Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 07, pp.18231-18235, July, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

COMPARATIVE STUDY BETWEEN MOLECULAR AND CONVENTIONAL DETECTION METHODS OF *MALASSEZIA FURFUR* ISOLATED FROM IRAQI PSORIATIC PATIENTS

[1]Abbas M. Al-Ammari, [*,2]Saife D. Al-Ahmer, [3]Azhar A.F. Al-Attraqhchi and [2]Noor I. Al-Baiyati

[1]Department of Biology, College of Science, Baghdad University, Iraq
[2]Institute of Genetic Engineering and Biotechnology for Post Graduate Study, Baghdad University, Iraq
[3]Department of Medical Microbiology, College of medicine, Al-Nahrain University, Iraq

ARTICLE INFO

Article History:
Received 09th April, 2015
Received in revised form
22nd May, 2015
Accepted 09th June, 2015
Published online 31st July, 2015

Key words:
M. furfur,
Psoriasis Disease,
ITS Region.

ABSTRACT

Background: Psoriasis is a common, chronic, relapsing/remitting, immune-mediated skin disease characterized by red, scaly patches, papules and plaques which usually itch. *Malassezia furfur* is a fungus, specifically yeast that is approximately 1.5-4.5 µm wide and 2-6 µm long. *Malassezia fufur* is a coccal and their cells contain a plasma membrane, a thick and multilaminar cell wall composed of chitin with an invagination characteristic of *Malassezia*, mitochondria, a nucleus, and all of the other vital organelles. The role of *Malassezia* yeasts in psoriasis is still undetermined, but there are several reports indicating that these microorganisms are able to elicit psoriasis form lesions in both human and animals.

Aims and objectives: The aims of the present study were to find out the prevalence of psoriasis disease, along with *Malasseiz furfur* and evaluation of the ITS PCR method sensitivity comparison with conventional methods.

Materials and Methods: In this study vaginal swabs from 180 individuals (60 psoriatic patients and 120 Healthy volunteers) were used for lacto phenol cotton blue stain, culture, PCR and RFLP methods. PCR was performed with primer pair targeted the internal transcribed spacer (ITS) region of *Malasseiz furfur*. The result of the PCR was compared with conventional methods. The PCR positive samples were identified by presence of ~509 bp amplicon of the ITS region.

Results: Conventional methods of lacto phenol cotton blue stain and culture methods showed a positive results in 16 (26.7%) out of 60 psoriasis patients and 26 (21.7%) out of 120 healthy control whereas PCR and RFLP methods showed 20 (33.3%) out of 60 psoriasis patients and 29 (24.2%) out of 120 healthy control.

Conclusion: PCR method is sensitive, specific and useful technique to detect *Malassezia furfur* in both of psoriatic patients and healthy control.

Citation: Abbas M. Al-Ammari, Saife D. Al-Ahmer *et al.* 2015. "Comparative Study Between Molecular and Conventional Detection Methods of *Malassezia furfur* Isolated from Iraqi Psoriatic Patients", *International Journal of Current Research*, 7, (7), 18231-18235.

INTRODUCTION

Psoriasis is a common, chronic, relapsing/remitting, immune-mediated skin disease characterized by red, scaly patches, papules, and plaques, which usually itch. The skin lesions seen in psoriasis may vary in severity from minor localized patches to complete body coverage (Menter *et al.,* 2008). The disease affects 2-4% of the general population (Shlyankevich *et al.,* 2014). *Malassezia furfur* is a fungus, specifically a yeast, that is approximately 1.5-4.5 µm wide and 2-6 µm long (Larone, 2002).

Corresponding author: Saife D. Al-Ahmer
Institute of Genetic Engineering and Biotechnology for Post Graduate Study, Baghdad University, Iraq.

Malassezia fufur is a coccal, and their cells contain a plasma membrane, a thick and multilaminar cell wall composed of chitin with an invagination characteristic of *Malassezia* (Gaitanis *et al.,* 2012), mitochondria, a nucleus, and all of the other vital organelles (Marcon and Powell, 1992). Rivolta made the first association of the lipophilic yeasts and psoriasis in 1873 (Rivolta, 1873). The role of *Malassezia* yeasts in psoriasis is still undetermined, but there are several reports indicating that these microorganisms are able to elicit psoriasis form lesions in both human and animals. The higher detection rate of *Malassezia furfur* was observed using molecular determination method than by conventional culture methods. An important molecule produced by *M. furfur* is tryptophan aminotransferase, which converts L-tryptophan to indolepyruvate (Slonczewski and Foster, 2011) by transferring an amino group.

Also produced is B-Glucosidase, which allows *M. furfur* to free up glucose by breaking down cellulose, the primary carbohydrate in cell walls. This allows *M. furfur* to break down the cell walls of other microorganisms on our skin cells which kills the organism. This can help prevent infection. *M. furfur* produces various cytokine, chemokine, and adhesion molecules. The cytokines help to regulate the inflammation response of the host; the chemokines help direct cell movement, and the adhesion molecules help *M. furfur* stick to the epithelial layer. The internal transcribed spacer (ITS) and large subunit (LSU) regions are now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic at the species level, and even within species (e.g., to identify geographic races). Polymorphisms in the internal transcribed spacer (ITS) region and intergenic spacer of fungal ribosomal DNA repeat units, at both the inter- and intraspecific levels, have provided practical epidemiological markers for typing a range of clinically important species (Gupta *et al.,* 2000).

MATERIALS AND METHODS

Samples collection

One hundred eighty samples were collected from patient's clinically diagnosis with psoriatic disease and healthy volunteers in Hospital of Medical Imammian kadhamain city, Baghdad, Iraq for a period of 5 months. Studying groups aged from 1 to 70 years old. This study was carried out after obtaining the approval from the Department of Biology/ College of Science/ Baghdad University and Ministry of Health/ Iraq. A questionnaire was provided based on dermatological care program including name, age, demographic data, symptoms of disease, treatment history and disease complications. Skin scraps were taken from 60 patients clinically diagnosis with psoriasis by a sterile surgical blades and skin swabs were obtained from 120 healthy volunteers (As control) by sterile swabs. The lacto phenol cotton blue stains were performed for all samples and then the samples were cultured on Sabouraud's dextrose agar plus chloramphenicol (SC) (Merck, Germany) overlaid with olive oil and incubated at 37°C for 1-2 weeks under aerobic condition. The samples were cultured on chrom agar Malassezia medium (Biomerieux, France) and incubated at 37°C for 48 hours under aerobic condition. Also the samples were cultured on pigment induction medium and incubated at 32°C for 1 week under aerobic condition as deferential medium for *M. furfur* from other *Malassezia spp*.

DNA extraction

Genomic DNA was extracted from the *Malassezia furfur* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of *Malassezia furfur* culture grown for 20 hours at 37°C in YPD broth (Sigma, USA) was transferred to a 1.5 ml microcentrifuge tube. The samples were centrifuged at 13,000 rpm for 3 minutes to pellet the cells and the supernatant was removed, then the cells were resuspended thoroughly in 300 µl of 50 mM EDTA (Sigma, USA). 20 µl of 20 mg/ml proteinase (Sigma, Missouri, USA) was added and gently pipet 4 times to mix, then the samples were incubated at 37°C for 30 minutes to digest the cell wall and cooled at room temperature. The samples were centrifuged at 13,000 rpm for 3 minutes, the supernatant was removed and 300 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added to the cell pellet, then gently pipet to mix. 100 µl of protein precipitation solution (wizard genomic DNA purification kit) was added and vortex vigorously at high speed for 20 seconds, then the samples were sit on ice for 5 minutes and centrifuged at 14,000 rpm for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 ml microcentrifuge tube containing 800 µl of cold absolute ethanol and gently mixed by inversion until the thread-like strands of DNA form a visible mass, then centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully decanted and the tubes were drained on clean absorbent paper and 300 µl of room temperature 70% ethanol were added, then the tubes were gently inverted several times to wash the DNA pellet. The samples were centrifuged at 14,000 rpm for 2 minutes and all the ethanol was carefully aspirated. The tubes were drained on clean absorbent paper and the pellet was allowed to air-dry for 10 minutes, then 50 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added. 1.5 µl of RNase solution (wizard genomic DNA purification kit) was added to the purified DNA sample and the sample was vortex for 1 second, then centrifuged briefly in a microcentrifuge for 5 seconds to collect the liquid and incubated at 37°C for 15 minutes. The DNA was rehydrated by incubating at 65°C for 1 hour and the solution was periodically mixed by gently tapping the tube, then the DNA sample was stored at -20°C until use.

DNA quantification

The extracted DNA from the *M. furfur* isolates was quantified spectrophotometrically at OD260/280 nm with ratios 1.4-1.5. The sensitivity of the ITS-F1and ITS-R4primers was evaluated by PCR amplification for serial diluted concentrations (10ng-100ng) of purified genomic DNA isolated from *M. furfur*.

Primers selection

The primers for ITS gene of *M. furfur* as the target gene for this study were selected according to (Zunaina *et al.,* 2008). This set of unique primers was designed based on the conserved region in *M. furfur*; primers were synthesized by Alpha DNA, Kanda. The primers sequence of ITS gene and their product size are shown in (Table 1).

Table 1. The primers sequences of ITS gene of *M. furfur* and their product size

Name of Primer	Sequence of Primer (5'-3')	Size of Product
ITS-F1	GCATCGATGAAGAACGCA(	~509 bp
ITS-R4	TCCTCCGCTTATTGATATG(	

PCR Master Mix

The PCR reactions for detection of ITS gene of *M. furfur* were performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing

(GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol ITS-F1 primer and 2.5 µl of 20 pmol ITS-R4 primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil.

PCR program

PCR was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Zunaina *et al.,* 2008), with some modification. Briefly, the Amplification of ITS gene of *M. furfur* was carried out with initial denaturation 94C for 5 min. was followed by 30 cycles of denaturation at 94C for 45S, annealing at 53 C for 45s, and extension at 72C for 1 min.

The thermal cycles were terminated by a final extension for 7 minutes at 72°C. Positive control and Nuclease free water as a negative control were used too.

PCR products analysis

The analysis of PCR products of ITS gene of *M. furfur* were performed on 2% agarose gels. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 60 minutes at room temperature.

The PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

RESULTS

Conventional methods

Of the 60 patients with clinical diagnosis of psoriasis and 120 healthy control, lacto phenol cotton blue stain and culture methods using Sabouraud's dextrose agar plus chloramphenicol (SC) (Figure 1), Chrom agar Malassezia Medium (Figure 2) and pigment induction medium (Figure3) yielded positive result of *M. furfur* in 16 (26.7%) out of 60 psoriasis patients and 26 (21.7%) out of 120 healthy control.

Analysis of extracted DNA of *M. furfur* isolates

After performing of the DNA extraction from *M. furfur* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1% agaros gel at 7volt /cm for 1 hour (Figure 4).

Analysis of PCR products of *M. furfur* ITS gene

On the basis of the ITS sequence region, a product of ~509 bp was amplified by PCR with ITS-F1 and ITS-R4 primers. PCR method showed a positive result in 20 (33.3%) out of 60 psoriasis patients and a positive result in 29 (24.2%) out of 120 healthy controls.

Figure 1. Culture of *M. furfur* on Sabouraud's dextrose agar plus chloramphenicol (SC) agar overlaid with olive oil

Figure 2. Culture of *M. furfur* on Chrom agar *Malassezia* Medium

The PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5 µl of the PCR product were loaded on 1% agarose gel and run at 7volt /cm for 1 hour. The gel was stained with ethidium bromide solution (0.5µg/ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system.PCR result was considered positive for *M. furfur* when there was presence of ~509 bp PCR product band on agarose gel electrophoresis. No amplification was observed with negative control (Figure 5).

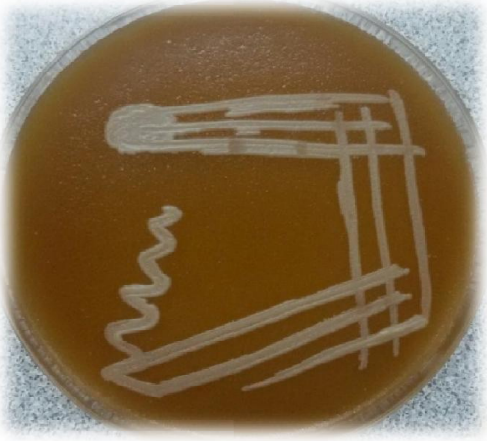

Figure 3. Culture of *M. furfur* on pigment induction medium

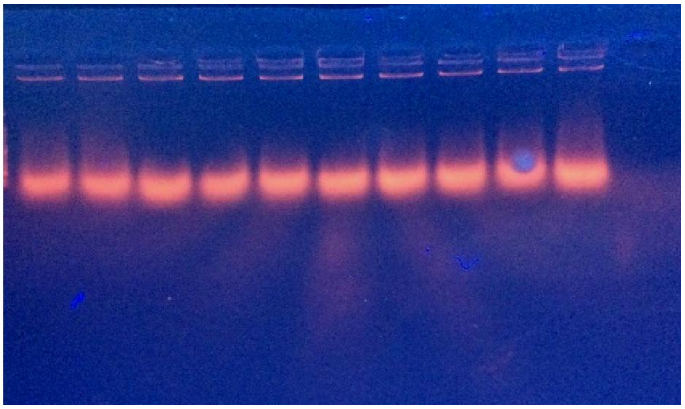

Figure 4. Gel electrophoresis of extracted DNA of *M. furfur* isolates using 1% agarose gel at 7volt /cm for 1 hour. Lane 1-10: Extracted DNA

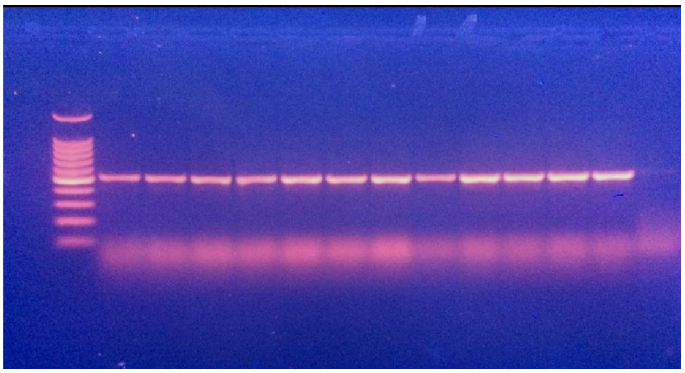

Figure 5. Gel electrophoresis of ITS PCR products of *M. furfur* isolates using 1% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-13: ITS PCR products of *M. furfur* isolates

DISCUSSION

In this study, the most common laboratory techniques done in diagnosis of *M. furfur* isolates were lacto phenol cotton blue stain and culture method using Sabouraud's dextrose agar plus chloramphenicol (SC), Chrom agar Malassezia Medium and pigment induction medium which were yielded positive result of *M. furfur* in 16 (26.7%) out of 60 psoriasis patients and 26 (21.7%) out of 120 healthy control. The advantages of conventional methods were non costly but the disadvantages of those methods were consuming time, contamination present, false positive result and require a large amount of sample (Chen *et al.,* 2000). Whereas performing of the PCR method for detection of *M. furfur* ITS region (~509 bp) using ITS-F1 and ITS-R4 primers showed a positive result in 20 (33.3%) out of 60 psoriasis patients and a positive result in 29 (24.2%) out of 120 healthy controls. The benefits of molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of molecular methods is costly. These explanations made molecular methods relatively more accurate than conventional methods (Kim *et al.,* 2013).

Conventionally, culture has been used to detect *M. furfur*, but takes two days to one week for final identification. The main problem with culture from skin swabs is the small amounts of *M. furfur* cells that can be obtained for diagnosis, increasing the negative results risk of *M. furfur* culture also some *M. furfur* isolates might be grow slowly. This may explain the negative culture results in 4 (6.7%) out of 60 psoriasis patients and 3 (2.5%) out of 120 healthy controls. In current study, ITS-F1 and ITS-R4 primers that amplify medically important *M. furfur* were selected and used. These primers are based on the conserved region of ITS gene which is designed to detect wide range of fungal strains with the PCR product size of ~509 bp (Ferrer *et al.,* 2001). The ITS gene is a multi-copy gene that is slowly evolving and highly conserved among fungi, making it an attractive target for the detection of *Malassezia* yeast in clinical specimens. Detection of *Malassezia* etiology by using ITS targeted PCR will be useful in early diagnosis of psoriasis disease and could help in early initiation of anti *Malassezia* therapy. Chen *et al.,* in 2000 have used ITS2 region specific primers to detect fungal species based on PCR product size identification by capillary electrophoresis (Latha *et al.,* 2014).

In most cases of dermatological disorders, the most important laboratory information that the dermatologists need to know is whether the infectious agent is fungal (*Malassezia* spp.) or bacterial. Positive PCR results that are available earlier than culture will justify the use of anti Malasseziasis, resulting in improved visual outcome. This study demonstrates that ITS gene-based PCR has high degree of sensitivity for the detection of medical significant *M. furfur*. The PCR diagnostic test had a sensitivity of 96.5%. PCR detected all of the 16 out of 60 psoriasis patients' samples and 26 out of 120 healthy control samples that were positive by conventional method. Two of the skin swabs samples that were negative by PCR are a case of other microbial psoriasis. This could be due to insufficient *M. furfur* elements present in skin swab or due to sequence variation of ITS gene of those two *M. furfur* isolates. The findings of this study agree with those of Kaneko *et al.* (2007), Gonzalez *et al.* (2009), Jagielski *et al.*, (2014) and Soares *et al.* (2015) founded that molecular diagnosis of *Malassezia* spp. were more sensitive and efficiency than conventional methods.

Positive PCR results that are available earlier than culture will justify the use of anticandidial agents promptly, resulting in improved visual outcome. This data agrees with the study by Anand *et al.,* (2001) and confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting.

Conclusion

This study has demonstrated the efficacy of the ITS gene to detect *M. furfur* in clinical samples. These molecular methods are sensitive, rapid and useful methods to detect *M. furfur* in the psoriatic patients when compared with conventional laboratory diagnosis of *M. furfur*. Further studies with larger sample size are needed to refine the technique, to calculate sensitivity and specificity, and to establish the value of the technique in managing of patients with psoriatic disease. Also, further studies using other molecular techniques are needed for detection of *M. furfur* in different cases of infections.

Acknowledgements

I would like to acknowledge Prof. Dr. Abdul-Hussein Al-Faisal, Dean of Institute of Genetic Engineering and Biotechnology for Post Graduate Studies and dermatologist Mr. Mohanad N. Kanaan.

REFERENCES

Anand, A., Madhavan, H., Neelam, V. and Lily, T. 2001. Use of polymerase chain reaction in the diagnosis of fungal endophthalmitis. *Ophthalmology,* 108(2): 326-330.

Chen, Y.C., Eisner, J.D., Kattar, M.M., Rassoulian-Barrett, S.L., LaFe, K., Yarfitz, S.L., Limaye, A.P. and Cookson, B.T. 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J. Clin. Microbiol.,* 38(6): 2302-2310.

Ferrer, C., Colom, F., Frases, S., Mulet, E., Abad, J.L. and Alio, J.L. 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J. Clin*. *Microbiol.* 39(8): 2873-2879.

Gaitanis, G., Magiatis, P., Hantschke, M., Bassukas, I.D. and Velegraki, A. 2012. The *Malassezia* Genus in Skin and Systemic Diseases. *Clinical Microbiology Reviews*. 1: 106-141.

Gonzalez, A., *et al.* 2009. Physiological and molecular characterization of atypical isolates of *Malassezia furfur*. *J. Clin. Microbiol.,* 47:48-53.

Gupta, A.K., Yatika, K. and Richard, C.S. 2000. Molecular Differentiation of Seven Malassezia species. *J. Clin*. *Microbiol.,* 38(5): 1869-1875.

Jagielski, T., Elżbieta, R., Aleksandra, Z., Katarzyna, R., Anna, B. and Jacek, B. 2014. Distribution of Malassezia species on the skin of patients with atopic dermatitis, psoriasis, and healthy volunteers assessed by conventional and molecular identification methods. *BMC Dermatology,* 14:3.

Kaneko, T., Koichi, M., Michiko, A., Ryoko, S., Yuka, N., Rui, K., Atsuhiko, H., Takashi, S., Shuichi, S., Shinichi, W., Hideyo, Y., Shigeru, A. and Noboru, O. 2007. Revised Culture-Based System forIdentification of *Malassezia* species. *lin Microbiol., 53* (11)*2*: 3737-3742.

Kim, J.Y., Hyung J.H., Yong, B.C., Yang, W.L., Kyu, J. A. and Kee, C.M. 2013. Molecular Biological Identification of Malassezia Yeasts Using Pyrosequencing. *Ann Dermatol*., 25(1): 73-79.

Larone, D.H. 2002. Medically Important Fungi: A Guide to Identification, 4ª ed.

Latha, R., Poongothai, G.K., Annie, R., Kavitha, K., Hemalatha, G., Nirmala, J. and Sethumadhavan, K. 2014. Phenotypic characterization and Antifungal Susceptibility Pattern to Fluconazole in *Candida* species Isolated from Vulvovaginal Candidiasis in a Tertiary Care Hospital. *Journal of Clinical and Diagnostic Research*, 8(5): DC01-DC04.

Marcon, M.J. and Powell, D.A. 1992. Human Infections Due to *Malassezia* spp. *Clinical Microbiology Reviews*. 5(2): 101-119.

Menter, A., Gottlieb, A., Feldman, S.R., Van Voorhees, A.S., Leonardi, C.L., Gordon, K.B., Lebwohl, M., Koo, J.Y., Elmets, C.A., Korman, N.J., Beutner, K.R. and Bhushan, R. 2008. Guidelines of care for the management of psoriasis and psoriatic arthritis: Section 1. Overview of psoriasis and guidelines of care for the treatment of psoriasis with biologics. *J. Am. Acad. Dermatol*., 58(5): 826-850.

Shlyankevich, J., Mehta, N.N., Krueger, J.G., Strober, B., Gudjonsson, J.E., Qureshi, A.A., Tebbey, P.W. and Kimball, A.B. 2014. Accumulating Evidence for the Association and Shared Pathogenic Mechanisms between Psoriasis and Cardiovascular-related Comorbidities. *Am. J. Med*. 127(12): 1148-53.

Slonczewski, J.L. and Foster, J.W. 2011. Microbiology: An Evolving Science 2 ed. Norton. 761-769; A-21-A-22.

Soares, R., Marcelo, B., Ana, C., Lucia, H. and Luciana, C. 2015. Malassezia Intra-Specific Diversity and Potentially New Species in the Skin Microbiota from Brazilian Healthy Subjects and Seborrheic Dermatitis Patients. *PLoS ONE journal,* 10(2): 179-190.

Zunaina, E., Wan Hazabbah, W., Chan, Y., Nur, H., Balqis, K., Siti, K., Sabariah, O., Zainul, F. and Manickam, R. 2008. Specific detection of fungal pathogens by 18S rRNA gene PCR in microbial keratitis. *BMC Ophthalmology,* 8(7): 1471.

****