OBSERVATIONS ON SOME HAZARDS OF WORK IN THE TUBERCULOSIS DIAGNOSTIC LABORATORY

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Walter A. Miller

1955

This is to certify that the

thesis entitled

Observations on Some Hazards of Work in the Diagnostic Tuberculosis Laboratory.

presented by

Walter A. Miller

has been accepted towards fulfillment of the requirements for

M.S. degree in Bacteriology

Major professor

Date May 20, 1955

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By
WALTER A. MILLER

A THESIS

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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Department of Bacteriology and Public Health

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The writer wishes to express his appreciation to Professor W. L. Mallmann, Department of Microbiology and Public Health, Michigan State University of Agriculture and Applied Science, for the suggestions and assistance given him on this study.

Appreciation is also expressed to Samuel R. Damon, Ph.D., Director, Bureau of Laboratories of the Indiana State Board of Health for making available the laboratory facilities to conduct this study.

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The occurrence of viable tubercle bacilli on the outside surface of specimen bottles used to submit sputum to a laboratory for tuberculosis examination were determined. The outside surface of 297 sputum bottles was washed with four percent sodium hydroxide. The sodium hydroxide was then neutralized with 2 N hydrochloric acid and a portion of this neutralized solution was cultured on Lowenstein-Jensen's tuberculosis medium. Twenty percent of all specimens containing tubercle bacilli in the sputum were found also to have virulent tubercle bacilli on the outside surface of the specimen bottle.

The ability of tubercle bacilli to survive two different acid-fast staining procedures was investigated. Duplicate slide preparations were made from twenty five cultures of Mycobacterium tuberculosis. One set was stained with the Ziehl-Neelson technique and a second set was stained by a method in which a wetting agent was added to the stain to aid penetration. Cultures of organisms removed from slides stained with the Ziehl-Neelson technique failed to produce growth. Cultures of organisms removed from slides stained by the method using the wetting agent produced eleven isolations.

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INTRODUCTION

Improved techniques in diagnostic procedures, for the laboratory diagnosis of infectious diseases, have been consistent with the progress achieved by research in allied scientific fields. These improved techniques have raised the quality of diagnostic tests, which in turn has increased the demand for these tests, by practicing physicians. To meet these demands it has sometimes been necessary to increase the work load of the laboratory beyond the limits dictated by the availability of adequately trained personnel. Adoption of these new and improved methods too often occurs without due consideration being given to the increased hazards of infection which are sometimes introduced by these newer techniques. Many laboratory workers are trained to perform diagnostic tests on the job, without adequate training in protecting themselves against the hazards involved in handling diagnostic specimens.

Protection of those who come in contact with infectious agents in the laboratory is of utmost importance. Anderson (1) states that "We must not take it for granted that scientists will invariably be infected by the materials with which we work. If the probability exists, greater efforts should be made to remove it".

REVIEW OF THE LITERATURE

A comprehensive survey has been made by Sulkin and Pike (10) of infection in this country among laboratory workers. A total of 1,334 infections in the United States was tabulated. The infections presumably were acquired as a result of laboratory exposure. Thirty-nine cases resulted in death, a fatality rate of 3.0 percent. Approximately one-third

of the infections had been reported in the literature. The remainder was detected through questionnaires mailed to approximately 5,000 laboratories including those associated with state and local health departments, accredited hospitals, private clinics, schools of veterinary science, under-graduate teaching institutions, biologic manufacturers, and various governmental agencies. The majority of the cases reported occurred during the last 25 years.

Of the laboratory-acquired infections, 775 were bacterial, 261 viral, 200 rickettsial, 29 parasitic and 61 fungi. The performance of diagnostic tests and research accounted for at least 668 of the 775 bacterial infections while the remaining 107 occurred in classwork, production of biologics or during the performance of a combination of those activities.

HAZARDS IN DIAGNOSTIC AND RESEARCH LABORATORIES

During the past few years considerable attention has been directed to the hazards of infection to which microbiologists are exposed during the course of routine work in diagnostic and research laboratories. Studies conducted at Camp Detrick, Maryland (9) showed that bacterial aerosols are produced by some of the common laboratory operations. Sieve type air samplers, placed at appropriate distances around laboratory working areas were used to demonstrate bacterial aerosols produced by the removal of stoppers from dilution bottles and removal of inocula from vaccine bottles with a hypodermic syringe as well as various pipetting and mixing procedures. A combination of high speed photography and the sieve type air sampler was used to demonstrate bacterial aerosols produced by the high speed blendor.

Sulkin and Pike (10) indicated that infections in laboratory personnel have occurred with much greater frequency in scientifically trained workers

than in animal caretakers and lessor trained laboratory assistants.

THE HAZARDS OF WORK IN TUBERCULOSIS

The incidence of laboratory acquired tuberculosis was second among bacterial infections with 153 cases reported. Trained scientific workers were involved in 136 cases. The remaining 17 cases involved students, animal caretakers, dishwashers, and janitors.

Most of the tuberculosis infections, with the exception of autopsy infections, were contracted from clinical specimens. Other sources of infections included laboratory accidents, aerosols, and handling discarded glassware whereas some sources were not indicated and were considered as undetermined. The infections are more often pulmonary than non-pulmonary. Long (3) pointed out that since most infections appear to be the results of diagnostic procedures they are perhaps to be explained on the grounds that greater danger exists when working with materials of unknown character than with those of known character.

Although the monetary loss involved in laboratory infections with tuberculosis is not the most important loss it cannot be overlooked. Plunkett (7) estimated that when one considers not only the actual cost of a case of tuberculosis, but also allied expenditures, the total cost is approximately $10,000 per case.

During the past few years, tuberculosis laboratories have adopted the procedure of culturing specimens to recover tubercle bacilli. This procedure has been shown to be superior to the simple acid-fast stained film examination. However, the adoption of the culture procedure has introduced hazards to the worker which were not experienced in the examination of stained film preparations. Failure to recognize these hazards

may well be the reason for the high incidence of laboratory infections with tuberculosis cited by Sulkin and Pike (10), Long (3), Fish and Spendlove (2).

ONE POSSIBLE SOURCE OF WORKER INFECTION IN THE TUBERCULOSIS DIAGNOSTIC LABORATORY

A common opinion shared by workers in the tuberculosis laboratory is that a possible source of infection is in handling contaminated specimen containers. Although this opinion is rarely denied it is not emphasized to the extent that adequate precautionary measures are usually taken during the processing of the specimens.

The specimen bottles generally used by health departments for the collection and submission of sputum specimens to the laboratory consist of a glass bottle 65 mm. high with a diameter of 35 mm. fitted with a cork or rubber lined metal screw cap. This bottle is of convenient size,and,when enclosed in a double container mailing outfit, complies with the regulations of the United States Postal Service.

Specimen collection outfits are usually furnished to physicians by the health department. The physicians then give the containers to the patient and instructs him to deposit the sputum in the bottle and mail it to the laboratory. The patient often holds the bottle before him as he coughs to produce the specimen. Aerosols of infectious material may be expelled by this action. Tiny droplets may dry on the outer surface of the bottle. Furthermore, a bottle is often contaminated directly by the patient as he deposits the specimen but, regardless of the manner in which the bottle becomes contaminated, tubercle bacilli dried in mucus remain viable for long periods of time. These contaminated bottles become a hazard to those who come in direct contact with them in the laboratory.

Before cultural techniques gained widespread acceptance as the

more efficient and exacting examination, sputum was examined by simply spreading a small amount of the material on a glass slide. The film was stained by one of several methods for acid-fast bacilli and examined microscopically. A specimen to be examined by this method could be sterilized by steam under pressure before it was opened. De-contamination of diagnostic specimens by steam under pressure is impossible when cultural procedures are to be employed and, thus, a contaminated specimen bottle becomes a hazard.

PURPOSE OF THIS STUDY

The following study was undertaken to demonstrate the possible occurrence of viable tubercle bacilli on the outside surface of specimen bottles which are used to submit sputum specimens to the laboratory for tuberculosis examination.

Specimen containers used for this study were those which were furnished to physicians throughout the state of Indiana by the State Board of Health for the collection of sputum for tuberculosis culture examination. The collection bottle has been described above.

Infectious organisms present on the outer surface of these bottles may be kept viable by the dried mucus. The bottle thus becomes a hazard to the worker unless some measure is taken to decontaminate it in the laboratory.

EXPERIMENTAL

When contamination does occur the number of tubercle bacilli present may be few. It was therefore necessary to devise a method to wash the surface of the bottle with a small amount of liquid which would be capable of removing the mucus but would not be toxic to the tubercle

bacilli. The washing solution must also be bactericidal for non-pathogenic organisms, present on the bottles. The effect of several washing solutions on several types of saprophytic mycobacteria for various times of exposure was first studied.

Test organisms used:

1. Mycobacterium phlei
2. Mycobacterium smegmatis
3. Mycobacterium designated as white saprophyte
4. Mycobacterium designated as radish bacillus

Washing solutions studied:

1. Physiological saline
2. Tri-sodium phosphate ($Na_3PO_4 \cdot 12\ H_2O$) 23 percent
3. Five percent solution of non-ionic detergent (Triton X-100) in 23 percent tri-sodium phosphate
4. Two percent solution of non-ionic detergent (Triton X-100) in 23 percent tri-sodium phosphate
5. Five percent solution of non-ionic detergent (Sharples 2181) in 23 percent tri-sodium phosphate
6. Two percent solution of non-ionic detergent (Sharples 2181) in 23 percent tri-sodium phosphate
7. Four percent solution of sodium hydroxide (without neutralization)
8. Four percent solution of sodium hydroxide (with neutralization)

I PROCEDURE FOR DETERMINING SUSCEPTIBILITY OF MYCOBACTERIA TO WASHING SOLUTIONS

1. Test organisms were transferred from original culture slants (Lowenstein-Jensen) to tubes containing 10 ml. of liquid Dubos medium and incubated at 37 C. for 72 hours.
2. The 72 hour cultures were placed in a mechanical shaker for 15 minutes to disperse organisms evenly throughout the liquid medium.

This procedure was repeated twice.

3. One-tenth ml. of the suspension of the third sub-culture of each test organism was transferred to a tube containing 9.9 ml. of each of the washing solutions listed above.

4. Immediately following mixing of the organisms with the first seven washing solutions listed above, 0.1 ml. was transferred to two tubes of Lowenstein-Jensen's tuberculosis medium slants. This procedure was following with all solutions except the 4 percent sodium hydroxide (see page 6). This sodium hydroxide solution was neutralized with 2 N hydrochloric acid with phenol red added as an indicator. One-tenth ml. of the neutralized sodium hydroxide was then transferred to two tubes each of Lowenstein-Jensen's medium.

5. One-tenth ml. of each washing solution containing the test organisms was withdrawn after exposure to each solution for 1, 2, 3, 4, 24, and 48 hours and placed in two tubes of Lowenstein-Jensen's medium.

6. The Lowenstein-Jensen slants were incubated and any colonial growth observed on the slants was recorded as 4+, indicating very heavy growth with no evidence of inhibition, 3+ indicating a detectable amount of inhibition, 2+ with 15-20 colonies, 1+ with 1-15 colonies and negative indicating no growth.

7. Physiological saline was used for a control. The growth obtained on slants from inocula transferred from saline for the various exposure times was used to compare the inhibition of the various washing solutions for the same exposure times.

The results are reported in Tables 1, 2, and 3.

TAPLE I

A. Results

1. GROWTH ON CULTURES TRANSFERRED IMMEDIATELY FOLLOWING EXPOSURE TO WASHING SOLUTION.

Washing Solution	M.phlei	M.smegmatis	White Saprophyte	Radish Bacillus
Saline (control)	4+	4+	4+	3+
TSP* - 23% ($Na_3PO_4 \cdot 12\ H_2O$)	4+	4+	4+	3+
TSP + Triton X-100 (5%)	3+	3+	4+	3+
TSP + Triton X-100 (2%)	2+	2+	4+	1+
TSP + Sharples 218l (5%)	1+	2+	3+	-
TSP + Sharples 218l (2%)	1+	-	3+	-
4% NaOH (not neutralized)	-	-	4+	-
4% NaOH (neutralized)	4+	4+	4+	4+

*TSP Tri-sodium phosphate

4+ very heavy growth with no evidence of inhibition

3+ a detectable amount of inhibition

2+ 15 to 20 colonies

1+ 1 to 15 colonies

- no growth

TABLE 2

2. GROWTH ON CULTURES TRANSFERRED AFTER 1 HOUR EXPOSURE TO WASHING SOLUTION

Washing Solution	M. phlei	M. smegmatis	White Saprophyte	Red Dish Bacillus
Saline (Control)	4+	4+	4+	4+
TSP* - 2% ($Na_3PO_4 \cdot 12 H_2O$)	3+	4+	4+	1+
TSP + Triton X-100 (5%)	-	2+	3+	1+
TSP + Triton X-100 (2%)	1+	2+	3+	-
TSP + Sharples 218l (5%)	1+	-	3+	-
TSP + Sharples 218l (2%)	-	-	3+	-
4% NaOH (not neutralized)	-	-	-	2+
4% NaOH (neutralized)	4+	4+	4+	4+

TSP* Tri-sodium phosphate

4+ very heavy growth with no evidence of inhibition

3+ a detectable amount of inhibition

2+ 15 to 20 colonies

1+ 1 to 15 colonies

- no growth

TABLE 3

3. GROWTH ON CULTURES TRANSFERRED AFTER 2 HOURS EXPOSURE TO WASHING SOLUTION

Washing Solution	M.phlei	M.smegmatis	White Saprophyte	Radish Bacillus
Saline (Control)	4+	4+	4+	4+
TSP* - 23% ($Na_3PO_4 \cdot 12\ H_2O$)	2+	1+	4+	2+
TSP + Triton X-100 (5%)	-	-	2+	-
TSP + Triton X-100 (2%)	-	1+	3+	-
TSP + Sharples 2181 (5%)	-	-	2+	-
TSP + Sharples 2181 (2%)	-	-	-	-
4% NaOH (not neutralized)	-	-	3+	-
4% NaOH (neutralized)	4+	4+	4+	4+

* TSP Tri-sodium phosphate

4+ very heavy growth with no evidence of inhibition

3+ a detectable amount of inhibition

2+ 15 to 20 colonies

1+ 1 to 15 colonies

- no growth

Although transfers were made also after 3,4,24, and 48 hours exposure to the washing solutions growth was observed only in the saline control and in the neutralized 4% NaOH tubes. Therefore, 4% NaOH was selected as the solution of choice and was neutralized immediately following the washing of each specimen bottle.

II PROCEDURE FOR WASHING THE OUTER SURFACE OF UNOPENED BOTTLE CONTAINING PATIENTS SPUTUM AS RECEIVED IN THE LABORATORY

The glass sputum bottle was removed from the mailing case and placed in a glass ointment jar. Ten ml. of 4% NaOH with phenol red indicator was added to the ointment jar. A metal screw cap was placed on the jar containing the sputum bottle and the 4% NaOH. This bottle was then rotated manually so that the 4% NaOH was allowed to wash the entire surface of the specimen bottle to remove viable tubercle bacilli which might be present. The washing solution was immediately neutralized with 2 N HCl and a portion of the neutralized washing was then placed on slants of Lowenstein-Jensen tuberculosis medium. The washed specimen bottle with the diagnostic specimen was then returned to the tuberculosis laboratory and the sputum was cultured in the routine manner. Cultures from these washings were observed weekly for six weeks for colonial growth of tubercle bacilli.

A. Observations

The outer surface of 297 sputum specimen bottles was washed in the manner described above. Sixty isolations of tubercle bacilli were obtained from these 297 specimens in the course of routine culture examination. The washings from the outer surface of the 297 specimen bottles yielded in 12 cultures of tubercle bacilli. Therefore, 20 percent of those specimens found positive in the routine examination were shown to

have viable tubercle bacilli on the outer surface of the bottle.

THE VIABILITY OF TUBERCLE BACILLI AFTER ACID-FAST STAINING

Several modifications of the original Ziehl-Neelson method for staining smear preparations of tubercle bacilli have appeared in the literature (8). Although no claim is made for sterilization of bacterial cells stained by these methods, some bacteriologists depend upon the alcohol and phenol used in the staining solution to destroy the bacilli. The heat employed by the original technique is also relied upon to "kill" tubercle bacilli.

Middlebrook and his co-workers (5) have shown that M. tuberculosis has a tendency to grow in a corded or serpentine configuration and when such suspensions, consisting of clumps of bacilli are stained, it is possible that organisms in the center of these clumps are protected from the bactericidal substances in the staining solution.

The preparation of slides for microscopic examination of "raw" diagnostic specimens and of "live" cultures is common practice in many tuberculosis laboratories. Slides with the infectious material may be allowed to "air dry" before staining. The process of drying is sometimes hastened by placing the unprotected slide over a hot plate and convection currents produced by the heat may result in the flaking of particles from the slide and the dissemination of infectious material.

The ability of virulent tubercle bacilli to withstand two different methods of acid-fast staining was studied. The original acid-fast stain procedure described by Ziehl-Neelson in 1882 (8) was compared with a modification of the Kinyoun carbolfuchsin acid-fast stain reported by Muller and Chermock (6).

Duplicate slides were prepared from 25 cultures of virulent tubercle

bacilli grown on Lowenstein-Jensen medium. The slides were placed in covered Petri dishes and dried in an incubator at 37 C. for 24 hours. One slide from each culture was placed on a staining rack and stained by the Ziehl-Neelson technique.

Procedure

1. Each slide was flooded with a solution made of 10 ml. (10 percent alcoholic solution) of basic fuchsin and 100 ml. of a 5 percent aqueous solution of phenol.
2. Slides were steamed gently over a flame for 5 minutes. (The slides were not boiled). Stain was added to the slides as it evaporated.
3. The slides were then washed with water and decolorized with 3 percent HCl in acid alcohol until no color flowed from the slides.
4. The preparations were then counterstained with a solution of methylene blue.

The duplicate slides were stained by the modified procedure of Kinyoun as described by Muller and Chermock (6).

The carbolfuchsin for this stain was prepared as follows:

Basic fuchsin	4 gm.
Phenol	8 ml.
Alcohol (95 percent)	20 ml.
Distilled water	100 ml.

The basic fuchsin was dissolved in alcohol and added to the water while shaking. The phenol was melted in a 56 C. water bath and 8 ml. was added to the stain with a pipette.

Turgitol number 7, a wetting agent, was added to the Kinyoun carbolfuchsin stain in the ratio of 1 drop to 30-40 ml. of stain.

The slides were flooded with this solution for 5 minutes (the original instructions specify 1 minute), no heat was applied to the slides in this series. The slides were then washed with water and decolorized with 3 percent HCl in acid alcohol until no color flowed from the slides. The preparations were then counterstained with a solution of methylene blue.

After the film preparations were stained each slide was placed in an ointment jar to which had been added 5 ml. of 4 percent NaOH with phenol red added as an indicator. The jars were placed in a wire basket and placed on a Kahn shaker for 10 minutes to remove as much of the stained film as was possible.

The NaOH from each jar was titrated to the neutral point with 2 N HCl. A portion from each of the neutralized washings was placed on two tubes each of Lowenstein-Jensen's tuberculosis medium.

Cultures inoculated from washings of the 25 slides stained by the original Ziehl-Neelson failed to produce colonies of tubercle bacilli. Washings from slides stained with the modified techniques described by Muller and Chermock produced 11 positive cultures.

SUMMARY

No detrimental effect was observed after the test organisms had been in contact with physiological saline for as long as forty-eight hours.

Slight toxic effects were noted from the use of Tri-sodium phosphate for one hour. A sharp increase in toxicity was noted at two hours. No growth was obtained in the subcultures after twenty-four and forty-eight hour exposure periods. The toxicity of this solution is of particular interest since this chemical is commonly used as an agent for decontaminating and digesting sputum in many laboratories.

Combinations of non-ionic detergents and tri-sodium phosphate proved to be even more toxic than tri-sodium phosphate used alone.

Test organisms kept in four percent sodium hydroxide for approximately ten minutes before neutralization with 2 N hydrochloric acid were not affected.

Twelve isolations of M. tuberculosis obtained from the outer surface of contaminated specimen bottles clearly supports Long's (3) suggestions that greater dangers may exist when working with materials of unknown character.

A change of procedure was introduced in the tuberculosis laboratory of the Indiana State Board of Health as a result of the findings of this study. All tuberculosis specimens submitted to the laboratory for diagnosis are assumed to be contaminated and are transferred to clean sterile bottles before they are processed.

The recovery of viable tubercle bacilli from "cold" stained film preparations indicate a possible hazard not only to laboratory workers but to wash room workers as well. This hazard can be eliminated completely by sterilizing all film preparations with steam under pressure before staining.

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