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Polymerase Chain Reaction-Restriction Fragment Length Polymorphism as a Confirmatory Test for Onychomycosis

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Abstract

BACKGROUND: Onychomycosis is a fungal infection of one or more units of the nail caused by dermatophytes, or mould and nondermatophytes yeast. Investigations are needed to establish the diagnosis of onychomycosis before starting treatment. Several investigations methods for diagnosing onychomycosis are microscopic examination with 20% KOH, fungal culture, histopathology examination with PAS staining (Periodic acid Schiff) and PCR (Polymerase Chain Reaction). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is a method after PCR amplification allowing more specific results.

AIM: To determine the diagnostic value of PCR - RFLP in the diagnosis of onychomycosis using fungal culture as the gold standard and to find out the majority fungal species that cause onychomycosis.

METHODS: This study is a diagnostic test for the diagnosis of onychomycosis by using culture as the gold standard.

SUBJECTS: Thirty - five patients suspected of having onychomycosis from history and dermatological examination.

RESULTS: PCR - RFLP in the diagnosis of onychomycosis has a sensitivity of 85.71%, specificity of 28.57%, positive predictive value (PPV) of 82.76% and negative predictive value (NPV) of 33.33%. The positive and negative likelihood ratios are 1.20 and 0.5 with an accuracy of 74.29%.

CONCLUSIONS: PCR - RFLP may be considered for a faster and more accurate alternative examination in the diagnosis of onychomycosis.

Introduction

Onychomycosis is a fungal infection of one or more units of the nail caused by dermatophytes, or mould and nondermatophytes yeast [1]. 50 % of nail infections and 30 % of superficial fungal infections are caused by onychomycosis [1][2][3][4]. Factors that may influence the onychomycosis prevalence rate include age, predisposing factors, social class, occupation, climate, environment and travelling frequency [3][4]. 50% of world population suffer from onychomycosis [4].

There are 3 onychomycosis - related fungal groups: dermatophytes, non - dermatophytes/ mould and yeast [1][2][3][4]. Dermatophytes include *Trichophyton rubrum, Trichophyton mentagrophytes*, *Epidermophyton floccosum* [1] [2] [4] [7] [8] [9] [10] [11] [12]. Nondermatophytes/mould include *Acremonium sp., Alternaria sp., Aspergillus sp., Botryodiplodia theobromae,* and *Fusarium sp.* among others [4] [6] [7] [8] [9]. *Candida Albicans* is the most commonly found yeast [1] [2] [3] [4] [5] [6] [7].

The diagnostic test is needed to confirm onychomycosis diagnosis before starting the antifungal therapy. Known diagnostic tests for onychomycosis include microscopic examination with 20% KOH, PAS (Periodic Acid Schiff) - staining examination, microscopic immunofluorescence with calcofluor - stain. PCR (Polymerase Chain Reaction) and fungal culture [3] [4] [11] [12] [13].

Microscopic examination with 20% KOH and fungal culture are the two most important tests used to confirm fungal infection diagnosis. Fungal culture needs around four weeks to identify the etiological agent of onychomycosis [3] [14].

Specific and sensitive molecular techniques such as Polymerase Chain Reaction (PCR) can also be used to diagnose myriads of microorganism agents

including pathogenic fungi [3] [15]. PCR is an in - vitro DNA synthesis and amplification technique [18]. The technique was first proposed by Karry Mullis in 1985 [15] [16]. PCR can be used to amplify the DNA within hours exponentially. This discovery has revolutionised the medical science and technology especially for its high diagnostic value [15]. In this current study, considering the capability of PCR technique to do early and accurate identification of pathogenic microorganisms and viruses, we'd like to evaluate the diagnostic technique capability for onychomycosis and comparing the results to the culture as the golden standard [14].

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR - RFLP) is a PCR method with enzymes addition after the DNA amplification. Thus it may give a more specific result [18][19]. Previous study my Monod et al. in 2006 found that PCR - RFLP results are fast and reliable enough to identify nondermatophytes as the aetiology of onychomycosis [20]. A study by Elavarashi et al. in 2013 found that PCR - RFLP with Internal Transcribed Spacer (ITS) primer, *Mva*I and *Dde*I enzymes may give promising results [21]. Therefore, this study was conducted to evaluate PCR as a diagnostic test to diagnose onychomycosis.

Methods

This study was conducted from April 2014 until reaching the minimum sample requirement in the Mycology Outpatient Clinics of RSUP H. Adam Malik, Medan Dermatovenereology Department. Twenty-five nail samples were taken in Mycology Outpatient Clinic of RSUP H. Adam Malik Medan Dermatovenereology Department, and fungal cultures were done in Microbiology laboratory of the University of Sumatera Utara, Faculty of Medicine. PCR - RFLP was done in Integrated Laboratory of the University of Sumatera Utara, Faculty of Medicine. Instruments used include scalpels, envelopes, ice bags, PCR tubes (Biologix), microcentrifuge tube (Sorenson), white tip (Biologix), yellow tip (Biologix), blue tip (Sorenson), micropipet (Rainin), cold storage, centrifuge (Biofuge, Germany), incubator (Mammert), thermocycler (applied biosystem type Veriti 96 well thermal cycler, Singapore), electrophoresis apparatus with power supply (Scie - plans, UK) and vortex (Biosan). Perishables used include nail speciments, Saboraud's dextrose agar medium, buffer Tris - EDTA (Sigma), EDTA (Sigma), DNA extraction kit (Promega), lyticase enzyme (Sigma), PCR kit (Promega), Internal Transcribed Spacer 1 (ITS1) primer and Internal Transcribed Spacer 4 (ITS 4) (1st Base), 2 % agarose gel (Promega), isopropanol (Merck), ethanol 70 % (Merck), ethidium bromide (Promega), DNA marker (Promega) and restriction enzyme *Mva*I and *Hae* III (Fermentas).

Basic data (including history - taking and dermatology examination) inputs were done in RSUP H. Adam Malik, Medan, Dermatovenereology Department. Nail sampling was done by the researcher. Taken nail samples were divided into two envelopes in which the first was taken to the microbiology laboratory for the fungal culture and the second was taken to the integrated laboratory for the PCR - RFLP. The collected data were summarised in 2 x 2 table and were analysed. Sensitivity and specificity of PCR - RFLP then compared with the gold standard, which is culture. Moreover, we compare the accuracy, negative predictive value (NPV) and positive predictive value in both modalities.

Results

Female is the gender group with most counts at 25 people (71.4%) as seen in Table 1. From Table 2 we can see that *Candida* onychomycosis (14 people, 40%) are the most commonly found onychomycosis clinical appearance followed by distal and lateral subungual onychomycosis (10 people, 28.5%) and total dystrophic onychomycosis (11 people, 31.4%). Table 1 shows most subjects' onychomycosis are located in foot nails (21 people, 60 %) with hand nails location at 14 people (40%).

Table 1: Characteristics of subjects in RSUP H. Adam Malik Medan in 2014

	Frequency	Percentage (%)
Gender		
Female	25	71.4
Male	10	28.6
Total	35	100.0
Clinical Appearance		
Candida Onychomycosis	14	40,0
Total Dystrophic Onychomycosis	10	28,5
Distal and Lateral Subungual Onychomycosis	11	31,4
Total	35	100,0
Location		
Foot nails	21	60
Hand Nails	14	40
Total	35	100,0

The most common fungal species identified from the cultures was *Candida albicans* (15 people, 42.8%) with *Phaecylomyces sp.*, *Epidermophyton floccosum, Trichophyton tonsurans, Candida tropicalis* and Culvularia were the least common at one person each (2.9%) as shown in Table 2.

Table 2: Onychomycosis fungal culture frequency distribution in RSUP H. Adam Malik Medan in 2014

No.	Species	Frequency	Percentage (%)
1.	No Growth	7	20,0
2.	*Candida albicans*	15	42,8
3.	*Aspergillus niger*	5	14,3
4.	*Cladosporium sp*	3	8,6
5.	*Phaecylomyces sp*	1	2,9
6.	*Epidermophyton floccosum*	1	2,9
7.	*Trichophyton tonsurans*	1	2,9
8.	*Culvularia*	1	2,9
9.	*Candida tropicalis*	1	2,9
Total		35	100,0

The most common fungal species identified from the PCR - RFLP technique was *Candida* albicans at 15 people (42.8%) with *Epidermophyton floccosum, Candida tropicalis,* and *Trichophyton tonsurans* was the least common at one person each (2.9%) as shown in Table 3.

Table 3: Onychomycosis PCR - RFLP Fungal Species Frequency Distribution in RSUP H. Adam Malik Medan in 2014

No.	Fungi Detected from PCR-RFLP	Frequency	Percentage (%)
1.	Not detected	11	31,4
2.	*Candida albicans*	15	42,8
3.	Negatif	6	17,1
4.	*Epidermophyton floccosum*	1	2,9
5.	*Trichophyton tonsurans*	1	2,9
6.	*Candida tropicalis*	1	2,9
Total		35	100,0

Onychomycosis detection using PCR - RLP yields a sensitivity value at 85.71% when compared to fungal culture results as the golden standard which means that 85.71 % of onychomycosis patients in this study were detected using this method and this shows that the instrument yield a high sensitivity (Table 4).

Table 4: Analysis and statistical tests results

Sensitivity	$=\frac{a}{a+c}$	x 100 %	$=\frac{24}{28}$	x 100 %	= 85.71%
Specificity	$=\frac{d}{b+d}$	x 100 %	$=\frac{2}{7}$	x 100 %	= 28.57%
Accuracy	$=\frac{a+d}{a+b+c+d}$	x 100 %	$=\frac{26}{35}$	x 100 %	= 74.29%
PPV	$=\frac{a}{a+b}$	x 100 %	$=\frac{24}{29}$	x 100 %	= 82.76%
NPV	$=\frac{d}{c+d}$	x 100 %	$=\frac{2}{6}$	x 100 %	= 33.33%

Discussions

This study found that PCR - RFLP method yield 85.71% sensitivity value and 28.71 % specificity value. The results were lower than the PAS - staining method but the invasiveness of PAS - staining compared to PCR - RFLP method should be put into consideration.

Kardjeva et al. in 2004 done a study in Germany and the study found out that from 261 onychomycosis cases, PCR method as a confirmatory diagnostic test yield 84% sensitivity compared to the fungal culture at 22% sensitivity. This shows that molecular methods yield better results and less time-consuming at 2 - 3 days when compared to a fungal culture that may take time from 2 to 4 weeks [12].

Litz et al., in 2010 compared the PCR method with KOH test, fungal culture, and PAS - staining from 559 nail specimens with the results was 37%, 40%, 22%, and 54% respectively [14]. A study by Rizal in 2010 at RSUP Haji Adam Malik Medan found that PAS - staining yield better results than fungal culture in onychomycosis diagnosis with 96.8% sensitivity and 50% specificity [5]. Mirzahoseini et al., in 2009 in Iran showed that PCR - RFLP method is fast and reliable enough to identify most of the pathogenic fungal species [19].

A study by Arca et al. in 2004 in Turkey found 40 positive results (77%) using 20% KOH test, 12 positive results (23%) using fungal culture, and 20 positive results (38%) using PCR method within 44 onychomycosis nail samples [22]. PCR is a selective and highly valued diagnostic tool to detect fungal species especially in cases where they can't be detected using conventional methods [17]. Baek et al., from Korea stated that PCR - RFLP is a highly sensitive method to detect and identify onychomycosis, having a higher diagnostic value when compared to conventional methods [22].

Specificity test aims to evaluate the capability of an instrument / a method to bring negative results among people who are without the disease. The specificity value of 28.57% means that 28.57% of onychomycosis patient suspects who are without the disease can be excluded using the PCR - RFLP method.

A study by Mohamed LM et al in 2007 in Egypt showed that out of 30 onychomycosis cases 13 (43.3%) were found positive, and 17 (56.7%) were found negative using the fungal culture whereas 16 (53.3%) were found positive and 14 (46.7%) were found negative using the PCR method [13].

The ability of the PCR method to detect the genome of infectious fungi on onychomycosis patients may explain the high sensitivity of the method [14]. Presence of contaminants during sampling and sample processing may explain the low sensitivity of the PCR - RFLP method [13] [17].

Positive Predictive Value (PPV) gives the estimated probability of subjects with positive results. This study found the PPV was at 82.76%. This suggests that this tool has a high strength to determine true positive results. Negative Predictive Value (PPV) gives the estimated probability of subjects with negative results. This study found the NPV was at 33.33%. This suggests that this tool has a low strength to determine true negative results thus not suitable as an onychomycosis screening tool.

Accuracy is an ability of an instrument to give the correct results out of the subjects. The PCR - RFLP method in this study was found with an accuracy of 74.29%, which showed the high ability of the tool to detect onychomycosis correctly.

Positive Likelihood Ratio (PLR) is the ratio between true positive results with false negative results. The PLR value of the PCR - RFLP method in this study is 1.20. Negative Likelihood Ratio (NLR) is the ratio between false negative results with true negative results. The NLR value of the PCR - RFLP method in this study is 0.5. A diagnostic test with a great positive strength usually gives a ratio value much more than 1 and is deemed as significant if the ratio is

more than 10. A diagnostic test with a great negative strength will give a likelihood ratio closer to 0.

This study concludes that PCR - RFLP can be deemed as a good tool to diagnose onychomycosis. The high sensitivity value suggests that this tool may be used as an alternative diagnostic tool for onychomycosis.

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