INT J TUBERC LUNG DIS 12(1):108–110

TECHNICAL NOTE

Ziehl-Neelsen staining: theory and practice

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SUMMARY

The information provided in the guidelines of the World Health Organization and the International Union Against Tuberculosis and Lung Disease for Ziehl-Neelsen staining is not practical on a number of points. The advice given here is meant to supplement the guidelines. It is based on experiments on and field experience of basic fuchsin stain and staining solutions.

KEY WORDS: microscopy; Ziehl-Neelsen; staining; basic fuchsin

THE PURPOSE of this technical note is to provide practically relevant information supplementing the guidelines of the World Health Organization (WHO) and International Union Against Tuberculosis and Lung Disease (The Union) on Ziehl-Neelsen (ZN) staining.[1,2] It is based on a limited number of experiments and practical field experience. It is hoped that it may stimulate others to carry out additional research into these small yet relevant practical issues.

REQUIREMENTS FOR BASIC FUCHSIN

- WHO and Union guidelines specify basic fuchsin as 'pararosaniline chloride, minimum dye content 88%, SIGMA P1528 or equivalent'.[1,2]

National TB Programmes (NTPs) are not only often not in a position to procure specific brands, but generic specifications are also difficult to use. Countries increasingly tend to purchase ready-made staining solutions that frequently lack even basic declarations such as concentration, date of manufacture and expiry date. Little is known about the basic fuchsin dye, a mixture of various related compounds.[3,4] Harada et al. reported that the best fuchsins have a maximum absorption in alcohol at ⩾551 nm, corresponding to a colour index of 42 510 or 42 520.[3] While such basic specifications cannot always be obtained, the absorption maximum of a sample can be easily checked with a spectrophotometer during a tendering process.

The concentration of basic fuchsin in ready-made stains is estimated by measuring absorbance at 555 nm. Our experiments have indicated that various basic fuchsin brands have very similar standard absorbance curves (Figure 1), suggesting that its concentration can be estimated photometrically from a 1/1000 dilution based on such a curve. If the slopes are really that constant for all brands, it may even be possible to divide absorbance by about 0.15 to obtain the concentration in mg/l.

In addition, qualitative checks using control smears positive for acid-fast bacilli (AFB) are necessary to check staining properties. These can be deceptive, as even a poor quality reagent will stain some AFB in rich smears. Only 1+ (10–99 AFB/100 fields) smears should therefore be used for quality control of new stain batches, to increase the probability that they become negative with an inferior solution. A solution passes only if solid, completely stained (not too thin or beaded) and strongly red AFB are seen at the expected numbers.[5]

PREPARATION AND SHELF-LIFE OF CARBOLFUCHSIN

- WHO and Union guidelines recommend preparing carbolfuchsin by mixing a stock of basic fuchsin dissolved in 95% alcohol with phenol dissolved in water, and filtering the solution before use, or by placing paper strips on the smears.[1,2]

Few basic fuchsin brands dissolve well in alcohol, and filtering large quantities of carbolfuchsin solution or covering each smear with a filter paper strip is impractical. If one takes the trouble to probe, serious problems with carbolfuchsin preparations are regularly reported, and filtration is often simply neglected.

Figure 2 shows the results of hitherto unpublished experiments on the solubility of three brands of basic fuchsin. A stock solution was made in pure alcohol or phenol, in a mixture of both, or in a mixture of one of them with water, topping up the remaining components

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Article submitted 17 July 2007. Final version accepted 9 October 2007.

[A version in French of this article is available from the Editorial Office in Paris and from the Union website www.iuatld.org]

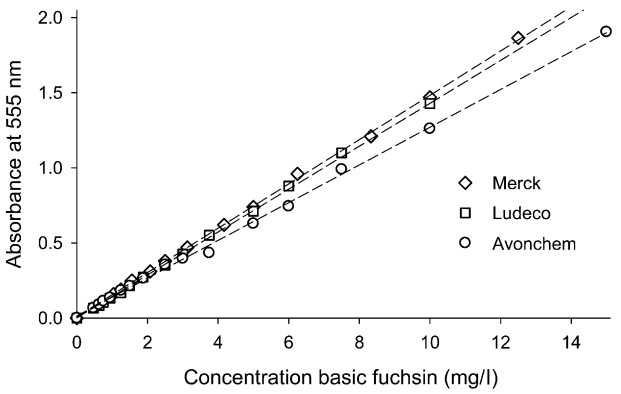

Figure 1 Standard absorbance curves by selected brands of basic fuchsin with completed staining solutions.

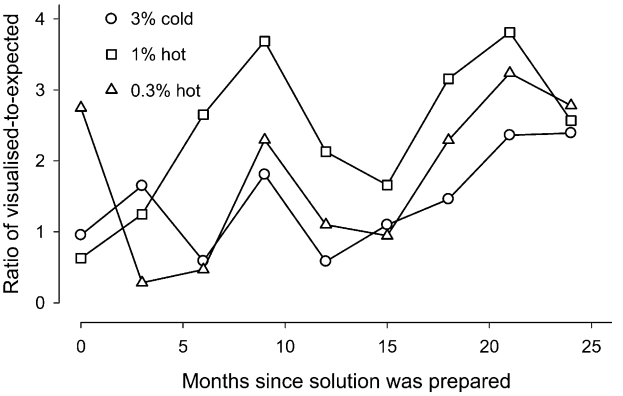

Figure 3 Ratio of visualised-to-expected number of AFB for various fuchsin concentrations and techniques by time since the solution was prepared. AFB = acid-fast bacilli.

afterwards. Merck New Fuchsin™ (Darmstadt, Germany) and Ludeco fuchsine Magenta™ (Brussels, Belgium) dissolved well in alcohol, but not the ubiquitous, inexpensive brand Avonchem Fuchsin basic™ (Macclesfield, Cheshire, UK). Absorbance at 555 nm was higher for a phenol/water or phenol/alcohol/water mixture than for other mixtures. Field experience shows that other brands, such as BDH Gurr Fuchsin basic™ (Poole, Dorset, UK), require the addition of water to the alcohol/phenol mixture to dissolve. A failsafe approach is to dissolve first the phenol in alcohol, then basic fuchsin together with 10–20% of the water. Once the basic fuchsin is completely dissolved, the solution is brought up to volume with the remaining water. Carbolfuchsin filtration is best and most conveniently done during staining, by pouring the solution on the smears through a funnel and filter paper. If the last 50 ml of carbolfuchsin is discarded, stocks do not need to be filtered.

- WHO and Union guidelines specify the shelf-life of carbolfuchsin solution stored in amber bottles at room temperature as being 6 to 12 months.[1,2]

Amber bottles are not commonly available. Furthermore, a high ambient temperature might adversely affect shelf-life. In a 2-year experiment at the Damien Foundation Bangladesh reference laboratory, carbolfuchsin staining solutions (0.3% and 1% basic fuchsin for hot ZN and 3% Kinyoun cold staining) were kept in the dark at about 35°C. Solutions were prepared as previously described.[6] Chemicals were provided by the NTP (Loba Fuchsin basic™, Mumbai, India). Two high-positive diagnostic smears, two low-positive follow-up smears and two negative smears, taken from lots of duplicate control smears, were stained quarterly. The ratio of visualised to expected AFB stayed above 1.0 for 2 years, indicating that there was no loss of potency in any of the solutions over time (Figure 3). These stained smears were then kept at ambient temperature in the dark and re-read weekly until complete fading of AFB. The speed of fading periodically accelerated during the monsoon seaso, but without a trend towards acceleration with ageing solutions, which might have indicated reduced staining capacity (Figure 4).

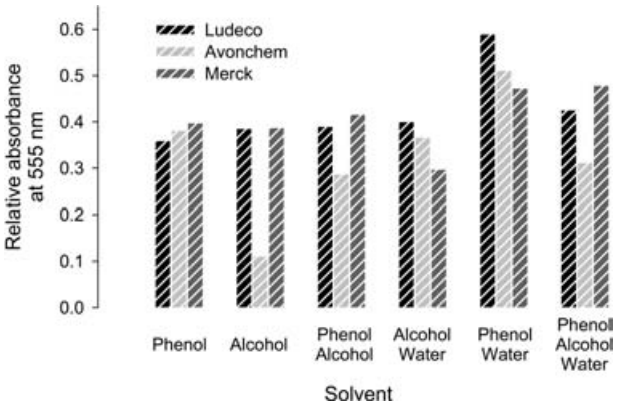

Figure 2 Solubility of three brands of basic fuchsin, showing the relative absorbance of the different brands by solvent.

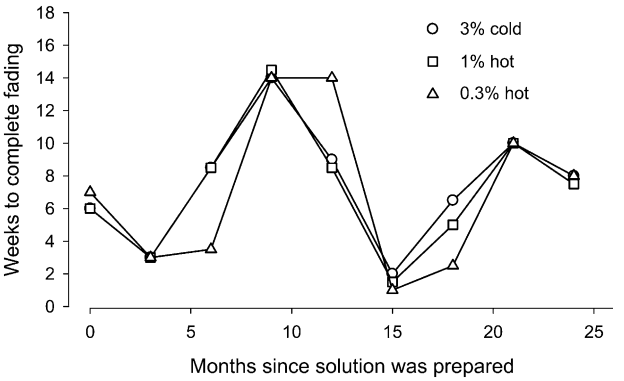

Figure 4 Fading of various fuchsin concentrations and techniques by time since the solution was prepared.

In the field, ready-made carbolfuchsin from renowned manufacturers was found to have deteriorated after about 5 years. Sunlight spoils stains rapidly, but amber bottles are not indispensable. Stocks can be stored in a cupboard in plastic containers, while solutions in use must be kept away from direct sunlight.

STAINING SMEARS

- The drawings in the Union guide illustrate the use of washing bottles for pouring staining solutions.

Bottles with a short spout are preferable for staining solutions, as washing bottles (plastic bottles that can be squeezed and have a long, bent spout) are untidy. Large, open water jugs are most practical for rinsing smears and are easy to clean.

- In the WHO guide illustrations, tubing attached to a tap is used for rinsing, and carbolfuchsin on the slides is heated with a Bunsen burner.

Tubing attached to a tap is not recommended, as it is rapidly colonised by environmental mycobacteria. These may become a source of contamination for staining solutions (which are often not prepared from distilled water in practice), or of confusion if restaining is unavoidable for correct rechecking.[7]

Bunsen burners or spirit lamps are very impractical for heating slides on a staining bridge. The flame of a spirit lamp is also too small, resulting in poor heating as the number of smears increases. For this purpose, the most efficient tool is also the most simple: an alcohol-soaked torch made from a metal rod and cotton wool.

SLIDES

- The Union guide recommends degreasing new slides before use.[2]

As degreasing is already done at the factory, this is unnecessary.[8] New slides may turn white-opaque and stick together due to humidity, not grease. The remedy is therefore to store slides in a dry place.

Acknowledgements

The authors wish to thank the Damien Foundation Belgium for their unconditional and continuous support. This publication was made possible through the support provided by the United States Agency for International Development (USAID) under the terms of Award No. GHS-A-00-03-00045-00. The opinions expressed herein are those of the author(s) and do not necessarily reflect the views of USAID.

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RÉSUMÉ

Les informations fournies dans les directives de l'Organisation Mondiale de la Santé et de l'Union Internationale Contre la Tuberculose et les Maladies Respiratoires en ce qui concerne la coloration de Ziehl-Neelsen ne sont pas pratiques à certains égards. Les avis donnés ici ont l'intention de fournir un complément à ces directives. Ils sont basés sur des expérimentations et sur l'expérience sur le terrain concernant la coloration à la fuchsine basique et les solutions de colorants.

RESUMEN

La información proporcionada por la Organización Mundial de la Salud y la Unión Internacional Contra la Tuberculosis y las Enfermedades Respiratorias sobre la tinción de Ziehl-Neelsen es poco práctica con relación a varios aspectos. Los consejos aportados en este artículo se consideran un complemento a tales recomendaciones y se basan en la investigación y en la experiencia sobre el terreno, especialmente sobre la tinción básica de fucsina y las soluciones de tinción.