DEMONSTRATION OF *MYCOBACTERIUM LEPRAE* IN SECTIONS IN 532 CASES OF LEPROSY

COMPARATIVE STUDY BETWEEN THE ZIEHL-KLINGMULLER AND THE WADE-FITE TECHNIQUES [1]

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Those who are accustomed to routine histopathological work in leprosy are aware of the difficulties of demonstrating *M. leprae* in sections by the usual methods of acid-fast staining. The difficulty of this demonstration may be increased by the effects of sulfone treatment. In the past several workers (1–5) attempted to improve the technique. In an article with Portugal (7) we showed the value of the Gram-Weigert method in this matter.

There is no doubt that the acid-fastness of the organisms of the genus Mycobacterium depends upon the presence of lipidic substances in its constitution. It is also a fact that a large part of these lipidic substances are extracted by such reagents as alcohol and xylol used in routine histological technique; this fact accounts for the partial or total loss of the acid-fastness of *M. leprae* when paraffin sections are used. As has recently been pointed out by Wade (8), in an article which should be read by those interested in this matter, there are two fundamental principles by which these difficulties may be overcome.

1. The principle of restoring the acid-fastness of the organisms. This principle was involved in a completely new technique published in 1938 by Faraco (1). This depends on refatting the germs after they have been rendered nonacid-fast in the imbedding and deparaffinizing sequences, by heating them with some substance such as a vegetable oil, or chicken fat, or even lubricating oil before staining them with fuchsin. While attempting to improve this technique, Fite, Cambre and Turner (4) hit upon a method which Lillie (6) calls the "Fite-Faraco" method. The appropriateness of this name is denied by Wade, as will be seen.

2. The principle of protecting the acid-fastness of the organisms. In the article referred to, Wade claims priority for this principle, one which he had employed for over 25 years but had not previously published. He points out that when the bacilli are subjected to the influence of any of the reagents used in clearing the tissues in the imbedding process, they are made liable to ("conditioned for") lipid extraction when they are again treated with a fat solvent in removing the paraffin. To avoid that

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[1] Presented at the VI International Congress of Leprology, Madrid, October 1953, in the Portuguese language. Translated, with certain modifications approved by the authors.

sequence ("double jeopardy") he originally used essential oils throughout the process—bergamont or origanum oil for deparaffinizing and anise oil for clearing the sections after staining.

This method is cumbersome and has been abandoned by Wade in favor of what he regards as an improvement of the one devised by Fite and associates (4). He holds that that method is not one of refatting but of protection; the sections are deparaffinized by a mixture of a paraffin solvent and an oil, the latter protecting the bacilli while the wax is being removed. Consequently the term "Fite-Faraco" is a misnomer. His own improvement, the "Wade-Fite" method, is given in detail below.[2]

In order to ascertain the value of this new technique we decided to undertake a comparative study of it with the Ziehl-Klingmüller method used routinely in this laboratory.

MATERIAL AND TECHNIQUE

The material studied consisted of 532 skin specimens from sulfone-treated and untreated patients. These specimens had been fixed in 10 per cent formol in physiological serum and imbedded in paraffin by the usual histological process. The staining techniques as we used them are as follows:

I. Ziehl-Klingmüller Technique

1. Xylene, two times until completely deparaffinized.
2. Alcohol sequence, 100%, 90%, 70%; wash in tap water.
3. Carbol-fuchsin, 25 to 30 minutes, at room temperature; wash.
4. Decolorize with 5% sulphuric acid; wash.
5. Stain with Carrazzi hematoxylin, 5-10 minutes.
6. Water, 10 minutes.
7. Alcohol sequence, 70%, 90%, 100%.
8. Clear with xylene.
9. Mount with Canada balsam.

II. Wade-Fite Technique

1. Deparaffinize with a mixture of equal parts of liquid petrolatum and rectified turpentine.
2. Blot with filter paper until of semi-dry appearance.
3. Wash with tap water, 5 minutes.
4. Carbol-fuchsin, 25 to 30 minutes, at room temperature; wash.
5. Blot with filter paper.[3]
6. Decolorize with 20% sulphuric acid; wash.
7. Stain with Carrazzi hematoxylin, 5-10 minutes.[3]
8. Water, 10 minutes.
9. Blot with filter paper and place in an incubator at 56° to dry.[2]
10. Mount with Canada balsam.[3]

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[2] In the same article Wade described the use of a water-soluble imbedding wax, a mixture of solid polyethylene glycols called Carbowax by the manufacturer, by which the use of fat solvents is avoided entirely, but that has nothing to do with the present investigation.

[3] In these steps we departed from the original technique as published by Wade, who (*a*) does not blot before decolorizing, (*b*) specifies dilute Loeffler's methylene blue for counterstaining, (*c*) allows the sections to dry at room temperature, and (*d*) mounts in any of the modern synthetic resins, which cause less rapid fading than does Canada balsam.

RESULTS

The findings in the comparison of duplicate sections stained by these two methods are given in Table 1. Statistical study of the figures there given shows that the differences are significant.

TABLE 1.—*Comparison of the Ziehl-Klingermüller (Z-K) and Wade-Fite (W-F) techniques in the demonstration of* M. leprae *in 532 paraffin-imbedded skin biopsy specimens.*

Findings	Number of cases	Percentage of cases
Both sections negative	237	44.6
Both positive and quantitatively equal	80	15.0
Both positive, more bacilli in W-F	115	21.6
Both positive, more bacilli in Z-K	9	1.7
W-F positive, Z-K negative	88	16.5
Z-K positive, W-F negative	3	0.6
Totals	532	100.0

The most interesting feature of these findings is the number of specimens which are found positive by the Wade-Fite technique but negative by the ordinary Ziehl-Klingmüller method. We repeated the examination two or more times with 19 of these 88 specimens and obtained positive results with the Ziehl-Klingmüller method in only three instances, although one which was Wade-Fite positive in the first examination was negative in the later ones.

Detailed study of these 88 cases showed: (*a*) Two which were 3+ with W–F and negative with Z–K were, on repetition, 1+ and 2+, respectively, with Z–K.

(*b*) Six were 2+ with W–F but negative with Z–K. One of these, on repetition, was 1+ with Z–K. Another changed from 2+ with W–F to 3+ with that method but remained negative with Z–K. It is to be noted that the original smear of the latter case, made from the biopsy specimen and stained by Ziehl-Gabbett, was positive.

(*c*) Eighty were 1+ with W–F and negative with Z–K. Smears from the biopsy specimens had been made in 38 of these cases, and 9 were positive (1+) while the other 29 were negative.

Referring to the 3 cases which, as shown in the table, were found to be positive with Z–K (1+) but negative with W–F, the following was found on repetition:

(*a*) One became positive (1+) with W–F.

(*b*) One gave the same results as before.

(*c*) One had revealed only very rare acid-fast bacilli in one small nerve twig found in the Z–K section.

Mention may be made of the marked difference in size of the two groups of cases in which the bacilli were more numerous in one section than the other. This was the case in no less than 115 cases—more than 20 per cent of the whole—with the W–F method but only 9 cases with the Z–K method.

SUMMARY AND CONCLUSIONES

The authors have made a comparative study of two methods of staining *M. leprae* in paraffin sections of biopsy specimens of the skin, namely, the Ziehl-Klingmüller method and the Wade-Fite in which, according to Wade, the bacilli are protected from extraction of their lipids during the deparaffinization. The data of the findings show differences which are statistically significant, and the following conclusions are drawn.

1. That the Wade-Fite method of staining gives better results in demonstrating *M. leprae* than does the Ziehl-Klingmüller method.
2. Repeated examinations of a number of specimens showed that in most cases the results remained the same, thus demonstrating that positivity actually depends on factors inherent in the method itself.
3. The fact that there were 38 cases found positive (1+) with the Wade-Fite method but negative with the other, of which only 9 were positive in smears made from the biopsy specimens, suggests that the new technique is not exclusively one of protection of the acid-fastness of *M. leprae* in the lesions but that it also has the property of restoring acid-fastness which has been lost in the tissues.

RESÚMEN

Los autores han hecho en estudio comparativo de dos métodos para la tinción de *M. leprae* en secciones de parafina en biopsias de piel. Los dos métodos fueron los de Ziehl-Klingmüller y Wade-Fite; en éste último, de acuerdo con Wade los bacilos son protegidos de la extracción de sus lípidos durante la de-parafinzación. Los datos obtenidos tienen significado estadístico y se puede llegar a las siguientes conclusiones.

1. Que el método de Wade-Fite dá mejores resultados en la tinción de *M. leprae* que el método de Ziehl-Klingmüller.
2. Exámenes repetidos en los mismas biopsias demostraron resultados comparables lo que significa que la "positividad" depende de factores inherentes en el método.
3. Debido a que hubo 38 casos positivos (1+) con el método Wade-Fite, pero negativos con el otro, de los cuales solo 9 fueron positivos en frotis de las lesiones, se sugiere que la nueva técnica no solo protege la acido-resistencia de *M. leprae*, sinó que también tiene la propiedad de restorar ésta propiedad la cual ha sido perdida en los tejidos.

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