DOI: https://doi.org/10.53350/pjmhs20221612647

ORIGINAL ARTICLE

Prevalence and Virulence Factors of Vaginal Candidiasis Among Females Collected in Tertiary Care Hospital, Khyber Pakhtunkhwa

SADIR ZAMAN[1], NAILA KHAN[2], NAZISH BABAR[3], AROOSA ZAMAN[4], MUHAMMAD NUGHMAN[5], AYESHA GUL[6]
[1]*Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan*
[2]*Women Medical Officer, Women and Children Hospital, Rajjar, Charsadda, Pakistan.*
[3]*Associate Professor of Microbiology and Head of Pathology Department, Gajju Khan Medical College, Swabi, Pakistan.*
[4]*Department of Microbiology, The University of Haripur, Haripur, Pakistan.*
[5]*Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, Pakistan.*
[6]*Department of Microbiology,Kohat University of science and Technology, Kohat, Pakistan*
Corresponding author: Ayesha Gul, Email: gulayesha743@yahoo.com

ABSTRACT

Background: The genus Candida includes about 200 different species, but only a few species are human opportunistic pathogens. Candida albicans is a commensal and opportunistic pathogenic agent that causes infection in immune compromised individuals. This work was conducted to study the detection of virulence factors of Candida spp. in clinical female samples from tertiary care hospitals of Kohat.

Methods: This cross-sectional study was conducted during April to July 2017. Patients data and samples were collected from Liaqat Memorial Hospital (LMH), Fauji Foundation Hospital, and Family Health Hospital, Kohat. Morphological identification was done by using different media i.e SDA and PDA. Further identification was done through gram staining and germ tube method.

Results: A total of one hundred and sixty (n=160) clinical samples were collected from outdoor patients, in which only 50 were Candida positive. Among all the 50 isolates of 13(26%) Candida spp. were germ tube positive. Further identification were done through Chrome Agar in which 17(34%) isolates were C. albicans, followed by 19(38%) were C. tropicalis, and 4(8%) isolates were C. glabrata. Detection of virulence factors is done by different methods in which 30(60%) isolates showed phospholipase activity in which 12(24%) of Candida spp. were large positive. In haemolytic activity showed 28(56%) strong haemolytic activity, 5(10%) were weak positive. Candida positive patients high 36 (72%) in 21 to 40 years.

Conclusion: In the present study, the results concluded that patient infected with Candida are highly virulent as about more than 60% of Phospholipase, Biofilm and Haemolytic positive. Candida species have high prevalence rate among 21 t0 40 years from the rest of very late and early age. Finding novel antibacterial chemicals is highly advised in view of the study's findings. Additionally, it is crucial to assess the candida resistant patterns at the genomic and proteomic levels in order to identify the genes causing the patterns of antibiotic resistance.

Keyword: Candida, Virulence Factors, Germ Tube, Chrome Agar, opportunistic pathogens, Haemolytic positive, Gram staining, Germ tube method.

INTRODUCTION

Fungi are eukaryotic microorganisms. Most fungal species, like moulds, have multicellular filaments called hyphae that form mycelium. Other fungi species, like yeasts, grow as single cells[1]. Candida species are one of the most important yeast, are normal inhabitants of the skin and mucosa (oral cavity, digestive tract, vagina)[2]. But Candida can become pathogenic in immune compromised person because of its dimorphic form. The dimorphic fungus Candida spp. can respond rapidly to environmental changes, and this flexibility could allow this organism to take advantage of impaired immunity and facilitate establishment of disease[3]. A distinctive characteristic of C. albicans is its ability to grow with three distinct morphologies – yeast, pseudo hyphae, and true hyphae, in tissues it may appear as yeasts or pseudo hyphae. Pseudo hyphae are elongated yeasts that visually resemble hyphae but are not true hyphae[4]. Over the last three decades, Candida spp. has emerged as an important cause of health care associated and opportunistic infection[5]. Around 17 different species of Candida are reported as disease causing agents[6]. The National Nosocomial Infections Surveillance System has reported Candida spp. are the fourth most common bloodstream isolates in nosocomial infections in USA. Over 95% of all fungal infections have been associated with C. albicans, A. fumigates and C. neoformans[7]. The most typical fungi infections in AIDS patients are also candida infections. Among these patients, oropharyngeal candidiasis predominates, which can cause malnutrition and hinder the absorption of medication[8]. The pathogens C. tropicalis, C. glabrata, Candida albicans, and C. parapsilosis are the most dominant[9]. C. albicans is the frequently yeast responsible for vaginal yeast infections[10]. Several virulence factors mainly contribute in the pathogenicity of Candida species which include evasion from host immune system, attachment, synthesis and secretion of tissue destroying hydrolytic enzymes such as proteases, phospholipases and haemolysin and finally biofilm formation[11]. Different risk factors make person more vulnerable for the Candida infections such as pregnancy, high estrogen levels in body, diabetic patient (commonly women), contraceptive containing a high estrogen dose and antibiotic drug treatment which suppress normal flora of vagina[12]. Recurrent vulvovaginal candidiasis (RVVC) is a disorder that affects roughly 5-8% of reproductive-age females, who experience four or more occurrences of clinical Candida infection year[13]. Drug-resistant biofilms is one of the significant attribute of Candida species for causing disease in humans. Among the different types of microbial biofilms[14], sessile cells within C. albicans biofilms are slighter vulnerable to antimicrobial substances than are planktonic cells[15]. It has been reported that the development of drug resistance within the Candida biofilms advances along with the maturation process[5]. So the growing number of drug resistant strains are demanding new antifungal targets to be focused[16]. This work was conducted to study the detection of virulence factors of Candida spp. in clinical samples from tertiary care hospitals of Kohat

METHODOLOGY

The research was carried out in the laboratory of the department of microbiology at Kohat University of Science and Technology in Kohat, Pakistan. The study was conducted for four months (April to July 2017). During this period, 160 samples of vaginal swabs were collected from tertiary care hospital of Kohat to isolate and identify the different Candida spp. causes Candidiasis. The collections of all the specimens were handled according to standard protocols.

Vaginal samples were collected and transported aseptically using swabs containing Amies medium (mwe, medical wire, UK). On SDA plates all the HVS samples were cultured and placed on both 25^{o}C for 24-48 hours[17]. Candida spp. were distinguished through their morphological characteristics by culturing them on SDA and differential medium i.e. Chrome agar. Further identification was done through Gram staining[18] and Germ tube test[19].

Biofilm production was checked by tube method. Cultures of Candida spp. were inoculated in sterile saline and incubated at 37ºC for 24 hours. In sterile test tubes containing 5ml SD broth, 0.5ml saline suspension was added and incubated at 37°C for 48 hours. After incubation, broth was discarded. Tubes were washed and then stained with 1% safranin for 10 minutes. Tubes were again washed and air dried. Biofilm production was scored as negative (-), weak (+), moderate (++), or strong (+++) positive based on the protocol given[20].

Sterile egg yolk medium was prepared by adding 6.2gm SDA, 0.1gm CaCl2 and 5.8gm NaCl in 100ml distilled water. Sterile egg yolk was centrifuged at 4000rpm for 30 minutes. Then 2ml supernatant of egg yolk was added in cooled media, mixed it well and then poured into sterile plates. A small drop (10µl) of culture suspension was poured on the plate containing medium with the help of pipette for 4-5 days at 37°C. The Phospholytic activity was calculated by measuring clear zone[21]. For hemolytic assay, sterile SDA medium with 3% glucose was prepared when temperature of medium came down to 45°C add 7% sheep blood. Sterile culture suspension was prepared and a small drop (10µl) of it was poured on the medium with the help of pipette and then incubated at 37°C for 48 hours in CO2 incubator. The Hemolytic activity was calculated by measuring clear zone[22,23].

All data were analyzed through statistical package for social sciences cersion 22 (SPSS).

RESULTS

A total 160 samples were collected from different patients visiting tertiary care hospital, Kohat from April 2017 to July 2017. After identification of Candida spp. on the basis of germ test tube, culture, microscopy and differential media i.e. Chrome agar 50 (31%) isolates were positive for Candida species. All positive samples produced whitish, glossy and smooth colonies on SDA which is the characteristic of Candida species (Fig-1).

Fig-1: Colonial morphology and growth of Candida species on SDA media

- Under microscopic examination with gram staining round to oval, purple coloured budding yeasts were observed in all positive samples (Fig-2) which were further identified by germ tube (GT) test. Among all 50 isolates, 13(26%) species produces germ tube and were positive for germ tube while 37(74%) were negative germ tube.

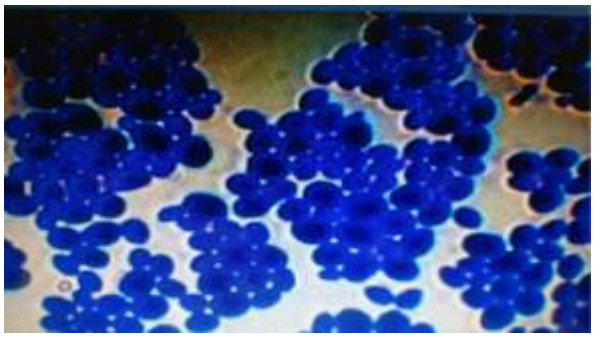


Fig-2: Gram staining of Candida spp.

For differentiation of isolates, the germ tube test was performed in which 26 (n-13) of the 50 Candida spp. isolates were found positive while 74% (n=37) were found negative. Differential media, such as Chrome agar, proved that C. albicans isolates with positive germ tube tests generated hyphae while their negative germ tube tests did not.

Table 1: Results of Candida positive germ tube test samples for all 50 clinically isolated

Samples	No of isolates	Percentage
Positive	37	74 %
Negative	13	26 %

It was confirmed by differential media i.e. Chrome agar that all the germ tubes test positive were C. albicans which produced hyphae while other species were found negative by germ tube test. After incubation of all Candida positive samples for 24-48 hours at 37°C, different colour of colonial growth were notified on Chrome agar. Purple colour shows C. krusei, blue colour were C. tropicalis, light green colour shows C. albicans, and pink colour indicates C. glabrata. Among positive samples, 38% (n=19) were C. tropicalis, followed by C .albicans 17(34%), C. glabrata i.e. 4(8%) and C. krusei i.e. 10(20%) (Tab-2, Fig-3).

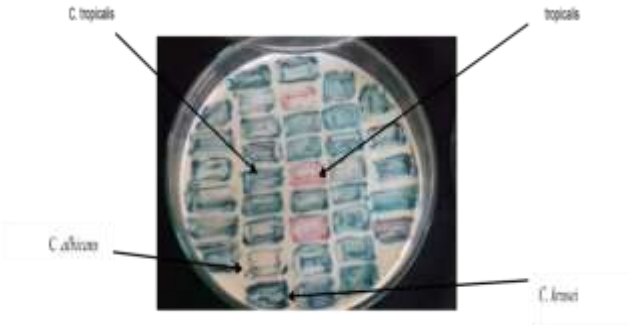


Fig-3: Identification of Candida spp. show colour on chrome agar.

Table 2: Results of Candida positive germ tube test samples for all 50 clinically isolated

Candida species	No. of isolates	Percentage%
C. tropicalis	19	38%
C .albicans	17	34%
C. glabrata	4	8%
C. krusei	10	20%

Among all 50 isolates of Candida spp. 34(68%) were biofilm producer in which 2(4%) isolates were strong positive, 6(12%) moderate positive, 26(52%) isolates were weak positive and 16(32%) isolates of Candida spp. were negative (, Fig-4, Tab-3).

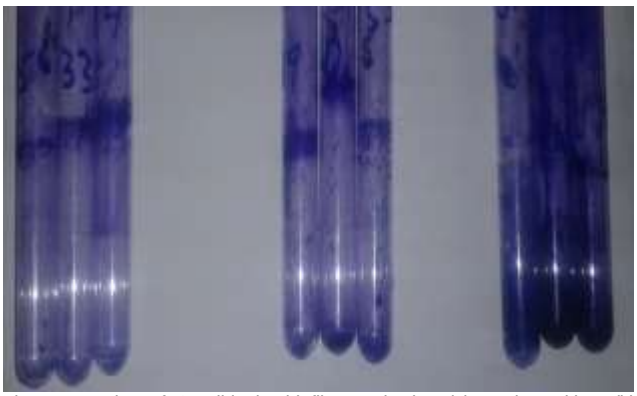


Fig-4: Detection of Candida by biofilm production (a) weak positive, (b) moderate positive (c) strong positive.

Table- 3: Results of Biofilm activity for all 50 clinically isolated Candida positive samples

Samples	Numbers	Percentage
Strong positive	28	56 %
Moderate positive	5	10 %
Weak positive	1	2 %
Negative	16	32 %

All 50 isolates of Candida spp. tested produced 34(66%) produced hemolysin, in which 28(56%) strong hemolytic activity, 5(10%) moderate positive, 1(2%) weak positive, 16(32%) were negative isolates of Candida.

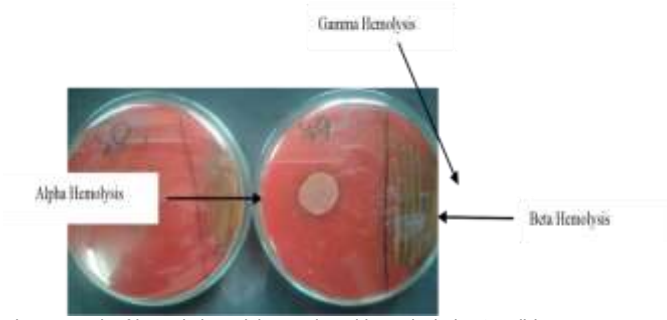
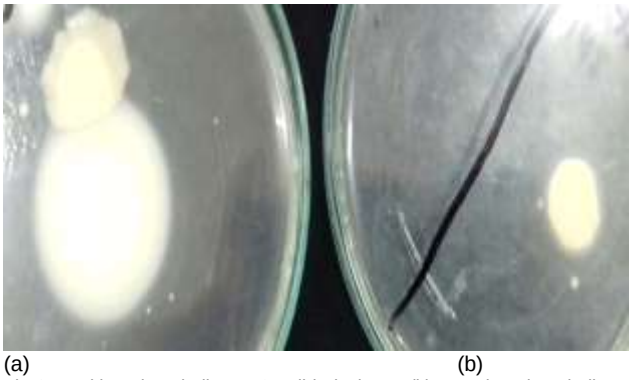


Fig-5: Result of hemolytic activity produced hemolysin by Candida spp.

Table- 4: Results of Hemolytic activity for all 50 clinically isolated Candida positive samples

Samples	Numbers	Percentage
Strong positive	28	56 %
Moderate positive	5	10 %
Weak positive	1	2 %
Negative	16	32 %

Phospholytic activity: All 50 isolates of Candida spp. were screened for phospholypase activity. Among these 30(60%) isolates showed phospholipase activity, in which 12(24%) isolates of Candida were strong positive, 7(14%) isolates were weak positive, 6(12%) isolates were weak positive, 4(8%) isolates of Candida spp. were moderate positive while 21(42%) were negative.



(a) (b)

Fig-6: Positive phospholipase Candida isolates, (b) negative phospholipase Candida isolates

Table-5: Results and percentage of phospholipase activity

Samples	Numbers	Percentage
Strong positive	12	24
Moderate positive	4	8
Weak positive	7	14
Negative	21	42

Further analysis of demographic data through questionnaire collected from patients revealed that Candida positive patients high 36 (72%) in 21 to 40 years, 11 (22%) in 21-40 years while lowest 3(6%) in 1 to 20 years.

Table-4: Demographic analysis age distribution of Candida positive patient

Group	Age (In years)	No. of samples	Percentage of Affected females
1	1-20	3	6%
2	21-40	36	72%
3	41-60	11	22%

DISCUSSION

All samples were cultured on SDA medium, colonies were observed after of incubation of 37°C for 24-48 hours. Smooth, creamy, pasty convex colonies of Candida spp. were formed, and they smelled unpleasant. Growth of Candida spp. on SDA medium, which encourages the growth of Candida, was utilised for the main isolation of Candida in the same investigation. Every HVS sample was inoculated into the sabouraud dextrose agar that Oxoid provided. However, SDA is not a differential medium, making it difficult to distinguish between various Candida spp. colonies produced on this agar[24]. With the help of gram staining that proved Candida species showed budding and oval yeast, having gram positive pseudo-hyphae under 100X of lense. This result is confirmed by[25]. The HVS was also examined by the gram stain. A smear was fixed on slide using 70% ethanol and then stained with: crystal violet that proved Candida species showed budding and oval yeast, having gram positive pseudo-hyphae under 100X of lense.

Germ tube positive samples were performed in which 13 were positive and 37 were negative. Germ tube result showed chlamydospores which indicate the positivity of Candida albicans while chlamydospores were absent in Germ tube negative \Candida species and same work was carried out by[26] and they also showed that GT give rapid result but they are unable to give further identification of different Candida species.

After the germ tube, further identification was carried out using Chrome Agar, one of Candida's differential media. All Candida positive samples were incubated at 37°C for 24-48 hours, and the colour of the colonial growth notified on chrome Agar. Light green colour shows C. albicans, blue colour were C. tropicalus, pink colour indicates C. glabrata and purple colour shows C. krusei, 17(34%) isolates were C. albicans, followed by 19(38%) were C. tropicalis, 4(8%) isolates were C. glabrata while 10(20%) isolates were C. krusei species. Same work was conducted by culture of Candida having mix culture of different species[27], were identified by using the selective media, HiChrom agar which shows different color colony for different species.

Among all 50 isolates of Candida spp. 34(68%) were biofilm producer in which 2(4%) isolates were strong positive, 6(12%) moderate positive, 26(52%) isolates were weak positive and 16(32%) isolates of Candida spp. were negative. Same work was conducted by[28].

All 50 isolates of Candida spp. were screened for phospholypase activity. Among these 30(60%) isolates showed phospholypase activity in which 12(24%) isolates of Candida spp. were large positive, 7(14%) isolates were weak positive, 6(12%) isolates were poor positive, 4(8%) isolates of Candida spp. were moderate positive while 21(42%) isolates of Candida spp. were negative same work as our study was done by[29].

All 50 isolates of Candida spp. tested produced 33(66%) hemolysin, in which 28(56%) strong hemolytic activity, 5(10%) weak positive, 1(2%) poor positive, 16(32%) were negative isolates of Candida and the similar work conducted by[30]. Further analysis of demographic data through questionnaire collected from patients revealed that Candida positive patients high prevalence 36 (72%) in 21 to 40 years while lowest 3(6%) in 1 to 20 years.

CONCLUSION

From the above current study it was concluded that all the C.albicans are germ tube positive and other species are germ tube

negative. Among all other isolated Candida spp. the major isolate is the C.tropicalus (38%) while the least C.glabarata (8%). For the identification of Candida spp. it was also concluded that chrome agar is the reliable method. In the present study the results concluded that patient infected with Candida are highly virulent as about more than 60% of Phospholipase, Biofilm and Haemolytic positive. After demographic data the current study also concluded that Candida species have high prevalence rate among 21 to 40(72%) years from the rest of very late and early age.

REFERENCES

1. Khan MSA, Ahmad I, Aqil F, Owais M, Shahid M, Musarrat J. Virulence and pathogenicity of fungal pathogens with special reference to Candida albicans. Combating fungal infections: Springer; 2010. p. 21-45.
2. Mujica M, Finquelievich J, Jewtuchowicz V, Iovannitti C. Prevalencia de Candida albicans y Candida no albicans en diferentes muestras clínicas: Período 1999-2001. Revista Argentina de microbiologia. 2004;36(3):107-12.
3. Perfect JR. Fungal virulence genes as targets for antifungal chemotherapy. Antimicrobial Agents and Chemotherapy. 1996;40(7):1577-83.
4. Coleman DC, Sullivan DJ, Bennett DE, Moran GP, Barry HJ, Shanley DB. Candidiasis: the emergence of a novel species: Candida dubliniensis. Aids. 1997;11(5):557-67.
5. Sardi J, Scorzoni L, Bernardi T, Fusco-Almeida A, Giannini MM. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. Journal of medical microbiology. 2013;62(1):10-24.
6. Papon N, Courdavault V, Clastre M, Bennett RJ. Emerging and emerged pathogenic Candida species: beyond the Candida albicans paradigm. PLoS pathogens. 2013;9(9):e1003550.
7. Richardson MD. Changing patterns and trends in systemic fungal infections. Journal of Antimicrobial Chemotherapy. 2005;56(suppl_1):i5-i11.
8. Kim D, Shin WS, Lee KH, Kim K, Young Park J, Koh CM. Rapid differentiation of Candida albicans from other Candida species using its unique germ tube formation at 39 C. Yeast. 2002;19(11):957-62.
9. Calderone RA, Fonzi WA. Virulence factors of Candida albicans. Trends in microbiology. 2001;9(7):327-35.
10. Strus M, Kucharska A, Kukla G, Brzychczy-Włoch M, Maresz K, Heczko PB. The in vitro activity of vaginal Lactobacillus with probiotic properties against Candida. Infectious diseases in obstetrics and gynecology. 2005;13(2):69-75.
11. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. Candida glabrata, Candida parapsilosis and Candida tropicalis: biology, epidemiology, pathogenicity and antifungal resistance. FEMS microbiology reviews. 2012;36(2):288-305.
12. Diba K, Rhaimirad M, Makhdoomi K, Khorshidvand Z. Identification of Candida species isolated from hospital acquired infections cases and hospital indoor environments. Afr J Microbiol Res. 2012;6(2):4164-8.
13. Williams DW, Lewis MAO. Oral Microbiology: Isolation and identification of candida from the oral cavity. Oral diseases. 2000;6(1):3-11.
14. Rajendran R, Robertson DP, Hodge PJ, Lappin DF, Ramage G. Hydrolytic enzyme production is associated with Candida albicans biofilm formation from patients with type 1 diabetes. Mycopathologia. 2010;170(4):229-35.
15. Kuhn DM, Ghannoum MA. Candida biofilms: antifungal resistance and emerging therapeutic options. Current opinion in investigational drugs (London, England: 2000). 2004;5(2):186-97.
16. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clinical microbiology reviews. 1998;11(2):382-402.
17. Hasan F, Xess I, Wang X, Jain N, Fries BC. Biofilm formation in clinical Candida isolates and its association with virulence. Microbes and infection. 2009;11(8-9):753-61.
18. Christopher K, Bruno E, editors. Identification of bacterial species. Proceedings of the 24th; 2003.
19. Lakshmana Kumar V. Characterisation and Speciation of Fungal Isolates in Neonatal Sepsis in a Tertiary Care Hospital: Madurai Medical College, Madurai; 2015.
20. Singhai M, Malik A, Shahid M, Malik A, Rawat V. Colonization of peripheral intravascular catheters with biofilm producing microbes: Evaluation of risk factors. Nigerian Medical Journal: Journal of the Nigeria Medical Association. 2012;53(1):37.
21. Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in Candida albicans. Sabouraudia: Journal of Medical and Veterinary Mycology. 1982;20(1):7-14.
22. Yigit N, Aktas E, Dagistan S, Ayyildiz A. Investigating biofilm production, coagulase and hemolytic activity in Candida species isolated from denture stomatitis patients. The Eurasian Journal of Medicine. 2011;43(1):27.
23. Neji S, Hadrich I, Trabelsi H, Abbes S, Cheikhrouhou F, Sellami H, et al. Virulence factors, antifungal susceptibility and molecular mechanisms of azole resistance among Candida parapsilosis complex isolates recovered from clinical specimens. Journal of biomedical science. 2017;24(1):1-16.
24. Kangogo M, Wanyoike M, Revathi G, Bii C. Phenotypic characterization of Candida albicans from clinical sources in Nairobi, Kenya. African Journal of Health Sciences. 2011;19(3-4):19-23.
25. Harrington A, McCourtney K, Nowowiejski D, Limaye A. Differentiation of Candida albicans from non-albicans yeast directly from blood cultures by Gram stain morphology. European Journal of Clinical Microbiology & Infectious Diseases. 2007;26(5):325-9.
26. Saigal S, Bhargava A, Mehra S, Dakwala F. Identification of Candida albicans by using different culture medias and its association in potentially malignant and malignant lesions. Contemporary clinical dentistry. 2011;2(3):188.
27. Baradkar V, Mathur M, Kumar S. Hichrom candida agar for identification of Candida species. Indian Journal of Pathology and Microbiology. 2010;53(1):93.
28. Dhanasekaran D, Vinothini K, Latha S, Thajuddin N, Panneerselvam A. Human dental biofilm: Screening, characterization, in vitro biofilm formation and antifungal resistance of Candida spp. The Saudi Journal for Dental Research. 2014;5(1):55-70.
29. de Souza Ramos L, Barbedo LS, Braga-Silva LA, Dos Santos ALS, Pinto MR, da Graça Sgarbi DB. Protease and phospholipase activities of Candida spp. isolated from cutaneous candidiasis. Revista Iberoamericana de Micologia. 2015;32(2):122-5.
30. de Melo Riceto ÉB, de Paula Menezes R, Penatti MPA, dos Santos Pedroso R. Enzymatic and hemolytic activity in different Candida species. Revista iberoamericana de micologia. 2015;32(2):79-82.