 10 ml distilled water, decontaminated with 5% sodium hypochlorite for 10 min, stained by the Ziehl-Neelsen method, and examined with a compound microscope (Olympus CBA, Micronal SA, Aparelhos de Precisão, São Paulo, SP, Brasil). The entire surface of the smear was scanned using a 10x objective (Fig. 1A), and the presence of cord-forming microcolonies i.e., a positive test, was confirmed using a 20x objective (Fig. 1B). The ocular magnification was 10x. A Olympus microscope model BX50F-3 (Olympus America Inc., Lake Success, NY) and Plus-X pan ISO 125/22 film (Eastman Kodak Company, Rochester, NY) were used to photograph a positive test.

Additional bacteriologic data - All sputum specimens studied were digested and concentrated by the 4% sodium hydroxide or trisodium phosphate methods (Strong & Kubica 1981, Brasil 1994), inoculated onto Loewenstein-Jensen slants, and incubated at 37ºC for eight weeks. Positive cultures were identified as *Mycobacterium tuberculosis* by routine tests performed at the National Tuberculosis Reference Laboratory (Rio de Janeiro, RJ, Brasil), including the following tests: growth rate, pigment production, niacin production, nitrate reduction, heat-stable catalase test (at pH 7.0/68ºC), and ability to grow in the presence of p-nitrobenzoic acid (500 mg/ml) and thiophen-2-carboxylic acid hidrazid (2 mg/ml) (Strong & Kubica 1981, Brasil 1994).

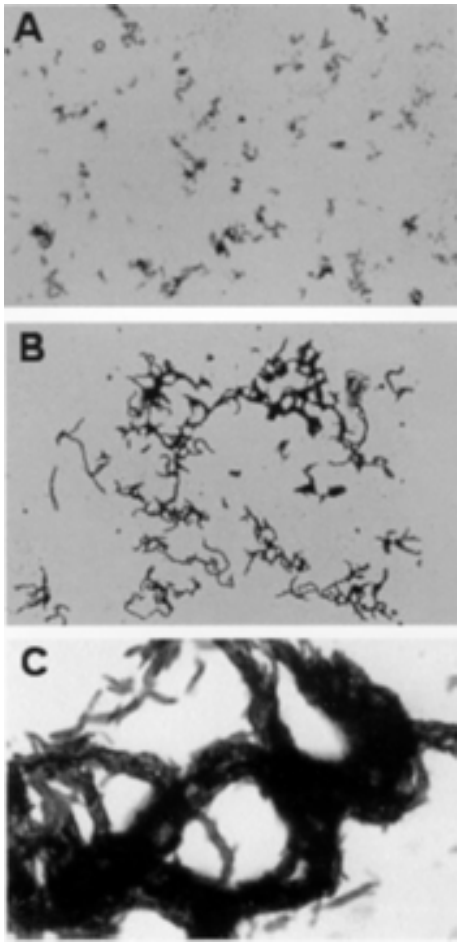

Fig. 1: positive-slide culture (test). A: sputum smear scanned with a 10x objective (approximately x100 magnification); B: sputum smear observed with a 20x objective (approximately x200 magnification); C: sputum smears observed with a 100x oil immersion objective (approximately x1,000 magnification)

In the first experiment, all the 46 air-dried control smears (not heat-fixed) and the 46 corresponding heat-fixed test smears were positive, i.e. produced cord-forming microcolonies when cultured in SLB medium (Fig. 1C). All the sputum specimes yielded positive cultures for *M. tuberculosis*. The

survival of tubercle bacilli at time zero to seven days post-heat fixation is shown in the Table. In approximately 13% (6 of 46) of the positive sputum smears, the tubercle bacilli survived on heat-fixed slides for up to seven days.

In the second experiment, all the 46 control smears produced growth on slide cultures. However, none of the corresponding Ziehl-Neelsen stained sputum smears showed growth on slides cultured in SLB medium.

Although some investigators (Blair et al. 1972, Allen 1981, Goldfogel & Sewell 1981, Peluffo & Kantor 1984) have reported that tubercle bacilli may survive on sputum smears immediately after heat fixation, our study describes the first attempt to investigate how long bacilli can survive on heat-fixed slides prior to staining.

In this study we demonstrated that heat fixation failed to sterilize all 46 sputum smears for which the corresponding control smears were positive. Our results also showed that tubercle bacilli may survive for at least seven days on heat-fixed sputum smears. Viable tubercle bacilli remaining in heat-fixed sputum smears may therefore present a potential risk of infection to laboratory personnel.

Allen (1981) found that 99% (184 of 186) of sputum smears were not sterilized by conventional flame fixation. In two other studies (Blair et al 1972, Goldfogel & Sewell 1981) in which sputum smears were heated at 75°C for 2 h and the material on the slide was removed with a sterile swab and cultured in a liquid medium, the growth of tubercle bacilli occurred in cultures from 6/10 (60%) slides (Blair et al. 1972) and from 8/10 (80%) slides (Goldfogel & Sewell 1981). Peluffo and Kantor (1984), using a procedure different from that employed in our study, found that 87 of 171 (50.9%) smear suspensions obtained from heat-fixed sputum slides yielded positive cultures for *M. tuberculosis*.

As expected, our results showed that Ziehl-Neelsen stained sputum smears failed to produce growth on slides cultured in SLB medium.

Probably the tubercle bacilli cells in these smears were killed by the action of phenol, present at a concentration of 5% in the carbolfuchsine used in our study (Smithwick 1976, Brasil 1994).

Similar results were described in a study in which a different procedure was used (Goldfogel & Sewell 1981). The sputum smears were heated at 75°C for 2 h and stained by the Ziehl-Neelsen method. The material on the slide was removed with a sterile dracon swab and cultured in Dubos broth at 35°C for six weeks. No specimen showed growth in broth cultures.

However, it is important to point out that our study demonstrates for the first time that Ziehl-Neelsen staining kills the tubercle bacilli in sputum smears after conventional fixation by heat, i.e., after the slide is passed for two to three times through the flame of a gas burner. Our results complement those of Allen (1981), who demonstrated that fluorescent staining by the auramina-phenol method killed the tubercle bacilli in sputum smears after conventional flame fixation.

As shown in the present study, viable tubercle bacilli remaining in heat-fixed sputum smears for at least seven days may present an infection risk to laboratory staff. In contrast, all tubercle bacilli were killed in sputum smears stained by the Ziehl-Neelsen method. Therefore, to render sputum smears safe for storage or shipment they should be stained immediately by the Ziehl-Neelsen method after air-drying and heat fixation or stored in a safe container to avoid transmission of tuberculosis.

ACKNOWLEDGMENTS

To Dr Janet W Reid for revision of the English text.

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TABLE

Growth frequency of tubercle bacilli in heat-fixed sputum smears cultured in selective lysed blood medium

Survival of tubercle bacilli after heat fixation[a]					
Time 0 no. (%)	24 h no. (%)	48 h no. (%)	72 h no. (%)	7 days no. (%)	Total no. (%)
22 (48)	7 (15)	7 (15)	4 (9)	6 (13)	46 (100)

a: the data are not cumulative.

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