CHAPTER FOUR

RESULTS
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Pathogens like bacteria and several viruses encounter the barrier formed by tight junction proteins on epithelial surfaces. These pathogens invade the hosts either by relocalization of the tight junction proteins and actin cytoskeleton or breaking down this barrier by the action of toxins or both. To understand the mechanism of modulation of tight junctions by *C. albicans*, invasion experiments were performed on epithelial cell lines to ascertain whether relocalization or breakdown of tight junctions is brought about.

4.1: *Candida albicans* invasion of epithelial cells is actin dependent

Host cell interaction with *C. albicans* is known to accumulate actin microfilaments at the site of invasion [Dalle et al, 2003; Atre et al, 2009]. This actin dependent invasion was verified when rabbit corneal epithelial cells were treated with a known inhibitor of actin polymerization, Cytochalasin D. Such cells when infected with *C. albicans* hyphae showed less sites of invasion in comparison to untreated cells. This decrease was found to be approximately 60%.

4.2: *Candida albicans* recruits Zonula Occludens-1 at the site of invasion

ZO-1 is a major protein that is known to interact with cytoskeletal proteins including actin [Suzuki, 2013] and also is cytoplasmic partner amongst the proteins involved in maintenance of barrier function. Therefore, it was essential to study ZO-1 modulation along with actin polymerization during the invasion of host cells by *C. albicans*. This was done by using two different
strategies, mainly using reporter protein and immunolocalization using specific antibodies.

**Figure 4.1:** Actin dependent cell invasion by *C. albicans* in SIRC cells. Control untreated and cytochalasin D treated (2uM for 30 minutes) SIRC cells were infected with *C. albicans* hyphae for 2 hrs. After fixing, permeabilizing and staining for actin, the cells were analysed for sites of invasion shown by arrowheads.

**Figure 4.2.1:** Peripheral localization of ZO-1-GFP chimera. HEK293T cells were transfected with plasmid expressing ZO-1-GFP fusion protein. The image was obtained using Leica SP5II confocal microscope and 488nm laser for excitation and acquired using Hybrid detector at 508-530nm.
When HEK-293T cells were transiently transfected with ZO-1-GFP chimera, green fluorescence was found to be localized at the periphery of the cells forming a distinct honeycomb like structure [Figure 4.2.1]. Therefore this system could be utilized to study the changes in ZO-1 localization during C. albicans invasion.

Upon infection by C. albicans, the peripheral localization of ZO-1 was altered and it was found to be colocalized with the newly polymerized actin at the site of hyphal invasion (as indicated by arrowheads) [Figure 4.2.2,a,b,c]. Co-localization of ZO-1-GFP and actin was confirmed by pixel locations in channel intensity graph, wherein ZO-1-GFP and TRITC-phalloidin positive pixels were present in the third quadrant indicating co-localization of the two proteins [Figure 4.2.2, d].

Figure 4.2.2: Colocalization of ZO-1-GFP and actin. ZO-1-GFP (a) and actin (b) localize at the site of invasion by C. albicans as shown by arrowheads. The overlay image shows intense yellow color at the invasion site (c). The quadrant 3 shows high number of pixels (d) indicating colocalization in the white boxed area at the site of invasion by C. albicans hypha (c).
It has been reported that actin and Rho GTPases form a uniform band or ring-like structure around the invading hyphae at the site of entry in epithelial cells [Atre et al, 2009]. In conjunction, the ZO-1-GFP reporter protein ring formed a similar ring-like structure (green) at the fungal invasion site analogous to that formed by actin as seen by red colored hollow circle in orthogonal sections of Z-stack of C. albicans infected ZO-1-GFP expressing cell stained by TRITC-phalloidin for actin [Figure 4.2.3,a,b,c].

![Figure 4.2.3: Orthogonal sections of colocalization of ZO-1-GFP and actin. The site of invasion of HEK 293T cells are represented as orthogonal sections with Zeiss Image Examiner software. The crosshair in (a) indicates the location of ring-like structure represented by green ring for ZO-1-GFP in (b) and red ring for actin in (c).](image)

4.3: Domains essential in recruitment of ZO-1 at the site of hyphal invasion

ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) homologue family and possesses three PDZ (postsynaptic density, disc-large, ZO-1) domains, a single SH3 domain (Src Homology-3) and a GuK (Guanylate Kinase) domain [Van Itallie and Anderson, 2014]. They share an
acidic domain, a basic domain, and a proline-rich C-terminus region which is not found in other MAGUK proteins. To understand the role of each of these domains in hyphal invasion and recruitment, chimeric proteins with GFP reporter were constructed having different domains of ZO-1. These chimeric proteins were transiently expressed in HEK-293T cells to identify the domains essential in ZO-1 recruitment and to ascertain the actin binding region (ABR) modulated by *C. albicans* invasion. HEK293T cells expressing only N-terminal region of ZO-1 (NZO-GFP; 1-506 amino acids, Figure 4.3.1 (1)) showed diffuse distribution of the chimeric protein within the cytoplasm. These cells, expressing N-terminal region which does not contain actin binding domain, when infected by the *C. albicans* hyphae, showed no recruitment at the site of cell invasion (Figure 4.3.1 (2) e,f,g). This was similar to the absence of localization in empty vector control (c1GFP) transfected cells (Figure. 4.3.1 (2) a,b,c). However, when HEK-293T cells transfected with CZO-GFP (C-terminal region of ZO-1, containing 507-1746 amino acids of ZO-1, Figure 4.3.1 (1)) were infected with *C. albicans*, they showed the typical co-localization (yellow color at the site of invasion) of GFP reporter chimeric protein (green) with actin (red) at the site of invasion (Figure 4.3.1 (2) i,j,k). This can also be confirmed from the colocalization graph, which shows pixels in the third quadrant in case of CZO construct (Figure. 4.3.1 (2) I) and are absent for c1GFP and NZO transfected cells (Figure. 4.3.1 (2) d, g).
Figure 4.3.1: Different domains of ZO-1 and their colocalization with Actin at the site of invasion by *C. albicans* in HEK293T cells. (1) Diagrammatic representation of complete ZO-1 molecule showing different functional domains and two deletion constructs made containing N-terminal (NZO) part containing the three PDZ domains and N-terminal (CZO) part having remaining domains. The numbers written in each domain show the number of amino acids from original ZO-1 molecule. (2) Representative images of localization of GFP chimeras (a,e,i) and actin (b,f,j) at the site of *C. albicans* invasion. Overlay images show colocalization of CZO construct with actin (k). The colocalized pixels marked by white box were assessed graphically (d,h,l) and are present in the quadrant 3 for CZO.
The CZO segment is known to contain actin interacting motif and therefore gets co-localized at the site of invasion and newly formed actin filaments. Similarly, GFP tagged ZO-1 deletion reporter proteins having C-terminal region (Figure 4.3.2 (1)), like CZO-ΔSH3 (CZO lacking SH3 domain; 592-1746 amino acids), CZO-ΔGUK (CZO lacking GUK domain but having SH3 domain) and PRR (CZO lacking SH3 and GUK domains; 762-1746 amino acids) co-localized with actin at the site of invasion (indicated by arrowheads, Figure 4.3.2 (2) a-i). At the same time, in cells expressing CZO-ΔPRR-GFP construct (CZO lacking both N- and C-terminal; 507-761 amino acids of ZO-1), due to absence of actin binding domain (PRR), the construct clearly did not co-localize with actin at the site of invasion by C. albicans (Figure 4.3.2 (2) j-l).

Figure 4.3.2: (1) Diagrammatic representation of deletion mutants tagged to GFP reporter protein from CZO domain.
Figure 4.3.2: (2) Localization of ZO-1-deletion constructs tagged to GFP reporter protein. GFP mutants (a,d,g) containing the actin binding region that is the PRR region show colocalization with actin (b,e,h) as seen by yellow color at site of entry by C. albicans (indicated by arrowheads) in overlay image (c,f,i). The mutant without PRR does not show colocalization (j,k,l).
4.4: Immunolocalization of Tight Junction Protein ZO-1

F-actin is known to bind to ZO-1 suggesting that these two interacting partners may mediate the assembly of different filaments at the TJs. To confirm this involvement of ZO-1 protein in cell invasion process by C. albicans, immuno-staining was performed using anti-ZO-1 monoclonal antibody on SIRC cell line infected by C. albicans. When C. albicans hyphae, invade the SIRC cells, distinct relocalization of ZO-1 was seen surrounding the hyphae (Figure 4.4, a) as seen by green fluorescence (pseudo-color used for Alexa 647). This immunostained area colocalized with the newly polymerized actin band (Figure 4.4, b).

<table>
<thead>
<tr>
<th>Construct of ZO1</th>
<th>ZO (507-1746)</th>
<th>NZO (1-506)</th>
<th>CZOΔGUK (507-1746-592-761)</th>
<th>CZOΔSH3 (592-1746)</th>
<th>CZOΔPRR (507-761)</th>
<th>PRR (762-1746)</th>
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</thead>
<tbody>
<tr>
<td>Localization after C. albicans infection</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4.1 Summary of localization different domains of ZO-1 upon infection by C. albicans.
Figure 4.4: Immunolocalization of ZO-1 at the invasion site of *C. albicans*. SIRC cells infected with *C. albicans* hyphae were fixed, permeabilized and after blocking, stained for ZO-1 and actin. Representative confocal microscopy image shows ZO-1 (a) colocalizing with actin (b) at the site invasion by *C. albicans*. Zoomed images and overlay image (c) shows accurate localization of ZO-1 and actin.

4.5: *Candida albicans* recruits Claudin-1 and -4 at the site of invasion

ZO-1 protein is known to interact with large number of soluble and membrane bound proteins. Consequently, re-localization of ZO-1 and actin during *C. albicans* infection may induce re-positioning of interacting proteins. Claudins are a group of trans-membrane proteins responsible for the cell-cell adhesion and paracellular permeability. Therefore it is likely that as actin and ZO-1 localization is altered upon *C. albicans* infection, claudins also may probably be relocalized at the site of invasion. Immuno-stained preparations using infected rabbit corneal epithelial cell line, SIRC, showed that claudin-1 and claudin-4 were co-localized with newly formed actin polymers at the site of *C. albicans* invasion [Figure 4.5 a,b,c and d,e,f respectively].
Figure 4.5: *C. albicans* recruits Claudin-1 and -4 at the invasion site. SIRC cells infected by *C. albicans* hyphae show colocalization of claudin-1 (a) and -4 (d) with actin (b,e) at the site of invasion (marked by arrowheads). Zoomed images and overlay images (c,f) show accurate localization of these proteins.
As described above, claudins were displaced to the site of infection during cell invasion by *C. albicans*. It is known that claudins are located at the apical end of polarized cells. The protein functionally similar to claudins, namely Junctional Adhesion Molecule-A (JAM-A) is located at the basal end of the tight junctions. JAM is known to interact with ZO-1 and is also responsible for regulating and maintaining the barrier function. And hence to validate this, immunostaining was performed on *C. albicans* infected SIRC cells. It was found that JAM-A (green) also localized along with actin (red) at the invasion site of *C. albicans* as shown in Figure 4.6 a,b,c. Involvement of JAM in invasion was validated by immunostaining using anti-JAM antibodies on SIRC cells infected with *C. albicans*. It was found that JAM-A (green) was localized along with actin (red) at the invasion site of *C. albicans* as shown in Figure 4.6 a,b,c.

![Image](image.png)

**Figure 4.6: Recruitment of JAM-A at the *C. albicans* invasion site.** SIRC cells infected by *C. albicans* hyphae also show colocalization of JAM-A (a) with actin (b) at the site of invasion. Zoomed images and overlay images (c) show accurate localization of these proteins.
Historically, the tight junctions were thought to form a simple and static paracellular seal, but now it has been shown that they dynamically regulate the paracellular flux in a size and charge selective manner. Such dynamics of tight junction structure can be determined by studying the diffusion of fluorescent tagged tight junction proteins. Using an approach known as fluorescence recovery after photobleaching (FRAP), a small (e.g., several microns) area of fluorescently tagged tight junction protein is transiently bleached [Liang and Weber, 2014]. As shown, ZO-1 localizes around the hyphae at the site of invasion. When ZO-1-

4.7: Dynamic Localization of ZO-1 at site of *C. albicans* Invasion

![Figure 4.7: Fluorescence recovery after photobleaching of ZO-1-GFP.](image)

Representative image of *C. albicans* infected HEK 293T cell transiently transfected with ZO-1-GFP (C) shows recovery of GFP at the bleaching site. The time-course images and fluorescence intensity and activity are shown graphically in A and B respectively.
GFP transfected HEK293T cells were infected with *C. albicans* hyphae, GFP fluorescence was seen around the invading hyphae. When a small area of such localized ZO-1-GFP was bleached (A), it showed complete recovery at the bleached site within 120 seconds as shown in the graph (B).

**Figure 4.8**: Loss of TJP ZO-1 in polarized MDCK-1 cells. Polarized MDCK-1 cells grown on coverslips were infected with *C. albicans*. *Candida* pseudohyphae (A,i) cause loss of ZO-1 as shown by arrow heads (A,ii) at the site of adhesion. Whereas infecting elongated hyphae show loss of ZO-1 in the infected plane (C) whereas ZO-1 in the plane above remains intact (B).

### 4.8: Immunolocalization of Tight Junction Protein ZO-1

Madin Darby Canine Kidney (MDCK) cell line is considered as a candidate cell line to study tight junction proteins and the barrier function [Balkovetz, 2006]. F-actin is known to bind to ZO-1 suggesting that these two interacting partners may mediate the assembly of different filaments at the tight junctions.

To confirm this involvement of ZO-1 in cell invasion process by *C. albicans*, immuno-staining was performed using anti-ZO-1 antibody on polarized MDCK cells infected by *C. albicans*. When pseudohyphae which immerge from the
yeast form of \textit{C. albicans}, invade the MDCK cells, distinct loss of ZO-1 was seen at the site of ingress of emerging tip in host cell membrane (Figure 4.8, A). When a z-stack of images of cell infected by an elongated invading hypha was done, the host MDCK cells showed localization of ZO-1 at that focal plane. However, the slices or levels above and below the plane of hyphal invasion showed intact ZO-1 immunocalization (Figure 4.8, B, C respectively).

4.9: Reduction in TEER by spent culture medium from hyphal stage of \textit{C. albicans}

Both the yeast and hyphal growth forms of \textit{C. albicans} are known to be pathogenic, however the hyphal form has been shown to be more invasive than the yeast form. This is linked to the expression of genes encoding virulence factors like the agglutinin-like sequence protein and the secreted aspartyl proteases. Therefore, it was crucial to check the effect of secreted products of hyphal stage, typically found in spent culture medium, on barrier function of epithelial cells. In addition to this only spent media could be tested because presence of hyphal or yeast cultures when tested on monolayers formed a thick biofilm which did not allow to assess the barrier function of host cell monolayers. The barrier function was evaluated by trans-epithelial electrical resistance (TEER) and transport of high molecular weight dextran. MDCK-1 cells were grown and allowed to form a fully confluent monolayer with polarized epithelial cells on trans-well filters with 0.4\(\mu\)m inserts. Such untreated control monolayers showed a very high TEER. When such MDCK monolayers were incubated for 24 h with the spent culture medium collected from yeast phase of \textit{C. albicans}, loss in TEER was not seen but it was slightly increased as compared to untreated control MDCK monolayers. While MDCK cells exposed to spent culture medium collected from hyphal phase of \textit{C. albicans}, showed time-dependent reduction in TEER indicating loss of tight junctions [Figure 4.9.1, (a)]. This decrease in TEER was significant and approximately 70\% reduction in TEER was found after 24 hour treatment of MDCK monolayers with the hyphal spent media compared to untreated monolayers [Figure 4.9.1, (b)]. This decrease in TEER can be confirmed by
another parameter like dextran flux which indicates paracellular permeability of monolayers. Dextran flux from \textit{C. albicans} hyphal spent medium treated monolayers was also found to increase significantly in comparison to untreated monolayers [Figure 4.9.1, (c)].

The loss in TEER or increase in paracellular permeability was not due to loss of cells in the monolayer as seen by the microscopic examination. Membrane inserts were stained by DAPI and TRITC-Phalloidin. When analyzed with confocal microscope, they showed uniform cell sheet with ideal actin and nuclear staining indicating intact cellular structure.

However, when these inserts with control and treated MDCK-1 monolayers were immuno-stained with anti-ZO-1 antibody and fluorescence quantified using 2.5D function of Zeiss Image Examiner, it was seen that control cells and cells treated with yeast spent medium had near comparable fluorescence indicating similar amount of ZO-1 was present in both cells. However, amount of fluorescence was highly reduced in MDCK monolayers treated with hyphal spent media, indicating decreased in ZO-1 quantity upon their treatment with secretory molecules of the more invasive growth phase of \textit{C. albicans} [Figure 4.9.2; 1, c; 2,c; 3,c]. The MDCK-1 monolayer did not show cell death as seen by intact nuclei [Figure 4.9.2; 1, a; 2,a; 3,a] and also intact actin staining [Figure 4.9.2; 1, b; 2,b; 3,b].
Figure 4.9.1: Loss of barrier function of MDCK cell monolayer upon treatment with spent media obtained from *C. albicans*. (a) A representative graph showing TEER measured for 24 hours for polarized MDCK cells grown on trans-well filters and treated with medium only (control, C, -●-), and spent media harvested from yeast (Y, -■-) or hyphal (H, -▲-) growth of *C. albicans*. (b) Changes in TEER of polarized MDCK cells as compared to controls measured after 24 hours of treatment of spent media harvested from yeast or hyphal growth of *C. albicans*. Each value represents
mean±SD of 3 independent experiments, each performed with 6 replicates. Statistical significance between groups was determined by one-way ANOVA test. (**P <0.01).

(c) 10kDa Alexafluor 647-dextran flux across the MDCK monolayers after treatment with treated with medium only (C), and spent media harvested from yeast (Y) or hyphal (H) growth of C. albicans. Each value represents mean±SD of 3 independent experiments, each performed with 6 replicates. Statistical significance between groups was determined by one-way ANOVA test (****P<0.001).

4.10: Alteration in ZO-1 levels in cells changes invasion frequency of C. albicans

In epithelial cells, TJ proteins create a barrier function by forming an unremitting and ribbon-like structure that is disrupted in many infections and inflammatory conditions. As seen by immuno-fluorescence and GFP chimeric protein expression analysis, C. albicans infection disrupts the seamless structure of TJ protein. Thus disruption or loss of TJ proteins may lead to higher rate of cell invasion and inversely increase in ZO-1 levels will decrease the invasion events.

To prove this, confluent MDCK cells were transfected with ZO-1-shRNA-GFP construct so that cells expressing GFP will co-express ZO-1 shRNA that will silence the ZO-1 gene expression via RNA interference. When monolayers were transfected with ZO-1-shRNA-GFP and immunostained using anti-ZO-1 antibody, it was seen that the ZO-1 expression was reduced in green fluorescent cells, due to the expression of shRNA. Such monolayers were infected with C. albicans and analyzed for invasion frequency by counting for number of invasion events by hyphal structures. Invasion by C. albicans was nearly five-fold more in green fluorescent cells expressing ZO-1-shRNA (+shRNA) than the non-fluorescent cells (-shRNA) that did not get transfected with ZO-1-shRNA-GFP (Figure 4.10.1). Non-transfected monolayers were also analyzed similarly (C) to assess the effect of transfecting agents on invasion. This indicated that loss of ZO-1 increases the invasion by C. albicans. The loss of expression of ZO-1 by ZO-1-shRNA-GFP was validated by immunostaining (Figure 4.10.2) as well as Western blotting (Figure 4.10.3).
Figure 4.9.2: Loss of ZO-1 upon treatment with *C. albicans* hyphal secretory products. Quantification of fluorescence as represented in 2.5D intensity graphs after immuno-staining of MDCK cells grown on transwell filters after treatment with spent media harvested from medium only (1, c), yeast (2, c) and hyphal (3, c) growth stages of *C. albicans*. The MDCK monolayer did not show loss of cells or monolayer as seen by intact nuclei (1, a; 2, a; 3, a) and also intact actin staining (1, b; 2, b; 3, b).
As against this, when ZO-1 expression was increased in MDCK cells by expression of ZO-1-GFP construct, the green fluorescence was indicative of cells having more ZO-1 than non-GFP expressing cells. Such ZO-1-GFP expressing cells showed far less [approximately 60%] invasion frequency than control cells that did not show any green fluorescence (Figure 4.10.4).

![Figure 4.10.1: Effect of expression of mZO1-1 shRNA and invasion frequency.](image)

**Figure 4.10.1: Effect of expression of mZO1-1 shRNA and invasion frequency.** mZO-1-GFP shRNA plasmid was transfected in MDCK cells and monolayers were infected with *C. albicans* hyphae. The numbers of invading hyphae (identified by actin staining) were counted in green colored cells expressing GFP and mZO1-1 shRNA (+shRNA) and cells not expressing green fluorescence (-shRNA). Nontransfected monolayers were also analyzed similarly (C) to assess the effect of transfecting agents on invasion. Each value represents mean±SD of 3 independent experiments after counting a minimum of 100 cells in each replicate. Statistical significance between groups was determined by one-way ANOVA test (P<0.001).
Figure 4.10.2: Expression of mZO-1-shRNA-GFP. A representative photomicrograph of mZO1-1-shRNA-GFP expressing MDCK-1 cells (i) showing more invasion frequency of *C. albicans* invasion corroborated by actin polymerization at the sites of invasion (ii). Phase contrast image of the same field (iii) with overlay (iv) is shown. The panel below shows a mZO1-1-shRNA-GFP transfected MDCK-1 cell (marked by *) showing loss of ZO-1 immunostaining by mouse anti-ZO-1 primary antibody followed by Alexa 647 labelled anti-mouse secondary antibody (red pseudocolor) in the marked cell whereas neighboring cells show ZO-1 surface staining (C).

Figure 4.10.3: Western blot analysis of MDCK cells expressing plasmid bearing mZO-1-shRNA-GFP construct. Total protein was extracted from control and transfected cells. The right lane shows reduced expression of ZO-1 in comparison to control untransfected cells. Actin was used as loading control.
**4.10.4: Effect of ZO-1-GFP over-expression on epithelial cell invasion by C. albicans.** SIRC cells transfected with ZO-1-GFP were infected with C. albicans hyphae. The number of sites of invasion in such GFP expressing cells and control untransfected cells were compared and found to be less in the former. Statistical significance (p<0.002) was determined by unpaired t-test.

**4.11: Effect of C. albicans infection and its secretory products on transcription**

Involvement of Tight Junction Proteins has been shown at the site of invasion by C. albicans of different epithelial cells. It was imperative to comprehend the activity of transcription machinery. Using primers specific for ZO-1 and GAPDH as control, mRNA levels of MDCK cells either infected for 6 hours or treated with yeast or hyphal spent media for 24 hours were analysed with untreated, uninfected cells as control. As shown in graph, upon infection for 6 hours, the transcription levels did not change significantly in comparison to uninfected cells (Figure 4.11, i, ii). When the polarized MDCK cells were treated with yeast spent medium for 24 hours the mRNA levels were increased but not significantly. In contrast, treatment of such layers with hyphal supernatant led to substantial increase in ZO-1 gene expression which was statistically significant with p<0.04 (Figure 4.11, ii, iii). This indicates that even though ZO-1 protein expression in reduced upon treatment with secretory products of the invasive hyphal stage of C. albicans, increase in mRNA level hints at the attempt of epithelial cells to recover their barrier function.
Figure 4.11: mRNA expression in MDCK cells either infected or treated with *C. albicans* spent media. (i) 6 h infection (I) of polarized MDCK cells with *C. albicans* hyphae did not change mRNA levels compared to uninfected cells (C). (ii) Representative gel image of 0.8% agarose gel stained with Ethidium bromide. (iii) Treatment of MDCK cells with spent medium from hyphal phase (H) of *C. albicans* resulted in significant increase in mRNA expression (p<0.04), but yeast spent medium (Y) did not cause significant difference in comparison to untreated cells (C).
4.12: *ex vivo* infection of buccal epithelium

Rodent models of oral, vaginal and gastrointestinal *Candida* infection are described and discussed in terms of their scientific merits. There is need for some level of host immunocompromise or exogenous treatment to ensure reproducible disease [Naglik et al, 2008]. Wistar rats immune-suppressed by administration of Cortisone [25mg per 1kg body weight, twice a week; Clarkson et al, 1988] were dissected and buccal mucosa obtained. When such *ex vivo* biopsies were subjected to *C. albicans* invasion, the tight junction protein showed distinct breakdown at the site of invasion as shown in the figure.

![Figure 4.12: *Ex vivo* infection of buccal mucosa by *C. albicans* hyphae.](image)

*Figure 4.12: *Ex vivo* infection of buccal mucosa by *C. albicans* hyphae.* GFP expressed *C. albicans* hyphae (A) upon invasion into buccal epithelium show relocalization of ZO-1 (B) and actin (C). (D) shows nuclear staining and (E) is the bright field image.

4.13: Saprophytic yeast isolates from clinics and their uptake by epithelial cells

Besides *Candida albicans*, many types of non-Candida yeasts have also emerged as common pathogens and are responsible for more than 50 % cases of yeast infections [Atre et al, 2009, Bhally et al, 2006]. Molecular
techniques, such as PCR and sequence information of amplified products of various genes have shown enhanced sensitivity and specificity in identifying the pathogenic yeast [Gargeya et al, 1990, Gonçalves e Silva et al, 2014]. After identifying yeast species isolated from variety of body fluids of different patients by amplification and sequencing ITS region, the invasive character of the saprophytic non-Candida yeast isolates using the Human embryonic kidney (HEK) cell line was assessed in vitro.

4.13.1: Identification of yeast isolates

A total of 40 yeast isolates from various clinical conditions were identified using morphology, biochemical test and colony color on Yeast chrome agar and found to be belonging to genus Candida. To confirm the identification of these isolates, the DNA sequence of PCR amplified ITS region was utilized. The identification revealed 70% of the isolates were belonging to genus Candida and were mainly belonging to C. albicans and non-albicans Candida like C. tropicalis and C. parapsilosis. Remaining isolates were novel and belonged to different species of yeast as shown in Table 4.2. Previous studies have shown that these yeasts have been isolated from various human infections from different organs and blood confirming their pathogenic trait [Gargeya et al, 1990, Bhally et al, 2006, Dooley et al, 1990, Vaughan-Martini et al, 2005, Pfaller et al, 2006, Hurley et al, 1987, Gross and Kan 2008, Paula et al, 2006, Park et al, 2008]. Most of these yeasts are normal flora of various plants, fruits, cereals or are common saprophytes found in soil environment.
<table>
<thead>
<tr>
<th>Species of yeast</th>
<th>Clinical history of the Yeast species</th>
<th>Normal Habitat of the Yeast species</th>
<th>Isolated from [in this study]</th>
<th>In vitro Cell Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavispora lusitaniae</td>
<td>Common yeast isolate from immuno-deficient patients</td>
<td>Cactophilic yeast</td>
<td>Sputum</td>
<td>+</td>
</tr>
<tr>
<td>Lindnera fabianii</td>
<td>Infection in neonates, Prostatitis</td>
<td>Industrial fermentations and starch based food</td>
<td>Urine</td>
<td>+</td>
</tr>
<tr>
<td>Hanesiapora opuntiae</td>
<td>No clinical cases</td>
<td>Cactophilic, flora of grapes and berries</td>
<td>Blood</td>
<td>+</td>
</tr>
<tr>
<td>Meyerozyma caribbica</td>
<td>An occasional human pathogen</td>
<td>Corn, sugar cane and soil</td>
<td>Sputum</td>
<td>+</td>
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<td>Meyerozyma guilliermondii</td>
<td>2% clinical yeast isolates</td>
<td>Insect, plant and food products</td>
<td>Urine</td>
<td>+</td>
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<td>Pichia kudriavzevii</td>
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<td>Rhizosphere of sugar beet</td>
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<td>Isolated from lungs, eye and Blood</td>
<td>Soil and plant material</td>
<td>Throat</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4.2: Saprophytic yeast isolates and their invasion properties.

4.13.2: In vitro cell invasion assay

Most of the novel saprophytic yeasts isolated in this study showed dimorphism. They germinated to pseudohyphae but did not differentiate to true hyphae. As development of true hyphae is linked to pathogenicity and invasive character of species like *C. albicans*, it was essential to check invasive character of these non-Candida isolates. During the *in vitro* study of invasion of mammalian cells by *C. albicans* hyphae, it was observed that hyphal tip induced a pseudopodia-like outgrowth from the nonphagocytic epithelial cells. The fungal hyphae penetrated and infiltrated the host cells at the tip of the pseudopodial structure with actin polymerization seen surrounding them [Atre AN et al, 2009]. In order to determine whether the yeast isolates in this study also behave similar to *C. albicans* and have invasive character, they were assessed using human embryonic kidney [HEK]
cell line. These epithelial cells were incubated with the blastospores of various isolates at 37°C and cell invasion was assessed by actin staining [Atre AN et al, 2009]. Host cell invasion or uptake can be seen in case of ovoid yeast cells of *Hanseniaspora opuntia* which invade cells not singly but in bunch [Figure 4.13, E]. Staining with TRITC-phalloidin and observation for actin structure, showed local actin polymerization surrounding the invading *Trichosporon asahii* [Figure 4.13, C] and *C. albicans* [Figure 4.13, D] indicating this process recruits host cell actin around the invading fungal structure. Some yeast such as pseudohyphae forming *Pichia kudriavzevii* invades cell singly and also shows actin reassembly [Figure 4.13, A]. The photomicrograph for this yeast clearly shows that a yeast cell attached to the cell surface and not invading cells did not induce any actin polymerization but another yeast cell present nearby is taken up by the epithelial cell inducing actin polymerization. Arrow heads indicate sites of actin remodeling. This clearly indicates that actin polymerization can be used as a dependable and explicit marker for study of invasion by yeast cells. Yeast isolates that are known to differentiate into hyphae also invaded HEK cells and invasion was marked by polymerized actin. Both yeast forms as well as hyphae were found to be penetrating and infiltrating the host cells from the tip of the pseudopodial structure. Results obtained for invasion capacity of different isolates are summarized in Table 4.2. Uptake of all the yeast isolates from various systemic infections by epithelial cells was observed and this included yeast species belonging to genera *Pichia, Wickerhamomyces, Clavispora* and *Lindnera*. These genera are usually known to be environmentally isolated saprophytes and reported as low virulent commensal organisms. Along with different yeast, even inert synthetic polymer beads were incubated with epithelial cells and were also found to be efficiently internalized with F-actin surrounding them [Figure 4.13, B]. This uptake of inert beads indicated that uptake of particulate matter, either living or inert, is a property of epithelial cells.
Figure 4.13: Actin remodeling in human embryonic kidney (HEK) cells during invasion or uptake of inert beads (A) pseudohyphae of P. kudriavzevii, (B) inert beads, (C) hyphae of Trichosporon asahii, (D) C. albicans hyphae and yeast form of Hanseniaspora opuntiae (E). Samples were stained with TRITC-phalloidin for actin and observed under fluorescence and phase contrast microscope. Arrow heads indicate sites of actin remodeling.