Technical method

Use of microwaves for acid and alcohol fast staining

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Certain bacteria that are characterised by a high content of lipid in their cell walls cannot be stained by simple stains, and either heat or prolonged contact is required to drive the stain into the cells; once stained they resist decolourisation by mineral acids or acid and alcohol. These organisms are designated acid fast or acid and alcohol fast. They include *Mycobacterium tuberculosis* and related mycobacteria, *Mycobacterium leprae*, and certain of the actinomycetes. Koch first stained the tubercle bacillus by immersion in an alkaline solution of methylene blue for 24 hours.¹ Erlich used warm solutions of magenta (fuchsin) or methyl violet for 15–30 minutes; the bacilli then resisted decolourisation with strong mineral acids.² He believed that the bacilli were resistant to ordinary stains because they were surrounded by a capsule permeable only to alkalis. Ziehl, however, showed that they could be stained with an acidic dye or 2% alcoholic methyl violet solution in carbolic acid water.³ Later, Ziehl used carbolic magenta. Neelsen increased the strength of phenol in the stain,⁴ and the Ziehl-Neelsen method became the standard one for general use.

The property of acid fastness seems to be due to the presence in the bacilli of unsaponifiable wax,⁵ first isolated by Aronson in 1898.⁶ It was referred to as one of the higher alcohols by Bulloch and Macleod.⁷ Tamura isolated an alcohol from mycobacteria that had the property of acid fastness and named it "mycol."⁸ It was at first regarded as an unsaturated fat but is now generally believed to be lipoidal or waxy in nature. Not everyone agreed on the simple chemical explanation of acid fastness. Sordelli and Arena, for example, stated that it depended on a semipermeable membrane around the organism that allowed magenta to diffuse inwards but did not allow acid magenta to diffuse outwards.⁹ The degree of acid fastness of an

organism depends on several factors but remains an important component in the identification of a restricted group of organisms.

The technical problems associated with the classic Ziehl-Neelsen method include the dangers of heating by flaming torch in laboratories in which volatile organic solvents are used, the deposits that accumulate on the underside of the slide as a result of heating, and the crystalline deposits of stain that collect on the film as a result of evaporation and drying. This method also requires 20–30 minutes of laboratory time. Numerous minor modifications have been made to the Ziehl-Neelsen method by increasing the concentration of carbolic magenta or by cold staining techniques,^{10–12} but the basic problems remain.

Some of the problems may be overcome by the use of microwave energy, a type of high frequency (2450 MHz) radiowave. It is absorbed by molecules of water, causing them to vibrate at a very high frequency and produce heat, which radiates out; consequently, there is an increase in the turgor pressure within the cells and the bacterial cell becomes more permeable, allowing easy passage of soluble products as shown by the increased permeability of bacterial enzymes.¹³ Prolonged exposure results in disruption and destruction of the cells.

We describe a new rapid approach to the Ziehl-Neelsen stain using a microwave oven for the stage of heating in carbolic magenta. The procedure also eliminates the need to treat histopathological section with xylol and alcohol and sheds some light on the way in which acid fast organisms react with stains.

Material and methods

MICROWAVE OVEN

A domestic oven (Sharp model 9410E) with a maximum output of 640 watts, four power settings, and a digital timer was used.

SPECIMENS

Clinical smears of sputum from patients with pulmonary tuberculosis and from non-tuberculous controls were prepared in the normal way and dried in

air. Histological sections of lung from patients with tuberculosis and patients with infections with *Nocardia asteroides* prepared in paraffin wax were provided by the department of histopathology.

ORGANISMS

Pure cultures of *M. tuberculosis*, non-acid fast bacteria including *Bacillus spp.*, clostridia, corynebacteria, staphylococci, streptococci, *Haemophilus spp.*, enterobacteriaceae, and neisseriae, *N. asteroides* NCTC 11293, and *Actinomyces israeli* NCTC 10236 were cultured on recommended media and grown in optimum conditions. Smears were made in the normal manner and dried in air. Pure cultures of *M. tuberculosis* were mixed with other organisms and smears made.

STAINS FOR MYCOBACTERIA

Carbolic magenta, magenta, and saffron were used for initial staining. Carbolic magenta was prepared according to the method described by Cowan and Steel.¹⁴ A solution of saffron (1%) was made in distilled water. Magenta was made by dissolving 1 g magenta in 10 ml absolute alcohol, which was then made up to 100 ml with distilled water.

COUNTER STAIN

Malachite green was prepared according to the method described by Cowan and Steel.¹⁴

STAINING METHODS

Standard Ziehl-Neelsen method Smears were flooded with carbolic magenta and heated until steaming with a torch for 10 minutes, washed with water, decolourised with acid alcohol (hydrochloric acid 3% in 95% alcohol), and counter stained with malachite green.

Microwave method Smears were flooded with carbolic magenta, placed in a sandwich box with the lid on, and exposed to microwave irradiation for 30 seconds at full power (640 watts). They were then decolourised with acid alcohol and counterstained with malachite green.

A series of 15 sets of slides was made from the cultured organisms. One set was stained by the standard Ziehl-Neelsen method, two sets were stained with magenta or saffron, heated by the conventional torch method, decolourised by acid alcohol, and counter stained by malachite green, four sets were flooded with one of three stains (carbolic magenta, magenta, or saffron) and exposed to microwave irradiation in the oven for 30 seconds at full power; one set treated with each stain was then decolourised by 1%, 5%, and 20% sulphuric acid and by acid alcohol.

On the basis of the results with pure cultures of

organisms, the limited number of clinical slides were stained by the following methods: one set of sputum smears was stained by the standard Ziehl-Neelsen method; a duplicate set was flooded with carbolic magenta, placed in the microwave oven for 30 seconds at full power, decolourised with acid alcohol, and counter stained with malachite green. One set of histological sections from patients with tuberculosis was stained by the standard Ziehl-Neelsen method. A second set was flooded with carbolic magenta without having been treated with xylol and graded alcohol, placed in the microwave oven for 30 seconds at full power, decolourised with acid alcohol, and counterstained with malachite green. The third set was treated as the second, except that the primary stain used was saffron.

Sections of lung from a patient with nocardia infection were flooded with carbolic magenta, placed in the microwave oven for 30 seconds at full power, decolourised with acid alcohol, and counter stained with malachite green.

Results

Table 1 shows the results with pure cultures, sputum smears, and histological sections. There were no differences between the results obtained with the standard Ziehl-Neelsen staining technique and the method based on microwave treatment in terms of proof of acid and alcohol fastness. The slides were cleaner, however, after treatment with microwaves, and there were no crystalline deposits of stain in the smears. Table 1 shows only the results obtained when the decolourising agent was acid alcohol. The mycobacteria were consistently acid and alcohol fast, unlike the other bacteria examined.

Table 2 records the effects of different decolourising solutions. Only *Mycobacterium spp.* were resistant to decolourising by acid alcohol or 20% sulphuric acid. *N. asteroides* was resistant to 1% sulphuric acid and about 50% of cells in the smears were resistant to 5% sulphuric acid. *A. israeli* and other non-acid fast species were decolourised by 1% sulphuric acid. Identical staining reactions were achieved by substituting magenta or 1% saffron for carbolic magenta and exposing the smears to microwave energy, although the acid and alcohol fast species were not stained by these particular stains when conventional heat was used.

The staining of sections of tissue (without dewaxing) by the microwave method with carbolic magenta or magenta was comparable with the results obtained by the conventional Ziehl-Neelsen method (after dewaxing), but 1% saffron was not suitable for histological sections as the dye was not

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Table 1 Comparison of acid and alcohol fast staining by standard Ziehl-Neelsen and microwave methods

Type of specimen	No of specimens examined	No positive by:	
		Ziehl-Neelsen method	Microwave method
Tuberculous sputum	30	30	30
Non-tuberculous sputum	30	0	0
Nocardia sputum	1	0	0
Cultures of <i>Mycobacterium tuberculosis</i>	30	30	30
Cultures of <i>Mycobacterium bovis</i>	1	1	1
Cultures of <i>Mycobacterium kansasii</i>	1	1	1
Cultures of <i>Nocardia asteroides</i>	1	0	0
Cultures of <i>Actinomyces israeli</i>	5	0	0
Cultures of non-acid fast bacteria	10	0	0
Histological sections of tuberculosis	4	4	4
Histological sections of nocardiasis	2	2	2

retained when the sections were treated with acid alcohol.

Discussion

The microwave method of acid fast staining was as reproducible and reliable as the standard Ziehl-Neelsen staining method. The advantages for the routine diagnostic laboratory over the standard method were that the slides were cleaner; there were no crystalline deposits on the smears; processing of smears was much quicker as the time was reduced by 15 minutes for each batch; no flames were required, and thus there was no risk of the slides cracking as a result of direct heat or of fires starting due to naked flames in the laboratory; and the smears were safe because mycobacteria are killed by microwave irradiation in 30–60 seconds in the absence of phenol (unpublished data). The characteristics of decolourisation remained the same, and a particular advantage for staining histological sections was that the tedious stages of dewaxing were eliminated.

This study also suggests that the acid fastness is probably a process in two stages. In the first stage, as a result of heating or exposure to microwaves, the stain penetrates the cell wall of acid fast organisms; penetration of stain during exposure to microwaves

is not simply a result of heating but also reflects increased permeability of the cell wall. In the second stage the stain probably forms a complex with mycol or other components of acid fast organisms that is insoluble in acid alcohol and therefore indelible; even the exposure of smears of acid fast bacteria to microwave radiation during decolourisation failed to decolourise the organisms, showing that it is not merely a matter of permeability.

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Table 2 Acid fast staining of smears from bacterial cultures by microwave method using different decolourising agents

Organisms	Acid fastness after decolourisation with:			
	Sulphuric acid			Acid alcohol
	1%	5%	20%	
<i>Mycobacterium tuberculosis</i>	+	+	+	+
<i>Mycobacterium bovis</i>	+	+	+	+
<i>Mycobacterium kansasii</i>	+	+	+	+
<i>Actinomyces israeli</i>	–	–	–	–
<i>Nocardia asteroides</i>	+	± (50%)	–	–
Non-acid fast bacteria	–	–	–	–

¹² Hok TT. A simple and rapid cold staining method for acid fast bacteria. *Am Der Resp Dis* 1962;**85**:753-4.

¹³ Spencer RC, Hafiz S, Cook C. Effect of microwave energy on the metabolism of Enterobacteriaceae. *J Med Microbiol* 1985;**19**:269-72.

¹⁴ Cowan S, Steel KJ. *Manual for the identification of medical bac-*

teria. 2nd ed. Cambridge: Cambridge University Press, 1974:161-3.

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Letters

Measurements for haemoglobin

During the meeting of the International Committee for Standardisation in Haematology (ICSH) secretariat it was noted that an increasing number of papers published in both monographs and journals are now using the World Health Organisation International Committee for Standardisation in Haematology/International Society of Haematology recommendation to express haemoglobin as gram/litre (g/l). Furthermore, haemoglobinometers, which use this scale, are being introduced.

The ICSH secretariat would like to point out the need for a rationalisation of the expression of haemoglobin results that would conform with the principles of SI units, and which should be encouraged as quickly as possible.

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(Will authors please note. Editor)

Convulsions and encephalopathy in a patient with leukaemia after treatment with metronidazole

Metronidazole is an antimicrobial agent commonly used to treat anaerobic bacterial infections. We consider that the side effect of convulsions resulting from treatment with metronidazole¹ may often be unrecognised, particularly as a consequence of cumulative exposure^{2,3} in a patient receiving many other drugs and suffering many complications of treatment. We describe a patient with acute myeloid leukaemia, who developed epileptiform seizures, encephalopathy, and sensory neuropathy after treatment with metronidazole.

A 43 year old woman presented with acute myeloid leukaemia (FAB classification M4) in December 1984. She was treated with Cytarabine 100 mg/m² intravenously for seven days and daunorubicin 50 mg/m² for three days.

During the course of chemotherapy she developed a fever, but no causative organism was identified. Metronidazole (a total of 18.5 g) was given intravenously over the next 12 days in combination with a penicillin and gentamicin. The induction treatment was repeated on 17 December for a further week. Ten days later she became feverish again and *Staphylococcus epidermidis* was isolated from blood cultures. Treatment with fucidin and netilmicin was started and there was an initial improvement, although the temperature never settled. Metronidazole (a total of 10 g) was added with the onset of diarrhoea because pseudomembranous colitis was suspected. A few days later the patient became acutely unwell, had rigors, and the parenteral antibiotics were changed to erythromycin, rifampicin, and ceftazidime. There was dramatic improvement and after five days all antibiotics were stopped.

The patient was clinically well, without fever, and ready for discharge when she was found on the floor having a grand mal fit. Over the next 24 hours she had several more fits despite treatment with chlor-methiazole and phenytoin. A computed tomographic scan showed no focal lesion. An electroencephalogram showed encephalopathy. The urea and electrolyte concentrations sampled 30 minutes before the first fit were normal. The platelet count had not fallen below $60 \times 10^9/l$.

The patient made a recovery to normal within 48 hours, but during this period she suffered some retention of urine. A repeat electroencephalogram four weeks later showed moderate abnormality. At this time the patient was in complete remission awaiting consolidation of treatment.

The patient received a total of 29 g metronidazole and developed epilepsy, encephalopathy, and, probably, sensory neuropathy manifested by retention of urine. Acute encephalopathy and epilepsy secondary to metronidazole probably resulted from a high cumulative dose.³ It is known that the drug crosses the blood brain barrier; in dogs it has been shown that high doses of metronidazole induce Purkinje cell lesions.⁴ It is especially important to be aware of this side effect in the leukaemic patient to avoid unnecessary invasive investigations in the search for

hidden leukaemic deposits or foci of infection.

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Enzyme markers in acute non-lymphoid leukaemia

We read with interest the recent review in the journal by Dr Drexler *et al.*¹ It is stated that "the main group AML did not show one distinct enzyme marker phenotype". While this may be true for the purine salvage enzymes, we would like to draw attention to the use of certain lysosomal enzymes in the diagnosis and classification of acute myeloid leukaemias in both adults and children.² We have now studied 50 patients with acute non-lymphoid leukaemia and have measured the activities and isoenzyme profiles for the enzymes: β hexosaminidase and α mannosidase. A summary of our results is shown in the table. Patients with AML, AMOL, and AMOL showed significant increases in these enzyme activities when compared with peripheral blood granulocytes. In leukaemic cells of lymphoid origin. Moreover, in patients with AML in particular there was a reduction in β hexosaminidase B isoenzyme peak when compared with the A component.