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Reproducibility of sputum smear examination for acid-fast bacilli: practical problems met during cross-checking

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SUMMARY

SETTING: A TB control programme in Bangladesh with proficiency testing of sputum smears for acid-fast bacilli (AFB) using cross-checking of routine smears.

OBJECTIVE: To document factors that may adversely affect repeatability of the AFB smear, and which should be taken into account for interpretation of cross-checking.

DESIGN: A number of simple experiments falling within the scope of small routine laboratories in a developing country.

RESULTS: Fuchsin staining is not stable, fading quickly in direct sunlight and combinations of high temperature and humidity. Diffuse daylight, immersion oil or xylene did not have the same effect. Contamination of smears by saprophytic mycobacteria may occur during staining or rinsing, with contaminants becoming visible only after restaining and cross-checking. Finally, AFB may be dislodged from smears during soaking in xylene or restaining, especially if the smears are made from thin, liquefied sputum.

CONCLUSIONS: These possible interfering factors should be taken into account when organizing proficiency testing and interpreting its results.

KEY WORDS: mycobacterium; quality control; stains and staining

REREADING at a higher level, including a counter-check of slides with discordant results, is the most effective method of proficiency testing of sputum smears examined for acid-fast bacilli (AFB) in peripheral centres.[1] However, technical problems can and do occur.

We have already reported that fuchsin-stain is not stable in hot, humid climates, causing erroneous declaration of false-positive results.[2] Other reasons for fading may also exist. Restaining before cross-checking is the only way to make sure that gross deficiencies of staining do not stay undetected, but this may cause new problems. We occasionally found over 50% false-negative results, clearly impossible in randomly selected smears coming mainly from tuberculosis suspects. AFB thus detected had an atypical morphology, hence contamination with saprophytic mycobacteria was suspected. On the other hand, it also happened that AFB was identified by both the peripheral microscopist and the first controller, but could not be found during extensive counter-checking. A loss of AFB during cleaning in xylene and/or restaining was suspected.

A number of simple experiments were therefore set up to obtain a better understanding into these phenomena.

OBJECTIVES

Answer to the following questions were sought: 1) What factors cause rapid fading of the fuchsin stain after Ziehl-Neelsen? 2) How can contamination of smears with saprophytic AFB occur, and can it be prevented or undone? 3) Can AFB be lost from well-fixed smears?

METHODS

Sputum smears were freshly smeared and stained, or restained at the start of the study. Heat fixation was used unless otherwise indicated. Staining was done according to the Ziehl-Neelsen (ZN) hot method, on individual slides. Solutions were freshly prepared according to standard formulations. The carbolfuchsin contained 1% basic fuchsin and 5% phenol. Sulphuric acid 25% in water was used for decolorization, and the usual counterstain was watery methylene blue 0.1% (or potassium permanganate 0.5% where specified). Distilled water was used for preparation of reagents for the experiments, and tap water for rinsing (except where otherwise indicated). Acids and potassium permanganate were of technical grade. Phenol and methanol were of analytical grade. Meth-

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ylene blue (LOBA Chemie, Bombay, India) and basic fuchsin were of reagent grade. Other brands of fuchsin (UCB, Belgium, Nentech, UK, and Fisons, UK) were used as well as New Fuchsin (Merck, Germany). In one trial slides stained with Victoria Blue (Victoria Blue R, Sigma, USA), using a variation of the Hallberg method,[3] were included. Hydrochloric acid 3% in acid alcohol 70% had to be used for decolorization after New Fuchsin or Victoria Blue. Non-drying synthetic immersion oil (Type B, Cargille, USA) was used. Repeat reading of smears was always done by the same examiner, followed by soaking in xylene (Nentech, UK) for 5 to 15 minutes and restaining when appropriate. At least 300 fields were screened for low positives or before declaring a smear negative.

Fading experiments

1) Three positive smears (1+ to 3+, IUATLD scale[4]) of different thickness were kept in a relatively dry, dark place at ambient temperature, under a thick layer of immersion oil. They were read at intervals during the course of one year, without cleaning or restaining in between.

2) Two series of each three positive smears (1+ to 2+) of different thickness were kept under transparent polythene, exposed to direct sunlight in the open, or alternatively in diffuse natural light.

Experiments 1 and 2 were both started during the winter time, with temperature (T°) around 20°C and relative humidity (RH) around 60% during the first months; these subsequently rose to around 35°C and 80% RH or higher.

3) Several trials were designed to study the influence of T° and RH, using a total of 154 different smears. In the main experiments, pairs of smears made from manually mixed sputum samples from untreated cases were kept under a variety of conditions. Smears differed in bacillary content and thickness. Fixation by heat or alcohol was compared. The main variables were the stains applied—besides Victoria Blue, all of the previously listed brands of basic fuchsin were used. For New Fuchsin, a freshly prepared and a one-year-old stain were compared. Decolorization was as described above. The usual counterstain was methylene blue, but some smears were included for comparison with potassium permanganate or no counterstain. Distilled water for rinsing was compared with tap water.

For the 100% RH trials, smears were kept at different RH on wet filter paper in tightly closed containers. Other RH values were obtained by keeping slides over watery solutions of potassium hydroxide (KOH) at various concentrations, checked by densitometer readings, prepared according to Solomon.[5] One series was kept over a saturated watery NaCl solution. The containers were then kept at fairly constant temperatures (room or incubator). Series of control slides were kept in containers with silicagel (control by hygrometer indicated 25% RH), at the same T° as the containers with test smears, or in a refrigerator at 4°C.

AFB were counted at the start of the trial and at weekly or monthly intervals thereafter until no AFB (even barely stained) could be identified any longer. For series kept at lower temperature and humidity, the experiment was stopped after one year. Only at that time were slides restained. In between readings they were cleaned in xylene before being returned to their containers, with the exception of the control slides exposed to the highest T° and RH; immersion or cedar oil was left on these to check for protection against fading.

4) Twelve thick smears (New Fuchsin stained) were soaked in xylene for 24 hours at about 30°C, with AFB counts of the fields before and after.

Contamination with saprophytic AFB

1) Saprophytic AFB was looked for in smears made from water taps and their rubber tubing in the laboratories, and from the filter compartments, candles and tap of a household ceramic candle-type water filter that had been used to purify water for preparing stains.

Fresh stains prepared with distilled water and absolutely clean glassware, checked for the absence of AFB using known negative control smears, were used to restain negative smears from peripheral laboratories where contamination was suspected. Stains used in routine at these centres were checked by means of negative control smears and distilled water for rinsing. To increase the probability of contaminants adhering to the smears, in these trials the staining cycle was repeated three times before reading.

2) Removal of adhering contaminants was attempted by soaking in methanol or methanol two parts/acetone one part, for 2 hours or overnight. Later trials used sulphuric acid 25% in water or 0.5% Tween 80 in xylene for half an hour. AFB were counted before and after soaking.

3) Removal of saprophytes using a locally available household-type water filter was checked using intact but old, or new ceramic filter candles.

A suspension of *Mycobacterium gordonae* was made in sodium hypochlorite 5%; the clumps of bacilli were broken up by vigorous manual shaking every few minutes for half an hour. After dilution with distilled water and sedimentation, smear confirmed that most AFB were lying single. A resuspended amount of AFB, representing growth of one Löwenstein-Jensen slant, was transferred to the water filter and 5 litres of water were added and left to filter through. The filtrate was again vacuum-filtered through a 0.2 micron filter membrane (Anodisc 47, Whatman, UK). Any bacilli that were present were transferred from the membranes to microscope slides using 1% agar as described by Smithwick and Stratigos.[6] All slides were stained with ZN and checked for the presence of AFB.

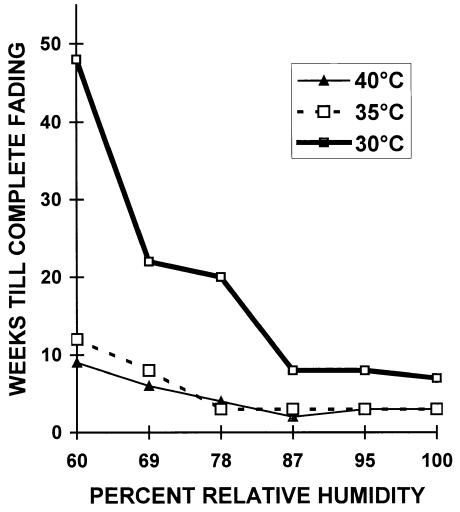

Figure 1 Fading of fuchsin stain relative to temperature and humidity.

Loss of AFB from heat-fixed smears

1) Loss of AFB during repeated short xylene soaks was studied by comparing counts after restaining with the original counts obtained in the fading trials described above. Smears of differing thickness and sputum consistency were compared.

2) The effect of restaining and xylene soaks combined was checked by repeat reading, removal of oil in xylene and restaining of seven low-positive smears of normal thickness and sputum consistency.

3) The effect of acids used for decolorization was checked comparing 10% hydrochloric and 25% sulphuric acid in water. Thick smears made from liquefied sputum samples were first stained, using 3% hydrochloric acid in water for decolorization, and AFB were counted. They were then divided into two groups of 98 smears each and restained, using 10% hydrochloric or 25% sulphuric acid, respectively, for decolorization, and AFB were counted again.

RESULTS

Fading experiments

1) Smears kept under a thick layer of immersion oil started to fade a little after 5 months; after one year, however, AFB were still clearly visible at about one third of their original numbers.

2) All smears exposed to direct strong sunlight became completely negative after 4 to 6 days, depending on smear thickness and the original quantity of AFB. After restaining AFB reappeared in their original numbers.

Smears kept in daylight but out of the sun did not fade for 2 months (during the colder and dryer winter season). AFB then started to fade and numbers began to decline when they became exposed to higher T° and RH as well. For this reason the experiment was stopped.

3) Fading was rapid at high degrees of RH (78 to 100%), combined with temperatures of 35 or 40°C (Figures 1 to 3). In these series, endpoints (when no more AFB could be found) were reached between 2 and 5 weeks. At a lower T° of 30°C and RH of at least 78%, this took 8 to 20 weeks (Figure 4). RH in these experiments was kept fairly constant over hygroscopic solutions of KOH, although the higher concentrations must have become more diluted with time. However, smears kept over a saturated NaCl solution (73% RH) gave comparable results, with fading also taking about 6 weeks at 40°C. At still lower levels of RH, the time to complete fading became much longer, and at 30°C and RH 50% (data not shown) AFB were still seen after one year. By that time it had become clear that AFB were increasingly disappearing from the smears, and the experiment was stopped.

AFB in thick smears seemed to fade more slowly,

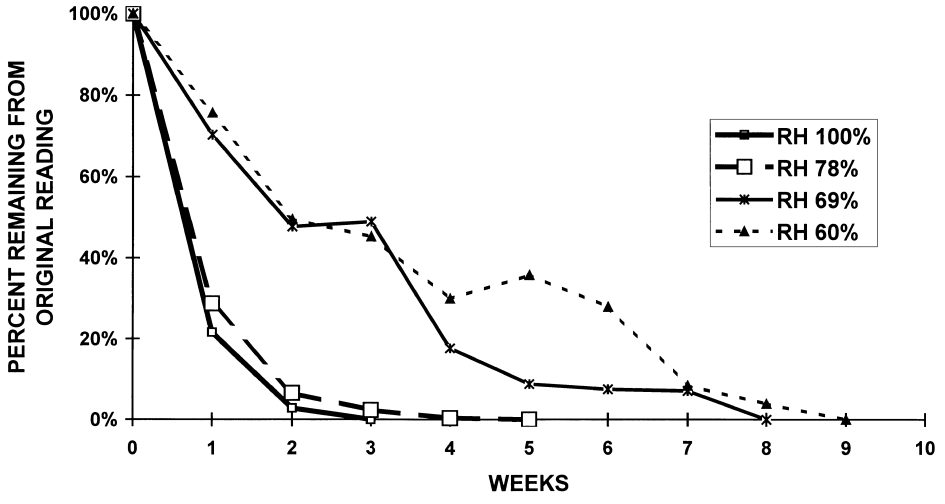

Figure 2 Fading of fuchsin stain, AFB still visible at 40°C.

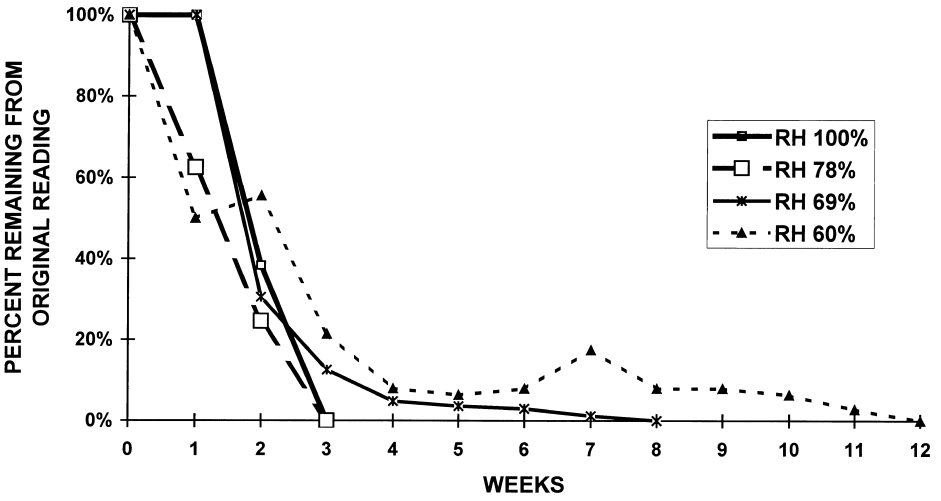

Figure 3 Fading of fuchsin stain, AFB still visible at 35°C.

but when liquefied sputum was used (autolysis by standing) this appeared to be due to the consistency of the sputum rather than the thickness of the smears. Very thin smears with scanty to 1+ AFB, as well as very thick, high 3+ smears, made from two liquefied sputum samples, all faded within 2 weeks at 40°C and 100% RH (other series, data not shown).

Fading of alcohol-fixed smears was equally rapid compared to heat-fixed smears. Use of distilled versus tap water for rinsing did not influence fading time.

Contrary to expectations, using different brands of fuchsin did not result in different speeds of fading, either at extreme or moderate T° and RH. Smears stained with fresh stain compared to one-year-old carbolfuchsin varied only slightly in brightness of colour. Fading of smears stained with Victoria Blue was even more rapid. No difference was observed when the counterstains were varied, between methylene blue, potassium permanganate or none at all.

Controls stained in the same way and kept over silicagel at the same T° as the test slides also showed a very slow decrease in numbers of AFB. At 40°C it took about 4 months for thin smears to become negative; thick smears did not change even after 12 months. Smears kept over silica at 4°C changed even more slowly.

Control smears kept at the same T° and 100% RH without removal of the immersion or cedarwood oil between readings showed no reduction in speed of fading. In the case of cedarwood oil, furthermore, fading was consistently complete one week earlier than when no protection or an immersion oil cover were used.

After restaining of faded smears, AFB reappeared in the same quantities as at the start, with the exception of thin smears from liquefied sputum: for these often only a fraction of the original count reappeared, and some even remained negative. The results shown

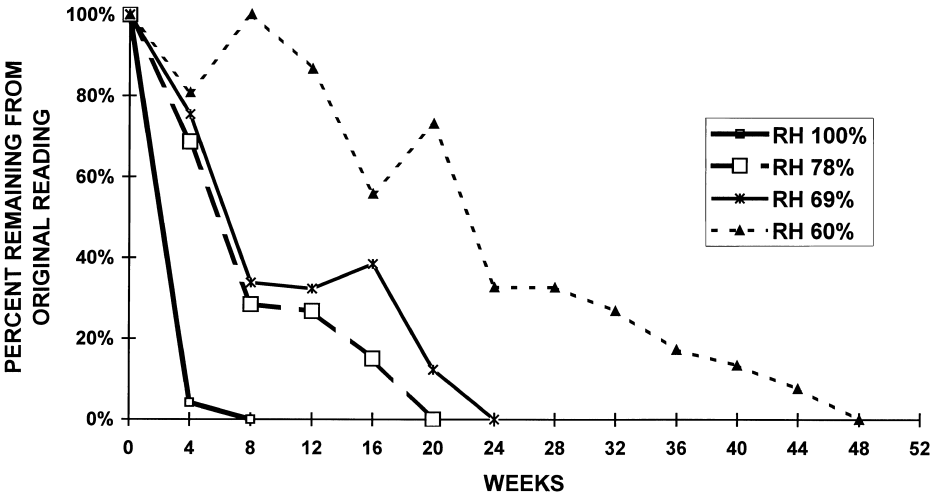

Figure 4 Fading of fuchsin stain, AFB still visible at 30°C.

in Figures 1–4 include only smears where AFB reappeared in high numbers.

4) After a 24-hour xylene soak generally the same numbers of bright red AFB were counted.

Contamination with saprophytic AFB

1) Wherever we looked for saprophytic AFB, we found them. Especially high numbers were present in the rubber tubing of a tap and at the bottom of a water filter compartment.

About half of the negative controls stained using water from this filter turned weakly positive. Furthermore, about 50% of the smears declared negative by the centres using these stains could be shown to be positive by repeat staining using uncontaminated stains and distilled water for rinsing.

2) Soaking in methanol or methanol/acetone failed to remove adhering contaminants. Their numbers were reduced, but this occurred also with positive controls (TB bacilli), and was finally found to be due mainly to decolorization.

Soaking in xylene with Tween 80 reduced contaminants to very low numbers, but could not consistently eliminate them. Soaking in xylene only for about half an hour already removed adhering clumps of contaminants. Prolonged treatment with sulphuric acid 25% had the same effect. None of the treatments affected the positive controls.

3) None of the smears made from candle-filtered water contained AFB. This was true for new as well as used but intact candles.

Loss of AFB from heat fixed smears

1) Evidence from the fading trials: most smears kept in a high humidity atmosphere for a prolonged period showed considerably less AFB upon restaining. In a number of smears kept over KOH solutions, none at all reappeared. Smears kept under oil at 100% RH in the same study also faded; however, their original counts reappeared after restaining.

Thin smears made from liquefied sputum kept on silicagel at 40°C or 4°C lost most or all of their AFB after a longer period with repeated readings.

2) No consistent loss of AFB was observed for seven low-positive smears of normal thickness made from fresh sputum samples after up to nine cycles of reading, soaking in xylene and restaining.

3) Mean AFB counts were slightly higher after restaining using 10% hydrochloric or 25% sulphuric acid, compared to the first reading after a staining cycle including 3% hydrochloric acid.

DISCUSSION

All of the trials were performed using the facilities of a routine TB project in a low-technology country. For this reason, not all results were obtained under carefully controlled conditions. Also, due to the nature of AFB microscopy itself, they should be considered indicative rather than 100% accurate. However, the same effects have been observed repeatedly, using different approaches, and we believe that the reported phenomena are real.

Fading

The hypothesis that fuchsin-stained AFB fade when kept under synthetic immersion oil is encountered in different countries and continents, and is also found in the handbook by Smithwick.[7] However, this was not shown to happen in a few months of continuous exposure. The fading finally observed in our trials was minor and might very well have been caused by changing climatic conditions alone, so it was not possible to conclude that the oil had any effect at all. Nevertheless from our limited observations, cedarwood oil may have had an accelerating effect on fading even at high T° and RH.

Repeated or prolonged xylene soaking could not be shown to cause appreciable fading.

As has been known for a long time,[3] strong direct sunlight causes a complete loss of colour of the AFB in a matter of days. On the other hand, subdued daylight indoors did not have the same effect over a period of months. No conclusion on long-term effects under these conditions could be drawn because of interfering climatic changes.

Complete fading can also occur within a few weeks because of high RH combined with high T°. The time to complete fading was much longer at 30°C than at 35° or 40°C, and independently of T°, also at lower RH. Some confusion in these experiments resulted from the fact that bacilli were also washed out of the smears, as discussed further on. Our final finding was that thick smears kept dry on silicagel showed no evidence of fading even after one year at high T°.

As hypothesized earlier,[2] this might be due to unbinding of fuchsin and diffusion of primary stain out of, and background stain into, the bacilli under these conditions. The fact that this occurred with all of the fuchsin brands tested, using fresh as well as old stain, and also with such chemically different compounds as New Fuchsin or Victoria Blue, may support this conclusion.

Thick smears seemed to take longer to fade. However, they also had much higher bacillary counts, and often contained clumps of AFB which resisted for the longest period. As smears made from liquefied sputum did not show much difference in fading as regards thickness or grading of smears, the sputum consistency and clumping of AFB, rather than the thickness of smears, may have been the protecting factors. Other variations of stains, rinsing water or fixation made no appreciable difference.

Contamination with saprophytic AFB

Saprophytic AFB were found in taps, tubing and containers, and in a water filter used to prepare water for

stains, as has been reported by others.[8–10] Other sources of contamination, such as slides, are possible.[11]

We demonstrated that smears could be contaminated via the stains. We found no evidence of contamination after a single first staining: this would interfere with clinical decision-taking, but is only possible with contaminated carbolfuchsin stain (or slides or sputum containers). In our experience, contamination became apparent only upon repeat staining, due to the presence of AFB in the methylene blue counterstain.

Even after stains were made exclusively with distilled water, however, contaminated smears continued to be seen occasionally in cross-checking after restaining. On checking taps and containers, this contamination was found to originate in the rinsing water of peripheral centres, and seemed to occur seasonally in certain areas. No single fool-proof method could be found to prevent this. Removing adhering contaminants before restaining was made possible to a large extent by prolonged soaking in xylene, Tween 80 or sulphuric acid 25%, and frequent cleaning of utensils and buckets used for rinsing water was sufficient to control contamination in most centres. Household ceramic candle-type water filters were shown to be able to remove saprophytic AFB, and proved effective in eliminating contamination in a few badly affected centres. However, contamination of these filters may be difficult to prevent consistently. Using boiled water for staining would kill, but not remove, still visible mycobacteria. On its own, boiled water would not be a sufficient measure, but it might delay colonization of a water filter subsequently used for purification. Distilled water should clearly be preferred for preparation of stains, but it is too expensive for rinsing. Water de-ionising installations may also become contaminated easily.[10]

Loss of AFB from heat-fixed smears

From the fading trials above, we assumed that AFB were washed out of thin smears by condensing water. However, since thin smears kept on silicagel also showed a loss of AFB roughly proportional to the number of times they had been re-examined, another factor in the loss of AFB was likely to have been the repeated soaks in xylene, and possibly also the final restaining. No such losses could be shown for smears of normal thickness made from fresh sputum—the AFB are therefore presumably safely fixed in a matrix of mucoid material. No losses of AFB could be attributed to the use of stronger acids for decolorization.

CONCLUSIONS

A number of important conclusions can be drawn concerning the reproducibility of sputum smear examinations for AFB. Many TB programmes have installed a system of proficiency checks by rereading peripheral smears, and the present findings are relevant in this regard.

1) The red fuchsin colour is not always stable; fading is very rapid under direct sunlight or high humidity combined with high T°. The first can be prevented by keeping smears out of the sun, but the second may not be easy to delay under conditions in high prevalence countries where refrigerators and climatisers are not widely available. A minimum approach would be to restain supposedly false positives systematically before counter-checking. However, poor staining is an important cause of serious false negative errors that may be discovered only after restaining. For this reason it may be better to restain systematically all smears prior to cross-checking, without taking any precautions to prevent fading.

Neither immersion oil nor xylene affected staining rapidly, but xylene soaks did wash AFB out of thin, fragile smears. Xylene is expensive and a known carcinogenic. For these reasons its use was discontinued in our peripheral centres in Bangladesh. Excess oil is simply removed by light wiping or blotting with toilet paper, slides are kept free of dust in boxes, and only the central laboratory uses xylene soaks before restaining. Xylene should be provided if cedarwood oil is used, as this oil seems to cause more rapid fading, and necessitates frequent cleaning of microscope lenses. As there is also a greater danger of damage to the lense (Olympus Optical Company LTD, personal communication), purchase of cedarwood oil should be considered a false economy.

2) Contamination of smears by saprophytic mycobacteria may have a disrupting effect on AFB microscopy, as they are in principle indistinguishable from *M. tuberculosis*. In some centres, very high rates of low-grade false negatives in cross-checking were due to contamination of counter stains or rinsing water; in fact they constituted 'false false negatives'.

To prevent contamination, distilled or bacteriologically filtered water should be used for the preparation of stains. In some areas, it may be advisable to use a household-type water filter for the preparation of pure rinsing water, although proper maintenance will certainly be critical. Even in Bangladesh, however, the regular cleaning of water containers and prolonged soaking in xylene or xylene with 0.5% Tween 80 were often sufficient precautions.

3) 'False false positives' may occur in cross-checking. Apart from being a limitation of cross-checking, where rare AFB do not always reappear, this may happen when AFB are washed out of the smear between the peripheral and cross-check readings. This was consistently observed with thin smears from liquefied sputum, during xylene cleaning and/or restaining, but it rarely seems to happen with smears of normal thickness and consistency. This is not sufficient reason, however, to refuse the examination of older, already liquefied sputum. Such specimens are entirely suitable for

microscopy, and there is evidence that because of the dispersion of clumped bacilli they may yield more positives (own data, not shown).

In practice, occasional scanty false-positive results should simply be disregarded for analysis of cross-checking results.

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RÉSUMÉ

CADRE : Programme de lutte antituberculeuse au Bangladesh avec tests de qualité des bacilloscopies par contrôle croisé des lames de routine.
OBJECTIF : Documenter les facteurs qui pourraient influencer défavorablement la reproductibilité de la bacilloscopie et qui devraient être pris en compte pour l'interprétation du contrôle croisé.
SCHÉMA : Un certain nombre d'expériences simples exécutables dans de petits laboratoires de routine dans un pays en développement.
RÉSULTATS : La coloration par la fuchsine n'est pas stable ; elle s'affaiblit rapidement sous lumière solaire directe et en cas de combinaison d'une température élevée et d'une humidité importante. La lumière du jour diffuse, l'huile d'immersion ou le xylène n'entraîne pas ce même effet. La contamination des lames par des mycobactéries saprophytes peut se produire au cours de la coloration ou du rinçage, les contaminants ne devenant visibles qu'après recoloration et contrôle croisé. Finalement, les bacilles peuvent être détachés des lames au cours du trempage dans le xylène ou de la recoloration, particulièrement lorsque les lames proviennent d'une expectoration fluide et liquéfiée.
CONCLUSION : Ces facteurs d'interférence possibles devraient être pris en compte pour l'organisation du test de qualité et l'interprétation de ses résultats.

RESUMEN

MARCO DE REFERENCIA : Un programa de control de TB en Bangladesh, con evaluación de la calidad de la baciloscopia utilizando controles cruzados de láminas de rutina.
OBJETIVO : Documentar los factores que pueden afectar en manera adversa la repetibilidad de la baciloscopia y que deben ser tenidos en cuenta para interpretar el control cruzado.
MÉTODO : Un número de experiencias simples, compatibles con la actividad de pequeños laboratorios de rutina en un país en vías de desarrollo.
RESULTADOS : La coloración con fucsina no es estable, se diluye rápidamente a la luz solar directa y en caso de combinación de alta temperatura y humedad. La luz del día difusa, el aceite de inmersión o el xileno no tienen el mismo efecto. La contaminación de los esputos por micobacterias saprófitas puede suceder durante la tinción o el lavado y los contaminantes se hacen visibles sólo después de una nueva tinción y control cruzado. Finalmente, los bacilos pueden ser desalojados de las láminas durante la inclusión en xileno o en la recoloración, especialmente si los extendidos se hacen con esputos muy licuados.
CONCLUSIONES : Estos factores que pueden interferir deben ser tenidos en cuenta cuando se efectúan controles de calidad y se interpretan sus resultados.