

“Determination of potential virulence factors in clinical isolates of various *Candida* species to assess its clinical implication in a tertiary care hospital in Eastern India”.

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Abstract: Candidiasis is the commonest fungal disease in human caused by various species of the yeast like fungi belonging to the genus *Candida*. It is commonly encountered as an endogenous infection due to its commensal nature. Transition from commensal to a pathogen is attributable to complex array of different virulence factors which directly interacts and damages mammalian host cells.

Aim: The present study was intended to determine different virulence factors expressed by clinical isolates of *Candida* species and to consider its clinical implication which will help in providing rational and justified therapy to the patients.

Methods and Material: 240 clinical isolates of *Candida* species obtained from various clinical specimens like nail (86), blood (44), deep tracheal aspirate (DTA) & bronchoalveolar lavage (BAL) (28), oral swabs & buccal washings (36), urine (30), corneal ulcer (10) and ear discharge (06) were included in the study and processed for speciation following standard protocol. The virulence factors detected were phospholipase activity in egg yolk agar, haemolysin production in Sabouraud's Dextrose Agar (SDA) supplemented with blood, esterase activity in Tween 80 media, adherence assay and production of biofilm.

Results: Among 240 *Candida* isolates, 80 were *C.albicans*, 60 *C.tropicalis*, 44 *C.parapsilosis*, 24 *C.lusitanae*, 16 *C.glabrata*, 12 *C.krusei*, and 04(four) *C.stellatoidea*. 118 (49.1%) isolates showed haemolysin production, 106 (44.1%) expressed phospholipase, 76 (31.6%) expressed esterase, 94 (39.1%) strains gave positive result in adherence assay and 116 (48.3%) *Candida* isolates were found to be biofilm producers.

Conclusions: The above study emphasizes on detecting virulence traits of *Candida* isolates obtained from clinical samples as contaminant commensal *Candida* strains are not infrequent isolation from clinical samples.

Key-words: *Candida*, virulence, phospholipase, esterase, haemolysin, adherence, biofilm.

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I. Introduction:

Candidiasis is the commonest fungal disease in humans caused by various species of the yeast like fungi belonging to the genus *Candida*^[1,2]. *Candida* colonizes several anatomically distinct body sites including skin, oral cavity, gastrointestinal tract, vaginal mucosa^[1,2]. It is commonly encountered as an endogenous infection due to its commensal nature. The clinical spectrum of candidiasis ranges from mucocutaneous overgrowth to disseminated infection like candidemia^[3]. Over the last three decades, *Candida* species has emerged as an important cause of healthcare associated and opportunistic infection^[4]. The increased use of intravenous catheters, total parenteral nutrition, broad spectrum antibiotic, cytotoxic chemotherapy and an increase in the population of immunocompromised patients have contributed to the increase of these infections^[5]. Transition from commensal to a pathogen is attributable to complex array of different virulence factors which directly interacts and damages mammalian host cells^[1]. Hence expression of these virulence factors contributes to the pathogenesis of candidiasis. Different virulence traits of *Candida* species are, germ tube formation, its ability to adhere to host cells as an initial step, yeast hyphal morphogenetic transformation facilitating penetration, phenotypic switching, immunomodulatory effects of fungal determinants and specific hydrolytic enzyme production^[6,7]. One of the major factors contributing to the virulence of *Candida* is its versatility. It adapts to a variety of different habitats for growth and can form surface-attached microbial communities known as biofilm. Biofilm formation is thought to be one of *Candida*'s most important growth adaptations because biofilms allow it the possibility to colonize oral surfaces^[8]. *Candida* species liberates several extracellular enzymes like phosphatase, coagulase, protease, phospholipase, lipase, esterase etc, which helps to evade the

tissue^[1]. Furthermore *Candida* also procures iron with the help of haemolysin production to establish infection within humans^[8]. Among *Candida* species expression of virulence factors may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection and host reaction. Knowledge of these virulence factors will be an important tool to understand pathogenesis of candidiasis and in addition will help explore new antifungal drug targets for improved therapeutic regimens^[9]. The present study was intended to identify different species of *Candida* isolated from clinical specimens and to examine their potential virulence determinants in vitro which might help in clinical correlation to administer rational and justified therapy.

II. Material and Methods:

The study was carried out in the Mycology section of the department of Microbiology at a tertiary care teaching Hospital in Kolkata. 240 *Candida* spp. were isolated from various clinical specimens following standard protocol. Among all the isolates 86 were obtained from nail samples, 44 from blood, 28 from deep tracheal aspirate (DTA) and bronchoalveolar lavage (BAL) samples, 36 from oral swabs or buccal washings of immunocompromised patients, 30 from urine samples, 10 from corneal ulcer and six (06) from ear discharge samples. ATCC *Candida parapsilosis* 90661 and ATCC *Candida albicans* 40028 were taken as controls. Speciation was done depending on the results of germ tube test¹, Colony morphology on HiCHROM *Candida* differential agar (HiMedia Laboratories, Mumbai, India), microscopic morphology on Dalmau plate culture on corn meal agar^[1] (HiMedia Laboratories, Mumbai), and also sugar fermentation tests by using HiCandida identification kit (HiMedia Laboratories, Mumbai, India).

Preparation of yeast suspension: Yeast suspension was prepared to evaluate different virulence factors. Small amount of a colony of a 24 – 48 hour culture of the organism was suspended in sterile Phosphate buffer solution (PBS; pH 7.2). The turbidity was matched to 0.5 McFarland standards^[10].

Examine for Phospholipase activity: For determination of phospholipase activity Egg yolk agar media was used^[10,11]. 13.0 g Sabouraud's Dextrose agar (SDA), 11.7 g NaCl, 0.11 g Calcium Chloride (CaCl₂) were dissolved in 184 ml of distilled water and sterilized by standard autoclave. Egg yolks were mixed and centrifuged at 500 g for 10 minutes at room temperature. 20 ml of the supernatant was added to the sterilized media for final preparation^[11]. 10 µl of yeast suspension was inoculated over the agar surface. The plates were incubated at 37°C for 5 days. Presence of enzyme activity was assessed by formation of precipitation zone around the yeast colonies^[10,11] (Fig. 1)

Examination for Haemolytic Activity: SDA containing 7% blood and 3% glucose was used to determine haemolytic activity. The pH of the media was adjusted to 5.6 ± 0.2. 10 µl of yeast suspension was inoculated over the agar surface and the media was incubated for 2 days at 37°C. Presence of haemolytic activity was assessed by the formation of a transparent /semitransparent zone around the inoculation site^[6] (Fig. 2)

Examination for Esterase activity: Tween 80 opacity test media was used for detection of esterase activity. 1% peptone, 0.5% Sodium Chloride (NaCl), 0.01% CaCl₂ and 1.5% agar were dissolved in distilled water and sterilized by standard autoclave. 0.5% Tween 80 was added to the media after cooling it to 50°C. The final pH was adjusted to 6.8. 10 µl of yeast suspension was inoculated and media was incubated at 37°C for 10 days. Esterase activity was assessed by presence of a halo pervious to light around the yeast colonies^[10](Fig 5).

Adherence assay: The adherence assay described by Kimura et al^[12] was used with minor modification in the present study. Buccal epithelial cells (BEC) from healthy donor were collected by the help of sterile cover slips and washed thrice and suspended in sterile Phosphate Buffered Solution (PBS, pH 7.2). 0.5 McFarland standard matched suspension of BEC and yeast suspension were mixed and incubated at 37°C for 45 minutes with gentle shaking. The suspension was centrifuged at 3000 rpm for 5 minutes and the sediment was repeatedly washed in PBS. A smear was prepared from the sediment and stained by Gram's Method. Adhered yeast cells over the epithelial cells were examined under microscope^[12,13] (Fig. 3,4).

Determination of biofilm production: Biofilm production was determined by visual method. Colonies from surface of SDA plate were inoculated into a polystyrene tube (Falcon conical tube with screw cap) containing 10 ml of Sabouraud-dextrose broth (SDB) supplemented with glucose (Final concentration 8%). After incubation at 35°C for 48 hours, the broth in the tubes were gently aspirated. The tubes were washed with distilled water twice and then stained with 2% safranin for 10 mins. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (-), weak (+), or strong (++) . The biofilm producer *Staphylococcus epidermidis* ATCC 35984 was used as a positive control^[14,15] (Fig 6)

III. Results:

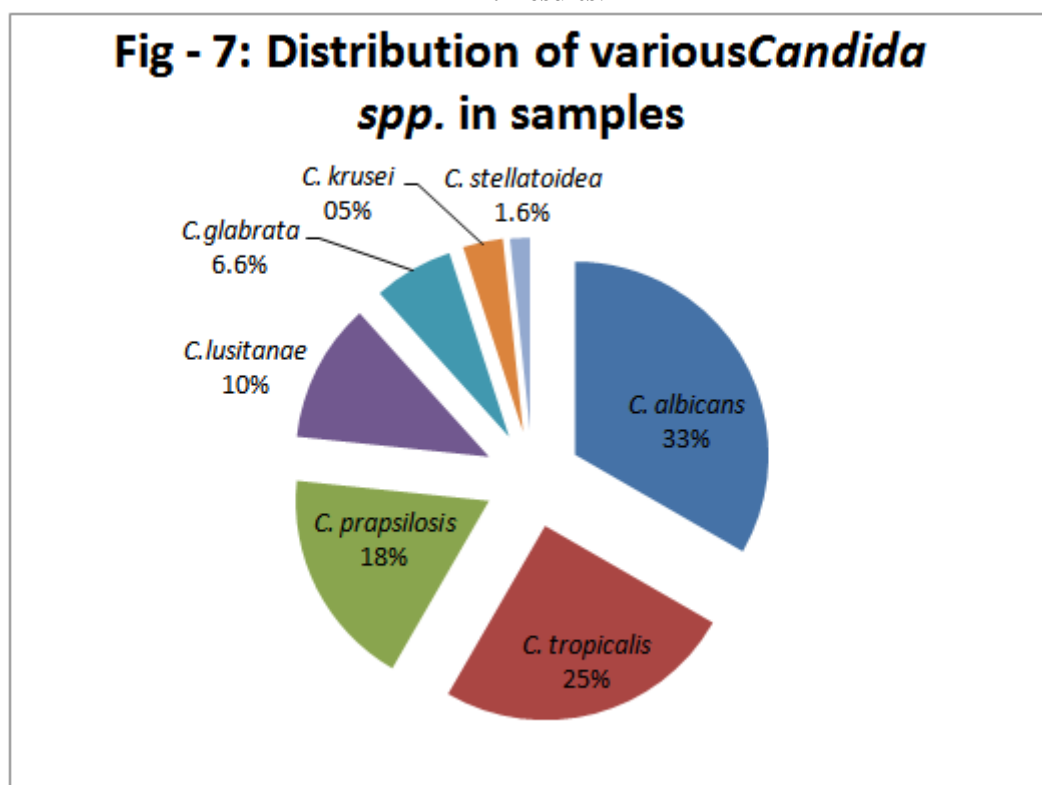


Table 1: Distribution of different virulence traits among the isolates (n=240) and controls (n=02):

Virulence determinants	Phospholipase No. (%)	Haemolysin No. (%)	Esterase No. (%)	Adherence No. (%)
Isolates (n= 240)	106 (44.1)	118(49.1)	76(31.6)	94(39.1)
Controls (n = 2)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

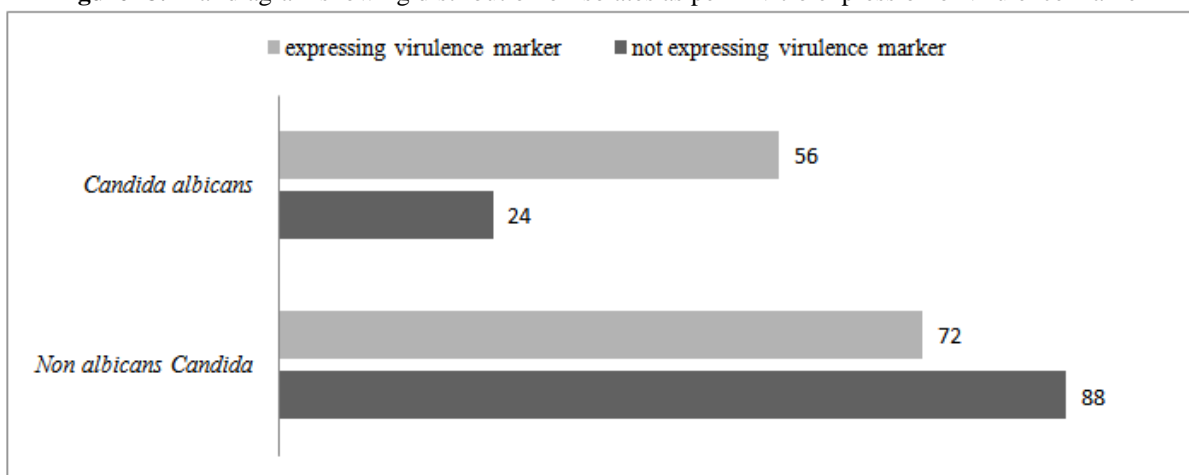
Table 2: Distribution of isolates as per different virulence factors (n==240)

Isolate	Phospholipase No. (%)	Haemolysin No. (%)	Esterase No. (%)	Adherence No. (%)
<i>C. albicans</i> (n = 80)	42 (52.5)	46 (57.5)	20 (35.71)	32 (42.86)
<i>C.parapsilosis</i> (n = 44)	08(18.1)	08 (18.1)	06 (13.6)	06(13.6)
<i>C.tropicalis</i> (n = 60)	36 (60.0)	40 (66.67)	30 (50.0)	36 (60.0)
<i>C.lusitaniae</i> (n = 24)	06 (25.0)	06 (25.0)	02 (0.83)	04 (16.67)
<i>C.glabrata</i> (n = 16)	08 (50.0)	10(62.5)	10 (62.5)	10 (62.5)
<i>C.krusei</i> (n = 12)	06 (50.0)	08 (66.6)	08 (66.6)	06 (50.0)
<i>C.Stellatoidea</i> (n = 4)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total(n= 240)	106 (44.1)	118 (49.1)	76 (31.6)	94 (39.1)

Table –3: Biofilm production by different clinical isolates of *Candida* species (n=240)

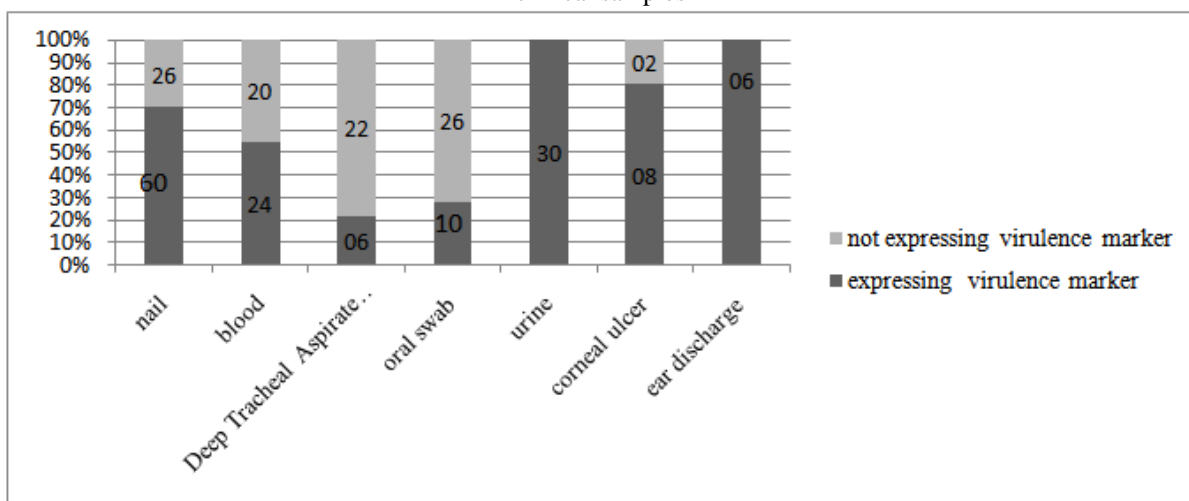
Species	Biofilm strong (++)	Biofilm weak(+)	Biofilm negative (-)
<i>C. albicans</i> (n=80)	26 (32.5%)	20(25%)	34(42.5%)
<i>C. tropicalis</i> (n=60)	24(40%)	10(16.6%)	26(43.3%)
<i>C. parapsilosis</i> (n=44)	14(31.8%)	04(9.09%)	26(59.09%)
<i>C. glabrata</i> (n=16)	06(37.5%)	02(12.5%)	08(50.0%)
<i>C. krusei</i> (n=12)	06(50.0%)	00	06(50.0%)
<i>C. lusitanae</i> (n=24)	04(16.6%)	00	20(83.3%)
<i>C. stellatoidea</i>	00	00	02(100%)
Total (n=240)	80(33.3%)	36(15%)	124(51.6%)

Figure- 8: Bar diagram showing distribution of isolates as per in vitro expression of virulence marker



Statistically significant difference was observed between the proportion of strains expressing virulence markers among *C. albicans* and *non albicans Candida spp.* (Fisher’s exact test; p 0.0118, OR 2.852, 95% CI 1.272 to 6.393).

Figure-9: 100% stack column showing distribution of *Candida spp.* Expressing virulence markers as per clinical samples



IV. Discussion:

There is significant increase in the incidence of Candidiasis over the past two decades. *Non- albicans Candida spp.* is replacing *Candida albicans* at most of the clinical sites presently^[1]. Falaqas M. E performed a systematic review to find the distribution of relative frequency of *albicans* and various *non albicans Candida spp.* among candidemia isolates in various parts of the world by searching Pub med and Scopus. *C. albicans* was the most common isolate in all the studies. *Non-albicans Candida* species were more common in South America, Asia and South Europe where *C. parapsilosis* and *C. tropicalis* were predominant^[16]. *C. albicans* (33.3%) was the most commonly isolated species in the present study. *Non albicans Candida spp.* (66.7%) exceeds the total isolated *Candida albicans* (33.3%). Similar to the present study Pethani J. et al in Delhi, 2011 also found higher isolation of *non albicans Candida spp.* (69%) than *Candida albicans* (31%)^[17]. This also corroborates well with the study of Basu et al, 2003^[18].

Significant statistical valuation was arrived at when *C. albicans* and *non albicans Candida* strains were compared for expression of virulence traits ($p=0.0118$, OR 2.852, 95% CI 1.272 to 6.393). This is similar with the previous studies of Inci et al, 2012^[10] and Gokce G et al, 2007^[19]. They also found significantly higher expression of virulence factors among *albicans* than *non albicans Candida*. In our study, haemolysin was the major virulence factor expressed by *Candida isolates* 118(49.1%) irrespective of its species. But the results of interspecies expression of haemolysin reveals highest haemolytic activity in *Candida tropicalis* followed by *C.krusei*, *C.glabrata* and *C. albicans* which is in accordance with the result of Yigit et al,2011. In the present

study phospholipase activity was demonstrated in 106(44.1%) *Candida* isolates, most commonly expressed by *C. tropicalis* and *C. albicans*. This observation was similar to the study of Deorukhkar et al, who also found *C. tropicalis* to be the most common virulent *non albicans Candida* isolate [20]. In the present study, biofilm production by *Candida* species revealed that 46/116(39.65%) strains of *Candida albicans* were biofilm producers while 70/116(60.34%) non-albicans *Candida* strains were biofilm positive, an observation which well corroborates with Yigit et al [81].

In the present study, the isolates from urine, ear discharge (100%) and nail (72.77%) were mostly expressing different virulence markers. *C. tropicalis* and *C. albicans* were most commonly isolated species from nail. *Candida* does not colonize nails and thus whenever it is isolated from clinically suspected onychomycosis, it is undoubtedly a pathogenic strain. But 26 out of 86 isolates obtained from nail samples (all *C. parapsilosis*) were found to be not showing any virulence attributes. Those isolates might possess other virulence traits which were not included in this study. Again these 26 samples were isolated along with moulds, which may be the causative pathogen. Moreover *C. parapsilosis* is one of the fungi, which are most frequently isolated from subungual space of human hands [21]. So, those isolates may be endogenous commensals / contaminants from other body sites. Isolates from eleven (22) DTA samples collected from patients did not express any virulence marker. Culture of BAL fluid of the same patients did not yield any growth of *Candida spp.* suggesting commensalism. Among 46 isolates from oral swabs / buccal washings of immunosuppressed patients only five (05) expressed virulence markers. So, it can be concluded that, even though immunosuppression is an important predisposing factor for Candidiasis, and there is increased isolation rate of *Candida spp.* from these patients, the isolates are not always pathogenic. Again the lower proportion of *Candida* species expressing virulence markers isolated from blood samples may be due to the fact that the strains were isolated after 6/7 days of incubation, probably suggesting contamination of commensal flora.

V. Conclusion:

Virulence of *Candida* may vary as it depends upon the type of species and the clinical sample from which it has been isolated. The above study emphasizes on detecting different virulence factors among *Candida* isolates in different clinical samples before starting therapy as commensal *Candida* strains (not expressing virulence traits) may be present in samples for which antifungal therapy is unjustified. Hence determinations of virulence factors are essential and should be mandatory to all clinical isolates of *Candida spp.* in the Mycology laboratory. Of course this will be an additional support to clinical correlation as well as to institute rational therapy.

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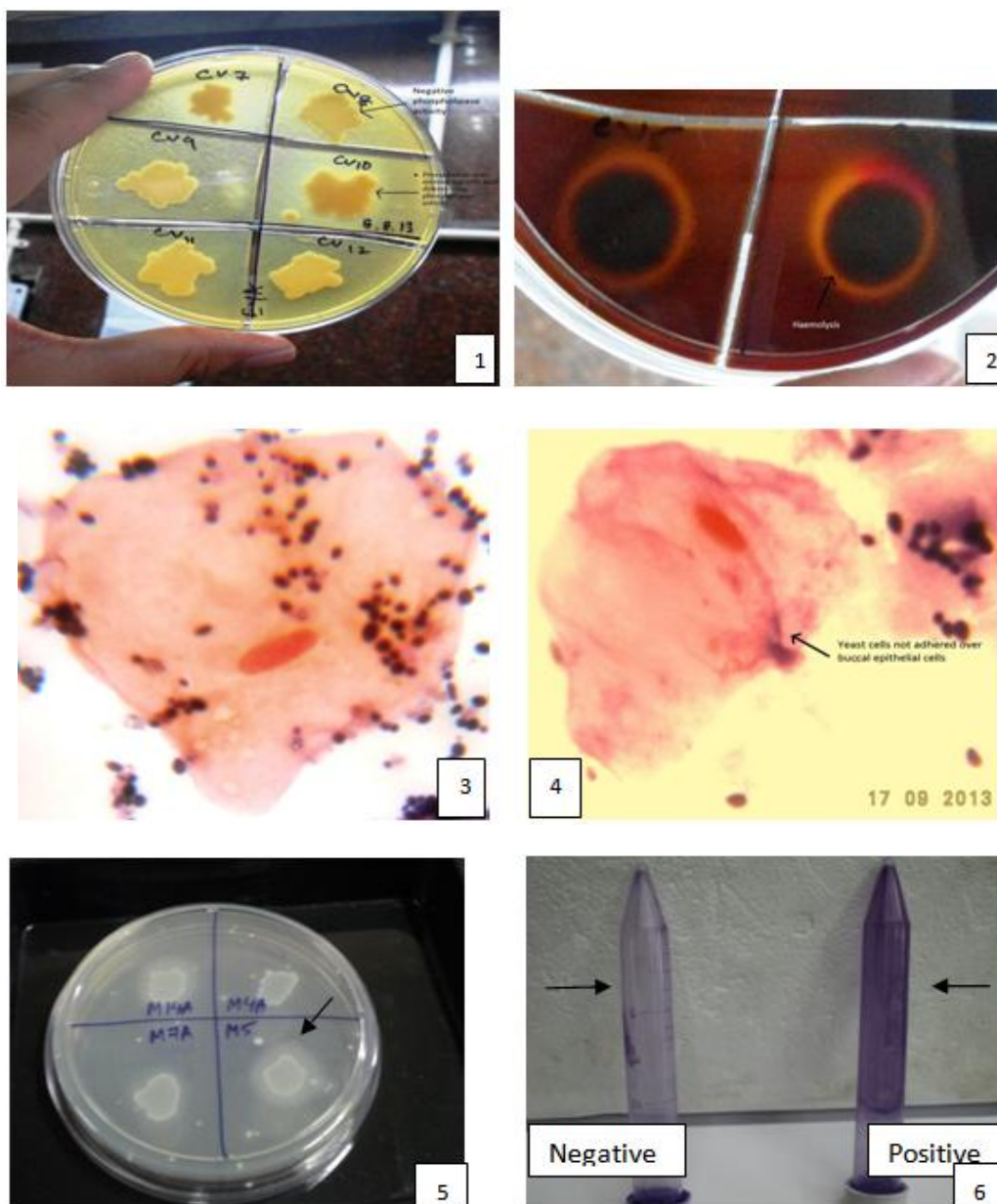


Fig 1: Positive Phospholipase activity on Egg yolk agar (Zone of precipitation around yeast colonies, CV₇, CV₁₀).

Fig 2: Hemolysis around Candida colonies on SDA Blood agar (Transparent/semitransparent zone around yeast colonies).

Fig 3: Adhered yeast cells on buccal epithelial cells (Gram stained smear x 1000)

Fig 4: Yeast cells not adhered on buccal epithelial cells (Gram stained smear x 1000)

Fig 5: Positive Esterase activity(halo around yeast colonies).

Fig 6: Biofilm formation.