A Pilot Study for the Evaluation of PCR as a Diagnostic Tool in Patients with Suspected Dermatophytoses

Abstract

Context: The conventional diagnostic tools for dermatophytoses suffer from several limitations including low sensitivity, specificity, and long turn-around-time. Aims: The present study was, therefore, performed to evaluate the performance of a polymerase chain reaction (PCR)-based method for the diagnosis of this condition. Settings and Design: The study was conducted in the Dermatology outpatient department of a tertiary care teaching hospital in central India over a period of 3 months from July to September 2015. Materials and Methods: Forty participants, including 25 cases and 15 controls, were recruited in this observational study. Direct microscopy and fungal culture were performed from skin scrapings and nail clippings collected from the participants. PCR was also performed to amplify the chitin synthase 1 and internal transcribed spacer 2 genes from DNA samples extracted from the same clinical materials, using the method reported by Brillowska-Dabrowska et al. The diagnostic performance of fungal culture and PCR was compared using OpenEpi software. Results: We observed a significant male predominance among patients with dermatophytoses. The sensitivity of fungal culture and dermatophyte PCR to diagnose dermatophytoses was 24% and 48%, respectively, whereas the specificity of the two assays was 100% and 93.3%, respectively. The likelihood ratio of a positive PCR assay was 7.2 and the negative likelihood ratio was 0.5. PCR assay also delivered a significant shortening of the time-to-diagnosis, with the mean turn-around-time being 8 hours and 14 days for PCR and culture, respectively. Conclusion: This study, thus, highlights the potential merits of the dermatophyte PCR assay in achieving a rapid diagnosis of dermatophytoses and underscores its utility as a complementary test to improve the sensitivity of the conventional diagnostic tools for this condition, as well as to reliably differentiate this condition from other similar skin conditions.

Keywords: Dermatophytoses, dermatophyte PCR, fungal culture, Tinea

Introduction

Although the community prevalence of dermatophytoses is not known for certain, it constitutes a common cause of skin disease in India.[1,2] The clinical presentation at times may have nonspecific manifestations such as itching, rash, erythema, pigmentation, alopecia, scaling, follicular papules, pustules, and nail discolouration, thereby making its diagnosis difficult. Differentiation of this condition from similar lesions in psoriasis, seborrhoeic dermatitis, eczema, superficial candidiasis, erythrasma, etc., is often challenging. Frequent use of topical corticosteroid creams further alters the clinical presentation by obscuring the signs of inflammation.[1]

The conventional procedures used in the laboratory diagnosis of this condition include potassium hydroxide (KOH) mount examination of samples from skin, hair, and nail tissues; fungal culture; and phenotypic identification of recovered isolates. These methods suffer from the limitations of being less sensitive, time-consuming, and expertise-dependent. Dearth of competent mycology laboratories in many parts of the country further precludes a proper diagnostic work-up.

Polymerase chain reaction (PCR)-based diagnosis, though practiced in many infective conditions, has not yet been widely employed as a diagnostic tool. Optimization of PCR for dermatophytoses holds the promise of delivering a sensitive, specific, and quick diagnostic tool that is not skill-based. In view of this, the present pilot study was designed to evaluate the potential of a PCR-based method to aid the diagnosis of dermatophytic fungal infections.
infections and to compare the same with the diagnostic performance of fungal culture.

Materials and Methods

A total of 40 participants attending our dermatology outpatient department were recruited in this observational study over a period of 3 months from July to September 2015. They included 25 patients, who were clinically diagnosed as suffering from dermatophytoses based on the characteristic features of their presenting lesions such as the presence of annular, erythematous, itchy plaques; peripheral scaling; centrifugal spread; subungal debris; yellowish or greenish discoloration of nails; onycholysis; thickening of nail plate; onychoschizia; and total dystrophy of nails. The control group (n = 15), comprised participants in which the diagnosis of dermatophytoses was ruled out and an alternative clinical diagnosis could be arrived at. This group of controls included patients suffering from a diverse range of conditions such as seborrhoeic dermatitis, allergic reactions, lichen planus, annular psoriasis, and polymorphic light eruptions. Skin scrapings (n = 11) and nail clippings (n = 4) were also collected from the controls. The clinical diagnosis of both the cases and the controls was made by a dermatologist who was blinded to the outcomes of the laboratory tests. Dermatophytoses was ruled out in controls based on clinical presentation, confirmation of alternative diagnoses, and negative results on KOH and fungal culture. Patients with doubtful clinical diagnosis and those who had taken systemic or topical antifungals/antibiotics/steroids in the previous 2 weeks were excluded from the study. The cases and controls were recruited consecutively following their presentation in our dermatology outpatient department, subject to fulfilment of the inclusion and exclusion criteria.

The study protocol was approved by the institutional ethics committee, and the clinical samples were collected after obtaining proper informed consent. The clinical samples including skin scrapings (n = 26) and nail clippings (n = 14) were collected depending on the lesion involved. A part of each of these samples was used for KOH mount examination and fungal culture, whereas the remaining samples were used for fungal DNA extraction and dermatophyte PCR. Direct microscopy of the samples for the presence of fungal elements was performed by placing the sample onto a clean glass slide with a drop of 10% KOH. Fungal culture was performed by inoculation onto Sabouraud’s dextrose agar (SDA) (HiMedia) and Dermatophyte test agar (DTA) (HiMedia) supplemented with antibiotics. The culture tubes were incubated at 25°C for 3–4 weeks, and the isolates were identified by examination of the cultural characteristics and microscopic morphology.

The genomic DNA was extracted from a part of the clinical samples using the dermatophyte PCR (Statens Serum Institut, SSI Diagnostica, Denmark) kit. In brief, DNA extraction was done from the clinical samples by a 2-step 10-min incubation of the sample in 100 µl of extraction buffer (60 mM sodium bicarbonate [NaHCO₃], 250 mM potassium chloride (KCl), and 50 mM Tris (hydroxymethyl) aminomethane (Tris), pH 9.5) at 95°C and subsequent addition of 100 µl anti-inhibition buffer (2% bovine serum albumin). After vortex mixing, the DNA-containing solution was used for PCR. A conventional multiplex PCR was performed using two sets of primers aimed at: (a) chs1 chitin synthase 1 for detecting dermatophytes; and (b) its2 (internal transcribed spacer) for detecting Trychophyton rubrum. The primer sequences were, as reported by previous authors, viz. (a) 5’-GAAGAGATTGTCTTGTGCATCGTCTC-3’ and 5’-CTCGAGGTCGACAAGCCAGAG-3’ for amplifying chitin synthase gene from all dermatophyte genomes, and (b) 5’-TCTTTGAAGCGCATTGCCGC-3’ and 5’-CGGTCTCTGAGGCGCGTGA-3’ for amplifying its2 gene from T. rubrum genome.[4,5] The PCR mix include 10 µl and 8.0 µl of PCR ready mix and primer mix, respectively, (both provided in working concentration by the manufacturer) and 2.0 µl of DNA template. The primer mix included an internal plasmid control that served as a template for the T. rubrum specific primers.[6] The amplification was performed in a thermal cycler (Applied Biosciences) and the PCR conditions consisted of one initial cycle of denaturation for 5 min at 94°C and 45 cycles of 30 s at 94°C; 30 s at 60°C; 30 s of extension at 72°C; and a final extension of 3 min at 72°C. After the PCR, the amplicons were electrophoresed in a 2% agarose gel and stained with ethidium bromide.

For calculating the sample size, we considered a sensitivity of 44% for dermatophyte PCR test[7] and a prevalence of 20% for dermatophytic infections among patients presenting to our dermatology outpatient department (based on our hospital records). With these considerations and an error rate of 10%, the optimum sample size was found to be 24.4. Accordingly, this time-bound pilot study was conducted with the recruitment of 25 cases and 15 controls. Chi-square test was applied to ascertain if the proportion of categorical variables between the two groups was statistically significant or not. Independent sample t-test was performed to examine if the continuous variables were significantly different between the two groups. These tests of significance were performed using the Statistical Package for the Social Sciences (SPSS) Version 21.0. (IBM Corp, 2012).[8] The performance characteristics of the diagnostic tools were calculated using OpenEpi software.[9] P value of <0.05 was considered significant.

Results

We recruited two groups of participants in this study based on the clinical evaluation of their presenting lesions by a dermatologist. The first group, viz. the case group (n = 25) comprised of patients who were clinically diagnosed to be...
suffering from dermatophytoses. The second group \((n = 15)\) comprised patients in whom dermatophytosis could be clinically ruled out based on the examination of the lesions, which was further corroborated by negative findings on microscopy and fungal culture.

The mean (±SD) age of the patients in the case group was 38 (±18) years, which was similar to the age of patients included in the control group (40 ± 15 years). However, the former group included a significantly higher proportion of males (73%), compared to the latter group (27%) \((P = 0.005)\). The clinical characteristics and the nature of presenting lesion among the cases and controls are compared in Table 1. A representative sample of lesions observed among the recruited participants is depicted in Figure 1.

We observed a sensitivity of 24% (6/25) for the samples positive for fungal culture, whereas the corresponding figure for dermatophyte PCR was 48% (12/25) positive samples. The specificity of the fungal culture and PCR assays were 100% and 93.3% (14/15 control samples were negative). The isolates recovered on fungal culture were identified as \(T. rubrum\) \((n = 2)\), \(T. mentagrophytes\) \((n = 2)\), \(T. verrucosum\) \((n = 1)\), and \(Epidermophyton floccosum\) \((n = 1)\) based on microscopic and cultural characteristics. KOH mount examination was positive in 11 out of 25 cases (44%) and in none of the 15 controls. The likelihood ratio of a positive PCR assay was 7.2 and the negative likelihood ratio for PCR was 0.5. The performance characteristics of fungal culture and dermatophyte PCR assays are shown in Table 2. The PCR assay also delivered a significant shortening of the time-to-diagnosis, with the

| Table 1: Profile of presenting lesions and risk factors among recruited participants |
|--------------------------------------|--------------------------------------|------|
| Age (Mean±SD)                        | 38±18                                | 40±15|
| Gender (% males)                     | 73                                   | 27   |
| Risk factors                         |                                      |      |
| Diabetes mellitus                    | 3                                    | 0    |
| Proximity to animals                 | 3                                    | 3    |
| Use of steroids                      | 2                                    | 4    |
| Shared bathing space                 | 8                                    | 3    |
| History of contact                   | 7                                    | 4    |
| Contact sports                       | 2                                    | 1    |
| Nature of lesions                    |                                      |      |
| Nail dystrophy                       | 6                                    | 5    |
| Crusting                             | 1                                    | 0    |
| Itchy lesions                        | 12                                   | 8    |
| Erythema                             | 9                                    | 8    |
| Scaling                              | 6                                    | 4    |
| Hyperpigmentation                    | 4                                    | 3    |
| Discharge                            | 2                                    | 0    |
| Blister                              | 0                                    | 1    |
| Inflammation                         | 1                                    | 2    |
| Clinical diagnosis                   |                                       |      |
| Tinea unguium:                       | 10                                   |      |
| Tinea cruris:                        | 7                                    |      |
| Tinea corporis:                      | 5                                    |      |
| Tinea manuum:                        | 3                                    |      |
| Seborrhoeic dermatitis:              | 4                                    |      |
| Allergic Reactions:                  | 4                                    |      |
| Lichen planus:                       | 3                                    |      |
| Annular psoriasis:                   | 2                                    |      |
| Polymorphic light eruptions:         | 2                                    |      |
mean turn-around-time being 8 hours and 14 days for PCR and culture, respectively. A representative gel picture of the PCR amplicons is shown in Figure 2.

Comparing the results of the culture and PCR techniques, we observed concordance between the two techniques in 60% (15/25) of the cases. In 10 patients (40%), PCR was positive for the presence of dermatophytes (chs1), whereas the culture results were negative. In 2 patients (8%), PCR results were negative, though fungal culture revealed growth of dermatophytes. However, 100% concordance was observed between the culture and PCR results for T. rubrum, with both the culture-positive samples yielding positive results in PCR for its2 gene.

### Discussion

In this study, we evaluated the potential of PCR assays in diagnosing dermatophytosis directly from clinical specimens. We observed sensitivity and specificity of 48% and 93.3%, respectively, with the PCR assay, which made it appear as an attractive complementary test for the diagnosis of this condition.

Despite the obvious advantages associated with a PCR assay for dermatophytosis, evaluation of such an assay is often challenging due to the lack of an appropriate “gold standard.” Use of fungal culture as the standard and considering the results of the same for calculation of “true positives” and “false positives” is inappropriate because its suboptimal sensitivity leads to incorrectly low values of diagnostic specificity and positive predictive value for the PCR assay.[10] Similarly, the use of microscopy results as gold standard is also unjustified owing to the nonspecificity of its findings. In view of this, we sought to evaluate the diagnostic performance of the PCR assay by recruiting a group of cases and controls on the basis of the clinical characteristics of their lesions. Consideration of clinical symptoms alone has been reported to result in misdiagnosis presumably in 50% of the cases.[11] However, we surmise correct identification of the group of cases in our study because microscopic and/or cultural evidence of dermatophytosis was obtained in all our patients. On the other hand, the control group was selected on the basis of observing conclusive evidence of an alternative diagnosis. None of the controls were culture-positive for dermatophytic moulds. Thus, we evaluated the PCR assay, vis-a-vis other diagnostic modalities, based on the “realistic” approach of using clinical diagnostic criteria as the “gold standard.”

Though PCR assays have been evaluated in dermatophytosis since the last decade, most of the studies have focussed on species identification of dermatophyte isolates recovered on fungal culture.[12] Studies evaluating the diagnostic performance of PCR assays directly on clinical samples have been relatively rare.[13] Some of these studies have targeted the amplification of the Internal Transcribed Spacer (its2) and 18S rDNA regions, chitin synthase 1 gene, and large subunit of rDNA.[13-15] Similar to our results, all authors have reported a significantly higher sensitivity for the dermatophyte PCR assay compared to fungal culture. However, to the best of our knowledge, no study has evaluated the diagnostic specificity of the PCR assay in clinically simulating dermatological conditions. The finding of specificity value of 93.3% in this pilot study suggests the successful use of this assay in ruling out the diagnosis of clinically similar nondermatophytic conditions. Despite efforts taken to minimize amplicon contamination, false positivity among controls could have resulted from cross-contamination occurring during DNA extraction.

We observed PCR- negative results in 33.3% (2/6) of culture-positive samples. The PCR results in these samples could be affected by the inhomogeneous distribution of fungal DNA in the clinical material. In addition, as observed by Gräser et al.,[12] the sensitivity of the PCR assays could be improved by the selective accumulation

| Table 2: Comparison of the performance characteristics of fungal culture and dermatophyte PCR assay |
|---------------------------------------------------------------|
|                                                                 |
| **Fungal culture**  | **Dermatophyte PCR** |
|---------------------|----------------------|
| **Sensitivity (95% CI)** | 24% (11.5-43.4) | 48% (30-66.5) |
| **Specificity (95% CI)** | 100% (79.6-100) | 93.3% (70.2-98.8) |
| **Positive predictive value (95% CI)** | 100% (61-100) | 92.31% (66.7-98.6) |
| **Negative predictive value (95% CI)** | 44.12% (28.9-60.5) | 51.85% (34-69.2) |
| **Diagnostic Accuracy (95% CI)** | 52.50% (37.5-67.1) | 65% (49.5-77.8) |

Figure 2: Agarose gel electrophoresis of PCR amplicons. Lane 1: PCR-negative sample showing 600 bp band for Internal Control only. Lanes 2 and 3: PCR-positive samples showing 366 bp (base pair) band, conserved across all dermatophytes (Pan-dermatophyte band). Lanes 4 and 5: PCR-positive samples showing 203 bp band, specific for *Trichophyton rubrum*. Lane 6: Positive Control for Pan-dermatophyte band. Lane 7: Positive Control for *Trichophyton rubrum*. Lane M: 100 bp DNA ladder.
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