

Calcofluor white: in search of a better diagnosis

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Abstract

Objective: Calcofluor white (CFW) is a fluorescent stain that allows observing fungal structures in different clinical samples thanks to its affinity for chitin. Darkfield microscopy facilitates the correct visualization of pathogens, favoring patients' timely and correct diagnosis. therefore, this work aims to evaluate the capacity for identifying mycotic structures in different biological samples of CFW staining.

Materials and methods: Thirty-six biological samples (vaginal fluid, bronchoalveolar lavage, cerebrospinal fluid, scales, urine, cornea, blood culture, and biopsy) were evaluated for fungi. All samples were processed by the three techniques: potassium hydroxide 20% (KOH), mycological culture and CFW.

Results: KOH technique gave a positive result in 58.3% of the cases, culture in 69.4% and CFW staining in 72.2%. The sensitivity and specificity of the CFW technique against KOH were 95% and 67%, while against mycological culture was 100% and 91%.

Conclusions: This study demonstrates that the CFW technique is a suitable method for identifying fungal structures in clinical specimens because it showed high sensitivity and specificity in relation to the traditional method and culture.

Keywords: Calcofluor white; KOH; Mycological culture; Diagnosis; Fungi.

Resumen

Introducción: Blanco de calcoflúor (BCF) es una tinción fluorescente que permite observar estructuras micóticas en distintas muestras clínicas gracias a la afinidad que tiene por la quitina. La microscopía en campo oscuro facilita la visualización correcta de los patógenos lo que favorece el diagnóstico oportuno y correcto de los pacientes. Por lo tanto, este trabajo tiene como objetivo evaluar la capacidad de identificación de estructuras micóticas en diferentes muestras biológicas de la coloración de blanco de calcoflúor.

Materiales y métodos: se evaluaron 36 muestras biológicas (flujo vaginal, lavado broncoalveolar, líquido cefalorraquídeo, escamas, orina, córnea, hemocultivo y biopsia) en busca de hongos. Todas las muestras fueron procesadas por medio de las tres técnicas: hidróxido de potasio (KOH) al 20%, cultivo micológico y blanco de calcoflúor.

Resultados: la técnica de KOH dio un resultado positivo en 58,3% de los casos, el cultivo en el 69,4% y la tinción con blanco de calcoflúor en el 72,2%. La sensibilidad y la especificidad de la técnica de BCF frente al KOH fue de 95% y 67% respectivamente, mientras que frente cultivo micológico fue de 100% y 91%.

Conclusiones: este estudio demuestra que la técnica de BCF es un buen método para la identificación de estructuras micóticas en las muestras clínicas debido a que demostró una alta sensibilidad y especificidad en relación con el método tradicional y el cultivo.

Palabras claves: Blanco de calcoflúor; KOH; Cultivo micológico; Diagnóstico; Hongos.

Introduction

In microbiological studies, the aim is to obtain accurate results in the shortest possible time, which allows patients to receive the appropriate treatment and improve their quality of life. Specifically in mycoses, diagnostic procedures in the laboratory are based on direct microscopy and culture of the microorganism. Therefore, the use of stains for the identification of fungal structures has a high importance in clinical diagnosis, the identifica-

tion is based on the physicochemical properties of these substances and the reaction that triggers on microbial cells (1).

The potassium hydroxide (hereafter KOH) technique is a useful, simple, fast, and low-cost method, which is based on the digestion capacity of KOH on keratin dissolving proteins and lipids (2), while the calcofluor white (hereafter CFW) technique is a fluorescent staining with high affinity for chitin and cellulose of the cell wall (3, 4). Although it is very sensitive and specific, it requires the use of a fluorescence microscope.

By using the CFW technique, a rapid presumptive diagnosis can be made in clinical samples, which allows the visualization of fungal structures and decreases the time for the observer, since it does not require experience for the visualization of fungal structures and the background of the stain is colored black and the fungi fluoresce; there are reports of this technique that allow the rapid observation from candidiasis (5), pythiosis (6), sporotrichosis (7) to aspergillosis (8). Previous studies have even suggested that direct fluorescence microscopy may be a sensitive technique, which could increase detection rates compared to KOH in the diagnosis of fungal infections (9-11).

The aim of this work was to evaluate the ability to identify fungal structures in different biological samples with CFW.

Materials and methods

An observational study was conducted, including 36 samples analyzed at the SYNLAB-Colombia clinical laboratory (<https://www.synlab.co/>) from June to December 2021, from vaginal fluid, bronchoalveolar lavage, nail scales, cerebrospinal fluid, urine, blood, cornea, and tissue.

Each sample was evaluated by direct examination using CFW, 20% KOH and mycological culture to determine the number of fungal structures.

The gold standard was shown in culture media, except for the samples that were positive for *Pneumocystis jirovecii*, which were standardized with the polymerase chain reaction (PCR) method due to the impossibility of culturing.

Two control strains (ATCC *Candida albicans* 14053 and ATCC *Escherichia coli* 25922) were used during CFW assembly to ensure the proper functioning of the technique.

Evaluation by 20% KOH

40 uL of each liquid sample, or nail scales, were deposited on a slide and 40 uL of 20% KOH was added. It was homogenized and covered with a

slide, allowed to stand for 1 minute and then observed under a light microscope (OLYMPUS CX31) at 10X and if mycotic structures were observed, they were identified at 40X.

Evaluation by calcofluor white

For all samples, 40 uL of the sample, or a representative amount in solid samples, was deposited on a slide. Subsequently, 40 uL of 20% KOH and 40 uL of CFW were added, homogenized, and covered with a slide, allowed to stand for 1 minute and then examined under ultraviolet (UV) light in 40X objective with a fluorescence microscope (Nikon, Eclipse E400, series: 672999).

Evaluation by mycological culture

Mycological diagnosis by culture in genital, urine and blood samples was performed using chromogenic medium and Sabouraud supplemented with chloramphenicol, in respiratory samples, biopsies and scales. In the case of nail and skin detritus, it was necessary to change the chromogenic medium to Mycosel, while sterile liquids were cultured in Sabouraud dextrose and Sabouraud medium supplemented with chloramphenicol.

Statistical analysis

Descriptive statistics were performed and the sensitivity and specificity of the 20% KOH and CFW techniques were calculated according to the culture result.

Results

The number of samples analyzed and the presence of fungal structures in relation to the 20% KOH, CFW or culture techniques are shown in Table 1. The percentage of positive samples was highest when using CFW (72.2%), followed by culture (69.4%), while the 20% KOH staining (58.3%).

Table 1. Type of samples tested, positivity status by 20% KOH, CFW and mycological culture techniques.

Type of samples	Number of samples	20% KOH positive	Positive culture	Positive calco-fluor white
Vaginal fluid	5	5	5	5
Bronchoalveolar lavage	7	1	3	3
Cerebrospinal fluid	3	2	2	2
Scales	12	8	10	11
Urine	2	1	1	1
Cornea	2	1	1	1
Blood	4	3	3	3
Biopsy	1	0	0	0
TOTAL	36	21	25	26

The sensitivity of direct examination with CFW was 95% and specificity 67%, while the sensitivity of culture to CFW was 100% and specificity 91% (Table 2).

Table 2. Specification and sensitivity comparison of fungal culture and microscopic examination after staining with potassium hydroxide or CFW.

	Culture and potassium hydroxide	Culture and calco-fluor white	Potassium hydroxide and calcofluor white
Sensitivity	80%	100%	95%
Specificity	91%	91%	67%

Macroscopic and microscopic observation of samples

The 36 clinical samples were processed by the three techniques. Figure 1 and 2 show two examples of identification with each of the methodologies used for their evaluation.

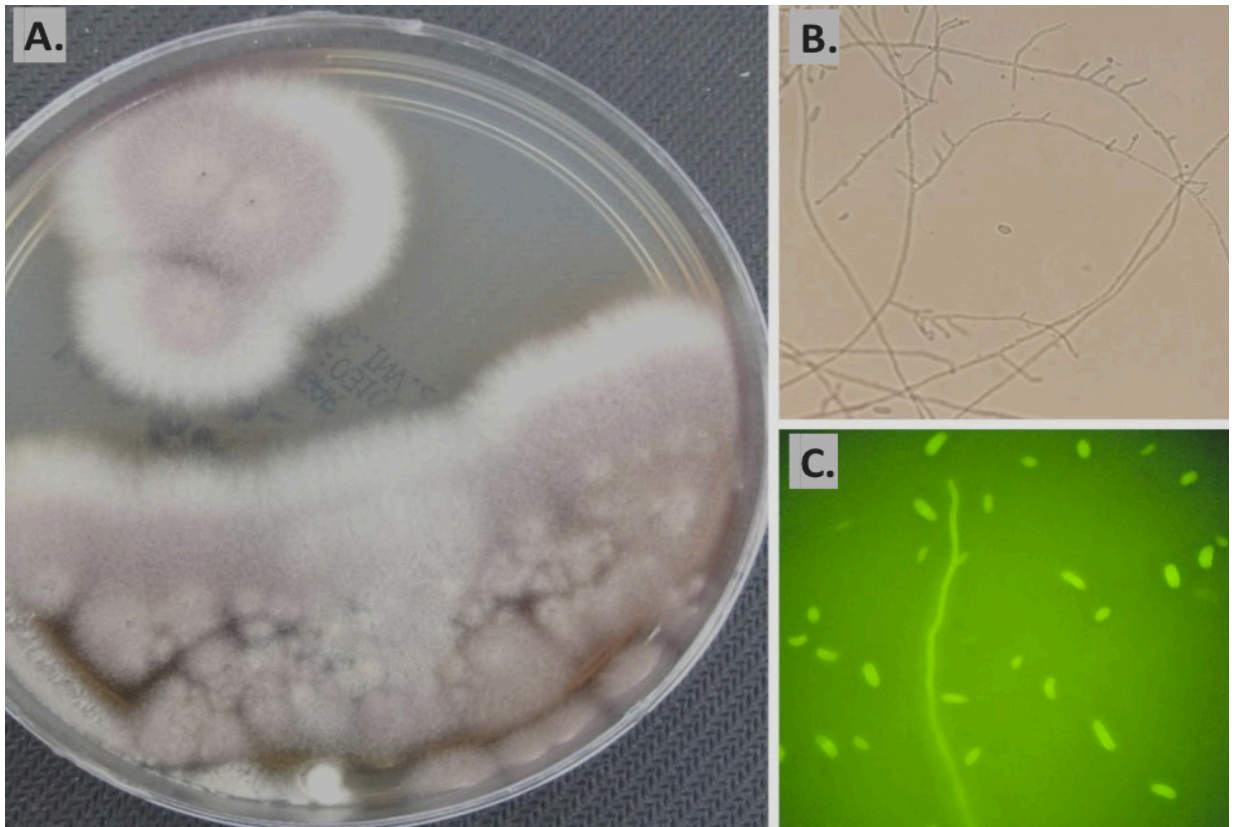


Figure 1. Comparison of the three techniques evaluated in a blood sample in which growth of *Fusarium sp.* complex was obtained.

- A) Mycological culture, growth of filamentous fungus was obtained in sabouraud agar.
- B) Calcofluor white, hyaline septate hyphae and crescent-shaped macroconidia are observed.

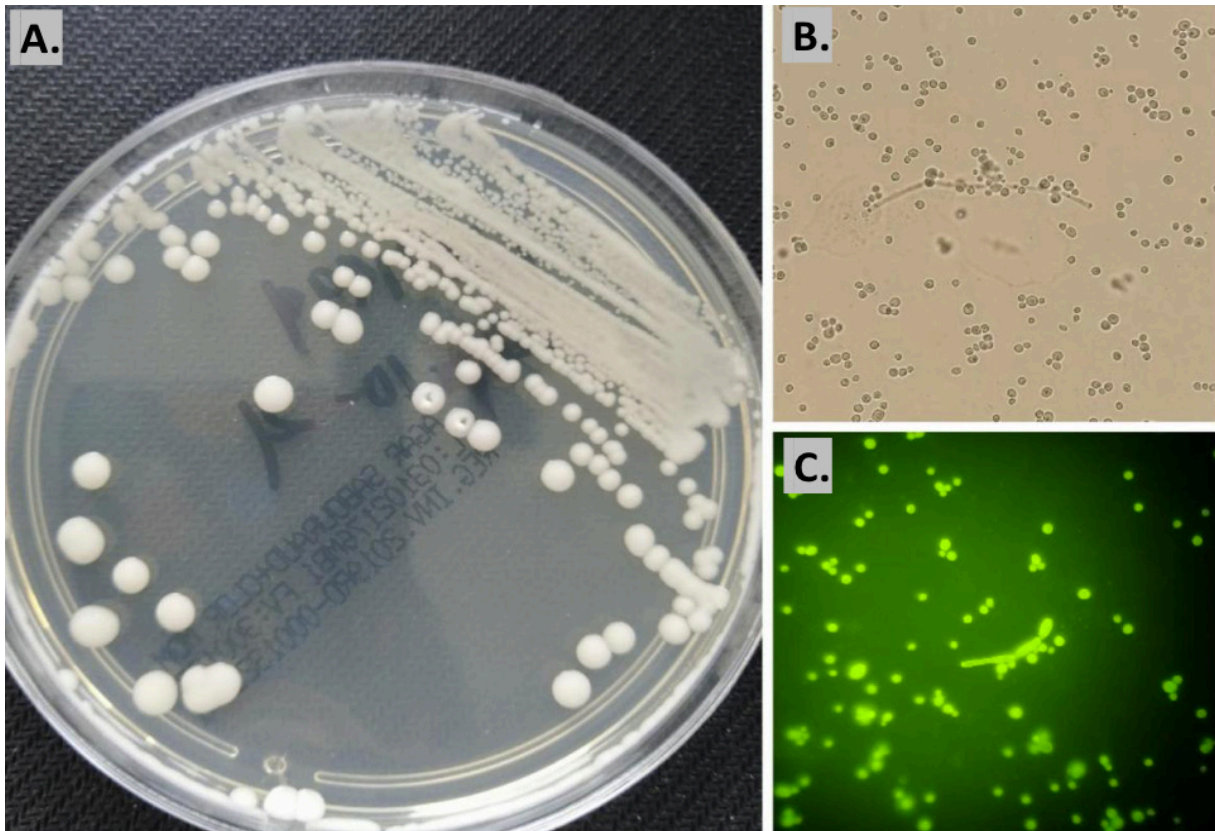


Figure 2. Comparison of the three techniques evaluated in vaginal fluid samples with positive growth for *Candida albicans*.

- A) Mycological culture, growth of creamy, yeast-like colonies was obtained.
- B) K20% KOH, abundant blastoconidia and pseudohyphae were observed.
- C) Calcofluor white, abundant blastoconidia and pseudohyphae were

Discussion

A comparison between the 20% KOH technique and CFW staining showed a percentage of positivity of 58.3% (21 of 36 samples), compared to 72.2% obtained with the CFW technique (26 of 36 samples), which showed a higher specificity and sensitivity of CFW (Table 2). Similar results were reported by Abdelrahman et al. (12) in 2006 and Sánchez Armendáriz et al. (4) in 2013. However, a study by Bonifaz et al. (9) in 2013 on samples from 33 patients with onychomycosis in Mexico City did not observe that the diagnosis using CFW (58% of the samples were positive) was better than KOH (67%) or culture (33%) (9).

In agreement with the results of the present study, Bao et al. (13) confirm the importance of the use of fluorescence since it improves the sensitivity and specificity of direct examination.

Even though 20% KOH is a useful and highly used technique for the identification of fungi, it presents some problems as the possibility of interpreting some non-mycotic structures as fungi and producing false positives (2), besides being difficult to observe fungal structures in samples with low counts of microorganisms (2, 4); microscopy constitutes, due to its low cost and availability, a diagnostic method accessible to laboratories of low complexity, but it could be improved by implementing the CFW technique (9). Pihet et al. (14) evidenced, as in the present study, better diagnostic results when using the CFW technique for the processing of clinical samples.

One of the major inconveniences found in the latter is the fluorescence that can occur in very thick samples, mainly in nails, since it does not allow a good visualization of the mycotic structures. It has been reported that the sensitivity of CFW varies among three microbiologists, due to it is a subjective test and depends on the experience of the observer (6), but the fluorescence in a dark field allows inexperienced observers to identify them easily, which decreases the percentage of false negative results (10, 15, 16). Recently, two pediatric cases were reported in which CFW was used to detect invasive pulmonary aspergillosis in bronchoalveolar lavage fluid, allowing rapid treatment decisions to be made (8).

On the other hand, since each fungus has different growth requirements, clinical samples should be cultured on solid media (Sabouraud Dextrose agar and Mycosel), some containing cycloheximide and chloramphenicol to inhibit the growth of environmental fungi and bacteria (17). However, growth periods of 2 to 6 weeks and despite a positive 20% KOH preparation, pathogen culture may not obtain growth, especially if antifungal treatment has already been initiated (18), and the instant identification of a specific pathogen allows the initiation of appropriate therapy in a short period of time (18), therefore, it is necessary to implement other diagnostic tools such as the CFW.

The results obtained from the present study show a percentage of positivity in the CFW technique of 72.2% corresponding to 26 samples, compared to 69.4% of positives obtained in the mycological cultures; similar results were reported by Biao et al (19). Even, with the advance of technology, in recent years, attempts have been made to perform fluorescence

microscopy by cell phones for the identification of fungal pathogens, which could be useful for the purpose of making diagnoses in more remote places at low cost and in a simple manner (20).

Additionally, *Pneumocystis jirovecii* were found in some of the clinical samples, these could be observed by the CFW technique where their asci were evidenced, the opposite occurred in the 20% KOH staining where no fungal structures were observed. This showed that the technique to be evaluated presents an excellent performance compared to the standard PCR test, which identified the pathogen in the biologicals analyzed. These results agree with the study performed by Abastabar et al. (21) who were able to identify the structures of *Pneumocystis jirovecii* by the CFW technique and then by the PCR technique and were in perfect concordance.

To conclude, the CFW technique, although it is more expensive, is emerging as the most rapid and sensitive technique for the diagnosis of mycoses. The costs of implementation are minimal when compared to the increased hospitalization of patients due to delays in reporting or lack of timely mycological diagnosis.

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