probable source was bacterial translocation through the intestinal wall. The two cases of thrombophlebitis of a vein catheterised with a deep catheter device were probably caused by a secondary catheter infection.

Mortality associated with fusobacterium bacteraemia was 11.5%. Considering the poor underlying medical condition of most of the patients, death may have been secondary to the severe underlying disease rather than fusobacterium infection. *Fusobacterium* spp. remain highly susceptible to first-line antibiotics such as β-lactams [7], but penicillinase production has been reported for some strains of *F. nucleatum* [8].

Only one patient presented with classic Lemierre's syndrome, perhaps because many cases of undiagnosed fusobacterium tonsillitis are treated with β-lactams before the onset of Lemierre's syndrome. In other countries, restricted use of antibiotics for tonsillitis has been associated with an increased risk of *F. necrophorum* sepsis [9].

Twelve patients presented with a haemostasis disorder associated with both *F. nucleatum* and *F. necrophorum*. This may reflect a non-specific haemostasis disorder, although *F. necrophorum* has been shown to display platelet-aggregating activity [10]. This has not been demonstrated for *F. nucleatum*. The present data indicate that both *Fusobacterium* spp. have a direct and specific effect on haemostasis.

Fusobacterium spp. should be considered as opportunistic pathogens in patients with impaired health status, caused by recent surgery (especially of the gut), evolving neoplasm, myelotoxic chemotherapy and perhaps deep venous catheterisation. Lemierre's syndrome, which was once a classic clinical presentation of fusobacterium infections, may become infrequent in developed countries. Secondary haemostasis disorders are frequently associated with fusobacterium bacteraemia. Nevertheless, the mortality rate of fusobacterium bacteraemia remains low.

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RESEARCH NOTE

Comparison of two fluorescent whiteners, Calcofluor and Blankophor, for the detection of fungal elements in clinical specimens in the diagnostic laboratory

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ABSTRACT

Fluorescent whiteners, such as Blankophor and Calcofluor white, bind to chitin and cellulose, and fluoresce when exposed to UV light. Detection of fungal elements from skin and nail samples was faster and more accurate using Blankophor compared with potassium hydroxide preparations and

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Calcofluor (sensitivity and specificity 100% and 86% vs. 83–90% and 84–88%, or 80% and 84%, respectively). Visibility was improved, and the procedures were simple, inexpensive and rapid, all of which are important considerations in a busy diagnostic laboratory.

Keywords Blankophor, Calcofluor white, dermatology, diagnosis, fluorescent whiteners, fungal elements

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Laboratory diagnostic procedures in dermatological mycology are based on direct microscopy and culture of clinical material. The most widely used and accepted method involves a wet preparation made using potassium hydroxide (KOH) 10% w/v. KOH dissolves most cellular debris readily without affecting the chitinous cell wall of fungi. However, KOH preparations often contain artefacts which are difficult to distinguish from fungal elements, thus limiting interpretation.

Fluorescent whiteners, such as Blankophor and Calcofluor white, have long been used in the paper industry and as fabric whiteners in washing powders [1]. These compounds bind to chitin and cellulose, and fluoresce when exposed to UV light [1]. Neither Blankophor nor Calcofluor have any health and safety implications when used in the laboratory, and both are inexpensive to purchase. The addition of such whiteners to KOH preparations has been shown previously to improve detection of fungal elements by increasing the contrast from background debris [2–6]. The present study examined the ability of Calcofluor and Blankophor to detect fungal elements in clinical specimens in a diagnostic laboratory setting, and compared the results with those obtained using KOH alone.

Routine dermatological specimens were examined in parallel. A sequential cohort design was adopted. KOH was compared initially with Calcofluor, and then with Blankophor. A clinically significant culture result was used as the confirmation of a positive preparation. Results for *Pityriasis versicolor* were omitted from the calculations as these could not be confirmed with a positive culture. Calcofluor (Sigma, Poole, UK) was dissolved (0.025% w/v) in deionised water, as permanent precipitation occurred at the extreme pH of KOH. One drop of 10 M NaOH was added to clear the solution. Examination of clinical specimens (n = 183) using Calcofluor was a two-step process. First, a drop of KOH 10% w/v (Sigma) was placed on a slide and a small amount of clinical material was added; then 5–10 μL of Calcofluor solution was introduced under the coverslip using a capillary tube. Blankophor (Valeant Pharmaceuticals, Costa Mesa, CA, USA) was prepared directly (0.001% v/v) in KOH 10% w/v. Examination of clinical specimens (n = 138) using Blankophor was a one-step process in which clinical material was added directly to a drop of Blankophor/KOH solution. The effects of temperature and light on storage of both fluorescent solutions were examined over a period of 99 days.

All preparations were examined using a dual light and fluorescent microscope fitted with a 350–460 nm excitation filter. First, preparations were examined using light microscopy at ×100 and ×250 magnification (as is routine for KOH preparations) and fungal elements were recorded. The fluorescent filter was then switched on and the slides were re-examined using fluorescence microscopy. This technique ensured that results for KOH and each fluorescence method were directly comparable, and that sampling errors and bias were avoided. A *Candida albicans* positive control was prepared for each batch of preparations being examined. Stained preparations of *C. albicans*, stored in a wet box at room temperature in the light, remained stable for at least 72 days.

All specimens were then dissected and cultured on Sabouraud glucose agar (Oxoid, Basingstoke, UK) and malt extract agar (Oxoid), and incubated for 4 weeks at 30°C. Sensitivity, specificity, positive and negative predictive values and efficiency were calculated. The standard error of the differences in percentages was calculated using the Z-test, and the statistical significance was evaluated. Ease of use of Calcofluor was also evaluated. Sixteen laboratory personnel, with varying levels of scientific training and experience, examined six preparations in a blinded fashion (three containing fungal elements). Results and comments were recorded.

Specimens of skin scrapings, nails and hair (n = 321) were examined in two sequential study cohorts with one observer during a 4-month period. The performance of Calcofluor, Blanko-

Table 1. Comparison of the performance of Calcofluor, Blankophor and KOH for the detection of fungal elements in clinical specimens

Method	Total specimens	True positives	False positives	True negatives	False negatives	Prevalence %	Sensitivity %	Specificity %	Efficiency %	Positive predictive value %	Negative predictive value %
Calcofluor	183	24	24	129	6	16.3	80	84	83	50	95.5
cKOH		25	24	129	5	16.3	83	84	84	51	96.2
Blankophor	138	30	15	93	0	21.7	100	86	89	66	100
bKOH		27	12	96	3	21.7	90	88	89	69	96

cKOH, KOH examined in parallel with Calcofluor; bKOH, KOH examined in parallel with Blankophor.

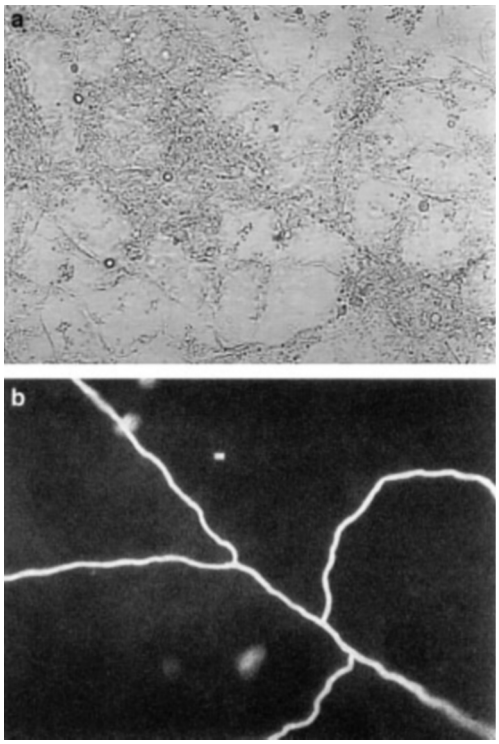

Fig. 1. (a). KOH 10% w/v preparation examined with ×250 magnification light microscopy; (b) the same field stained with Blankophor 0.001% v/v and examined using UV microscopy. The culture of this specimen grew *Microsporum canis*.

phor and KOH is summarised in Table 1. Blankophor appeared to be significantly more effective at revealing fungal elements than both KOH (p 0.0202) and Calcofluor ($p < 0.0001$). This appeared to be true for a broad range of fungi from multiple specimens. An example is shown in Fig. 1. The Calcofluor and Blankophor working solutions were both unaffected by light and temperature, and both were stable for >3 months. Experienced staff found the Calcofluor preparations to be easier and more rapid to use than KOH alone, as the slides could be screened at a lower magnification. Those with less scientific training and experience had some problems distinguishing artefacts from fungal elements with Calcofluor.

Overall, the use of fluorescent whiteners provided an accurate alternative for the detection of fungal elements in clinical specimens in a routine laboratory setting. The technique is safe, uncomplicated, quick, reliable and inexpensive, all of which are important considerations in a busy diagnostic laboratory. The study further demonstrated that the addition of a fluorescent whitener to KOH preparations increases the contrast between background debris and fungal elements (Fig. 1). A key advantage was the fact that it can typically take up to 3 min to screen a KOH sample, compared with 30–60 s to screen a fluorescent whitener sample, as the latter preparation can be screened easily on low power (×10).

Haldane and Robart [4] have previously compared Calcofluor and KOH for the examination of dermatological specimens. Calcofluor was found to have a higher sensitivity (92%), compared with KOH (88%), with equivalent specificity (95%). In the present study, Blankophor had increased sensitivity compared with both Calcofluor and KOH. However, the Calcofluor study was undertaken first, and thus the possibility of learning bias cannot be excluded. Furthermore, the Calcofluor method was a two-step process, whereas that of Blankophor only involved one step. Nevertheless, fluorescent staining of dermatological specimens appears to be a feasible and rapid method for use in the clinical laboratory setting.

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RESEARCH NOTE

Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR

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ABSTRACT

A real-time PCR assay for *Clostridium difficile* was developed, based on the *tcdB* gene, which detected all known toxinogenic reference strains (n = 45), within 30 serogroups and 24 toxinotypes. The analytical sensitivity was 1×10^3 CFU/mL, and the detection limit in faeces was 1×10^5 CFU/g. The optimal protocol for DNA extraction from faecal samples involved use of the MagnaPure system with a Stool Transport and Recovery (STAR) buffer pre-treatment. In a 1-month prospective study of 85 patients with diarrhoea, the sensitivity, specificity and positive and negative predictive values of the assay were 100%, 94%, 55% and 100%, respectively, compared with the standard cell cytotoxicity assay.

Keywords Assay, *Clostridium difficile*, faecal samples, real-time PCR, *tcdB* gene

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Clostridium difficile has been recognised as the causative agent of antibiotic-associated diarrhoea (CDAD) and pseudomembranous colitis (PMC). Enteropathogenicity depends on the production of enterotoxin A (TcdA; 308 kDa) and cytotoxin B (TcdB; 270 kDa) [1,2]. TcdA has been regarded as the most important factor causing enteropathogenic disease [3,4], but there have been an increasing number of reports of disease caused by TcdA-negative, TcdB-positive strains [5]. Therefore, the present study designed a real-time PCR assay for *tcdB* to enable rapid diagnosis of CDAD associated with toxinogenic *C. difficile*. An optimal DNA extraction protocol for faecal samples was established, and an internal control was included to verify amplification.

Primers and probe (Table 1) were designed from the non-repeat region of a known *tcdB* sequence (accession no. X53138) using the Primer3 program (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplification reactions were performed in a 50-μL final volume, containing 25 μL IQ Supermix (Bio-Rad, Veenendaal, The Netherlands), 5 pmol forward primer, 10 pmol reverse primer, 4 mM $MgCl_2$, 0.2 μM probe, and 5 μL DNA extract. Following an enzyme activation step for 3 min at 95°C, amplification comprised 50 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C in an iCycler IQ real-time detection system (Bio-Rad). The assay was optimised using *C. difficile* strain ATCC43594, and had an analytical sensitivity in saline 0.9% w/v of 1 CFU/PCR, corresponding to 1×10^3 CFU/mL. In addition, ten-fold dilutions of ATCC43594 (1×10^7–1 CFU) were spiked into 1 g of pooled *C. difficile* culture-negative faeces to determine the sensitivity of the real-time PCR assay in comparison with culture.

For *C. difficile* culture, faecal samples, with and without ethanol-shock treatment, were inoculated on to selective media as described previously [6]. Colonies of Gram-positive bacilli with sub-terminal spores were tested for L-proline-aminopeptidase production and aesculin hydrolysis [7]. Two separate experiments revealed that the sensitivity of the real-time PCR assay with faeces was less