Viability of Mycobacteria in Formalin-Fixed Lungs

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It is generally accepted that the risk of contracting tuberculosis is relatively high among medical laboratory workers and pathologists. Nevertheless, there is an assumption that once tissue is fixed in formalin, the risk for transmission and subsequent infection of mycobacteria is greatly reduced, if not altogether eliminated. To test the viability of potentially infectious mycobacteria in formalin-fixed tissue, tissue specimens from autopsy lungs fixed in formalin were cultured for mycobacteria. Of 138 cases with histologic evidence of acid-fast bacilli, 12 grew mycobacteria, including 3 *Mycobacterium tuberculosis* isolates, suggesting that there is a risk of contracting tuberculosis from tissue that has been fixed in formalin, if aerosolization or accidental inoculation should occur. HUM PATHOL 35: 571-575. © 2004 Elsevier Inc. All rights reserved.

Key words: **formalin, autopsy, lungs, mycobacteria, tuberculosis.**

Abbreviations: **TB, tuberculosis; AFB, acid-fast bacilli; HPF, high-power microscopic fields; MAC, *Mycobacterium avium* complex; MTB, *Mycobacterium tuberculosis*; NCOH, National Centre for Occupational Health; NHLS, National Health Laboratory Service; NTM, nontuberculous mycobacteria; PCR, polymerase chain reaction.**

The risk of contracting tuberculosis (TB) from pathology specimens, both in laboratories and in autopsy settings, is well recognized and documented.[1-3] Infection is said to occur through inoculation or aerosolization of mycobacteria during the examination of tuberculous tissues. There are also 2 documented cases of embalmers who have contracted TB during the embalming process.[4,5] These were the first cases in which direct infection was demonstrated, by using DNA fingerprinting.

There appears, however, to be an assumption that formalin-fixed tissue poses little or no risk of infection. This is evident from the paucity of information on this topic in the medical literature and from the lack of guidelines concerning the safe handling of formalin-fixed tissue that is infected with TB. The guidelines on preventing the transmission of TB in health care settings of the Centers for Disease Control and Prevention (CDC)[6] and the Occupational Safety and Health Administration (OSHA)[7] both fail to mention a risk posed by formalin-fixed tissue.

As early as 1949, reports in the literature described the growth of Mycobacteria from embalmed bodies,[8-10] although not much detail was provided. The chemical composition of embalming fluid is not standardized, and it is difficult to ascertain the formalin concentration used. Meade and Steenken[8] and Johnson et al[10] comment on the chemical makeup of embalming fluid, but the information is not very helpful. That first group of investigators[8] states that a formalin (10% commercial formaldehyde solution or 4% HCHO) concentration of 3% to 25% is used, whereas the latter[10] believes that the formalin concentration is 8.5%. It appears that commercial 40% formaldehyde solution, diluted 9:1, is a common ingredient of all embalming fluids.

Most specimens handled by pathologists and technologists in histology laboratories are fixed in 10% buffered formalin; this practice has continued for more than a century.

The specimens are examined and dissected, and appropriate samples are taken for microscopic examination. The efficacy of formalin as a bactericidal agent for *Mycobacterium tuberculosis* (MTB) and nontuberculous mycobacteria (NTM) such as *Mycobacterium avium*-complex (MAC) is nevertheless uncertain, and its role as a disinfectant remains unclear. Much of the uncertainty stems from the fact that formalin has been shown to have bactericidal properties in vitro, but the in vivo activity is much less apparent.[11]

The first study that attempted to culture MTB from formalin-fixed lung tissue was negative[11]; no growth occurred after 72 hours to 9 years in formalin. Those investigators[11] stated that the number of cases, namely 5, was too small for definitive conclusions. The editorial[12] accompanying that article suggested that the risk of infection from formalin-fixed tissue samples could not be ignored. The authors of the current article reported in 1998 on the isolation of MTB from formalin-fixed lungs.[13] The current article reports on a larger sample of 138 formalin-fixed lungs that were cultured for MTB.

The (South African) Mines and Works Act of 1973 stipulates that lungs and hearts of deceased mine and foundry workers be removed postmortem and sent to the National Centre for Occupational Health (NCOH) for examination, for compensation purposes, provided the next of kin agrees. Should a worker die within a 100-km radius of the NCOH, the cadaver is sent for a complete autopsy examination. The lungs are gravity inflated with formalin so that a whole lung section may be prepared and evaluated for emphysema. The majority of lungs examined come from outlying areas. The

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TABLE 1. Cases Yielding Viable Mycobacteria

Case No.	Clinical Diagnosis	No. Bacilli per 5 HPF	Days fixed in Formalin	Incubation Time Until Growth Detected (d)	Type of Acid-Fast Bacilli
1	Cavitary PTB	>20	31	16	*Mycobacterium tuberculosis*, confirmed
2	Miliary PTB	>20	45	39	*Mycobacterium tuberculosis*, confirmed
3	HIV	15	41	38	Mycobacterium species
4	Multi-drug resistant PTB	>25	74	38	Mycobacterium species
5	Miliary PTB	>25	22	31	*Mycobacterium avium*, confirmed
6	Miliary PTB	30	>10	86	Mycobacterium species
7	Miliary PTB	20	>10	56	Mycobacterium species
8	HIV	15	32	72	*Mycobacterium tuberculosis*, confirmed
9	Miliary PTB	20	27	21	Mycobacterium species
10	Miliary PTB	>25	80	36	Mycobacterium species
11	HIV	>25	36	36	Mycobacterium species
12	Fibrocaseous TB	30	38	56	Mycobacterium species

Abbreviations: HPF, high-power fields; HIV, human immunodeficiency virus; PTB, pulmonary tuberculosis; TB, tuberculosis.

lungs are removed locally and sent in 2 to 3 L of 10% buffered formalin (4% HCHO) that is prepared and supplied by the NCOH. The fixation time in formalin varies from 2 weeks to several months, and all specimens are inspected for adequate fixation upon receipt (criteria are hardening, firmness, and gray discoloration). However, it is not possible to determine fixation of the central areas of the lungs, so on arrival, all specimens are placed in approximately 10 L of 10% buffered formalin for an additional 10 days.

In South Africa, the incidence of TB has been rising concurrently with the number of patients infected with the human immunodeficiency virus (HIV). Consequently, the number of tuberculous lungs received by the NCOH has increased markedly over the past decade.

We performed a study to address the viability of mycobacteria in formalin-fixed autopsy lungs. We propose recommendations that we believe will reduce the risk of infection in laboratory personnel.

MATERIALS AND METHODS

Preparation of Tissue Samples

Over a 2-year period, from 1997 to 1999, we selected formalin-fixed lungs (as described above), with macroscopic evidence of TB of moderate to severe degree with respect to fibrocaseous areas, caseating granulomas, cavitation, necrosis, and miliary lesions. We obtained three 20-mL portions of tissue from the affected areas in each case and minced the tissue with a clean scalpel blade. The divided portions from each case were placed in 3 appropriately labeled glass vials that were stored at 4°C at the National Health Laboratory Service (NHLS) Tuberculosis Laboratory within 3 hours of the specimens being obtained. Tissue from these cases was processed at the NCOH, and Ziehl Neelsen stains were available on the paraffin sections 1 week later. All cases with more than 15 acid-fast bacilli (AFB) per 5 high-power microscopic fields (HPF), as assessed with 10-mm ocular lenses and 40× objective lenses, were processed for culture at the NHLS within 2 weeks of the microscopic findings. A total of 138 cases were cultured.

Preparation of Tissue Pellets

Approximately 3 mL of minced tuberculous tissue was taken from each vial and ground, by hand, with sea sand. A small quantity of phosphate buffer (pH 6.8) was added to the resulting paste, and the material was further ground. Tissue–sand suspensions were placed in a centrifuge tube and spun at 3000 rpm for 15 minutes. The supernatant was discarded, and the pellets were resuspended in 1.5 mL of phosphate buffer. A 0.6-mL aliquot of the pellet suspension was cultured with the BACTEC 460 radiometric system (Becton Dickinson, Sparks, MD) by inoculation into Middlebrook 7H12 medium (Becton Dickinson), to which 0.1 mL of PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Becton Dickinson) was added to prevent fungal and bacterial overgrowth. An additional 0.2-mL aliquot was planted on Lowenstein Jensen medium (Diagnostic Media Products, Rietfontein, South Africa) as a backup. The BACTEC system was monitored at weekly intervals for the presence of $^{14}CO_2$, indicative of bacterial growth.

Culture of Tissue Pellets

Samples were incubated for up to 90 days and were discarded if no growth occurred. When growth was detected, smears were prepared and examined for features of AFB by using Ziehl Neelsen staining. If AFB were detected, a polymerase chain reaction (PCR) was performed with specific primers for MTB complex (IS 6110). If the PCR was negative for MTB, the specimen was subjected to biochemical tests for the identification of NTM. When a positive mycobacterial culture was contaminated with other bacteria, an attempt was made to decontaminate the colonies with 4% NaOH before reculturing, in the hope of obtaining pure mycobacterial growth. Every precaution was taken to prevent cross-contamination with mycobacteria from other sources. In the TB laboratory, all NCOH cases were processed separately from the routine specimens, and single-use vials of decontaminating fluid and buffer were used.

RESULTS

Of the 138 cases (including those of our pilot study[13]) with histologic evidence of AFB, 12 cases (9%) grew mycobacteria (Table 1). All lungs processed for

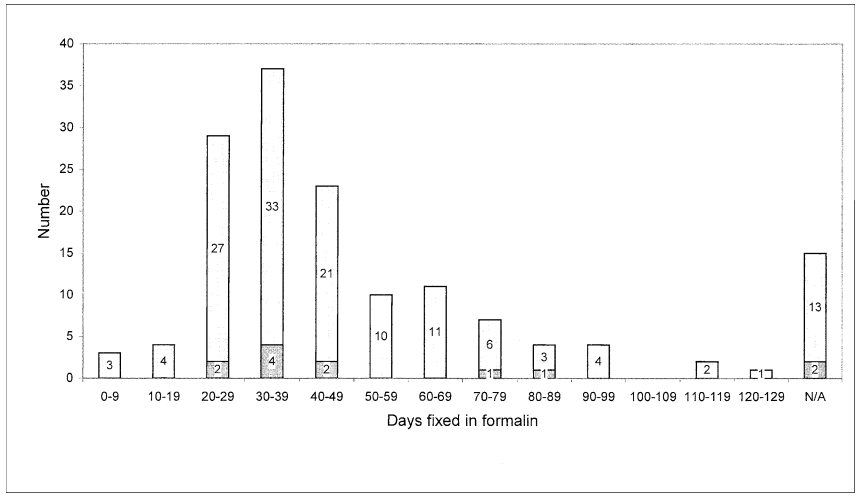

FIGURE 1. Cases with detectable growth, by period fixed in formalin. Bars indicate total number of samples, with shaded portions indicating number of cases with viable mycobacterium. NA, not available but longer than 10 days.

culture had the same macroscopic and microscopic characteristics, namely that tissues were adequately fixed and had at least 15 AFB per 5 HPF. The 12 cases with viable organisms had no special microscopic features.

Industrial Exposure

All lungs were from gold miners except 1 case (no. 11), which was from a platinum miner. Two cases (no. 4 and 10) were from the same mine but were obtained in 1998 and 1999, respectively.

Bacteriologic Species Identified

Polymerase chain reaction results and biochemical testing confirmed MTB in 3 cases (no. 1, 2, and 8) and MAC in 1 case (no. 5). On subculturing, these cases grew pure mycobacterial colonies. In 7 cases (no. 3, 4, 6, 9, 10, 11 and 12), both the mycobacteria and contaminants grew on reculturing after treatment with 4% NaOH. In 1 case (no. 7), both the mycobacteria, as demonstrated on Ziehl Neelsen preparations, and the contaminants were killed by the NaOH.

Some of the remaining 126 tissue samples grew nonmycobacterial contaminants only, and no AFB were detected on smears prepared from the colonies.

Effect of Formalin on Viability of Organisms

The number of days that the tissue was exposed to formalin did not appear to influence the viability of mycobacteria in any way. The majority of lungs (81 of 138) were fixed in formalin from 20 to 49 days; 8 of the 12 viable cases were fixed for this period (Fig 1). Two of the 12 viable cases were in formalin for 74 and 80 days (no. 4 and 10, respectively). Information about the date of death and commencement of formalin fixation in 2 cases (no. 6 and 7) was not available (Fig 2). Of special interest was a case (no. 12) in which lungs removed at autopsy at NCOH were gravity inflated with 10% buffered formalin upon removal and packed in 15 L of formalin for 38 days. Mycobacterium species grew despite the extensive treatment of the lungs with fresh formalin.

Rate of Bacteriologic Growth

We would have expected those samples with the greatest number of AFB per HPF to show the earliest signs of growth, but this was not observed. Seven samples yielded colonies of mycobacteria in 21 to 39 days, and of these, 4 cases (no. 4, 5, 10, and 11) had more than 25 AFB per 5 HPF. Two cases had 15 and 20 AFB per 5 HPF (no. 3 and 9, respectively). It is notable that

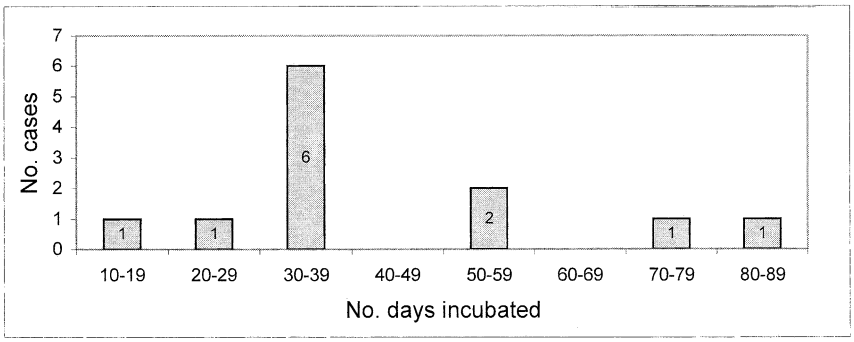

FIGURE 2. Cases with detectable growth, by incubation period.

2 cases (no. 12 and 6) with 30 AFB per 5 HPF showed growth after 56 and 86 days' incubation, respectively (Table 1).

Antibiotic Treatment Before Death

Information about antibiotic treatment before death was not obtainable in all cases. However, tissue from 3 cases (no. 3, 8, and 11) was from HIV-infected patients. One case (no. 4) was multidrug-resistant TB, and 1 case (no. 12) was fibrocaseous TB. The remainder were clinically diagnosed as miliary TB.

Contaminant Organisms

All cases had contaminant organisms that also grew on the media. These contaminants were non-AFB, and we assume that they were present in the tissue samples as a result of either contamination during removal or infection during life. However, on microscopy there was no evidence of additional infection other than TB in the viable cases.

DISCUSSION

In a small study using gravity-inflated tuberculous lungs that were perfused with 10% buffered formalin from which 1- to 1.5-cm-thick sections were prepared and stored in formalin-filled plastic bags, Kappel et al[11] sampled 5 cases. The first case was sampled after 3, 14, and 30 days' fixation. The remaining cases were sampled between 45 days and 9 years after fixation. Smears from each tissue sample were positive for AFB by Ziehl Neelsen staining. One-milliliter portions were planted on various appropriate media and incubated for an unspecified time. None of the samples yielded any growth. To our knowledge, this is the only reported investigation of separated formalin-fixed lungs before our study.

In previous investigations, no mention was made as to the abundance of AFB in tissue sections, as expressed as number of bacilli per HPF. Albeit crude and imprecise, it is a useful index of infection. Whereas Kappel et al[11] used a single 1-mL tissue aggregate, we used three 20-mL aggregates for each case and found that only 1 or 2 produced mycobacterial colonies for each case that turned out positive for viable organisms. Many of the patients in the current study may have been on TB treatment, rendering the bacilli nonviable, but the details of this and the duration of treatment were largely unknown. Nevertheless, we believe that our extensive sampling reduced the possibility of missing viable bacilli and that, had more aggregates per case been obtained, we would probably have had more positive cases.

Although uncertainty prevails with respect to the infectivity of fixed tuberculous tissues, direct and indirect evidence from fresh tissue is not disputed. Bogen[14] notes that many anatomists in the past avoided tuberculous postmortem material and mentions that Laennec became infected while performing an autopsy in 1800. Aware of a high incidence of newly acquired TB among interns on autopsy services, Bogen[14] kept caged guinea pigs "a few feet" from the dissecting table. In a series of 600 tuberculous autopsies, 3 animals became infected.

In an epidemiologic study, Meade[15] found that most tuberculin conversions in medical students took place during autopsy rotations. When handling of infectious tissues was halted, a reduction in conversions was noted. Lundgren et al[16] reported the infection of 3 medical students and 1 technician after participation in 2 tuberculous autopsies.

Reid[17] proved, by way of an epidemiologic study, a higher incidence of TB in pathologists and technologists as compared with a control group of engineers and clerks. Sterling et al[4] reported a case of TB in an embalmer who handled an infected cadaver. Those investigators[4] pointed out that handling of fresh body fluids might have resulted in aerosolization and subsequent transmission of infection.

Routine preventive measures are the rule in the handling and processing of fresh tuberculous tissues, but there is a false sense of safety with respect to fixed specimens. Smith,[12] in an article previously published in Human Pathology, questioned the procedures used in culturing mycobacteria from fixed tissue, suggesting that false-negative results might be obtained in the processing. He raised doubts as to the bactericidal efficacy of formalin fixation because of its degradation due to reaction with tissue, thereby permitting tubercle bacilli to survive. We believe that our use of larger tissue samples and the detection of viable mycobacteria confirm Smith's comment.[12]

A major limitation of the study was the inability to identify the species of mycobacteria in the AFB-positive cultures, which gave negative TB PCR results. Because TB PCR is not 100% sensitive for MTB, we may have underestimated the presence of this species in the acid-fast cultures. Bacterial contamination was a major problem and difficult to deal with because mycobacteria in paucibacillary specimens may be compromised by the standard decontamination methods, which can destroy up to 60% of viable bacilli. It is most difficult to achieve a balance between adequate decontamination with NaOH and preservation of live mycobacteria.

Mycobacterial cross-contamination is a well-recognized problem in tuberculosis laboratories. However, specific precautions were taken to reduce the possibility of MTB cross-contamination. Specimens were processed individually, and separate vials of reagents and buffer were used for each specimen. These liquids were not, however, cultured after use, to exclude contamination.

Although NTM found in soil, water, food, dust, and laboratory stock solutions may be a contaminant, the majority of our positive cultures were from deceased patients diagnosed in life as having TB (cases were confirmed as granulomatous inflammation with AFB on microscopic examination of lung tissue). Therefore, contamination is highly unlikely as a source of viable bacilli.

Although viable MTB raises concern for transmission to pathologists and technologists through inoculation or aerosolization, the potential danger of NTM is not clear. Three of the patients were known to be infected with HIV, and some of the mycobacteria recovered may not be pathogenic in people with an intact immune system. Nevertheless, it is well to consider that many NTM do indeed cause disease, such as *Mycobacterium marinum, M. kansasii,*and *M. avium* complex. Perhaps the real significance of our study lies in the fact that if mycobacteria species can survive formalin fixation of infected tissue, then surely MTB, when present, will remain alive in a significant percentage of such specimens and pose a threat to personnel who are charged with handling and processing the tissue. It is recommended that the use of gloves, gown, and mask be a standard procedure when handling known or suspected cases of infected tissues, even when the tissues have been formalin fixed.

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