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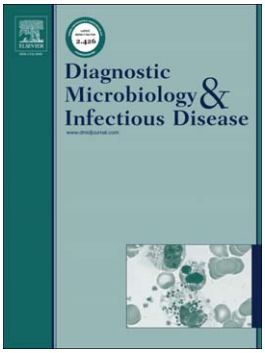

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Diagnosis of dermatophytosis: an evaluation of direct examination using MycetColor® and MycetFluo®

Running title: Evaluation of MycetColor® and MycetFluo®

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Abstract

Dermatophytes are an important cause of superficial fungal infection. Direct examination of skin, nail or hair samples remains essential in diagnosis as it provides a quick response to the clinician. However, mycological analysis, including direct examination and culture, often lacks sensitivity. The use of stains or fluorochromes may enhance the performance of direct examination. We analysed 102 samples from patients with suspected dermatophytosis in four different diagnostic mycology laboratories. Two reagents, MycetColor® and MycetFluo®, which use Congo red and calcofluor dye, respectively, were evaluated for the direct microscopic examination of skin, hair and nail specimens. The results were compared to those of culture and conventional direct examination. Both reagents were able to clarify the specimens and also to specifically stain fungal elements. Microscopic examination of the specimens was greatly facilitated with MycetFluo®, which allowed a higher number of positive cases to be detected compared to the other methods.

Keywords: dermatophytes, dermatophytosis, mycological diagnosis, direct examination, stain, fluorochrome

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1. Introduction

Dermatophytoses are superficial mycoses affecting human skin (*tinea faciei*, *tinea corporis*, *tinea cruris*, *tinea pedis*), hair (*tinea capitis*), beard (*tinea barbae*) or nails (onychomycosis or *tinea unguium*). Onychomycoses are particularly common, as they represent about 30% of superficial mycoses in dermatology consultations and 50% of aetiologies of nail disease (Moreno and Arenas, 2010). According to the recent study of Fahri *et al.* (2011), the prevalence of onychomycosis among patients consulting their general practitioner is 16.8%. Although rare in children, the frequency of onychomycoses increases with age, with a prevalence of about 48% in patients over 70 years of age (Levy, 1997).

Nail disease is not synonymous with onychomycosis and examination of biological samples is essential to make a diagnosis and establish a specific treatment (Hainer, 2003). The diagnostic approach, which includes examination of the patient, must be completed by mycological analysis and/or histology of nail samples (Welsh *et al*., 2010). Histological analysis of nail biopsies, which is considered the "gold standard" for the diagnosis of onychomycoses, is seldom performed and routine diagnosis of dermatophytosis involves direct microscopic examination of samples, followed by culture. Due to the low sensitivity of direct microscopic examination and the slow growth of dermatophytes *in vitro*, molecular methods such as polymerase chain reaction (PCR) have been developed to enhance and speed up the diagnosis of these superficial mycoses (Verrier *et al*., 2013). However, microscopic identification of fungal elements directly in clinical specimens is still the first approach in most laboratories because of its simplicity and rapidity (Feuilhade de Chauvin, 2005).

In this study, we evaluated two reagents, MycetColor® and MycetFluo® (SR2B, Avrillé, France), for their ability to clarify preparations of skin, nail and hair samples obtained from patients with clinically suspected dermatophytosis and to stain fungal elements in the samples.

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2. Materials and methods

2.1. Clinical samples

Clinical specimens, including skin and nail scrapings, and hair, were collected by physicians (mycologists or dermatologists) and sent to one of three different mycology laboratories (Parasitology-Mycology laboratory, University Hospital, Angers (Lab-1); private Tharreau laboratory, Segré (Lab-2), and Parasitology-Mycology laboratory, University Hospital, Poitiers (Lab-3)). In these laboratories, each sample was inoculated onto Sabouraud's agar for mycological culture and treated with the reagents used routinely for direct examination, namely chloral-lactophenol (CL), MycetColor® and potassium hydroxide with chlorazol black (KCB) respectively.

The remaining samples were stored in closed glass tubes at room temperature for up to 2 months and then sent to the Parasitology-Mycology laboratory of the Pharmacy faculty, Angers (Lab-4), which performed a comparative study between four tests for direct examination: CL, KCB, MycetColor® and MycetFluo®. For this evaluation, only 102 specimens with a sufficient quantity to allow these four additional direct microscopic examinations were selected. They included 51 nail specimens, 38 skin scrapings and 13 epilate hair samples. In this laboratory, each sample was divided into four equal parts: one part for each test. The observers for these four microscopic examinations were unaware of the previous results direct examination and culture results obtained by Labs 1, 2 and 3.

2.2. Direct microscopy

Small amounts of each specimen were deposited into a drop of CL (chloral hydrate, 20 g; phenol, 10 mL; lactic acid, 10 mL) or KCB (KOH, 20 g; glycerol, 10 mL; chlorazol azole E black (0.1%) 10 mL; sterile distilled water, 80 mL) (both from Polysciences Europe Eppelheim, Germany), on a glass microscope slide. The preparations were covered with a

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coverslip and pressed gently to remove air bubbles. Fungal elements were then visualized under a light microscope and by phase-contrast microscopy for CL preparations. Samples were considered positive when fungal hyphae and/or arthroconidia were present.

MycetColor® and MycetFluo® (SR2B, Avrillé, France) contain sodium dodecyl sulphate (SDS) and a dye or fluorochrome (Congo red and calcofluor white, respectively). These reagents are able to digest keratin in skin, nail and hair samples and also to stain hyphae or spores which are detected with a conventional microscope for MycetColor® or with a fluorescent microscope for MycetFluo®. Staining was performed according to the manufacturer's instructions. Briefly, the skin, nail or hair samples were deposited onto a microscope slide placed on a black card. Twenty-five microliters of dissociating solution was added and the sample was squashed gently with a stick to ensure complete immersion in the dissociating solution. After 15 to 30 min, 50 µL of dye was added and the whole preparation was homogenized (sample + dissociating solution + stain) and then covered with a coverslip. After incubation for 15 min at room temperature, the slide was examined under a microscope. For MycetColor®, an Olympus BH2™ microscope or Nikon Fluophot™ microscope with white light (blue filter) was used. With this reagent, samples were considered positive when fungal elements (hyphae or conidia) appeared red on a pink or light orange background. Skin or nail cells usually remain unstained or faintly stained. For MycetFluo®, a Nikon Fluophot™ microscope equipped with an epifluorescence attachment was used. By using a combination of UV-excitation filters (excitation wavelength 365 nm) or a combination of V-excitation filters (excitation wavelengths 410-420 nm), samples were positive when fungal elements (hyphae or conidia) harboured blue-white or green-blue fluorescence, respectively.

All direct microscopic examinations were carried out using 10X or 40X objective lens amplification. Images were acquired using a Nikon M35™ automatic exposure camera with Kodacolor Gold™ ISO 400 film.

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2.3. Cultures

For dermatophyte cultures, three to five fragments of nail, skin or hair sample were placed on Sabouraud's dextrose agar plates containing antibiotic (chloramphenicol) and supplemented with cycloheximide (Actidione®, Sigma-Aldrich) to prevent contamination by moulds. Plates were incubated for up to 3 weeks at 30°C. Isolates were identified to the species level by macroscopic and microscopic examination.

2.4. Performance calculation and statistical analysis

Samples were considered positive when either direct microscopic examination (whatever the technique used) or culture or both were positive. The other samples were considered negative. These positive/negative results were used as the "gold standard" to evaluate the performance of MycetColor® and MycetFluo®.

Statistical analysis was performed using McNemar's test. The χ^2 test was performed to determine whether there was a statistically significant difference between the four reagents used for microscopic examination and between microscopic examination and culture. $p<0.05$ was considered statistically significant.

3. Results

Among the 102 samples tested, cultures were positive for 20 (39.2%), 24 (63.1%) and 13 (100%) nail, skin and hair samples, respectively. Dermatophytes were identified to the species level using morphological examination (macro- and microscopic characteristics).

Sixty-seven of the specimens (65.7%) were positive by at least one method of direct examination or by culture. These results of direct examination by Lab-1, Lab-2 and Lab-3 were confirmed by Lab-4 (data not shown). Out of 51 nail specimens examined, 28 were positive by microscopy or culture (54.9%) (**Table 1**). The results of the comparative study

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carried out by Lab-4 showed that the CL, KCB and MycetColor® methods were positive for 14 (27.4%), 13 (25.4%) and 13 (25.4%) of nail samples, respectively (**Table 1**). The differences were not statistically significant. MycetFluo® was positive in significantly more cases than the other methods: 25 (49%) vs. 13 or 14 samples ($p<0.05$). Twenty-six out of 38 skin samples (68.4%) were positive by direct microscopy or culture (**Table 1**). A total of 16 (42.1%) skin samples were positive using each of the CL, KCB or MycetColor® techniques vs. 20 (52.6%) positive samples using MycetFluo® ($p=0.125$). Among the 42 positive cultures from skin and nail scrapings, *Trichophyton rubrum* was the predominant species isolated (52.3%), followed by *T. interdigitale* (35.7%) and *T. mentagrophytes* (7.1%). *Epidermophyton floccosum* and *Microsporum canis* were isolated only once each (Table1). No correlation was observed between the performance of the different methods used for direct examination and the genus or species of dermatophyte isolated. Thirteen hair samples (100%) were positive by each of the four methods of direct examination. The species identified in culture were: *T. soudanense*, *T. tonsurans*, *M. audouinii*, and *T. mentagrophytes*.

The sensitivity of CL, KCB, MycetColor®, MycetFluo® and culture, was 64.2%, 62.7%, 62.7%, 83.6% and 85% respectively (**Table 2**) and the negative predictive value (NPV) was 59.3%, 58.3%, 58.3%, 76% and 77.7%, respectively. As samples were considered positive when direct microscopic examination or culture was positive, no false positive result was therefore noted and consequently all techniques had a specificity of 100%.

Figures 1 to **3** show the images obtained using MycetColor® (**Fig. 1**) and MycetFluo® (**Fig. 2-3**) with different samples (skin, nails and hair). Depending on the specimen, staining of the filaments appeared more or less pronounced with MycetColor®. Observation was greatly facilitated using MycetFluo® and identification of the fungal elements was easier, as they appeared fluorescent blue or green.

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4. Discussion

Treatment of dermatophytosis is frequently prolonged, often weeks or months in the case of *tinea capitis* or onychomycosis. For this reason, a definitive diagnosis is essential before starting treatment (Hainer, 2003). Despite recent progress in molecular techniques, conventional mycological analysis by direct examination and culture is the standard method used in most laboratories.

For direct examination, which allows a quick response to clinicians, specimens need to be dissociated between a slide and a coverslip in a drop of dissociating agent such as KOH (10-20%) with or without dimethyl sulfoxide (DMSO) or CL. To facilitate observation and increase the sensitivity of microscopic examination, selective staining can be used. The best method is to use a fluorescent dye such as calcofluor white.

In this study, we evaluated the efficacy of MycetColor® and MycetFluo®, two commercially available reagents, to detect fungal elements and diagnose dermatophytosis in 102 skin, hair and nail specimens. As our baseline, we considered samples that were positive by direct microscopy (by any method evaluated) or by culture or by both as true positive samples. The percentage positive results for nail, skin and hair specimens was 54.9%, 50.9% and 100% respectively.

Regarding the fungal cultures, the 20% rate of false-negative cultures can be explained by the presence of non-viable fungal elements (i.e. non-cultivable) or by previous antifungal treatment, despite the presence of fungal hyphae on direct microscopic examination.

The 13 epilate hair samples were positive with all the techniques used for direct examination and by culture. This 100% positivity rate can be explained by correct clinical diagnosis by mycologists or dermatologists.

There was no significant difference in the results obtained for skin samples using MycetColor® and MycetFluo® and the in-house direct examination techniques (CI and KCB).

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For the 51 nail specimens, a positive result was obtained with CL, KCB and MycetColor® in 27.4%, 25.4% and 25.4% of cases, respectively. These differences were not statistically significant. However, these rates were significantly lower than that obtained with MycetFluo® (49%).

Figures 1, 2 and 3 show how the two staining reagents, MycetColor® and MycetFluo®, can help the technician recognize fungal elements during the direct examination of skin, nail or hair samples. Septate mycelium and arthroconidia were easy to find using these stains.

Our results concur with those of Haack *et al.* (1987) who showed that using a fluorescent dye, Blankophor®, was superior to KOH for the detection of dermatophytes, and those of Abdelrahman *et al.* (2006), who reported better specificity and sensitivity using calcofluor white compared to KOH alone. Like these authors, we also found that preparation of the samples with MycetFluo® was quick and observation was easy. With this reagent, the location of fungal elements during microscopic examination can be carried out with 10X lens amplification and confirmation performed with 20X or 40X lens amplification. It has been known for many years that fluorescent dyes greatly assist in the direct examination of skin, hair and nail preparations. Thus, in 1986, Holmberg proposed the use of Blankophor® for diagnosis in the mycology laboratory.

Monod *et al*. (1989) compared the principal techniques used for direct microscopic examination in mycology. These included methods without staining, methods with staining (including fluorochromes) and conventional light microscopy. For Congo red, the authors used a 1-step procedure by combining this dye with SDS as a dissociation reagent. They reported that incubation for 20 min was required before observation and that the results were satisfactory for dermatophytes. The same components are present in MycetColor® but the test procedure involves two steps: (i) 15 min for dissociation; and (ii) 15 min for staining. During the first step, the manufacturer recommends to "tap on the sample with a stick (provided in

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the kit), to ensure that the sample is completely immersed in the dissociating solution". As Congo red is soluble in SDS, a reagent with a 1-step technique could have been developed. In our opinion, pre-incubation with the detergent is carried out to ensure that dissociation is complete before staining. Like these authors, we did not find that the sensitivity and specificity of Congo red associated with SDS was superior to that of CL or KCB. However, we noted that the detection of fungal elements, which were stained, was easier than with CL and KCB, and that the use of thin pieces of specimen, especially nails, allowed complete dissociation and good clearing of the preparation. Regarding the fluorochromes, the advantage of Blankophor® is its solubility in Na_2S, KOH or NaOH, so that it can be used in a 1-step procedure, unlike calcofluor which precipitates in KOH and must be used in a 2-step procedure: dissociation of the sample followed by staining (Monod *et al*., 1989). These authors obtained better results with Blankophor® in Na_2S. MycetFluo® contains SDS and calcofluor, which was probably guided by the toxicity of KOH and the disagreeable smell when Na_2S is used, and by the good solubility of calcofluor in SDS. MycetFluo® was also used in a 2-step procedure as outlined in the package insert, probably for the same reasons as those invoked above for MycetColor®. This double incubation increases the time of the procedure and could be a disadvantage of these commercially available reagents; however, it is offset by the quality and facility of reading, especially for MycetFluo®. In addition, the presence of glycerol slows down dehydration of the specimen allowing microscopic observation to be delayed and retention of the slides for teaching purposes.

In the last 15 years, PCR methods have been developed for the detection and/or identification of dermatophytes in samples obtained from patients with clinically suspected dermatophytosis. An update on the currently available molecular techniques has recently been published by Jensen and Arendrup (2012) and Gräser *et al.* (2012). However, despite the high sensitivity of these techniques, false positive or negative results were observed. For this reason, other

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authors such as Nenoff *et al*. (2012) have advocated a combination of conventional and molecular procedures to increase the sensitivity and specificity of the diagnosis of dermatophytosis. Direct examination of samples for fungal elements can be carried out by conventional microscopy with white light. Without colouration, the sensitivity of direct examination can be increased by using phase-contrast microscopy, with fungal structures more clearly delineated. Thus, many laboratories use this method with KOH and/or CL preparations. In the absence of a phase-contrast microscope, the use of stains or fluorochromes improves reading comfort with easier detection of fungal structures, and increases the sensitivity of direct examination (Elewski, 1996; Robert and Pihet, 2008). Several dyes, including lactic blue, Parker™ ink or Chlorazol black E, have been proposed (Panasiti *et al*., 2006; Robert and Pihet, 2008). Other stains such as Congo red (found in MycetColor®) or Chicago Sky Blue 6B (CSB stain®), which allow better contrast along with lower potential toxicity compared to black chlorazole, have been used in many laboratories (Robert and Pihet, 2008; Tambosis and Lim, 2012). When the laboratory possess a fluorescence microscope with adequate filters (blue filter 400-440 nm), fluorochromes such as Blankophor® (Bayer) or calcofluor white (Sigma) (found in MycetFluo®) can be used (Monod *et al*., 2000; Hamer *et al*., 2006).

Although the advantages of detecting fungal elements in dermatology specimens using fluorescence microscopy have been well known for many years, this technique is not commonly used in many laboratories. The main reason, apart from the lack of a fluorescence microscope, is that preparing in-house reagents poses problems regarding the safety of reagents and validation and stability of the batches. These problems are solved by using MycetFluo®, which is an in vitro diagnostic medical device (IVDMD) and is CE marked.

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5. Conclusion

Although commercially available PCR kits for the detection of dermatophytes currently exist, few laboratories use them routinely. Direct microscopic examination of samples remains essential as it allows a quick response to clinicians. MycetColor® and MycetFluo® are both able to clarify skin, hair and nail samples and to stain fungal elements using Congo red and calcofluor dyes, respectively. MycetFluo® detected a higher number of positive cases compared to the other methods. In addition, according the Material Safety Data Sheet (MSDS), calcofluor white is not considered a hazardous substance so the use of MycetFluo® does not require special safety measures. In contrast, if the other reagents are used, stringent precautions have to be taken. These products are potential carcinogens, may cause irritation of the skin, eyes and respiratory tract, and are harmful if swallowed. Moreover, CL and KCB may potentially have more dangerous acute health effects due to the mixture of chemical products they contain (combination of chloral hydrate, phenol and lactic acid for CL; KOH and chlorazol azole E black for KCB).

MycetFluo® is an IVDMD and is therefore CE marked. It is non-toxic, readily available and easy to use and could lead to greater use of calcofluor in many diagnostic mycology laboratories.

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Conflicts of interest

Raymond Robert is a scientific adviser to SR2B.

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Figure legends

Fig. 1. MycetColor® preparation of skin scales (**A**), nail scraping (**B**) and hair (**C**) showing septate branching mycelium breaking up into arthroconidia (**C**, courtesy of Dr. Yohann Le Govic)**.**

Fig. 2. Direct examination of nail scraping using MycetFluo®. Samples with fungal elements were observed using fluorescent light only (combination of UV-excitation filters: 365 nm, **C**; 410/420 nm, **F**), white light (**A**, **D**) or both (**B**, **E**).

Fig. 3. MycetFluo® staining of mycelium in an infected hair (endothrix).

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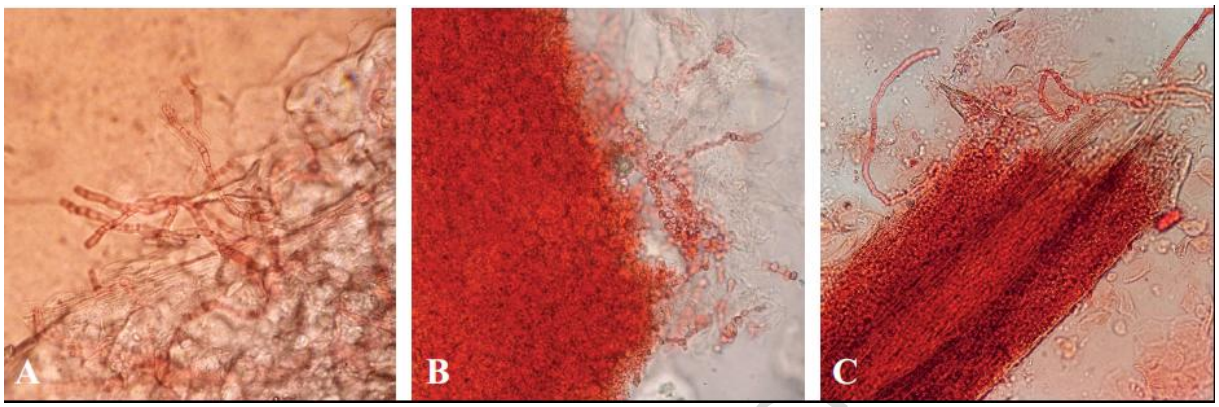

Figure 1

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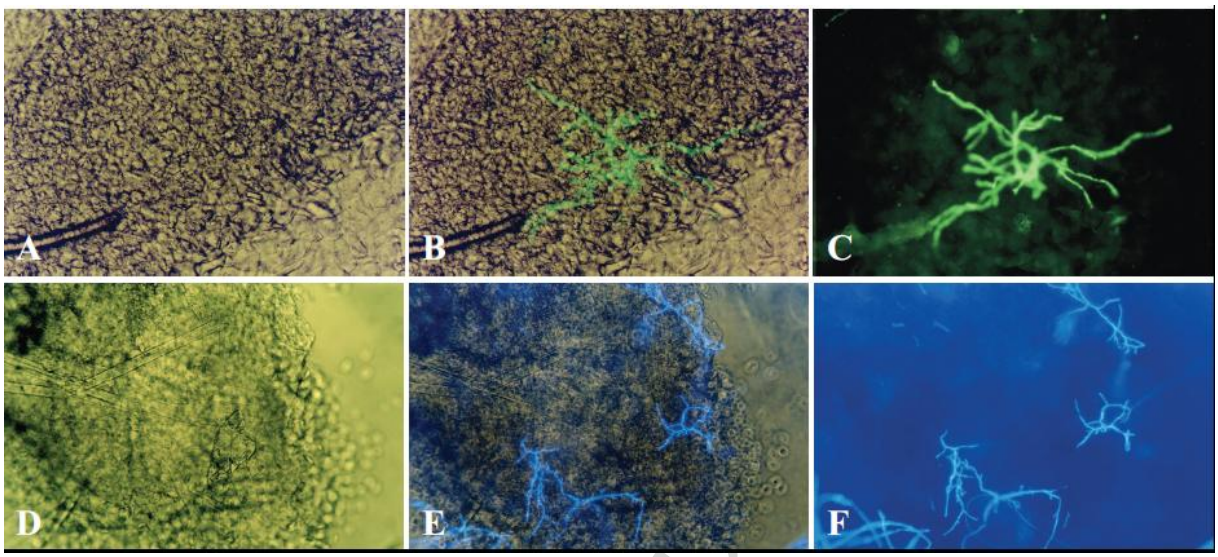

Figure 2

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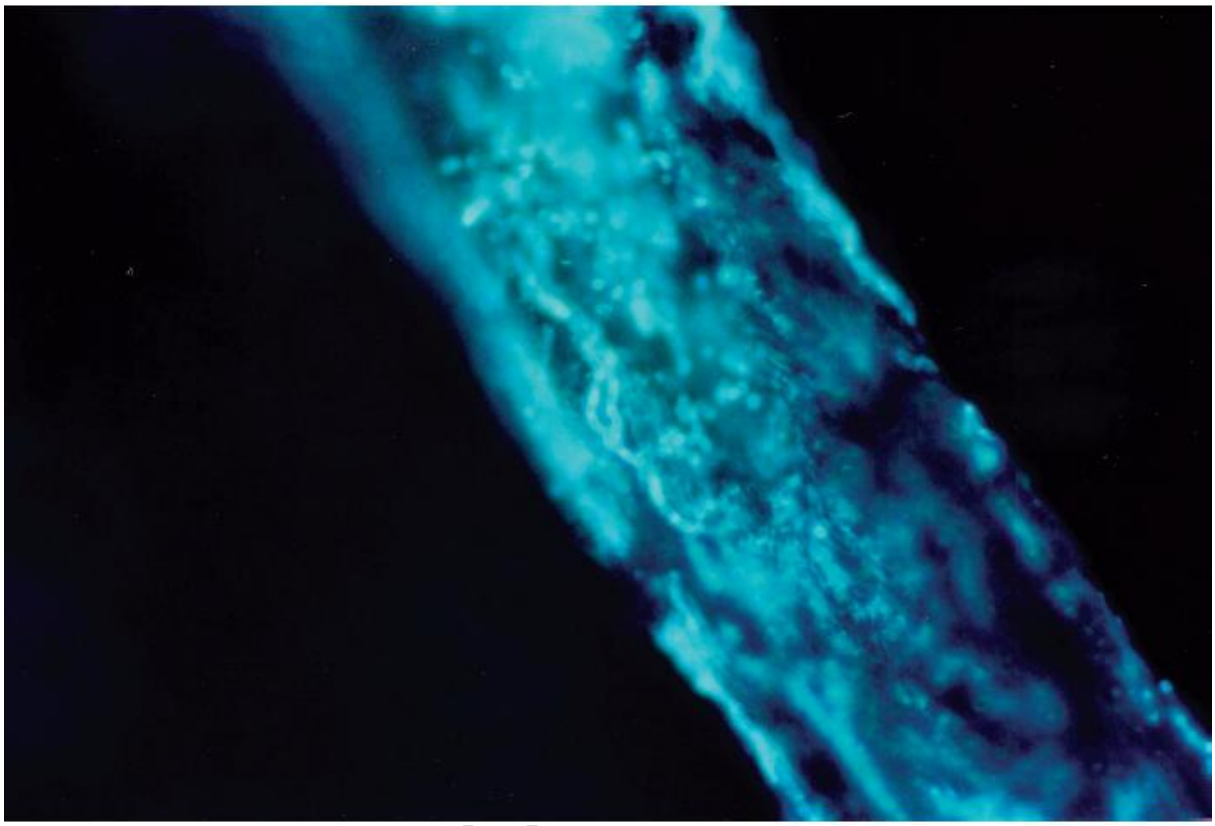

Figure 3

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Table 1. Detection of hyphae by direct microscopic examination using CL, KCB, MycetColor® and MycetFluo® and culture results for the 102 samples (skin, nail or hair) included in the study.

	Direct microscopic examination positive: n (%)				**Positive cultures n (%)**	**Frequency n (%) Species isolated in positive cultures**
Samples (n)	**CL**	**KCB**	**MycetColor®**	**MycetFluo®**		
Nail (51)	14 (27.4%)	13 (25.4%)	13 (25.4%)	25 (49%)*	20 (39.2%)	28 (54.9 %)
	Positive	Positive	Positive	Positive	Positive	12 (23.5%)
						T. interdigitale (6)
						T. rubrum (6)
	Positive	Positive	Negative	Positive	Positive	1 (1.9%)
						T. rubrum
	Positive	Negative	Positive	Positive	Positive	1 (1.9%)
						T. rubrum
	Negative	Negative	Negative	Positive	Positive	3 (5.9%)
						T. interdigitale (2)
						T. rubrum (1)
	Negative	Negative	Negative	Positive	Negative	8 (15.7%)
	Negative	Negative	Negative	Negative	Positive	3 (5.9%)
						T. interdigitale (2)
						T. rubrum (1)
	Negative	Negative	Negative	Negative	Negative	23 (45.1%)
Skin (38)	16 (42.1%)	16 (42.1%)	16 (42.1%)	20 (52.6%)	24 (63.1%)	26 (50.9%)
	Positive	Positive	Positive	Positive	Positive	16 (42.1%)
						T. mentagrophytes (2)
						T. interdigitale (2)
						T. rubrum (10)
						E floccosum (1)
						M canis (1)
	Negative	Negative	Negative	Positive	Negative	2 (5.3%)
	Negative	Negative	Negative	Negative	Positive	*6 (15.8%)*
						T. mentagrophytes (1)
						T. interdigitale (3)
						T. rubrum (2)
	Negative	Negative	Negative	Negative	Negative	12 (31.6%)
Hair (13)	13 (100%)	13 (100%)	13 (100%)	13 (100%)	13 (100%)	13 (100%)
						T. soudanense (8)
						T. tonsurans (3)
						M. audouinii (1)
						T. mentagrophytes (1)
Total (102)	43 (42.1%)	42 (42.1%)	42 (42.1%)	58 (56.8%)	57 (55.8%)	102 (100%)

* statistically significant ($p<0.05$)

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Table 2. Determination of positive results, negative results, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for direct microscopy using CL, KCB, MycetColor® or MycetFluo® and culture (P, positive; FN, false-negative).

Samples (n)	Method	Positive (n)	Negative (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Nail (51)		28	23				
	CL	14	23	50	100	100	62.1
	KCB	13	23	46.4	100	100	60.5
	MycetColor®	13	23	46.4	100	100	60.5
	MycetFluo®	25	23	89.3	100	100	88.4
	Culture	20	23	71.4	100	100	74.1
Skin (38)		26	12				
	CL	16	12	61.5	100	100	54.5
	KCB	16	12	61.5	100	100	54.5
	MycetColor®	16	12	61.5	100	100	54.5
	MycetFluo®	20	12	76.9	100	100	66.6
	Culture	24	12	92.3	100	100	85.7
Hair (13)		13	0				
	CL	13	0	100	100	100	100
	KCB	13	0	100	100	100	100
	MycetColor®	13	0	100	100	100	100
	MycetFluo®	13	0	100	100	100	100
	Culture	13	0	100	100	100	100
Total (102)		67	35				
	CL	43	35	64.2	100	100	59.3
	KCB	42	35	62.7	100	100	58.3
	MycetColor®	42	35	62.7	100	100	58.3
	MycetFluo®	56	35	83.6	100	100	76
	Culture	57	35	85	100	100	77.7

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Highlights

- Dermatophytes are an important cause of superficial fungal infection.
- Mycological analysis, including direct examination and culture, often lacks sensitivity.
- The use of stains or fluorochromes enhances the performance of direct examination.
- MycetFluo® allows a higher number of positive cases to be detected.
- MycetFluo® is CE marked and could lead to greater use of calcofluor in many laboratories.

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