Choosing the decolourizer and its strength to stain *Mycobacterium leprae*. Does it actually matter?

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Summary Leprosy bacilli are more easily decolourized during staining than tuberculosis bacilli, so a weaker concentration of decolourizer is usually recommended. In Indonesia, the same 'strong' decolourizer is used for identifying both organisms. In a study to compare the results using different concentrations of different decolourizers, no difference could be found in the bacterial index (BI). It is suggested that the same staining technique can be used for tuberculosis and leprosy.

Introduction

Ideally, all leprosy patients should have one skin smear examination before starting treatment, if reliable facilities are available.[1] The Ziehl–Neelsen method involves staining with carbol fuchsin, followed by decolourization then counterstaining with methylene blue.[2] If the decolourization process is excessive, bacilli may be rendered invisible. Concerns regarding which decolourizer to use, what strength and for how long, have resulted in several investigations.[3–6]

In Indonesia, the same staining technique is normally used for leprosy and tuberculosis, despite the widely accepted opinion regarding the reduced acid and alcohol 'fastness' of *Mycobacterium leprae*.[2] The objective of this study was again to find out if (i) different decolourizers and (ii) different concentrations of the same decolourizer had an effect on the BI of skin smears.

Although the Ziehl–Neelsen stain is used universally to stain *M. leprae*, there are many modifications.[7] The main difference between the technique for leprosy and tuberculosis is the strength of the decolourizer, but in addition, the duration of decolourizing varies. For tuberculosis, this is usually either 25% sulphuric acid or 3% hydrochloric acid in 70%

ethanol for 3 min.[8] For leprosy, decolourization or differentiation using 1% hydrochloric acid in 70% ethanol is favoured by most,[2,7,9,10] but 5% sulphuric acid by some.[11] Dharmendra surprisingly recommends 3% acid alcohol.[12] The counterstain is usually methylene blue, although the concentration varies between 0·2%[11] and 1%.[6] Vettom and Pritze found 10 different techniques from 29 projects, the most notable variations being in decolourization time.[13] They ranged from 1% acid alcohol for 5 s to 20% acid alcohol for 1 min and from 5% sulphuric acid for 1 min to 25% sulphuric acid for 10–20 min.[13]

Despite this, several research laboratories use the same concentration of decolourizer for leprosy as for tuberculosis (A. McDougall, personal communication). The Indonesian leprosy manual describes two possible Ziehl–Neelsen methods, one with acid alcohol (concentration not specified) for 3–5 s and the other with 25% sulphuric acid for 8 s.[14]

Materials and methods

Skin smears were taken from 40 multibacillary (MB) leprosy patients, with a range of BI. The selection was by the technician who took the smears, did the staining and read the results. The patients were registered cases and were on WHO MB multidrug therapy (MDT).[1] All patients had smears taken from four standard sites with four identical slides being made from each patient, each slide with the four sites on it.

Apart from the decolourizer, the same Ziehl–Neelsen method was used.[15] The decolourizers were 1% hydrochloric acid in 70% ethyl alcohol (HCl), 3% hydrochloric acid in 70% ethyl alcohol, 5% sulphuric acid (H_2SO_4) and 25% sulphuric acid. Slides were stained in batches. Carbol fuchsin was filtered onto the slide, allowed to act for 2 min, heated gently until steam rose, allowed to act for a further 10 min, washed with tap water until clean, transferred to a staining rack and dipped in decolourizer for 8 s. If any slide was still red, this was repeated, washed, and counterstained with 0·3% methylene blue for 1 min. Most slides only received one immersion in the decolourizer.

The intention was that the examination of the smears would be blind, but it was possible to distinguish immediately between the slides decolourized with HCl and those with H_2SO_4, by microscopy of the smear. It was not possible to distinguish between 1% and 3% HCl or between 5% and 25% H_2SO_4.

The BI was reported for each of the four smears on the slide and the average was calculated. The morphological index (MI) is not routinely used in Indonesia.

The results were compared using the Student's paired *t*-test.[16] Differences were considered significant at the 95% level of confidence.

Results

Forty patients had slit skin smears taken, resulting in 160 slides. Each slide had four smears, except in the case of patient 6, who fainted after smears had been taken from two sites (ears). Thirty-four patients were positive with at least one staining technique. An average BI was calculated for each of the 160 slides (see Table 1). Comparison was also made using individual smears.

Eleven patients had a higher BI with 1% HCl than with 3%. Twelve patients had a lower BI with 1% HCl than with 3%. Eight patients had a higher BI with 5% H_2SO_4 than with 25%. Thirteen patients had a lower BI with 5% H_2SO_4 than with 25%.

When looking at low BIs (i.e. >0 but <2), the same pattern was observed. Eleven patients had a low BI using 1% HCl. In two cases, the BI was greater using 1%, and in five cases, the BI was less. Twelve patients had a low BI using 5% H_2SO_4. In three cases, the BI was greater using 5% and in five cases, the BI was lower with 5%.

Table 1. BI results for 40 patients using four decolourizers

Patient	1% acid alcohol	3% acid alcohol	Difference	5% sulphuric acid	25% sulphuric acid	Difference	Difference between 1% HCI & 5% sulphuric acid
1	1·75	2·25	−0·5	2	1·75	0·25	−0·25
2	1	1	0	0·75	0·75	0	0·25
3	4·75	4·5	0·25	4	4·25	−0·25	0·75
4	1	1	0	1	0·75	0·25	0
5	0	0	0	0	0	0	0
6	4	4	0	3	4	1	1
7	2	1·75	0·25	1	1·25	−0·25	1
8	3·75	3·5	0·25	4	4	0	−0·25
9	4·5	3·75	0·75	4	4·25	−0·25	0·5
10	1·5	1·75	−0·25	1·25	1·25	0	0·25
11	2·75	2·5	0·25	1·75	2·75	−1	1
12	0·5	1·5	−1	0·5	0	0·5	0
13	1	1·75	−0·75	1·25	1·25	0	−0·25
14	0	0	0	0	0	0	0
15	4·5	4	0·5	0	3·25	−3·25	4·5
16	1·75	2·75	−1	0	3·25	−3·25	1·75
17	2·5	2·75	−0·25	0	2·5	−2·5	2·5
18	1·25	1	0·25	1	1	0	0·25
19	0	0	0	0	0	0	0
20	4·25	4·25	0	4·25	3·5	0·75	0
21	2·75	2·25	0·5	1·5	3·5	−2	1·25
22	0·5	0·5	0	0·25	0	0·25	0·25
23	0	0	0	0	0	0	0
24	4·75	4·75	0	4·5	4	0·5	0·25
25	0	0	0	0	0	0	0
26	3·25	4	−0·75	3	3·25	−0·25	0·25
27	2	2·25	−0·25	0	0	0	2
28	3·75	3·75	0	3·75	3·75	0	0
29	4·75	4·75	0	4·5	4·5	0	0·25
30	3·5	3·75	−0·25	3·5	2·25	1·25	0
31	3·5	4	−0·5	3	2·5	0·5	0·5
32	4	3·75	0·25	4·25	4·25	0	−0·25
33	5	5	0	5	5	0	0
34	1·25	0·5	0·75	0·25	0·75	−0·5	1
35	0	0	0	0	0	0	0
36	3·5	4·25	−0·75	3·75	3·75	0	−0·25
37	2	3·5	−1·5	3·75	3·75	0	−1·75
38	0	1·25	−1·25	0·75	1·25	−0·5	−0·75
39	0·5	0·5	0	0	1·25	−1·25	0·5
40	4·75	4·25	0·5	4·75	4·75	0	0
Total	92·5	97	−4·5	76·25	88·25	−12	16·25
Average	2·31	2·43	(0·11)	1·91	2·21	(0·30)	0·41
SD			0·50			0·94	0·98
p value			<0·2			<0·1	<0·02

The average of all the BIs using 1% HCl was 2·31 (with seven negatives). The average BI using 3% HCl was 2·43 (with six negatives). The difference between the BIs was not statistically significant ($p < 0.2$ and >0.1). The average BI using 5% H_2SO_4 was 1·91 (with 11 negatives). The average BI using 25% H_2SO_4 was 2·21 (with nine negatives). Again, the difference between the BIs is not statistically significant ($p < 0.1$ and >0.05). When the differences between the BIs with 1% acid alcohol and 5% sulphuric acid were examined, there was an average fall of 0·41. This was significant at the 2% level ($p < 0.02$), suggesting that there might be a real decrease in BI when using sulphuric acid.

There was generally good correlation between the four slide results from each patient. An exception was found in patients 15, 16 and 17, where the slides stained with 5% H_2SO_4 were negative, whereas the other three were moderately or strongly positive. The most likely explanation for this was that these slides were wiped clean on the wrong side of the slide, hence removing all four smears from the slide. If the results from these three patients are excluded, the average BIs are 2·26 and 2·36 for acid alcohol and 2·06 and 2·14 for sulphuric acid. The differences between these figures (between the two acid alcohols, the two sulphuric acids and between 1% acid alcohol and 5% sulphuric acid) all fail to reach statistical significance ($p < 0.5$, $p < 0.5$ and $p < 0.1$, respectively).

In 38 of 158 sites examined, there was a variation of two or more BI units (i.e. more than a 10-fold difference in the number of bacilli) from the same site using different decolourizers. No pattern was observed.

The slides which were stained using an acid alcohol decolourizer were generally easier to examine than those using sulphuric acid. Irrespective of concentration, the bacilli were sharper, clearer, more red, the background had accepted more blue for differentiation and there was little or none of the background pink 'fuzz' that is a feature of the slides which were decolourized using 5% or 25% H_2SO_4.

Discussion

POSITIVITY

This study failed to show that a stronger decolourizer resulted in a lower BI. The BI was slightly higher with the stronger concentration, but not reaching statistical significance (see Figure 1). This was also noted when looking at the results from patients with a low (<2) BI. The BI was slightly lower with sulphuric acid, this reaching significance only when the questionable data from patients 15, 16 and 17 were included.

POSSIBLE BIAS

All patients were purposefully selected. No attempt was made to have a representative sample of leprosy patients, new or otherwise. Since the purpose of the study was to compare results patient by patient, analysis using an average BI for all patients per technique is open to question. The difference in BI from the same site could also be affected by the quality of the smear and within-observer variation. Three patients had results which suggest a major laboratory error. Analysis was also performed excluding results from these three patients.

DOES IT REALLY MATTER?

Most new leprosy patients can be competently diagnosed and commenced on appropriate
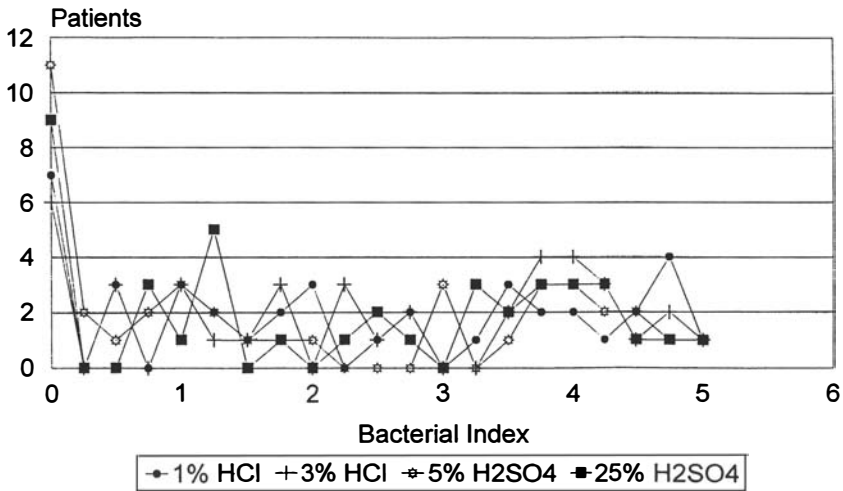
Figure 1. Comparison of BI results using four decolourizers.

treatment without a skin smear. However, there are some patients who present with single or few lesions, but who have early multibacillary disease.[17] There are other patients, either new or old, who have no clearly demonstrable clinical cardinal signs, but who have a positive smear. There are suspicions that some multibacillary patients, who are released from fixed duration treatment but still with a BI of >3, may have a significant relapse rate. It has been suggested that regular clinical and bacteriological review after release from treatment is indicated for these patients.[18] Where communications are difficult, it may not be practical to refer all smear examinations to a referral laboratory.[19] Hence, there is still be a need for skin smear examination to be made available, at least at district level.

The success of the WHO Elimination Strategy suggests that there will gradually be fewer positive smears. Leprosy will not, however, be eradicated by the year 2000. It is therefore essential that there be accessible centres with trained staff who will be able to carry out this procedure accurately. As the world tuberculosis situation deteriorates, it becomes increasingly necessary to have laboratories in peripheral health units capable of bacteriological examination of sputum. Tuberculosis diagnosing centres (possibly one such laboratory per 100,000 of the population[20]), with an established system of quality control, can also be responsible for leprosy microscopy. This is easier if the staining technique for both organisms is identical. From this study, it does not appear likely that the choice of decolourizer or its strength is a major factor in the general unreliability of skin smear services in some parts of the world.

It is possible that this is a much more robust technology than is generally thought. This study suggests that the same technique for staining leprosy and tuberculosis bacilli can be used, furthermore, that acid alcohol is an improvement on sulphuric acid.

Acknowledgements

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