organism correctly. The long ITS had an advantage over the short ITS region, as it was more specific, thus allowing more reliable speciation of target organisms.

Health service laboratories adopting molecular rDNA methods for the correct identification of medically important fungi from culture are faced with the dilemma of choosing which rDNA region, and hence which primer pair or pairs, to use from the numerous options reported in the literature. The present study demonstrates that each primer pair has its own sets of advantages and disadvantages in terms of the criteria described above (Table 3).

Overall, the results of this study support the use in tandem of both the short and the long ITS rDNA regions for the purposes of molecular (rDNA) identification and reconfirmation of fungi of medical importance.

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Use of heat provides a fast and efficient way to undertake melanin bleaching with dilute hydrogen peroxide

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Heavily pigmented melanocytic lesions can often be difficult to interpret especially when attempting to define nuclear atypia and therefore substantiating a benign or malignant diagnosis. The presence of melanin pigment poses two fundamental problems. First, it has a direct physical masking effect on antigen–antibody interactions, due to the intracellular nature of the pigment. Second, 3,3-diaminobenzidene (DAB), the most widely employed chromogen for demonstrating antigen/antibody reactions, is also brown in colour and therefore can be difficult to distinguish from melanin pigment.

Table 1. Antibodies used to assess the effects of melanin bleaching using dilute hydrogen peroxide.

Antibody	Source	Dilution	Pretreatment
S100 protein	Dako	1:4000	Trypsin
HMB 45	Launch	1:40	Microwave
NKIC3	Launch	1:40	Microwave
Melan A	Launch	1:30	Microwave
CD3	Vision Biosystems	1:100	Microwave
CD20	Dako	1:500	Microwave
CD68	Dako	1:100	Trypsin
CD34	Vision Biosystems	1:25	None
CD45	Dako	1:50	Microwave
CD31	Dako	1:10	Trypsin
SMA	Dako	1:250	None

Dako, Denmark House, Angel Drove, Ely, Cambridge, UK.
Launch Diagnostics, Ash House, Ash Road, New Ash Green, Longfield, Kent.
Vision Biosystems (Europe). Balliol Business Park West, Benton Lane, Newcastle upon Tyne.

Traditional techniques have used coloured chromogens such as alkaline phosphatase (red)[1] or have employed tinctorial techniques to stain melanin pigment a different colour from the antigen–antibody final reaction product (e.g., azure B).[2] These methods have merit in certain circumstances, but, in the author's experience with cutaneous and metastatic deposits of heavily pigmented lesions, they do not always enable accurate assessments of the exact localisation of antigen–antibody interactions (e.g., membrane or cytoplasmic labelling profiles). This problem may be exacerbated if the antigenic epitope is co-expressed on cell membranes and in the cytoplasm (e.g., S100 protein).

The two widely used bleaching procedures, permanganate/oxalate and dilute hydrogen peroxide (H_2O_2), have advantages and disadvantages.[3–7] In the author's experience, the two most significant factors are bleaching time and the range of antibodies employed. Permanganate/oxalate has a clear advantage, as bleaching can be achieved in most cases within an hour and can be incorporated easily in conventional daily immunostaining protocols. In contrast, the H_2O_2 method requires 24 hours.

However, the range of antibodies that can be used following these two bleaching procedures varies. The H_2O_2 method allows extensive application of antibodies, whereas permanganate/oxalate permits a useful but restricted range.[8]

The use of heat-mediated antigen retrieval techniques in immunocytochemistry is now well established, and whether or not such methods can be utilised to increase the speed of

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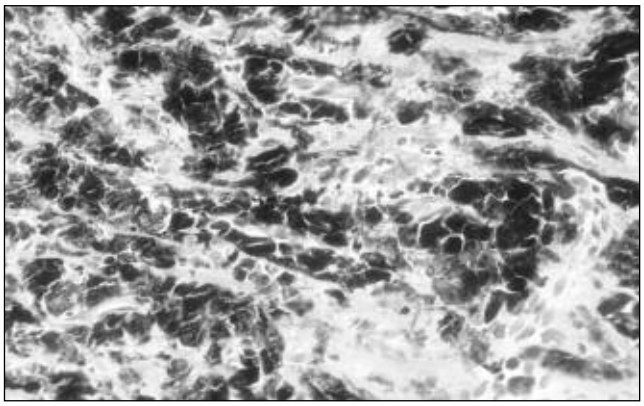

Fig. 1. A dysplastic naevus prior to bleaching showing extensive dermal melanosis due to the presence of foamy cells filled with melanin (H&E, original magnification x100).

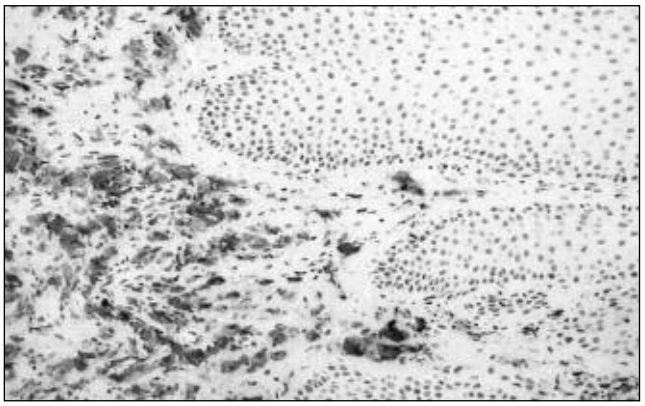

Fig. 2. Dermal foamy melanophages labelling with anti-CD68 following treatment with dilute H_2O_2 bleaching in a conventional 60˚C oven for two hours (original magnification x60).

bleaching melanin in tissue sections is worthy of further investigation. In this study, an appraisal of bleaching procedures using dilute H_2O_2 heated in a variety of ways is investigated.

Five cases of melanocytic lesions with extensive dermal melanosis were assessed for two criteria: speed of completion of bleaching, and the effectiveness of subsequent immunocytochemical investigations.

Sections were cut from all five cases and mounted on superfrosted poly-L-lysine-coated slides (VWR International). The sections were dewaxed and taken to water. The bleaching procedure was performed using dilute H_2O_2, as previously described.[8] Briefly, sections were placed in 10% H_2O_2 in phosphate-buffered saline (PBS; pH 7.6) in a Coplin jar. Sections were then heated by one of two methods: in a conventional drying oven at 60˚C, or in a water bath at 60˚C. In addition, slides were left for 24 hours in dilute H_2O_2 at room temperature as a control procedure. Optimal times for the completion of bleaching defined microscopically were then determined for each method.

Following bleaching, slides were placed in running tap water before undergoing antigen retrieval using either heat-mediated retrieval in 0.01 mol/L sodium citrate buffer (pH 6.0) in a conventional microwave oven for 12 or 18 min, or enzyme digestion with 0.1 g trypsin at 37˚C for 5 min. The antibodies employed following bleaching are listed in Table 1. In all cases, negative controls involving the omission of the primary antibody and incubation with PBS were included. The Dako Real HRP detection system was used for all antibodies.

Visualisation of the final reaction product was achieved using DAB and all sections were counterstained with Harris' haematoxylin (1 min). Positive controls for each antibody were included on unbleached immunostained sections of known positive tissues.

The bleaching procedures using conventional heating in an oven or water bath increased the speed of bleaching considerably. All cases examined by both processes achieved completion of bleaching within 150 min. In comparison, sections left at room temperature required 24 hours for completion of bleaching.

All immunolabelling results achieved with a panel of markers following bleaching were comparable, irrespective of the bleaching process used (Figs 1–3). No discernable loss of antigen demonstration was seen in any one of the three

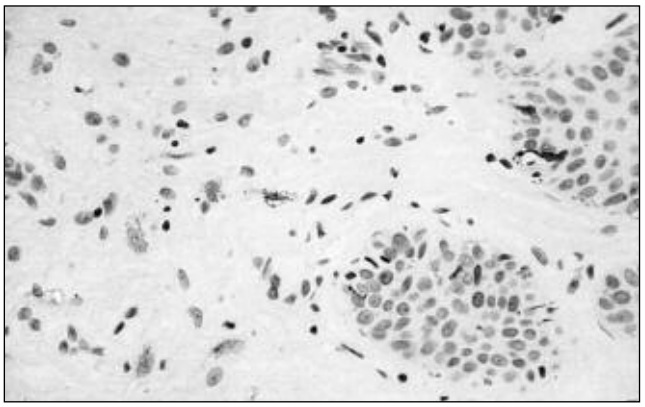

Fig. 3. Labelling with HMB45 following bleaching with dilute H_2O_2 in a waterbath at 60˚C, showing unstained dermal melanophages but positive labelling of melanocytes in the epidermis (original magnification x70).

methods used. There was also no evidence of non- specific labelling with any of the antibodies tested.

This study shows that bleaching times using dilute H_2O_2 can be reduced from an average of 24 hours at room temperature to 150 min at 60˚C. This enables bleaching and immunolabelling to be completed on the same day. The immunolabelling achieved was not compromised in any way when compared to slides bleached for 24 hours at room temperature.

In conclusion, bleaching with dilute H_2O_2 in PBS at 60˚C using a conventional drying oven or a water bath method significantly increases the speed of completion of bleaching and also allows the application of a wide selection of antibodies. This additional modification to a well-documented bleaching method has significant benefits in terms of increased speed and efficiency and is recommended when performing immunocytochemical investigations.

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Alcohol and its influence on the survival of *Vibrio cholerae*

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Microbiologists in Inverness have become interested in the great cholera outbreak in Britain in 1831–32.[1] In 1832 the immoderate drinking of alcohol was considered a predisposing cause in the acquisition of cholera, as its incidence was much higher in the heavy drinker than among the general population.[2] In response to cholera's impending arrival in the capital of the Highlands, the newly formed Inverness Board of Health advised the population "to have great moderation in the use of fermented and spirituous liquors".[3] Was this advice correct?

More recently, in 1979, restrictions on alcohol also formed part of the public health advice during an outbreak of El Tor cholera in Tanzania, where locally made alcoholic beverages, which were made largely with untreated water, were banned for fear that these would act as vehicles of infection.[4]

The drinking water in Inverness, which was drawn from the River Ness during the first cholera epidemic in the late summer of 1832, would have been contaminated with the excreta of cholera victims. The spirits that would have been drunk in Inverness may have been diluted with this water before being sold.

There was no legal definition of proof spirit until 1879 and retailers in the 1830s diluted spirits with water for extra profit.[5] Thus, this study is designed to examine the effects of alcohol on *Vibrio cholerae* survival.

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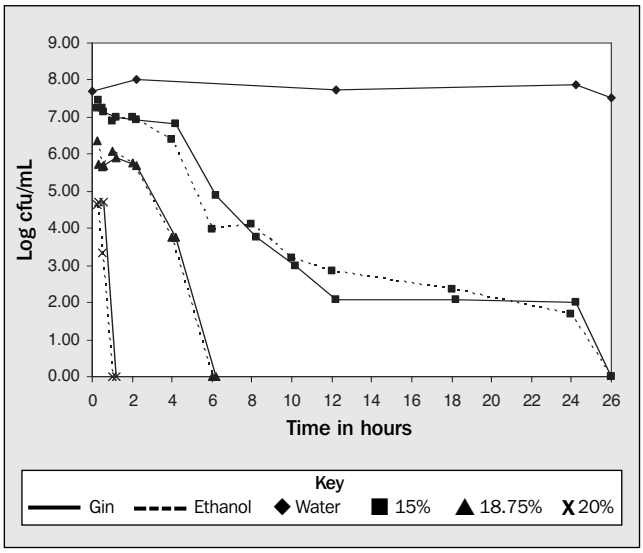

Fig. 1. Survival of *V. cholerae* in gin and ethanol.

The organism used was *V. cholerae* (NCTC 11348). Dry London Gin (37.5%, Tesco) was tested in parallel with equivalent concentrations of ethanol in a series of four experiments. A 16- to 18-hour culture of *V. cholerae* in alkaline peptone water was suspended in sterile de-ionised water (pH 7.0) to give a density of 10^7 colony forming units (cfu)/mL.

Contaminated water (5 mL) was added to an equal volume of the gin and the ethanol to give a final alcohol concentration of 20%, 18.75% and 15% (5.34 mL of gin to 4.66 mL of contaminated water for 20% gin). The test solutions were incubated at 13°C, which was the average local ambient temperature when cholera was present in Inverness in 1832.[6]

A sample (1 mL) was removed and decimal dilutions were performed using 9 mL 0.1% peptone (Oxoid CM 0733) with NaCl (pH adjusted to 8.0) at 15 min, 30 min, 1 h, 2 h and at two-hourly intervals thereafter until an end point was reached. Aliquots of 200 µL were removed from each decimal dilution, plated on two thiosulphate citrate bile salt (TCBS) agar (Oxoid CM 0333) plates and incubated at 37°C for 18–24 h. The density of contaminated water was tested at the beginning and the end of the experiments.

If sparse or no growth was apparent at 24 h, the plates were re-incubated for a further 24 h and bacterial growth was assessed again at 48 h. Bacterial growth was measured as the number of yellow colonies seen with the naked eye, and the results of the duplicate plates were averaged and multiplied by the dilution factor. The lower limit of detection was 20 cfu/mL.

Figure 1 shows the survival of *V. cholerae* in 20%, 18.75% and 15% gin over 26 h. In 20% gin there was a 3 log reduction in the number of *V. cholerae* isolated at 15 min, with a total reduction in numbers after 1 h. The reduction was less dramatic in 18.75% gin, with no *V. cholerae* surviving after 6 h. In 15% gin, the numbers fell slowly to zero after 26 h. Identical survival times were found in all three ethanol dilutions tested (Fig. 1). In contrast, the number of *V. cholerae*