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Comparing real-time PCR and Calcofluor-white with conventional methods for rapid detection of dermatophytes: Across-sectional study

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ABSTRACT

In this study, the performance of conventional methods, Calcofluor-white, and Real-time PCR methods were compared to establish an effective method for screening dermatomycosis. Our results showed excellent agreement between direct examination with Calcofluor -white (kappa = 0.97) and real-time PCR (kappa = 0.89) in 307 clinical samples.

Dermatophytes are fungi that infect keratinized tissues. Although dermatophytosis is considered to be an inconsequential disease, its psychological and esthetic effects are highly significant (Hay, 1990).

The diagnosis of dermatophytosis can be done through conventional methods. Microscopic examination of fungal elements is a rapid and inexpensive technique, but it cannot differentiate species and might yield false-negative results in up to 15% of cases. Culture is more specific than direct examination, but it is time consuming and might yield negative result in about 40% of positive specimens (Westerberg and Voyack, 2013).

In addition to conventional methods, molecular techniques are used to detect and identify dermatophytes (Verrier and Monod, 2017). Real-time polymerase chain reaction (PCR) has been recently used to detect dermatophytosis. This method has been widely employed for the detection of dermatophytes species (Dorak et al., 2007). Mirhendi et al. designed two specific primers sets and one probe, based on the beta-tubulin gene to detect almost all dermatophytes (pan-dermatophyte), except the subspecies (Mirhendi et al., 2016). The rapid processing of multiple samples, high specificity and sensitivity, no need for specialized skills and saving time in comparison to culture are advantages of these molecular methods. Since the real-time PCR displays excellent analytical results, we compared real-time PCR results of clinical specimens with modified DNA extraction method with those of direct microscopic examination, culture, and Calcofluor-white.

This study was conducted on 307 clinical specimens (241 skin scale, 62 hair and 4 nail scrapping) obtained from lesions of outpatients suspected of dermatophytosis referred to Faghihi Hospital, Shiraz, Iran, from June 2017 until July 2018. The collected specimens were divided into four portions. The first portion of the specimens was used for direct microscopic examination with 20% KOH, the second for culturing on two sets of media including sabouraud dextrose agar (SDA, Himedia) with/without cycloheximide, the third for staining with Calcofluor white, and the last for DNA extraction. Calcofluor-white for working solution was prepared with 9 ml of PBS (pH 7.2) and 1 ml of Calcofluor-white 0.1% solution. The samples were incubated for 20 min with working solution and KOH10% (1:1) and observed with fluorescent microscope (Olympus-BX61, Japan) (Harrington et al., 2003).

For DNA extraction, approximately 25 mg of specimen was transferred to a 2 ml tube and treated with 500 µl of a digestion buffer (containing 400 mM Tris-HCl[PH:8], 60 mM EDTA [PH:8], 150 mM NaCl, and 1%SDS) for 30 min, using a commercial kit (Yekta-Tajhiz Azma, Iran). The primers and probe in this study were used as described by Mirhendi et al. (2016). All real-time PCR reactions were performed in a final volume of 20 µl using Taq DNA polymerase master-mix (Ampliqon, Denmark), 3 µl DNA templates, 0.6 µM of each forward and reverse primers, and 0.3 µM of the TaqMan™ hydrolysis probe(Pishgam Biotech, Tehran, Iran). PCR program consisted of an initial step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, and 60 s at 60 °C. Appropriate positive and negative controls were included in every run.

Dermatophytosis was confirmed by direct microscopic examination with KOH20% in 190 (61.4%) out of the 307 clinical specimens. Amongst the 134 (43.3%) positive cultures, all were found positive by direct examination, while 56 (18.2%) positive samples by direct microscopy yielded negative culture result (Table 1).

In order to detect dermatophytes, Calcofluor-white staining has to bind with the chitin of the fungal pathogen cell walls (Fig. 1). Dermatophytes were detected in 189 (61.1%) by Calcofluor-white. Out of 307 samples tested by the real-time PCR, 185 (59.9%) were positive. In

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Table 1
Comparison between microscopic examinations with KOH and three other methods (culture, Calcofluor white, and real-time PCR) for the detection of dermatophytes.

	Pos-Pos	Neg-Neg	Pos(KOH)-Neg	Neg(KOH)-Pos
Culture	134 (43.6%)	117 (38%)	56 (18.3%)	0
Calcofluor-white	188 (61.2%)	116 (37.7%)	2 (0.6%)	1 (0.3%)
Real-time PCR	180 (57%)	112 (34.8%)	10 (4.8%)	5 (3.2%)

Pos: positive, neg: negative, direct: direct examination.

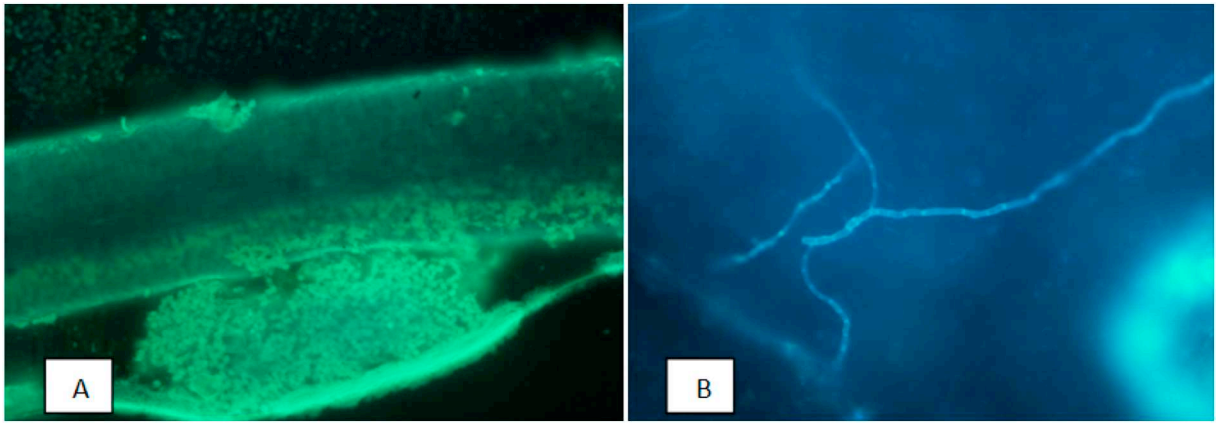

Fig. 1. Fluorescent images of Calcofluor white stained arthroconidia around hair shaft (ectothrix) (A) and hyphae in the skin scales (B).

Table 2
Diagnostic performance of the tests by considering hyphae at the direct examination.

Test	Sensitivity	Specificity	PPV	NPV	Kappa index
Culture test	70.5%	100%	100%	61.5%	0.646
Calcofluor white	98.9%	99.1%	99.4%	98.3%	0.979
Real-time PCR	94.7%	95.7%	97.2%	91.8%	0.897

PPV: positive predictive value, NPV: negative predictive value.

total, 10 samples were positive by direct examination, but could not be confirmed by real-time PCR (Table 1).

Considering the Kappa-index shown in Table 2, the classifications of concordance between the methods were: significant between direct microscopic examination and culture (kappa = 0.64), excellent between direct examination and both calcofluor-white (kappa = 0.97) and real-time PCR (kappa = 0.89). The positive and negative predictive values (PPV and NPV), specificity, and sensitivity were 99.4%, 98.3%, 99.1%, and 98.9%, for Calcofluor-white and 97.2%, 91.8%, 95.7%, and 94.7% for real-time PCR, respectively (Table 2).

The Calcofluor-white stain is a rapid and easy method to perform, and the results are generally easy to interpret and require less experience (Pihet et al., 2016). In the present study, Calcofluor-white stain clearly appears to be a good candidate for diagnosis of dermathophytosis with 99.1% and 98.9% specificity and sensitivity, respectively. Haldane et al. demonstrated a sensitivity of 92% and a specificity of 95% with Calcofluor-white compared to a sensitivity of 88% and a specificity of 95% with potassium hydroxide (Haldane and Robart, 1990).

In the real-time PCR, the suitability of a partial beta-tubulin gene was selected as a target for diagnosis of causative agents of dermatophytosis. This gene has been used successfully for species identification in other fungi (Balajee et al., 2009; Serra and Peterson, 2007; Gilgado et al., 2005; Mostert et al., 2005; Motamedi et al., 2017). Our results showed that 180 (57%) specimens were positive in both direct examination and real-time PCR, while 5 (3.2%) specimens were negative for direct examination and positive in real-time PCR. In ten samples (6 skin scales (2.4%) and 4 hair samples (6.4%)) real-time PCR yielded negative results. Although a false positive PCR result cannot be excluded, it is more likely that these were false negative results of the direct examination, which might be associated with an inadequate amount and preparation of specimens, skill of observer, and a quick examination of slides. Although our DNA extraction method was different from that of Motamedi et al., our results are similar with hers that found 9.96% positive PCR in specimens with negative microscopic results (Motamedi et al., 2017). We also compared Calcuflore-white staining with the KOH and molecular methods.

Altogether, Calcofluor-white stain appears to be an interesting, easy, and rapid alternative to the KOH20% for the diagnosis of dermatophytosis. Alternatively, based on significant efficacy results of the Real-time PCR, it might be used as a fast and convenient method for screening and detection of dermatophytosis.

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