International Journal of Molecular Medicine and Advance Sciences 2(3): 239-244, 2006

Rapid Diagnosis of *Candida albicans* Vaginitis Directly from High Vaginal Swabs

[1]Soo Fen Wong, [2,3]Boon Chong Tan, [1]Siti Rohani bt Abdul Hadi,
[4]Kee Peng Ng and [1]Pei Pei Chong
[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences,
University Putra Malaysia, 43400 Serdang, Selangor
[2]Brittania Women and children's Hospital, Kajang, Selangor
[3]Unit of Obstetric and Gynaecology, Department of Human Growth and Development,
University Putra Malaysia, 43400 Serdang, Selangor
[4]Department of Microbiology, Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia

Abstract: Candidiasis is an opportunistic fungal infection that has been rising in incidence due to the increasing number of immunocompromised patients. Various species of Candida are able to cause vaginal infections in women, but *Candida albicans* is the most common cause of vaginal candidiasis. Identification of *Candida albicans* still rely mostly on microbiological culture and biochemical tests that are time-consuming and requires trained mycologists. This study reports the rapid identification and detection of *Candida albicans* directly from high vaginal swabs without prior culture, using nested Polymerase Chain Reaction (PCR). A pair each of external primers and internal primers that are complementary to the IPC1 gene were designed. The specificity of the primers were tested against 4 non-albicans ATCC strains of Candida and also against 2 strains of baker's yeast (Saccharomyces cerevisiae). Seventy one High Vaginal Swabs (HVS) samples were collected in which one portion was cultured and subjected to the germ tube test while the other portion was subjected to direct DNA extraction and nested PCR. Out of 30 culture-positive samples, 19 were detected as *C. albicans* via the germ tube test (specificity=81.8%, sensitivity=89.5%, $p<0.05$) whereas nested PCR detected 21 (specificity=72.7%, sensitivity=94.7%, $p<0.05$). Among the 41 culture-negative samples, nested PCR detected 26. This study has produced a sensitive method that can detect *C. albicans* from vaginitis patients without the need for culturing.

Key words: *Candida albicans*, vaginitis, nested PCR

INTRODUCTION

Candida albicans is a predominant pathogenic opportunistic yeast in human beings which may cause superficial and rarely systemic candidiasis. It infects especially immunocompromised patients, surgical patients, prolonged hospitalized patients and newborn infants[1].

Oral candidiasis has been reported as one of the earliest and the most frequent infections associated with AIDS[2]. The incidence of candidaemia has risen by 219-487% between 1980-1989 and is currently ranked the fourth most important cause of septicaemia[3]. It is reported that the crude mortality rate for invasive or disseminated canidaemia is 50-80% even after adequate treatment[4]. As a consequence of the increased wide usage of antifungal drugs, in particular the azole group of drugs, the emergence of azole-resistant *Candida* species has been widely reported.

As for vaginal candidiasis, it has been reported that 75% of all women will experience at least one episode of Candida vaginitis in their life-time[5]. Vaginal candidiasis is caused by *C. albicans* predominantly (at least 80%), although other strains of *Candida* such as *C. tropicalis* and *C. glabrata*, are becoming more and more prevalent[6].

Therefore, an early and accurate diagnosis of *C. albicans* infection would be critical to allow a prompt and appropriate treatment in order to improve the prognosis and in the mean time decrease the unnecessary use of antifungal drug which can lead to emergence of drug-resistant strains. However, the current widely applied conventional methods, which is through culture followed by morphology identification such as germ tube or chlamydospore formation tests, are not only time-

Corresponding Author: Chong Pei Pei, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

consuming, but are neither specific nor sensitive. In fact, blood cultures can fail to detect about 25-50% of disseminated candidiasis even with the best available method[7].

The Polymerase Chain Reaction (PCR) method is increasingly used nowadays to conquer the weaknesses of conventional methods. It is amenable to most systems, rapid, sensitive and specific. Furthermore, the GenBank database has the DNA sequences of a large number of genes in the yeast and fungal genomes. Hitherto, quite a number of studies have been conducted in the attempt to detect and distinguish various *Candida* species using PCR-based methods[8-11], but there is still no report on direct detection of *C. albicans* from High Vaginal Swabs (HVS) via PCR without the need for culture.

Thus, this study aims to design PCR primers specific to the genomic sequence of *C. albicans*, to test for the specificity of the primers for *C. albicans* versus other closely related fungal species and to evaluate the potential application of the primers for detecting *C. albicans* DNA directly from vaginal swab samples.

MATERIALS AND METHODS

Clinical specimens: Subjects were female patients in O and G clinics of General Hospital Kuala Lumpur and Hospital Kajang, who complained of vaginal discharge or itchiness in vaginal area and asymptomatic patients who need speculum examination. Two Higher Vaginal Swab samples (HVS) were taken from each patient after obtaining informed consent. The first swab was placed in 1ml normal saline (0.9%) while the second swab was placed in Amies Transport Medium (ATM) with charcoal.

Isolation and characterization of microorganisms from specimens: Samples in ATM were cultured on Sabouraud Dextrose Agar supplemented with chloramphenicol (SCA), Sabouraud Dextrose Agar (SDA) and Nutrient Agar (NA) respectively, on the same day as samples collection and incubated at 37°C. The chloramphenicol supplement was to inhibit bacterial growth. Samples that grew smooth, creamy, bulging and yeasty-smelling colonies within 10 days were considered culture-positive. The germ tube test, which was and still is considered a simple definitive test for identification of *Candida albicans*, was performed on these samples[12]. Samples that had negative result for germ tube test were subjected to the sugar assimilation test for further identification of *Candida* species[13].

DNA extraction

Culture-positive specimens: A single colony was taken from each culture-positive sample and grown overnight in 2 mL of Sabouraud's dextrose broth at 37°C on a shaker. The cells were harvested the following day by centrifugation at 5000 rpm for 10 min and the pellet was resuspended in 1ml of Phosphate Buffered Saline (PBS). After washing with PBS twice, the pellet was then resuspended in 500 μL of lysis buffer and incubated at 37°C for 30 min on a rotary shaker. Twenty-five μL of 20% SDS and 2.5 μL of proteinase K were added to the pellet in lysis buffer. The mixture was incubated at 56°C for 60 min for samples kept in ATM and at 37°C for samples kept in saline. This was followed by boiling at 100°C for 5 min. DNA was extracted with 200 μL of phenol-chloroform-Isoamyl alcohol the upper aqueous phase was transferred to a clean tube. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol. The DNA pellet was washed with 250 μL of cold 70% ethanol and finally resuspended in 20 μL of sterile distilled H_2O.

Specimens in saline/Tris-HCl: Five hundred microlitres of specimen in saline/Tris-HCl was added with 25 μL of SDS and 2.5 μL of proteinase K and was then incubated in a shaker at 37°C for 1 h and followed by boiling at 100°C for 5 min. The remaining steps were the same as DNA extraction from specimens in ATM after boiling for 5 min.

Polymerase Chain Reaction (PCR): PCR was performed using 10 μL (for samples kept in saline) or 1 μL (for DNA extraction from culture-positive samples) of DNA template in a final volume of 20 μL. Final concentrations of components of the PCR were: 1×PCR buffer, 3.75 mM $MgCl_2$, 0.5 pmoles of each primers, 0.125 mM dNTP mixture and 0.75U Taq polymerase. Amplification was performed in a thermal cycler (Perkin-Elmer 2400). Hotstart PCR was done for culture-positive specimens whereas for direct PCR from HVS samples, nested PCR was performed. The cycling conditions for first round of nested PCR was Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 52°C for 30s, extension at 72°C for 1 min and final extension at 72°C for 10 min. In the second round PCR, 10 μL of the PCR product obtained from the first round PCR using outer primers (IPC5f/IPC2r) was diluted 20X and added to a new reaction mixture with inner primers (IPC3f/IPC4r) and was reamplified using the same cycling protocol as the first round.

Specificity and sensitivity tests: Specificity of the designed primer sets was evaluated using five American Type Culture Collection (ATCC) strains of *Candida* namely *C. albicans* (ATCC 14053), *C. glabrata* (ATCC

2001), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) and *Sacharomyces cerevisiae* strains 699 and 700 from NCYC (National Collection of Yeast Culture, UK). For the sensitivity test, one colony of *C. albicans* were grown overnight in 2 mL SDB in a shaker at 37°C. On the next day, serial dilutions of the culture was performed. In order to determine the viable cell count for each dilution, a 0.1 mL aliquot was used to inoculate duplicate SDA plates, then macerated with a glass spreader and incubated overnight at 37°C. On the following day, the number of colonies on each plate arisen from cells of each dilution were scored and the CFU/mL was calculated. The remaining yeast broth from each dilution was used for DNA extraction. Nested PCR using the IPC5f/IPCf2r and IPC3f/IPC4r primer sets was done.

Gel electrophoresis: Amplimers were analyzed by electrophoresis on 2% agarose gel (for PCR products using IPC1 and P_{450} primers sets) in 1× TAE buffer (0.04 M Tris-acetate; 0.01M EDTA) at 60V for 60 min. The results were visualised over a uv-transilluminator and the pictures was captured using the Gene Genius Gel Documentation and Analysis System-Genesnap.

Data analysis: Sensitivity and specificity were analyzed using 2×2 tables as in Jekel[14]. Reference method for *C. albicans* status is defined as germ tube positive and IPC1 nested PCR positive, or germ tube positive and either PCR using ITS3/ITS4 universal primers as reported by Fujita *et al.*[15] or sugar assimilation positive, or IPC1 nested PCR positive and either PCR using ITS3/ITS4 or sugar assimilation or positive with nested PCR using a previously described[16] cytochrome P_{450} primers. The significance level was evaluated using Fisher's exact test.

RESULTS

Clinical specimens: A total of 71 samples were collected from 70 individuals with age ranging from 18 to 61 years old. Among these individuals, 39 presented with vaginal discharge, 1 with itchy vagina, the remaining were either asymptomatic or presented with other symptoms. 30 of the samples were culture-positive.

Specificity of primers: The IPC5f/IPC2r outer primers and IPC3f/IPC4r inner primers showed bands of the expected sizes when tested with the genomic DNA of *C. albicans* ATCC 14053 (as shown in Fig. 1). They did not give any bands when tested with ATCC strains of other *Candida* species, namely *C. glabrata, C. krusei*, *C. tropicalis* and *C. parapsilosis*. Neither did they produce any bands when tested against two strains of *S. cerevesiae*, NCYC 699 and NCYC 700. The results indicate that the primer sets designed were very specific.

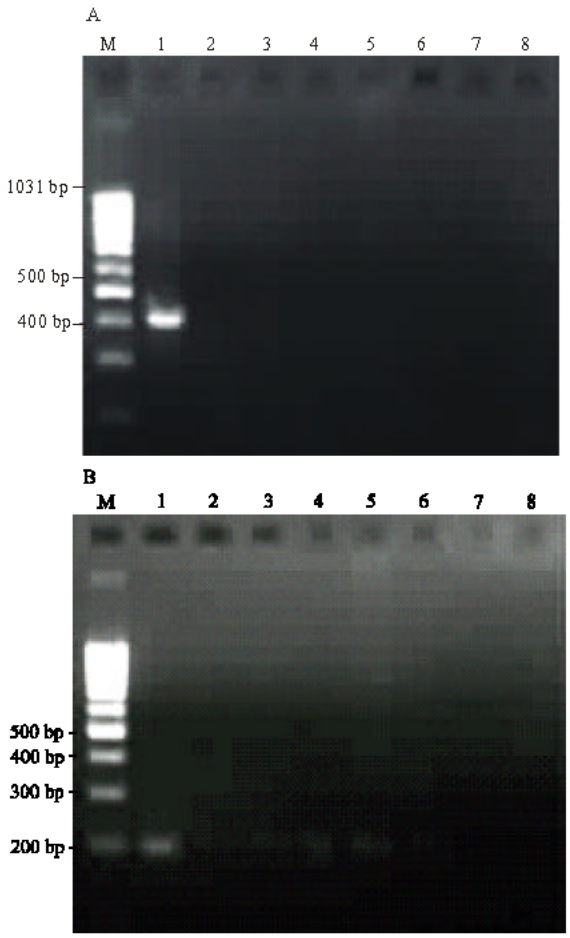

Fig. 1: Gel elctrophoresis of PCR products obtained from the genomic DNA of various *Candida* species and *Saccharomyces cerevisiae* using the designed IPC1 primers. A, primers used were outer primers IPC5f and IPC2r; results showed only *C. albicans* genomic DNA gave an expected band at 406 bp. B, primers used were inner primer pair IPC3f and IPC4r; only the genomic DNA of *C. albicans* gave an expected band at 200 bp. Lane M - 100-bp ladder marker, 1- *C. albicans* ATCC 14053, 2- *C. glabrata* ATCC 2001, 3- *C. krusei* ATCC 6258, 4- *C. tropicalis* ATCC 750, 5- *C. parapsilosis* ATCC 2019, 6 - *S. cerevisiae* NCYC 699, 7 - *S. cerevisiae* NCYC 700, 8 - negative control (no DNA added)

1: Specificity and sensitivity of germ tube test in detecting C. albicans compared to reference method

		Reference method	
		positive	negative
Germ tube test	positive	17	2
	negative	2	9

Specificity = 81.8%, Sensitivity = 89.5%, $p<0.05$

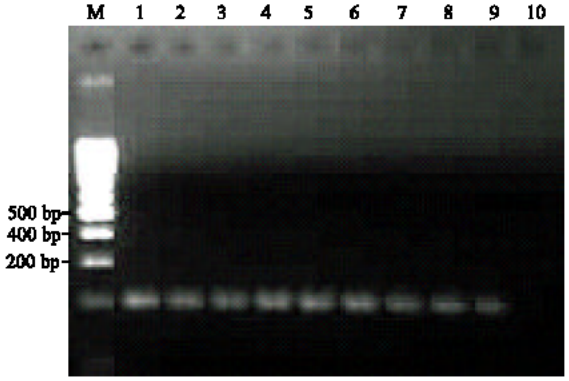

Fig. 2: Gel electrophoresis of PCR products obtained by nested PCR against a serially diluted culture of *C. albicans* ATCC strain. An overnight culture of *C. albicans* was serially diluted in Sabouraud dextrose broth and the DNA extracted from each serial dilution was subjected to nested PCR using IPC5f/IPC2r primer pair in the first round and IPC3f/IPC4r primer pair in the second round PCR. M-100 bp ladder marker (Fermentas), 1 - 10^2 dilution, 2 - 10^3, 3 - 10^4, 4 - 10^5, 5 - 10^6, 6 - 10^7, 7 - 10^8, 8 - 10^9, 9 - 10^{10} dilution; 10 - negative control

Table 2: Specificity and sensitivity of newly developed IPC1 nested PCR in detecting C. albicans compared to reference method

		Reference method	
		positive	negative
IPC1 nested PCR	positive	18	3
	negative	1	8

Specificity = 94.7%, Sensitivity = 72.7%, $p<0.05$

Sensitivity of primers: The PCR results for the sensitivity test is displayed in Fig. 2. In the 10^7, 10^6,.10^5 serial dilutions, the *Candida albicans* cell counts were 1, 25 and 168 colonies forming units (CFU)/mL respectively. The nested PCR method was able to detect all the dilution series tested upto 10^{10} dilution. Hence, the method is extremely sensitive and able to detect Candida cells at lower than 1 CFU/mL.

Culture-positive samples: Among the 30 culture-positive samples, we were able to detect 19 as *C. albicans* via germ tube test whereas 21 were detected as *C. albicans* via nested PCR using IPC5f/IPC2r and IPC3f/IPC4r. For three discrepant results that were germ tube-positive but IPC1 nested PCR-negative, we identified two of them as non-*albicans Candida* (false positive in Table 1) species and one (false negative in Table 2) as *C. albicans* via sugar assimilation test as well as PCR using ITS3/ITS4 primers. For 5 samples that were germ tube-negative but nested PCR-positive as shown, the actual status of two of them

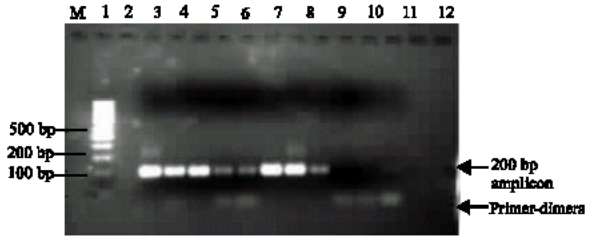

Fig. 3: Gel electrophoresis of PCR products amplified from culture-negative samples using nested PCR with IPC5f/IPC2r and IPC3f/IPC4r primers. Lane M-100 bp ladder marker. Lanes 1 to 11 are HVS samples in saline from patients. 1 - p19, 2 - p18, 3 - p15, 4 - p17, 5 - p16, 6 - p25, 7 - p21, 8 - p13, 9 - p20, 10 - p46, 11 - p52. Lane 12 is negative control

(false negative in Table 1) were *C. albicans* (one co-existed together with *C. glabrata*) while three others (false positive in Table 2) were non-*albicans Candida* species. The sensitivity and specificity of the two methods compared to the reference method are shown in Table 1 and Table 2.

Culture-negative samples: The remaining 41 out of the 71 samples were culture-negative samples. In order to see whether these were due to false negative result by culture method, the newly developed nested PCR was performed on these samples. In doing this, the sensitivity between nested PCR and culture were tested. Figure 3 is a representative result of the gel electrophoresis for the PCR amplicons obtained from culture-negative samples. We found that 26 samples were identified by IPC1 nested PCR as *C. albicans*. The result was further confirmed by performing nested PCR using *C. albicans* -specific P_{450} primers (results not shown).

The prevalence of *C. albicans* species among the *Candida* isolates cultured from the HVS samples determined via germ tube test (the definitive diagnostic test for *C. albicans*) was 26.8%. The non-*albicans Candida* species were present in 11 of the samples (15.5%). However, the nested PCR method was able to detect a much higher prevalence of *C. albicans* among the HVS samples than the germ tube test, even from those patients who did not show any clinical signs of vaginal infection, probably because *C. albicans* exists as commensal in some of the individuals.

DISCUSSION

The specificity of IPC5f/IPC2r and IPC3f/IPC4r primers were tested with *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *S. cerevisiae* 699 and

S. cerevisiae 700. The results showed the primers were very specific for *C. albicans* as no cross-species amplification was observed. The sensitivity test performed showed all ten fold serial dilutions gave an expected band of 200 bp. The ultra-sensitivity of the method was proven by its detection limit of <1 CFU/mL. As we had used 35 cycles of amplification for both the first and second round of the nested PCR, the amplification may have reached the plateau phase. This sensitivity is comparable with PCR-ELISA method.

Among the samples that were culture-positive, germ-tube negative but nested PCR (IPC1) positive, two were *C. albicans* (one existed at the same time with *C. glabrata*) but three were non-*albicans Candida* species. There is a possibility that it is a false-positive result from the nested PCR (IPC1) due to cross-contamination from true-positive samples, or the samples could have had *C. albicans* presented together with non-*albicans Candida* species but *the C. albicans* colonies were undeliberately not picked from the mixed culture when doing germ tube test. As there is no gold standard method for identification of *C. albicans*, we had arbitrarily assigned a sample as *C. albicans*-positive if it fulfilled two of the tests as outlined in the methods. We surmised that the sensitivity and specificity of the IPC1 nested PCR would be even higher than what we currently report if the true *C. albicans* status of each sample were known.

For the 26 culture-negative but nested PCR (IPC1) positive samples, nested PCR using the *C. albicans*-specific P_{450} primers[15] verified the *C. albicans* status. This clearly indicated that the IPC1 nested PCR method is more sensitive and specific than the conventional morphology-based identification method.

The prevalence of *Candida* species showed *C. albicans* was the predominant species in HVS. This is in concordance with the results reported universally. The nested PCR (IPC1) study showed 67.7% of the subjects presented with *C. albicans*. Nonetheless, whether the *C. albicans* acts as a commensal or a pathogen in these women cannot be ascertained since high sensitivity of the nested PCR method is able to detect minute quantities of *C. albicans* DNA even in asymptomatic women.

CONCLUSION

In conclusion, we have successfully developed a PCR assay to simultaneously detect and identify the most predominant species of *Candida* directly by species-specific amplification of the IPC1 gene. To our knowledge, we reported here for the first time the ability to *detect C. albicans* DNA directly from HVS without the need to culture, which means mixed organisms in the samples does not affect the reliability and efficiency of the PCR method. The ability to distinguish *C. albicans* from other *Candida* species is potentially useful because *C. krusei* and *C. glabrata* are emerging species that are innately less susceptible to azole drugs and were implicated as the causal agents for recurrent vaginitis. For future work, it is suggested that primers specific for other *Candida* species are designed from the IPC1 gene and applied using the multiplex PCR method for rapid and simultaneous identification of various *Candida* species.

ACKNOWLEDGEMENT

This study was partially funded by the Ministry of Science, Technology and Innovation, Malaysian government through an IRPA/BIOTEK grant scheme. We would like to acknowledge the guidance and facilities offered by Prof. Heng Fong Seow in this study.

REFERENCES

1. Polanco, A., O.E. Mellad, C. Castilla and J.L. Rodriguez-Tudela, 1999. Detection of *Candida albicans* in blood by PCR in a rabbit animal model of disseminated candidiasis. Diagn. Microbiol. Infect. Dis., 34: 177-183.
2. Greenspan, D., 1994. Treatment of oral candidiasis in HIV-positive patients. J. Am. Acad. Dermatol., 31: S51-S55.
3. Nishikawa, A., T. Sugita and T. Shinoda, 1999. Rapid identification of *Debaryomyces hansenii/ Candida famata* by polymerase chain reaction. Med. Mycol., 37: 101-104.
4. Pittet, D., L. Li, R.F. Woolson and R.P. Wenzel, 1997. Microbiological factors influencing the outcome of nosocomial bloodstream infections: A 6-year validated, population-based model. Clin. Infect. Dis.,24: 1068-1078.
5. O'Neil, P., T. Dornan and D.W. Denning, 1997. Medicine. Churchill Livingstone, New york.
6. Holmes, K.K. and H.H. Hansfield, 1998. Sexually transmitted diseases: Overview and clinical approach. In: Harrison's Principles of Internal Medcine (14th Edn.). Fauci, A.S., E. Braunwald, K.J. Isselbacher, J.D. Wilson, J.B. Martin, D.L. Kasper, S.L. Hauser and D.L. Longo, (Eds.). McGraw-Hill, London.
7. Berenguer, J., M. Buck, F. Witesbsky, F. Stock, A. Pizzo and T.J. Walsh, 1993. Lysis centrifugation blood cultures in the detection of tissue-proven invasive candidiasis: Disseminated versus single-organ infection. Diag. Microbiol. Infect. Dis., 17: 103-109.

8. Fujita, S., Y. Senda, S. Nakaguchi and T. Hashimoto, 2001. Multiplex PCR using Internal Transcribed Spacer 1 and 2 regions for rapid detection of yeast strains. J. Clin. Microbiol., 39: 3617-3622.
9. Irobi, J., A. Schoofs and H. Goossens, 1999. Genetic identification of *Candida* species in HIV Positive patients using polymerase chain reaction and restriction fragment length polymorphism analysis of its DNA. Molecular and Cellular Probes, 14: 401-406.
10. Loeffler, J., H. Hebart, S. Magga, D. Schmidt, L. Klingspor, J. Toller, U. Schumacher and H. Einsele, 2000. Identification of rare *Candida* species and other yeasts by polymerase chain reaction and slot blot hybridization. J. Clin. Microbiol., 38: 207-212.
11. Maaroufi, Y., C. Heymans, J.D. Bruyne, V. Duchateau H.M. Rodriguez-Villalobos and F. Crokaert, 2003. Rapid detection of *Candida albicans* in clinical blood samples by using a TaqMan-based PCR assay. J. Clin. Microbiol ., 41: 3993-3998.
12. Berardinelli, S. and O.J. Dennis, 1985. New germ tube induction medium for the identification of *Candida albicans*. J. Clin. Microbiol., 22: 861-862.
13. Evans, E.G.V. and M.D. Richardson, 1989. Medical Mycology -a practical approach: Identification of Yeasts; Buckley H.R. 97-109. IRL Press, England.
14. Jekel, J.F., J.G. Elmore and D.L. Katz, 1996. Epidemiology, Biostatistics and Preventive Medicine. W.B. Saunders Company, London.
15. Fujita, S.I., A.L. Brent, J. Timothy, Errol Reiss and J.M. Christine, 1995. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species in blood. J. Clin. Microbiol., 33: 962-967.
16. Chryssanthou, E., L. Klingspor, J. Tollemar, B. Petrini, L. Larsson, B. Christensson and O. Ringden, 1999. PCR and other non-culture methods for diagnosis of invasive *Candida* infections in allogeneic bone marrow and solid organ transplant recipients. Mycoses, 42: 239-247.