FIELD METHOD FOR CONCENTRATING *MYCOBACTERIUM LEPRAE* IN SKIN BIOPSY SPECIMENS[1,2]

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A new field method for concentrating mycobacteria from skin biopsy specimens has been developed which greatly improves chances for bacteriologic diagnosis. The potential utility of such a procedure lies in an increase in diagnostic precision in doubtful cases of leprosy, and the possibility of initiating epidemiologic studies to discover whether there are asymptomatic or subclinical carriers of acid-fast bacilli in areas where leprosy is endemic.

Several attempts have been made to improve present technics of finding and recognizing leprosy bacilli. The standard procedure (Wade) is generally called the "scraped incision" method, because a smear is made of tissue fluid scraped from a shallow skin incision ([6]).

Several concentration procedures have been reported, including one developed by Khanolkar and Rajalakshmi ([5]) in which the concentration of bacilli was sufficient to permit their discovery in cases of tuberculoid leprosy. The need for special equipment and somewhat complicated procedures made these methods impractical for general field use, however. Dharmendra and Mukherjee reported a concentration procedure in which skin biopsy specimens were ground directly in chloroform ([1]).

Figueredo and Desai ([2]) tried various complicated methods, but finally settled on a relatively simple technic in which biopsy specimens were ground with a glass rod in a mixture of chloroform and xylol (5%) to extract and concentrate the bacilli. An impressively large series of contacts of leprosy cases was studied and compared with outpatients in Bombay who were not leprosy contacts. They reported remarkable findings of positivity rates in leprosy family contacts, ranging from 10 to 33 per cent, while comparison groups were negative. No confirmation of such a high frequency of bacteriologically positive contacts has been reported by other investigators.

MATERIALS AND METHODS

A. *Guinea-pig experiments.*—Counting bacilli: Standardization of heated suspensions of *M. lepraemurium* was necessary in order to quantitate the numbers of bacilli injected into a series of sites along the backs of guinea-pigs for subsequent recovery

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by the biopsy methods. Using a standard-size pin head, dipped vertically into the suspension, uniform drops were spotted on a slide ([3]). The number of acid-fast bacilli was counted in a band, one oil-immersion field wide, across the transverse diameter of the spot. For injection in the first experiments, a suspension containing *M. lepraemurium* 8×10^8 bacilli per cc. was used (Table 1), which is the strength of the standardized Wade lepromin we have used in skin test experiments. Subsequently, this was diluted 20× to approximately 4×10^7 bacilli per cc. (Table 2).

TABLE 1.—*Bacillary counts with different methods of concentrating* M. lepraemurium *from skin biopsy specimens from guinea-pigs.*[a]

	Method of preparation of smears						
	I Tissue fluid squeezed from biopsy	II Impression smear	III Tissue from I teased in acetic acid	IV Centrifuged sediment from III	V Corium teased in acetic acid	VI Epidermis teased in acetic acid	VII Centrifuged sediment from V and VI
Average number of acid-fast bacilli per field	4	35.7	24.6	263.5	196	22.7	206

[a]Scalpel biopsy procedure.

TABLE 2.—*Bacillary counts at different stages of acetic acid concentration for* M. lepraemurium *in series of six biopsy specimens from guinea-pigs.*[a]

	Impression smear	"Scraped incision" method	Surface scum 20 min. after grinding	Supernatant 20 min. after grinding	Sediment 20 min. after grinding	Surface after standing 24 hours	Surface after chloroform & centrifuging	Chloroform sediment after centrifuging
Average number of bacilli per field (950×)	0.076	0.024	0.148	0.115	0.073	0.075	0.045	0.216
Probability of positive result in field examined	0.044	0.019	0.099	0.076	0.060	0.055	0.037	0.149

[a]Corneoscleral biopsy instrument.

Precise counts were made of the numbers of bacilli subsequently recovered by skin biopsy procedures. Standard-size spots were made on a glass slide. The acid-fast bacilli were counted along two diameters, transverse and vertical, at a magnification of 950×. Organisms were often clumped in tissue preparations; it was estimated that the average clump contained 20 acid-fast bacilli.

Injection into guinea-pigs: Aliquots of 0.1 cc. of autoclaved *M. lepraemurium* were injected intracutaneously into a series of sites along the backs of guinea-pigs. India ink, when mixed to make one part per thousand in the bacillary suspension, identified the area of injection and did not interfere with the resulting reaction.

Recovery procedures: Biopsy specimens were taken from the injection sites at varying intervals 3-4 days after inoculation. Several different instruments and methods of excision were tried, including the following: removal of a wedge-shaped biopsy specimen with a sharp scalpel, 2 and 4 mm. skin punches, to cut cylindric skin fragments, which were then cut free at the desired depth with a scalpel, and a 2 mm. corneoscleral biopsy specimen forceps.[4] The latter provided a uniform-sized fragment relatively painlessly and quickly, and its use has become our standard procedure.

The biopsy wound was scraped as in the standard "scraped incision" method and material from it was smeared on a slide. In some experiments, the biopsy specimens were divided and one-half placed in chloroform and 5 per cent xylol, as described by Figueredo and Desai (2). The other half was squeezed on a slide until a drop of tissue fluid was expressed. For concentration, each fragment was dropped into 0.4 cc. of 2 per cent acetic acid in an 8 × 30 mm. corked tube and left to stand for 3 hours or more, after which the epidermis was readily removed. Various concentrations of acetic acid were tested from 1 per cent, as used by Khanolkar and Rajalakshmi (5), to glacial. Comparisons were made also with hydrochloric acid and other chemicals. The most satisfactory preparation for softening tissues was 2 per cent acetic acid. The softened corium was macerated for five minutes with a glass rod, the end of which was flattened so as to leave one sharp edge. The heavier tissue elements settled in 30 minutes, although it was demonstrated also that when convenient the tubes could be stored in a refrigerator for 20 hours. The supernatant was decanted into an 8 × 30 mm. centrifuge tube, shaken with 0.04 cc. chloroform for three minutes, and centrifuged 10 minutes at 1,000 G. Top speed on a hand centrifuge was also adequate. The supernatant was pipetted off and the chloroform button was spread over a standard 15 mm. area.

All slides were stained by Hanks' carbol fuchsin staining method (3% basic fuchsin in ethyl alcohol, 1:9 in 5% phenol) for one minute while steaming, followed by 1% methylene blue 1:4 in 5% sulfuric acid for half a minute (4).

B. *Field trial.*—A field trial was carried out in a leprosy colony in Ludhiana, India. Burnt-out cases with low or negative counts by standard methods were selected.

The site selected for biopsy was cleaned first with ether and then with 70 per cent ethyl alcohol. The area was pinched tightly to dull pain and a bite of tissue measuring about 1 × 2 mm. was taken with the corneoscleral forceps. The wound was then scraped and smeared. The biopsy specimen was placed in an 8 × 30 mm. tube containing 0.4 cc. 2 per cent acetic acid and corked for transportation to the laboratory. The wound was dressed with a piece of cotton saturated with tincture of benzoin.

The concentration procedure described above for guinea-pig tissues was used. Smears were made from the final chloroform button and from the crude tissue sediment prior to its discard. Results with this concentration method were compared with the standard method.

All films were stained by Hanks' method and examined for five minutes. Blind comparisons were made of similarly prepared concentration slides from five leprosy patients and five nonleprosy controls.

RESULTS

A. *Guinea-pig experiments.*—Comparisons were made of various concentration procedures. In Table 1 it can be seen that squeezing juices out of a biopsy specimen did not carry out as many bacilli as were deposited on the slide from an impression smear. After the tissue residue was teased in acetic acid the centrifuged sediment contained a considerable concentration of bacilli. When the corium and epidermis were separated, most of the bacilli were found in the corium. Acetic

[4]Holtz Corneoscleral Punch. The Lawton Co., 425 Fourth Avenue, New York, N. Y. 10016.

TABLE 3.—*Comparison of acetic acid and chloroform-xylol methods of concentrating* M. lepraemurium *from skin biopsy specimens from guinea-pigs.*[a]

	Acetic acid method	Chloroform-5% xylol method	Improvement factor
Number of specimens	26	26	
Average number of bacilli per microscopic field (950×)	1.28	0.09	14×
Probability of field being positive	0.433	0.069	6.3×

[a]Corneoscleral biopsy instrument.

acid was found to be more effective than hydrochloric acid in softening tissue and preparing it for maceration. Eight dilutions from 0.8 per cent to glacial acetic acid were tested for ability to soften skin fragments. At concentrations greater than 25 per cent tissue was fixed. Maximum tissue softening occurred in about 1.5 per cent. Recovery of mycobacteria was almost equally good in the range from 1.5 per cent to 12.5 per cent. In comparison of the new acetic acid method and that of Figueredo and Desai (2) for extracting leprosy bacilli from tissue, the superiority of the new method was clearly demonstrated. Table 3 shows that there was 6.3 times as great a chance of positive findings in a microscopic field and 14 times more bacilli per microscopic field.

Having shown that acetic acid freed the bacilli effectively for dispersion in a fluid suspension, we next turned to the problem of finding the best way of recovering the mycobacteria from the suspension. Table 2 shows the average number of bacilli per microscopic field when various parts of the suspension were smeared, as compared with results when a drop of chloroform was used for final concentration. The chloroform sediment brought together bacilli scattered through the various phases of the suspension and increased the probability of a microscopic field being positive by 1.5 times more than any other method.

TABLE 4.—*Comparison of concentration method with standard "scraped incision" method in 31 "burnt-out" cases of leprosy.*

	New method		Standard "scraped incision"	
	Number	Per cent	Number	Per cent
Negative	10	32	26	84
Positive	21	68	5	16
TOTAL	31	100	31	100

B. *Field trials in patients.*—Thirty-one "burnt-out" cases of leprosy were selected because of the low probability of their being bacteriologically positive. With the corneoscleral biopsy forceps an ellip-

tical specimen of skin measuring 1 × 2 mm. was obtained from an appropriate skin site and treated by the new concentration method. From the crater left by the removal of tissue for biopsy a scraping of tissue fluid was smeared by the usual "scraped incision" method.

Comparative counts clearly showed the superiority of the new procedure. As shown in Table 4, 68 per cent were positive by the new method, as compared with 16 per cent by the old. The difference is highly significant, the probability of an equal or greater difference occurring by chance being less than P 0.001. In the five cases positive by both methods, the numbers of bacilli per microscopic field were increased by factors of 13, 28, 56, 82 and 127 times respectively. In the small series of leprosy patients and nonleprosy controls, on whom blind readings of the concentration slides were made, no leprosy bacilli were found in the three controls, while two of the five leprosy cases were positive.

DISCUSSION

The need for an improved laboratory method for bacteriologic diagnosis of leprosy has long been evident. Tuberculoid and borderline cases are often negative by standard procedures. It is impossible seriously to investigate the possibility that leprosy bacilli may be found in preclinical or asymptomatic infected persons without a concentration technic simple enough to be used under field conditions. Our position can be likened to that in the diagnosis of malaria when only thin smears of blood were available. With the introduction of thick smears it became technically feasible to conduct mass epidemiologic surveys to determine the distribution of malaria parasites in population groups.

The fundamental need in a concentration technic is to increase the probability of finding organisms per microscopic field. A reduction in the number of microscopic fields which must be examined materially reduces the investment of time and increases the chances of success.

Some of the specific advantages of this new concentration method are:

(1) Only a small fragment of skin is needed.

(2) The use of a corneoscleral biopsy forceps produces little more pain than a needle prick, and is not emotionally disturbing to others waiting in line to be tested.

(3) The instruments and equipment can be cleaned readily, thereby minimizing the chances of carrying bacilli from one test to the next and thus producing false positive results.

(4) Simple and readily available chemicals and equipment are used, which can be set up easily in a field laboratory.

(5) The time interval is flexible. The test can be completed in four hours or specimens can be held in the refrigerator for 24 or more hours.

(6) The epidermis is separated readily and discarded, thus reducing the possibility of introducing saprophytic bacteria and other artifacts.

SUMMARY

A new method of concentrating mycobacteria from skin specimens removed for biopsy has been described, which is particularly adapted to field use. Guinea-pig experiments showed 14 times more bacilli per microscopic field than were found with a previous concentration method. Field trials in burnt-out leprosy patients showed 13 to 100 times concentration of acid-fast bacilli, as compared with the standard "scraped incision" method.

RESUMEN

Se describe un nuevo método para concenrar micobacterias de especímenes de piel extraida para biopsia, la cual es particularmente adaptable para uso en campo. Experimentos con cobayos mostraron 14 veces mas bacilos por campo microscopico que los que se encontraron con métodos de concentración previos. Ensayos de campo en pacientes leprosos quemados, mostraron 12 a 100 veces mayor cencentración de bacilos acido-resistentes, si son comparados con el método standard de "scraped incision."

RESUMÉ

Une nouvelle méthode de concentration de mycobactéries dans les prélèvements de peau obtenus à partir de biopsies a été décrite. Cette méthode est particulièrement adaptée pour être utilisée sur le terrain. Des expériences menées chez les cobayes ont montré qu'il était possible d'obtenir 14 fois plus de bacilles par champs microscopique qu'avec une prócédente méthode de concentration. Des essais sur le terrain, chez des lépreux résiduels, ont permis d'obtenir des concentrations de bacilles acido-résistants dans un rapport de 12 à 100 comparison au nombre obtenu avec la méthode standard d'incision et grattage.

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