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Direct molecular analysis of *Malassezia* species from the clinical samples of patients with pityriasis versicolor

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A B S T R A C T

Background and Purpose: Species identification of *Malassezia* using culture-dependent methods is time-consuming due to their fastidious growth requirements. This study aimed to evaluate a rapid and accurate molecular method in order to diagnose the pityriasis versicolor (PV) and identify *Malassezia* species from direct clinical samples.

Materials and Methods: Skin scraping or tape samples from patients with PV and healthy volunteers as the control group were collected. Diagnosis of PV was confirmed by direct microscopic examination. The DNA extraction was performed according to the steel-bullet beating method. Polymerase chain reaction-restriction fragment length polymorphism assay using *Hha*I restriction enzyme was applied for the identification and differentiation of *Malassezi*a species.

Results: The PCR method was able to detect *Malassezia* in 92.1% of specimens which were also confirmed with microscopic examination. Statistically, a significant association was observed between the results of the two assays ($P < 0.001$). Moderate agreement was identified between the two methods to diagnose the PV in both populations (Kappa: 0.55). Considering microscopic examination as the gold standard method for confirmation of PV, the sensitivity, specificity, positive predictive value, and negative predictive value values of the PCR assay for recognition of PV were 85%, 75%, 92%, and 60%, respectively. *M. globosa* and *M. restricta* were the most prevalent species isolated from patients.

Conclusion: In this study, the two-step molecular method based on the amplification of the D1/D2 domain and digestion of the PCR product by one restriction enzyme was able to diagnose and identify *Malassezia* directly from clinical samples. Consequently, it can be said that the molecular-based method provides more facilities to identify fastidious species, such as *M. restricta*.

Keywords: *Hha*I enzyme, *Malassezia globosa*, *Malassezia restricta*, PCR− RFLP, Tinea versicolor

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Introduction

Malassezia species are lipid-dependent basidiomycetous yeasts with numerous functionally distinct strains that have recently been classified as Malasseziomycetes class. Among the different clinical manifestations of *Malassezia* species, Pityriasis versicolor (PV) is a well-known superficial infection of the skin due to overgrowth or morphogenesis of yeasts to filamentous forms [1].

During the past years, molecular-based assays [2-7] for *Malassezia* detection/identification have revealed the ubiquitous presence of *Malassezia* on the skin in addition to pathogenic roles in different skin disorders [8]. While these approaches have met with various degrees of success, most of them require cultivation to enhance sensitivity, which increases both the potential for cultural bias and the turnaround time for analysis.

In this regard, two culture-independent molecular methods for the detection and identification of *Malassezia* from clinical samples have been tested in recent years. However, these methods require either separate amplification with specific primer sets for each species [4, 5, 7] or sequencing which can be costly and time-consuming.

Therefore, the present study aimed to evaluate the rapid and accurate molecular method for the species identification of *Malassezia* directly from clinical samples by amplification of the D1/D2 domain using Polymerase chain reaction-restriction fragment length polymorphism (PCR−RFLP).

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Materials and Methods

In this cross-sectional study, samples from the skin of 125 patients with PV who referred to the Medical Mycology Laboratory of Razi Hospital in Tehran were collected. Moreover, 17 healthy volunteer individuals were included as the negative control group. The diagnosis of PV was confirmed by microscopic observation of scales using potassium hydroxide (10% KOH) examination, which demonstrated the typical ovoid clusters of yeast cells and short filamentous. Following the confirmed diagnosis of PV, a part of the sample was stored at 25 °C for molecular analysis by PCR assay.

To identify the *Malassezia* species by molecular method, fungal genomic DNA was extracted manually from each clinical sample using steel bullet disruption [9]. The D1/D2 domains of the 26S rRNA gene which produces approximately 580 bp fragments were amplified [6]. The PCR reactions were performed in a 25 µL final volume containing 2×Taq Master Mix (final conc. 1×) (Ampliqon RED, Denmark), 0.5 µM primer, and 4 µL (20 ng) of template DNA. Temperature conditions for PCR were 5 min at 95 °C, 37 cycles of 45 s at 95 °C, 45 s at 58 °C and 1 min at 72 °C, followed by 7 min at 72 °C. The PCR products were analyzed by electrophoresis on 1% (w/v) Tris–acetate–EDTA agarose gels containing 2.5-3% ethidium bromide, and visualized under a UV detector (UVITEC, UK).

The 142 PCR products of the D1/D2 domains were subjected to RFLP analysis with the *Hha*I enzyme (New England Biolabs, Beverly, MA, USA). Restriction reactions were carried out in 10 µL volumes containing 5 µL (200 ng) of PCR product, 2 µL of the restriction enzyme, 1µL of appropriate reaction buffer (final conc. 1×), and 2 µL of distilled water at 37 °C for 24 h. Restriction patterns were compared with those of reference *Malassezia* strains and species-specific patterns established in the original publications [6]. The DNA sequencing of D1/D2 domains was performed for 2 clinical samples that were confidently identified by PCR–RFLP, and for 8 clinical samples that could not be identified by PCR–RFLP.

The Chi-squared test was used to analyze the statistical difference between the ability of methods to detect *Malassezia* species in patients and healthy subjects. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the PCR assay were calculated.

Results

This study was performed on 142 participants from whom 104 (73.2 %) skin scales and 38 (26.8%) scotch tapes were collected. The PCR amplification of the partial D1/D2 region produced a single amplicon of the expected size (Figure 1A) for 102 samples. In comparison to a molecular analysis by PCR, more samples were positive by direct microscopic examination (102 vs. 110). The PCR method could detect *Malassezia* in 94 (92.1%) specimens which were also positive in microscopic examination.

Moreover, the PCR analysis detected *Malassezia* in eight samples that showed negative results using the direct microscopy method. Statistically, a significant association was observed between the results of the two assays (P<0.001). Considering microscopic examination as the gold standard method for confirmation of PV, the diagnostic performance of the PCR assay for recognition of PV was calculated. The sensitivity, specificity, PPV, and NPV values of the PCR were 85%, 75%, 92%, and 60%, respectively.

Upon digestion of the amplified products with the *Hha*I restriction enzyme, two different restriction patterns could be distinguished. Both patterns matched exactly the *Hha*I restriction patterns predicted for *Malassezia globosa* (129 and 455 bp), and *Malassezia restricta* (580 bp). In line with these patterns, *M. globosa* and *M. restricta* were detected in 80 (85.1%) and 14 (14.9%) samples, respectively (Figure 1B).

Based on the sequencing data of the D1/D2 region for eight clinical samples that could not be distinguished by PCR–RFLP, *M. globosa* and *M. restricta* were identified with 100% identity in seven and one samples, respectively. Representative sequences of the DNA-sequenced *Malassezia* are

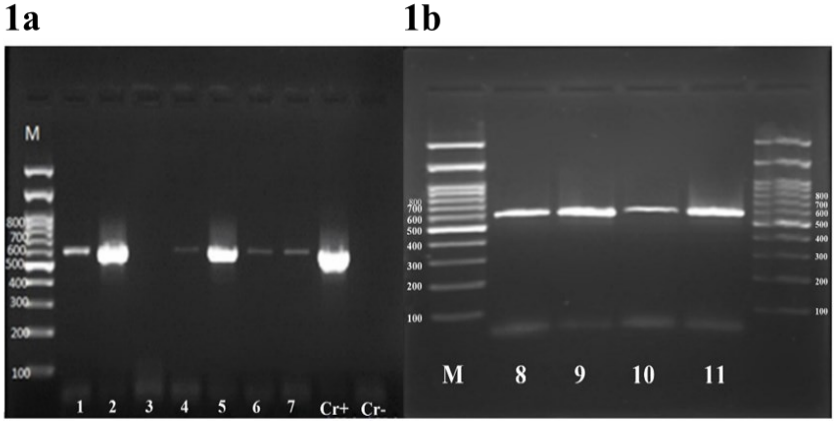

Figure 1A. Agarose gel electrophoresis of D1/D2 region of PCR products. Before digestion with *HhaI*: lanes 1-7 are example samples. Lanes cr+ and cr- are positive and negative controls, respectively, and lane M is a 100-bp molecular size marker.
Figure 1B. After digestion with *HhaI*: lanes 1 and 2 are *M. globosa*, lanes 3 and 4 are *M. restricta*, and lane M is a 100-bp molecular size marker.

deposited in the GenBank database with accession numbers of OR234714-OR234723.

The results of species distribution in the study population showed that *M. globosa* was found in 67.7% (n=84) of the patients with PV, and 11.7% (n=2) of the healthy population. It should be noted that *M. restricta* was just identified in patients with PV (15; 12.1%).

Discussion

According to the results, molecular identification involving PCR–RFLP analysis had a remarkable ability for the direct identification of *Malassezia* in clinical samples. As can be seen in previous studies [10, 11], the diagnosis of fungal infections using PCR-based methods, if performed directly on clinical samples, has a significant improvement, compared to the results obtained from the conventional methods.

The advantages of these methods include 1) the possibility of simultaneous detection and identification of the causative agent, 2) no need for culture in cases where the sample cannot be cultured for any reason, and 3) reducing the time required to obtain the result to one day. Prompt detection and identification of the causative agents of infection lead to the selection of the appropriate treatment by saving time, as well as the prevention of the induction of drug resistance. Therefore, in this study, it was attempted not only to diagnose PV infection directly from the clinical sample by PCR–RFLP method but also to identify the common *Malassezia* species that are the causative agents of this infection.

The PCR assay used in this study has high diagnostic characteristics, especially in terms of sensitivity and PPV that could be arising from the selection of suitable primers for PCR analysis. Regarding species identification of *Malassezia,* the general or species-specific primers are designed from different regions of the rRNA complexes so far [12, 13] or the *elongation factor 1 alpha* genes [14]. Consistent with previously reported results [15], the primers used in the current study amplified the D1/D2 region that was efficient for species identification of *Malassezia* yeasts.

Until now, many molecular methods have been introduced for the detection and identification of *Malassezia* species from clinical isolates. The most common methods include nested PCR [16], PCR–RFLP [17], and real-time PCR [3] with different levels of accuracy. Among the above-mentioned methods, the PCR–RFLP method is a sensitive, accurate, and fast (when using fast digest enzymes) method without requiring advanced tools that can detect microorganisms at the species and intra-species levels [18, 19].

Among the other advantages of PCR–RFLP assay, it can be pointed out that there is no need for complete gene sequence information, no influence from the environmental agents, and high reproducibility [20]. However, it should be noted that this method also has disadvantages, which include the high cost of some endonuclease enzymes and the reduction of sequencing accuracy if there is more than one mutation in the gene sequence detected by the enzyme.

It should be noted that some studies have used the D1/D2 domain for PCR–RFLP assays and identified *Malassezia* species by exposing the PCR product to two to three enzymes. It seems that the reason for the variety of species identified in the prior studies, compared to our study, is the use of more than one enzyme in the PCR–RFLP reaction.

In some studies, in order to identify *Malassezia* species, the isoschizomers of *Hha*I enzymes have been used to create breaks in the D1/D2 domain [6, 21, 22]. The *Hha*I has isoschizomers, such as *Cfo*I, *FnuD*III, *HinGU*I, *HinPl*I, *HinSl*I, *HinS2*I, *Mnn*IV, and *SciN*I [23].

Recent studies based on culture methods in different geographical regions of Iran have shown that *M. globosa* is the most prevalent species recovered from PV patients (frequency range: 36-43%) [24-27]. In view of these obtained results, it should be noted that *M. restricta* species could not easily recover by conventional culture methods. However, the independent culture methods are capable of identification of *M. restricta*. Consequently, it is worth noting that molecular methods are more efficient to determine the species distribution of fungal agents in epidemiological studies [5, 28].

Conclusion

In this study, the two-step molecular method based on the amplification of the D1/D2 domain and digestion of the PCR product by one restriction enzyme was able to identify *Malassezia* directly from clinical samples. Moreover, the suggested molecular-based method provides more facilities to identify fastidious species, such as *M. restricta*.

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Authors' contribution

M. M. contributed to the design of the study and writing of the research paper. Z. G. performed the clinical sample collection. S. K. performed the experiments. E. E. and S. Y. contributed to the acquisition, analysis, and interpretation of data. K. Z. advised the whole process of the study. All authors provided critical revisions for important intellectual content and also read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Financial disclosure

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