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Genotyping and subgenotyping of *Trichophyton rubrum* isolated from dermatophytosis in Iraqi patients using RFLP-PCR

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Abstract:
Aim: *To identify the genotypes and subgenotypes of Trichophyton rubrum isolates based on the molecular techniques.*
Methods: *Eight isolates of T. rubrum were isolated and identified phenotypically and genotypically using cultural, physiological and molecular tests; respectively, based on conventional and RFLP-PCR.*
Results: *Its noticed that only 8/24 (33.33%) isolates were classify as T. rubrum with amplicon size equal 601bp. RFLP-PCR showed the presence of two genotypes (I,II) belong to T. rubrum. The profile of RFLP-PCR showed that 6/8 (75%) of isolates represent the genotype I which contained subgenotypes (Ia-Id) while the other genotype symbolized as genotype II that contained the subgenotypes (IIa-IId).*
Keywords: *T. rubrum, genotypes and subgenotypes, dermatophytosis, RFLP-PCR.*

I. Introduction

The dermatophytes are a group of closely related fungi that have capacity to invade keratinized tissue (skin, hair, nail, feathers, horns and hooves) of human and other animals to produce an infection, dermatophytosis. About 40 species belonging to the genera *Microsporum*, *Trichophyton* and *Epidermophyton* are considered as dermatophytes, they possess two important properties: they are keratinophilic and keratinolytic. This mean they have the ability to digest keratin in vitro in their saprophytic state and utilize it as a substrate and some may invade tissues in vivo and provoke tineas (Kalinowska *et al*., 2009a).

Trichophyton rubrum, is the most common causative agent of dermatophytosis worldwide, mainly occupying the humans' feet, skin, and between fingernails. *T. rubrum* is known to be one of the most prominent anthrophilic species of dermatophtyes, a fungus commonly causing skin diseases (White *et al*., 2012), Very little is known about the mechanism of its invasion and pathogenicity (Maranhao *et al*., 2007). Though it is usually not life-threatening, infections are long-lasting, recurring, and incredibly difficult to cure. The fungal pathogen's ability to produce and secrete proteolytic enzymes is a major virulence factor (Chen *et al*., 2010).

The present study tried to use the molecular methods such as Polymerase chain reaction (PCR), Restriction fragment length polymorphism (RFLP) and sequencing of internal transcribed spacer (ITS) region of the ribosomal DNA in addition to classical methods due to that methods are more specific, precise, rapid and are less likely to be affected by external influences such as temperature variations and chemotherapy in the identification of T. rubrum isolates and detection the capability of these isolates to produce the protease and its gene expression.

Molecular typing of an infectious agent is important for epidemiological studies and for the development of appropriate infection control strategies. In Iraq, all clinical laboratories are not fully aware of the important of molecular typing of dermatophytes and how to detect them, laboratories may also lack the resources to epidemiological study and crub the spread of these types. To the best of the researcher knowledge, until now no published researcher has described the distribution of genotypes with *T. rubrum* isolates in Iraqi patients. However different types have been identified in this study but not phenotypic test can differentiate among them, a fact which creates problems for surveillance and epidemiological studies. Present study describe the use of conventional PCR for the purpose of identifying genotypes of the isolates.

II. Materials and Methods

Clinical specimens

Atotal of 150 clinical specimens (hair, nails and skin scrapings) were collected from patients who attended the Dermatology and Venereal disease center at Mergan Teaching hospital and private clinic in Babylon city from February 2014 to May 2014. The specimens were inoculated on Sabouraud's dextrose agar containing cycloheximide (0.5g/l) and chloramphenicol (0.05g/l) at pH 5.6 and incubated at 29±2ºC for 14-21 days (Burns et al, 2010).

Identification of Dermatophytes isolates

1: Morphological features

The following criteria were taken in consideration in the growth identification,(Kwon-Chung and Bennett, 1992; Irene and Richard,1998; Collee *et al*, 1996; De Hoog *et al*, 2002; Milne,1996; Burns *et al*, 2010).

1-Morphological features of growth colony which were including (color, texture, margin, consistency, colony reverse and pigments that reproduced).

2-Microscopic and Macroscopic features (size, shape, arrangement, of microconidia and macroconidia, their conidial ontogeny, types of specialized structure upon which spores were borne)

2: Physiological tests

2.1: Production of Urease test

This test was conducted according to de Hoog & Guarro, 1995, by taking two test tubes which containing urea medium, this medium was inoculated from the colonies grown in (10) days using sterile loop. Test tubes were incubated in (29±2ºC) for 7 days, and any change in the color of media that mean to analysis the urea. Changes the color of medium from yellow to red or dark pink when urea completely analyzed, but during the partially analysis of urea the color changed from yellow to faint pink.

2.2: Hair perforation test in vitro

Sterile human hair (hair from blond child) is suspended in sterile distilled water supplemented with (2-3 drop) of yeast extract. The tested organism (10-14 days) is inoculated onto the hairs. After 2 weeks' incubation at 29±2ºC , hairs are mounted with lacophenole stain to look for wedge-shaped penetrations perpendicular to the hair axis (Burns *et al*, 2010).

2.3: Growth in 37 ºC

To investigate the capability of the fungi to growth in (37^0C), fungal tissues was took from new produced colony by sterile loop and then make scraping on the (SDA) media. Then incubated at (37^0C) for (14 days), after that the growth results were recorded.

Molecular identification of *T. rubrum* by conventional PCR.

Culture and harvest the isolates

A portion of specimens was cultured on SDA by spot inoculation technique. The cultures were incubated at 29 ±2 ºC until visible fungal growth or (5-10) day-old for early PCR analysis. The genomic DNA of 24 dermatophytes isolates from young fungal colonies were extracted by using a grinder in presence of liquid nitrogen for initial breaking up of the mycelia. Final DNA extraction was achieved by special purification kit. The amount of 6 µl of DNA solution was used as a template in the following PCR, specimens were checked and quantified on 2% agarose gel and by using Nanodrop spectrophotometer.

PCR and preliminary screening by RFLP

The PCR-amplified internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was performed with primers ITS-1 forward (5'-ACGATAGGGACCGACGTTCC-3') and ITS-1 reverse (5'-CCCTACCTGATCCGAGGTCA3') under the following PCR conditions:

Amplification reactions were performed in final volumes of 20 µl containing 5 µl of template DNA, reaction buffer (10 mM Tris-HCl [pH 9], 30 mM KCl), 1.5 mM MgCl2, 250 µM (each) dATP, dCTP, dGTP, and dTTP, 1.5 µl of each forwared& reverse primer, and 1 U of DNA polymerase.

The samples were overlaid with sterile paraffin oil and PCR was performed for 32cycles in a DNA Thermal Cycler with 5 min of initial denaturizing at 95°C, 30 sec. of denaturation at°C, 30 sec. of annealing at 60°C, 1 min of extension at 72°C and 10 min of final extension at 72°C. PCR products were separated by electrophoresis in 2% agarose gels for 2 h. Amplification products were detected by staining with ethidium bromide and were visualized under UV light.

The amplicons were digested with restriction enzyme MvaI and then electrophoresed in a 2% agarose gel, stained with ethidium bromide, and observed under UV light.

III. Results

Identification of *Trichophyton rubrum*

Morphological features of suspected isolates demonstrated that their colonies were appeared on SDA as different criteria including (color, Texture, margin, consistency, Colony reverse and pigments that reproduced), T. rubrum characterized by cottony- or powdery- like, white or light beige, flat or elevated colony, with or without pigment on the reverse of the colonies.While the microscopic features of slides were prepared from

isolates colony and stained with lactophenol cotton blue were revealed a tiny structures of dermatophytes isolates (size, shape, arrangement, of microconidia and macroconidia, their conidial ontogeny, types of specialized structure upon which spores were borne), the microscopic evaluated characteristics were: fine regularly septate mycelium, clavate or piriform, scarce or abundant microconidia; eventually clavate or cylindrical, multiseptate, smooth thin wall, some with terminal appendages macroconidia; and hyphal structures similar to macroconidia (closterospores). (Figure 1 and 2).

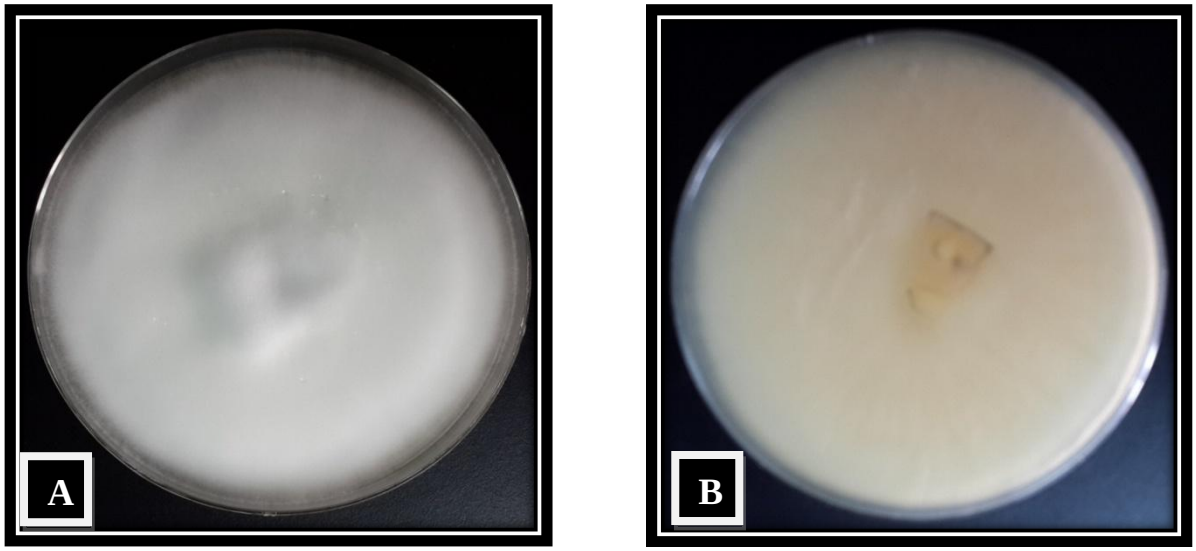

Figure (1): *Trichophyton rubrum* colony growing on (SDA) after 14 days of incubation. (A): Front side of the colony. (B): Reverse side of the colony.

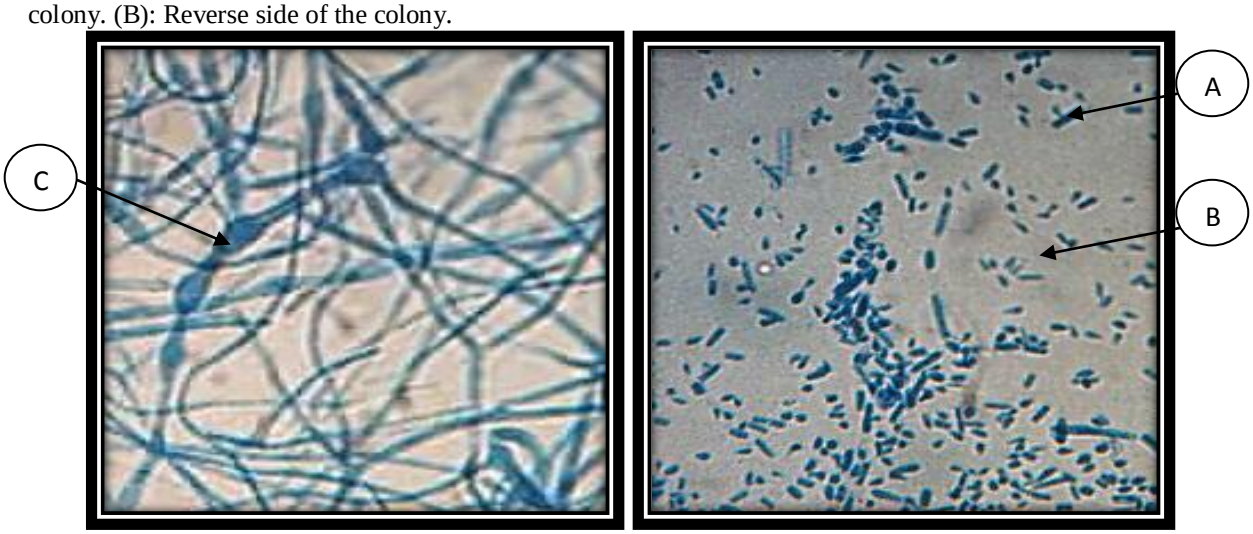

Figure (2): Macroconidia and Microconidia structures of *T. rubrum* isolates growing on (SDA) after 14 days of incubation. (A): Macroconidia, (B): Microconidia. (C): Clesteospores.

Molecular confirmatory diagnosis of *T. rubrum* isolates

In the present study, the confirmation process of the 8 *T. rubrum* isolates were also conducted by conventional PCR technique to detect the presence of a specific 5.8S rRNA gene. The extracted genomic DNAs of these isolates were used as a template for amplification with primers of Internal Transcribed Spacer1 (ITS1). The results revealed that only 8/24 (33.33%) isolates were classified as *T. rubrum* with amplicon size equal 601bp after band electrophpresis and UV-transilluminated of the product as shown in fig (3).

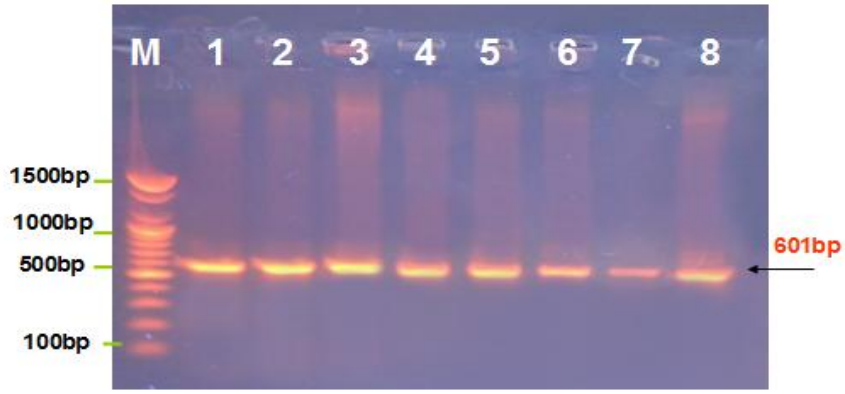

Figure (3): Ethidium bromide-stained agarose gel of PCR product amplified with ITS1 primer of *T. rubrum* (Amplicon size=601bp), Where laneM= Marker, 100-1500bp; Lane(1-8), T. rubrum isolates.

To determine the genotype of the 8 isolates of *T. rubrum* recovered from clinical specimens of dermatophytes. The PCR product (601bp) resulted from amplification of extracted genomic DNA with primers of target gene (5.8S rRNA) of the ITS1 region are digested by restriction endonuclease (MvnI) using RFLP technique showed the presence of two genotypes (I&II) belong to species of *T. rubrum* Figure (4-11). The profile of RFLP showed that 6/8 (75%) of isolates represented the genotype I which contained the subgenotypes (Ia-Id) with molecular size (95bp, 156bp, 457bp, 482bp), while the other genotype symbolized as genotype II that contained the subgenotypes (IIa-IId) with molecular size (80bp, 156bp, 482bp, 500bp) which represented 2/8 (25%) of isolates Table (1).

Table (1): Genotypes and subgenotypes of *T. rubrum* according to the RFLP patterns by MvnI restriction enzyme.

Genotypes	Subgenotypes	RFLP pattern (bp) by MvnI enzyme	Total isolates%
I	Ia	~95bp	6/8 (75%)
	Ib	~156bp	
	Ic	~457bp	
	Id	~482bp	
II	IIa	~80bp	2/8 (25%)
	IIb	~156bp	
	IIc	~482bp	
	IId	~500bp	

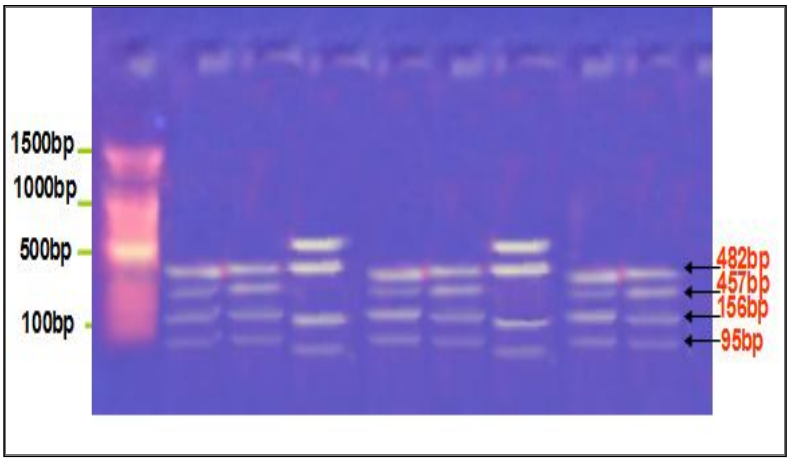

Figure (4): Agarose gel electrophoresis image that shown the RFLP-PCR product analysis of ITS1 gene in *Trichophyton rubrum* isolates that digestion by restriction endonuclease (MvnI) that digestion of the 601bp PCR product into 95bp and 156bp, 457, and 482bp as genotype I (Lane 1, 2,4,5, 7, & 8), 73bp and 151bp, 482, and 520bp as genotype I I (Lane 3 and 6).

IV. Discussion

Dermatophytes are the most common agents of fungal infections worldwide. Dermatophytic infections have been considered to be a major public health problem in many parts of the world. The infections are widespread in the developing countries and are of particular concern in the tropical and temperate regions, dermatophytosis has been reported to be encouraged by hot and humid conditions and poor hygiene (Richa *et al*, 2012).

In addition to the well known superficial infections caused by *Trichophyton* spp., such as Tinea capitis, Tinea corporis, Tinea pedis and Tinea unguium, dermatophytes species especially *Trichophyton rubrum* is the most common dermatophyte causing deep and invasive infections (Marconi *et al*., 2010).

Conventional laboratory diagnosis of dermatophytes based on micro- and macro-morphological examination of primary isolates and sometimes supplemented with physiological characters such as hair perforation and urease tests for atypical isolates in some laboratories in wealthier parts of the globe, the diagnostic therefore, tends to be stripped down to the minimal cost necessary to achieve targeted proficiency levels that are based mainly on the ability to identify typical isolates accurately (Graser *et al*., 2006; Yvonne *et al*., 2008).

Even though importance of the conventional method in the identification of dermatophytes but also it can be difficult or uncertain because there is considerable variation among isolates of some species and it is a time-consumimg procedure requiring even 30 day for final isolation and identification of etiologic agent at genus or species level. Likewise, in some instance, the causative dermatophytes fail to produce any obvious reproductive structure in culture (termed sterile mycelia) which make it impossible for ultimate definitive diagnosis (Malinovschi *et al*., 2009). In-vitro culture is capable of providing a species-specific determination of dermatophytes on the basis of morphological and biochemical criteria. However, for some unusual and atypical isolates identification may require a range of culture media and tests, these tests are not only costly and time-consuming, but also demand specialist skills. More importantly, because these conventional methods depend on measurements of the phenotypic characteristics of dermatophytes, they can be easily influenced by outside factors (such as temperature variations and chemotherapy) that may interfere with the metabolic process of the dermatophyte and affect the interpretation of in-vitro culture results (Li *et al*., 2008). In the last decades, genotyping approaches have proven to be useful for solving problems of dermatophyte taxonomy, as well as enhancing the reliability and speed of dermatophytosis diagnosis (Jackson *et al*., 2006; Borman *et al*., 2008; Malinovschi *et al*., 2009). The internal transcribed spacer (ITS) regions of the fungal ribosomal DNA (rDNA) has been used as one of the techniques for species identification because it is faster, accurate species determination, specific and are less feasible to be affected by exterior effects such as temperature changes and chemotherapy (Girgis *et al*., 2006; Kong *et al*., 2008). Studies revealed that morphological characteristics of colonies of all isolates of T. rubrum are similar to *T. rubrum* isolated from tinea cruris, tinea pedis and tinea capitis of human (Graser *et al*., 2000). Colonies of *T. rubrum* are fluffy to cottony and white to cream in color. Macroconidia are spare or abundant and microconidia are present in all isolates.

In the last few years genotypic approaches have proven to be useful for solving identification problems regarding dermatophytes; in fact, genotypic differences are considered more stable and more precise than phenotypic characteristics. In addition, species identification has a wide role in monitoring the demographic distribution and changes in frequency of specific dermatophyte infections (Garg *et al*., 2009; Gherbawy *et al*., 2010).

However different isolates have been identified in this study, but not phenotypic test can differentiate among them. Present study describe the use of conventional PCR for the purpose of identifying genotypes of the isolates. The study was conducted to determine the distribution and diversity of genotypes dermatophytes isolates recovered from dermatophytosis patients in Babylon city using 18S rDNA primers (ITS region).

The detection rate of dermatophytes by PCR technique has shown great deal of variation. It depends on the target gene used in the analysis (Kabir , 2004). Because the identification of dermatophytes by the conventional laboratory methods are cumbersome, laborious and often inconclusive due to fungal phenotypic variability and pleomorphism, application of advanced molecular diagnostics provide successful approaches for rapid diagnosis of pathogenic dermatophytes with high accuracy, sensitivity and specificity (Behzadi and Behzadi, 2012; Behzadi *et al*., 2014).

PCR-RFLP technique can be considered as a powerful technique for discrimination and identification of fungal isolates that is independent of morphological and biochemical characteristics especially when the proper restriction endonucleases are selected (Kanbe *et al*., 2003; Dobrowolska *et al*., 2006).

In the current study, PCR-RFLP of two universal primers (ITS1& ITS4) were used to amplify the ITS region of the rDNA in all fungal isolates studied followed by digestion of the PCR product with restriction enzyme (MvnI) that digest of the 601bp PCR product into 95bp and 156bp, 457, and 482bp as genotype I and 73bp and 151bp, 482, and 520bp as genotype II.

The identification results are in agreement with established and recent taxonomical insights into the dermatophytes; for example, highly related species also had closely related and sometimes difficukt-to-discriminate ITS2-RFLP patterns. Analysis of ITS region based on PCR-RFLP was used to identify and discriminate between 57 of T. rubrum clinical isolates (Hryncewicz-Gwóźdź *et al*., 2011) and between different species or varieties of Trichophyton, Microsporum and Epidermophyton (Gräser *et al*., 1999, 2000a,b; Mirzahoseini *et al*., 2009). They reported that PCR-RFLP serves as a rapid and reliable method for the identification of *T. rubrum* isolates and other species of dermatophytes.

Recently, Rezaei-Matehkolaei et al. (2012) used PCR-RFLP assay to find the exact differentiating restriction profiles for each dermatophyte species. They reported that the ITS-PCR cut by restriction enzymes MvaI-RFLP is a useful and reliable scheme for identification and differentiation of several pathogenic species and can be used for rapid screening of even closely related species of dermatophytes in clinical and epidemiological settings.

Although dermatophyte fungi are in great numbers, common pathogenic dermatophyte fungi are only few. Therefore, the attention was paid to the commom dermatophyte fungi (*T. rubrum*) during collection and the study aimed to seek a rapid and simple method to identify them for doctors. Additionally, the reliable specificity among interspecies and conservatism among intraspecies of ITS region can ensure the sensitivity and specificity of identification

On the other hand, in about 9 hours, we could obtain electrophoretic profiles starting from cultures, as the DNA extraction technique, while the only disadvantage of the use of PCR-RFLP for identifying dermatophytes is the relatively higher cost in comparison to the classical method, the advantages of its use are many, so provide the opportunity for dermatophyte identification at species level therefore this method can be of great utility when it is not possible to use (Faggi *et al*., 2001).It is concluded that this procedure can differentiate genera and occasionally species of medically important fungi and that following the necessary validation experiments; it can be used directly on clinical specimens to assist prompt diagnosis of systemic fungal infections.

The results obtained in this study demonstrated that by using restriction enzyme (MvnI), the differentiation of *T. rubrum* isolates could be easy. On the basis of this finding, they investigated these different isolates by restriction endonuclease and found four bands pattern for each genotype I and II symbolized numerically from Ia and IIa to Id and IId. This is the first report indicating subgenotypes differences within genotype I and II of T. rubrum in Iraq. In order to demonstrate the usefulness of this method in epidemiological studies and to determine the relation of these subgenotypes studies with different infection sites, much larger and epidemiologically sophisticated studies are needed. The differences in the subgenotypic patterns in this investigation with previos studies may be referring to the differences in race ethics geographical location and type of specimen. However many studies hint to genetic diversities in *T. rubrum* isolates according to these factors (Hryncewicz-Gwozdz *et al*., 2011; Mohammadi *et al*., 2015).

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