REGINALDO GONÇALVES DE LIMA NETO

CAPACIDADE DE ADERÊNCIA ÀS CÉLULAS EPITELIAIS DA CAVIDADE ORAL E PERFIL HISTOQUÍMICO DE CULTURAS DE *CANDIDA* ESTOCADAS NA MICOTECA URM

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RESUMO

A capacidade de aderência de espécies de *Candida* às células epiteliais, é um dos fatores relevantes de patogenicidade que está associado ao perfil histoquímico da superfície da célula. O objetivo dessa pesquisa foi avaliar a habilidade de aderência de espécies de *Candida* às células epiteliais da cavidade oral e correlacionar ao perfil histoquímico da parede celular. Foram testados 20 isolados de *Candida albicans*, seis de *C. krusei*, 15 *C. parapsilosis* e cinco de *C. tropicalis* frente às células epiteliais da cavidade oral. As células epiteliais foram incubadas com suspensões de leveduras sob agitação. Histoquímica foi estudada usando lectinas conjugadas a peroxidase. Resultados mostraram que *C. albicans* e *C. tropicalis* foram mais aderentes que *C. krusei* e *C. parapsilosis* ($P<0.01$), apresentando alta expressão de resíduos de α-L-fucose na superfície celular. Três isolados de *C. parapsilosis* apresentaram valores similares de aderência e perfil histoquímico ao observado em *C. albicans* e *C. tropicalis* ($r=0.8336$, $P=0.0001$). Todos os isolados de *C. krusei* foram fracamente aderentes, mostrando a baixa patogenicidade inerente a esta espécie. Adicionalmente, nossos resultados mostraram a presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico e D-galactose/*N*-acetil-D-galactosamina na parede celular das espécies estudadas. Carboidratos complexos sobre a superfície de espécies de *Candida* podem representar um meio pelo qual as interações entre os fungos e seus hospedeiros podem ser estabelecidas.

Palavras chaves: *Candida*, aderência, células epiteliais, histoquímica, lectinas.

ADHERENCE CAPACITY TO EPITHELIAL CELLS AND HISTOCHEMISTRY PROFILE OF *CANDIDA* SPP. STOCKED IN URM CULTURE COLLECTION

ABSTRACT

Several studies report the adherence capacity of *Candida* spp. to epithelial cells, but comparative studies based in this pathogenicity factor versus cellular recognition via cell surface carbohydrates are scarce. This study aimed to evaluate the adherence ability of 20 *Candida albicans*, six *C. krusei*, 15 *C. parapsilosis* and five *C. tropicalis* strains to human buccal epithelial cells according the expression of cell surface carbohydrates evaluated via lectin histochemistry. Adherence assays were carried out incubating epithelial cells with the yeast suspensions under stirring. Lectin histochemistry was examined using peroxidase conjugated lectins. Results showed that *C. albicans* and *C. tropicalis* were more adherents than *C. krusei* and *C. parapsilosis* ($P<0.01$), besides to present high α-L-fucose moieties in the cell surface. Three isolates of *C. parapsilosis* presented similar adherence values and histochemistry profile to that of *C. albicans* and *C. tropicalis* ($r=0.8336$, $P=0.0001$). All *C. krusei* strains adhered weakly, showing the low pathogenicity inherent to this species. Moreover, our results showed the presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid and D-galactose/*N*-acetyl-D-galactosamine in fungal cell wall. Complex carbohydrates on the surface of *Candida* species may represent additional motifs through which interactions of this fungus with host cells could be established.

Keywords: *Candida*; Adherence; Epithelial cells; Lectin histochemistry.

SUMÁRIO

1 Introdução Geral

1.1 Aderência celular

Os fungos através de seus mecanismos de patogenicidade, como a capacidade de se aderir às células do hospedeiro, podem causar danos ao homem de diversas maneiras, como processos infecciosos, hipersensibilidade imediata ou tardia, micetismo e micotoxicoses (de HOOG et al., 2000; FISHER; COOK, 2001).

As infecções de natureza fúngica, em especial as oportunistas, são predominantemente graves, persistentes e recidivantes, destacando-se as causadas por leveduras (ZAITZ et al., 1998; SIDRIM; ROCHA, 2004). Esses fungos unicelulares que se reproduzem predominantemente por brotação, fissão e/ou brotofissão, podendo apresentar micélio verdadeiro e/ou pseudomicélio, fazem parte da microbiota normal do indivíduo, bem como do meio ambiente (KREGER-VAN RIJ, 1984; KWON-CHUNG; BENNETT, 1992).

Em 1839, Langenbeck descreve pela primeira vez um microrganismo, atualmente conhecido como *Candida albicans*, considerada a mais importante levedura patogênica ao homem. Posteriormente, em 1842, Gruby descreveu a candidíase oral e classificou essa levedura no gênero *Sporotrichum*. Em 1853, Charles Robin denominou de *Oidium albicans* e Berkhout, 70 anos depois, denominou o gênero de *Candida* (KREGER-VAN RIJ, 1984).

Alguns atributos têm sido sugeridos, como responsáveis pela patogenicidade de diversos grupos de fungos, incluindo espécies de *Candida*. A maior parte destes incide na aderência às células epiteliais a partir do reconhecimento das células do hospedeiro por moléculas da superfície celular fúngica; conversão morfogenética dos organismos da forma unicelular (levedura) para uma forma multicelular (filamentosa) e a secreção de biomoléculas supostamente invasivas como proteases e fosfolipases (MARTÍNEZ et al., 1998; NAVARRO-GARCIA et al., 2001; MARCHAIS et al., 2005).

A capacidade de aderência dessas leveduras às células do hospedeiro é considerada complexa e importante, e um passo essencial para desencadear a infecção micótica. O reconhecimento dos ligantes da célula do hospedeiro por microrganismos patogênicos é essencial para a sua sobrevivência, seguido pela invasão da célula ou tecido (STURTEVANT; CALDERONE, 1997).

As espécies de *Candida* são as leveduras mais comumente relacionadas com doença humana, destacando-se *C. albicans*, a qual faz parte da microbiota da pele, mucosa oral e genital assim como do trato gastrintestinal (DEL BRUTTO, 2000). Estas leveduras podem passar oportunamente de sapróbios a patogênicos, provocando quadros clínicos variáveis, desde processos febris, benignos, a septicemias, algumas vezes fatais, se não forem diagnosticados e tratados de maneira correta (LACAZ et al., 2002).

Na década de 30, doenças do homem associadas às espécies de *Candida* eram raras, e candidíase profunda era praticamente desconhecida. Atualmente, tem se destacado como infecção fúngica mais freqüente em indivíduos imunocomprometidos, tornando-se um problema de saúde pública. Contudo a candidíase superficial está entre as infecções mais comuns de pele e mucosas (ZAITZ et al., 1998).

Vários autores afirmam que espécies de *Candida* apresentam baixa virulência e quando relacionadas com enfermidade no homem e em animais são incapazes de produzir infecção no indivíduo imunocompetente. Alterações nas defesas celulares do hospedeiro, na fisiologia ou na microbiota normal se faz necessário para que a colonização se torne doença. A candidíase aguda ou crônica, superficial ou disseminada, pode apresentar diversas variedades clínicas, causada por diversas espécies (LACAZ et al., 2002; SIDRIM; ROCHA, 2004).

Segundo Swoboda-Kopec et al. (2001), Mestroni e Bava (2003), Pfaller e Diekema (2004), Roilides et al. (2004), Liu et al. (2006), nas últimas décadas infecções causadas por *C. albicans* tem diminuído relativamente devido à incidência de infecções causadas por outras espécies como *C. glabrata*, *C. krusei*, *C. parapsilosis* e *C. tropicalis, C. guillermondii*, *C. lusitaniae*, *C. melibiosica, C. pelliculosa, C. pseudotropicalis*, *C. rugosa, C. uitilis* e *C. kefir*.

O aumento das micoses oportunistas deve-se ao avanço da tecnologia médica, através do emprego de novos agentes antibacterianos de modo empírico, introdução de técnicas agressivas no tratamento do câncer, transplantes de órgãos, pandemia da AIDS e outros fatores responsáveis pelo crescente número de pacientes imunocomprometidos (RUBIN; FARBER, 1999; CARRILLO-MUÑOZ et al., 2001).

O aumento do número de infecções por fungos oportunistas tem estimulado novos esforços, para desvendar os fatores e circunstâncias de patogenicidade de várias espécies.

Estudos têm demonstrado que há variações na capacidade de aderência por algumas espécies de *Candida*, o que explicaria o porquê de algumas serem encontradas mais freqüentemente colonizando um determinado sítio anatômico (ELLEPOLA; SAMARANAYAKE, 1998; JABRA-RIZK et al., 2001, DAGDEVIREN, et al., 2005).

A multiplicidade nos mecanismos de aderência celular depende de carboidratos, glicocorticóides, corticosteróides, antibióticos, drogas antifúngicas, detergentes, sais e soro sanguíneo, assim como concentração, idade e viabilidade da cultura do microrganismo (BYKOV; VELICHKO, 1988; GHANNOUM et al., 1992).

Gallardo-Moreno et al. (2003), descreve as interações envolvidas na aderência celular como inespecíficas, as hidrofóbicas e como específicas, as interações adesina-receptor.

Hidrofobicidade de superfície celular representa um importante papel na patogenicidade de microrganismos, incluindo espécies de *Candida* (MASUOKA; HAZEN, 2004). Células de *Candida* hidrofóbicas são mais aderentes que as hidrofílicas a uma variedade de tecidos do hospedeiro, além de serem mais resistente à fagocitose (HAZEN et al., 2000). De acordo com Hazen et al. (2001), *C. albicans* é o único patógeno fúngico observado até o momento, capaz de regular seu status hidrofóbico na superfície.

O "status" na hidrofobicidade tem sido correlacionado a características na arquitetura da parede celular. A camada superficial da parede celular de *C. albicans* contém estruturas fibrilares compostas de mananoproteínas. A habilidade desta levedura de regular a hidrofobicidade de sua superfície corresponde a uma capacidade de alterar a conformação das fibrilas mananoprotéicas. Fibrilas sobre a superfície de células hidrofóbicas são curtas e agregadas, enquanto que aquelas sobre células hidrofílicas são mais longas, espaçadas e irradiadas (MASUOKA; HAZEN, 1997).

Para Gallardo-Moreno et al. (2003), a hidrofobicidade é a principal forma de interação molecular envolvida na aderência dos microrganismos a superfícies poliméricas inertes, como próteses, válvulas e cateteres, mas a expressão desta característica é bastante influenciada pelas condições de crescimento. Por esta razão, torna-se difícil mensurar o valor absoluto da hidrofobicidade no processo infeccioso.

A maior parte dos experimentos sobre aderência dos fungos às células do hospedeiro, tem sido feita com *C. albicans*. A evolução deste organismo como um

comensal humano foi provavelmente resultado da habilidade de colonizar células epiteliais da mucosa e de selecionar vários outros mecanismos de patogenicidade que promovam a invasão (STURTEVANT; CALDERONE, 1997).

Reconhecimento específico entre os fungos e as células de seus hospedeiros é mediado pela interação entre moléculas biologicamente ativas de ambas as superfícies celulares. Comumente ocorre entre proteínas ligadoras de carboidratos, como lectinas, que estão presentes na superfície das células e se ligam a açúcares complementares, livres ou conjugados, na superfície da outra célula (MENDES-GIANNINI et al., 2000; ESQUENAZI et al., 2003). Essas moléculas que estão presentes na superfície celular das leveduras são chamadas de adesinas, e sobre as células do hospedeiro que são reconhecidas pelo patógeno, de ligantes ou receptores (STURTEVANT; CALDERONE, 1997).

Nesse contexto, entre o final da década de 70 e início de 80 surgem os primeiros experimentos *in vitro*, que tentam mensurar a capacidade de adesão celular de espécies de *Candida*, especialmente de *C. albicans*, além de determinar os fatores que interferem nesta característica de patogenicidade (KIMURA; PEARSALL, 1978; KIMURA; PEARSALL, 1980; KLOTZ et al., 1980; MCCOURTIE; DOUGLAS, 1981; SOBEL et al., 1981; SAMARANAYAKE; MACFARLANE, 1982; LEE; KING, 1983). Estes tipos de ensaios vêm sendo otimizados para refletir tão próximos quanto possível, as condições de adesão *in vivo* (SUNDSTROM, 2002).

Há mais de uma década, vários pesquisadores vêm detectando, isolando e caracterizando as adesinas envolvidas na interação de espécies de *Candida* a superfícies epiteliais, endoteliais e a matrix extracelular (CRITCHLEY; DOUGLAS, 1987; DOUGLAS, 1992; HOSTETTER, 1994; CORMACK et al., 1999; MORAGNES et al., 2003).

Segundo Sturtevant e Calderone (1997), entre os principais ligantes da célula humana que são reconhecidos por *C. albicans*, estão βGalNac(1-4β-Gal), Fuc(α1-2)Gal(β1-4)Glc, βGalNac(1-4β-Gal), Fuc(α1-2)Gal(β1-3)GlcNac(β1-4)Gal(β1-4), Arginina-glicina-ácido aspártico (RGD), lisofosfolipídio e Galβ1-4Gluβ1-1Ceramida. Esses ligantes são diversos, e apresentam todas as classes de biomoléculas incluindo carboidratos, proteínas e lipídios (CALDERONE, 1993; HOSTETTER, 1994; YU et al., 1994).

Poucos estudos têm demonstrado as implicações que a presença de carboidratos complexos na superfície celular fúngica pode trazer (JABRA-RIZK et al., 2001). Nesse contexto, glicoconjugados podem ter importante papel no reconhecimento de moléculas receptoras na célula do hospedeiro, mediando uma inicial adesão e posterior infecção.

Brassart et al. (1991) evidenciam a afinidade que as adesinas possuem por complexos glicosídicos. Assim, estas funcionam como lectinas, que reconhecem principalmente, entre outros açúcares, resíduos de α-fucose das células do hospedeiro. Esses pesquisadores ratificaram esta afirmação ao demonstrar o decréscimo da adesão de células de *C. albicans* às células epiteliais da cavidade oral, quando estas últimas foram pré-incubadas com lectinas que reconhecem α-fucose, mas não quando essas mesmas lectinas foram pré-incubadas com as leveduras. Esses experimentos permitem concluir que, as lectinas se ligam às células epiteliais no momento da pré-incubação, e originam um impedimento estérico às células de leveduras.

Burford-Mason et al. (1998) afirmam que atualmente, não há uma correlação clínica que confirme *in vivo* a ligação do fungo a sacarídeos específicos do hospedeiro. Essa correlação tem sido feita com a observação de pacientes com candidíase oral intensa, que foram descritos como secretores de saliva ineficientes, em comparação com indivíduos secretores. Os autores concluíram que a saliva de bons secretores contém ligantes que bloqueiam a adesão das leveduras, conseqüentemente, a baixa freqüência de candidíase oral neste grupo de pacientes.

Interessante associação clínica foi estabelecida com lectina ligadora de manose (MBL) e candidíase vulvovaginal (LIU et al., 2006). Foi demonstrado que um aumento na concentração desta lectina no epitélio vaginal favorece o início da defesa da hospedeira, uma vez que esta molécula liga-se a múltiplos sítios de carboidratos da superfície microbiana. Após aglutinação, MBL ativa o sistema complemento através de C1q, que se liga a um anticorpo. Este complexo opsoniza a levedura e promove a fagocitose. A modulação de macrófagos e ativação de C4 e iC3b constituem outros mecanismos imunorregulatórios descritos para a referida lectina (IP; LAU, 2004; PELLIS et al., 2005).

Vardar-Ünlü et al. (1998) utilizaram neoglicoproteínas, açúcares ligados a fluroceína através da albumina, para mapear as lectinas presentes sobre as células e o tubo germinativo de *C. albicans*. Nestes experimentos com microscopia de fluorescência,

espectofluorimetria e citometria de fluxo, foi possível concluir que a sonda de fucose apresentou uma alta afinidade pela levedura e principalmente pelo seu tubo geminativo, enquanto a sonda contendo manose apresentou baixa ligação. Em contrapartida, as sondas de glicose, galactose e lactose, não mostraram significância nos níveis de ligação exibidos.

Vários pesquisadores têm se empenhado em determinar a natureza bioquímica das adesinas, em várias espécies fúngicas, utilizando sondas específicas que irão mapear a superfície desses organismos. Tronchin et al. (1997) e Bouchara et al. (1997) identificaram o reconhecimento de laminina e fibrinogênio humano por sialoglicoconjugados presentes em conídios de *Aspergillus fumigatus*. Semelhante descrição foi realizada por Hamilton et al. (1999), que descreveram a interação entre conídios de *Penicillium marneffei* e fibronectina via ácido siálico presente na superfície deste organismo. Glicoproteínas de superfície celular de *Fonsecaea pedrosoi* também foram isoladas, parcialmente caracterizadas e correlacionadas com a sua possível influência de interação com o hospedeiro (LIMONGI et al., 1997; LIMONGI et al., 2001). Além desses, vários outros fungos patógenos como *Paracoccidioides brasiliensis*, *Sporothrix schenckii* e espécies de *Trichophyton* vêm tendo os componentes da parede celulares estudados e associados a interações com superfície do hospedeiro e estabelecimento de infecções (ALMEIDA et al., 1998; HANNA et al., 2000; LIMA et al., 2001; ESQUENAZI et al., 2003; ESQUENAZI et al., 2004; FIGUEIREDO et al., 2004).

Além de açúcares, *C. albicans* se liga a um extenso número de receptores de origem protéica, como colágeno do tipo I e IV, laminina, fibrinigênio, entactina e fibronectina, entre outras proteínas como componentes do complemento (ALAEI et al., 1993; WADSWORTH et al., 1993; LOPEZ-RIBOT; CHAFFIN, 1994; LOPEZ-RIBOT et al., 1994; PENN; KLOTZ, 1994; LOPEZ-RIBOT et al., 1995). Todas essas proteínas compõem a matrix celular endotelial e interação de *C. albicans* a essas moléculas contribui para sua virulência, uma vez que linhagens que apresentam mutações em genes codificadores de adesinas que reconhecem as proteínas da matrix endotelial, não têm sido isoladas clinicamente (STURTEVANT; CALDERONE, 1997).

Prakobphol et al. (1994) mensuraram a ligação de *C. tropicalis* a uma variedade de lipídeos, glicolipídios e glicoproteínas salivares, e observou que a ligação com a maior parte dos componentes foi mínima, exceto com o radical “liso” de vários lisofosfolipídeos.

O mecanismo não foi totalmente elucidado, entretanto é provável que uma hidroxila livre no carbono 2 seja responsável pelo reconhecimento. O benefício de tal experimento foi duplo; tanto por se tratar de uma espécie do gênero *Candida* que não fosse *C. albicans*, como por evidenciar a participação de lipídeos como ligantes da célula hospedeira.

Vários fungos, incluindo *C. albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Sporothrix schenckii* e *Saccharomyces cerevisiae*, se ligam ao glicoesfingolipídio lactosilceramida (Galβ1-4Gluβ1-1 Cer). A relevância biológica dessa observação foi estabelecer, *in vitro*, o bloqueio da aderência de *C. neoformans* às células nervosas humanas, pelo lipídio lactosilceramida (JIMENEZ-LUCHO et al., 1990).

Todas as propriedades eletrostáticas da superfície celular possuem um importante papel no processo de adesão. Soares et al. (2000) determinaram a contribuição do ácido siálico na carga iônica da superfície celular de *C. albicans* e identificaram sua ligação a sub-unidades galactosiladas. Rodrigues et al. (1997) e Soares et al. (1998) observaram por densidade, a similaridade entre resíduos deste ácido nesta espécie com de outros fungos patógenos, como *C. neoformans*, *F. pedrosoi*, *P. brasiliensis* e *S. schenckii*. Essas conclusões sugerem que esta molécula é responsável, pelo menos em parte, pela eletronegatividade da superfície da célula e pode representar um papel central na patogênese de *C.albicans*.

Para Varki (1997) ácidos siálicos são cadeias oligossacarídicas, com unidades terminais não reduzidas de glicoconjugados, pertencentes a uma família de açúcares carboxilados no carbono nove, e que possuem como estrutura comum o ácido *N*-acetil-neuramínico. Portanto, ácido siálico α2,3 e α2,6 ligados à galactose estão bastante presentes nos sialoglicoconjugados (POWELL; VARKI, 1996).

Além de todas as interações moleculares, os carboidratos comumente consumidos na dieta exercem uma significante influência nutricional sobre o aumento da aderência (PIZZO et al., 2000). Samaranayake e MacFarlane (1980) foram os primeiros a investigar esta hipótese quando mostraram uma maior adesão de *C. albicans* a dentaduras de acrílico, quando estas se desenvolveram em meios enriquecido de sacarose. McCourtie e Douglas (1981) relataram que sacarídeos específicos como sacarose e galactose poderiam aumentar a aderência de *C. albicans* a acrílico e células epiteliais da cavidade oral humana. O fato é que altas concentrações desses açúcares (500 mM) tornariam a superfície dessa levedura

mais fibrilar, e portanto, mais aderente que leveduras que se desenvolvem em um meio com, por exemplo, 50mM de glicose.

Similar observação foi verificada por Enache et al. (1996) que relatou o aumento da aderência de *C. albicans* a células esofágicas humanas, quando estas leveduras cresceram "overnight" em 500mM de galactose. Essas condições de aderência foram bloqueadas na presença de *N*-Acetil-glicosamina ou D-glicosamina.

Pizzo et al. (2000) demonstraram que a aderência de *C. albicans*, *C. tropicalis* e *C. krusei* a células epiteliais apareceu significantemente promovida quando estas espécies foram semeadas em meios ricos em sacarose, glicose, frutose e maltose. Em contrapartida, meios com sorbitol e xilitol provocaram uma diminuição na aderência. Esses resultados sugerem que o freqüente consumo de carboidratos como sacarose, glicose, frutose e maltose podem representar um fator de risco para candidíase oral. A limitação de seu consumo por substituição de sorbitol ou xilitol pode ser valorosa para o controle de infecção oral por espécies de *Candida*.

Embora muitas moléculas presentes na superfície fúngica podem promover a interação parasita-hospedeiro, outras ou essas próprias moléculas têm a capacidade de bloquear a adesão de leveduras a células humanas, como demonstrou Ollert et al. (1993), que os sacarídeos D-glicosamina, D-galactosamina e *N*-acetil-D-glicosamina atenuavam significantemente a adesão de *C. albicans*, a células esofágicas e queratinócitos humanos.

Como os glicídios, as proteínas também apresentam capacidade de inibir a ligação parasita-hospedeiro. Klotz et al. (1992) observaram que o peptídeo arginina-glicina-ácido aspártico (RGD), bloqueia a colonização aos tecidos de coelhos por *C. albicans*, quando este peptídeo é administrado antes da infecção induzida via hematogênica.

Sawyer et al. (1992) comparam um grupo de ratos tratado com RGD e um grupo não-tratado, e concluiram, após perfusão e análise do pulmão, que o primeiro grupo apresentou um alto grau de fagocitose e eliminação de *C. albicans*. Estas observações indicam que o peptídeo se liga ao parasita, atuando como opsoninas para as células de defesa do pulmão. Esses dois experimentos indicam, que o organismo possui em sua superfície, moléculas biologicamente ativas que reconhecem o referido peptídeo, todavia o papel destas adesinas na patogênese permanece desconhecido.

Em recente pesquisa, Polaquini et al. (2006) demonstraram os efeitos benéficos do extrato aquoso do vegetal *Azadirachta indica*, que possuiu um potencial efeito antiadesivo a dois isolados de *C. albicans* sobre resina. Este benefício pode ser atribuído a um ou mais componentes, incluindo oxidantes, flavinas e outras substâncias presentes no extrato. Todavia, adição do extrato alterou o comportamento da superfície celular, tornando-a mais hidrofóbica, que notadamente, deveria contribuir para interação entre a célula e superfícies como resina, e, portanto, está intimamente relacionado ao potencial patogênico do fungo (ANIL et al., 2001; SINGLETON et al., 2001). Os autores acreditam que um fator isolado, como a hidrofobicidade de superfície celular, não seja totalmente responsável pela aderência, mas uma conjunção de fatores estruturais e físico-químicos das células envolvidas no processo.

De acordo com Polaquini et al. (2006), os resultados obtidos reforçam a possibilidade do uso desse extrato na prevenção de infecção da cavidade oral por espécies de *Candida*, uma vez que agentes anti-sépticos, como clorexidina, possuem efeitos tóxicos a células humanas (ELLEPOLA; SAMARANAYAKE, 2001).

Em estudo desenvolvido por McCarron et al. (2004) foi possível verificar uma redução significante na aderência de blastosporos de *C. albicans* a células epiteliais da cavidade bucal, após serem tratadas com nanopartículas. As células da levedura absorveram as nanopartículas poliméricas, após incubação, o que levou ao aumento do diâmetro celular e diminuição na capacidade de adesão em cerca de 70% com relação a células não incubadas. Para os autores, isto sugere que aplicação tópica deste polímero pode ser usada na profilaxia de candidíase oral.

Além de estudos qualitativos, que apenas determinam os tipos de moléculas presentes na interface fungo-hospedeiro, ensaios quantitativos, com o objetivo de explorar a base molecular da aderência, estão sendo desenvolvidos. Nikawa et al. (2002) correlacionaram o conteúdo de ATP celular em isolados de *Candida* e sua capacidade de aderência a células epiteliais da cavidade oral. Utilizando-se desta quantificação, foi demonstrada a excelente correlação diretamente proporcional entre os níveis de ATP das células viáveis e sua habilidade no processo de adesão.

Atualmente, para estudar o papel de adesinas de *Candida* na patogênese de candidíase, modelos animais que refletem o estado imunodeficiente do hospedeiro humano

são requeridos (SUNDSTROM, 2002). De Repentigny et al. (2002), utilizando ratos transgênicos que expressavam HIV-1 em células do sistema imune, os quais desenvolviam uma patologia similar a AIDS, apresentaram extensa colonização do trato gastrointestinal, e tornaram-se mais susceptíveis a candidíase orofaríngea.

Sundstrom et al. (2002), com o intuito de estabelecer o papel da adesina Hwp1 na virulência do agente etiológico, utilizaram ratos imunodeficientes na imunidade inata e adquirida que se demonstraram extremamente susceptíveis a candidíase orofaríngea. Os autores concluíram que esses modelos prometem ser utilizado para avaliação do papel das adesinas de *Candida*.

Desta mesma forma, Glee et al. (2001) mensuraram através de um inovador sistema de análise, que foi originalmente desenvolvido para demonstrar o papel de selectinas e outras adesinas leucocitárias na vasculatura, a aderência de *C. albicans* às células endoteliais.

Conhecer todos os mecanismos e minúcias envolvidas no processo de adesão entre a célula fúngica e seu hospedeiro, tem-se demonstrado favorável para a procura de um alvo mais específico do fungo. Esse conhecimento irá conduzir para uma terapêutica antifúngica ideal, com redução dos efeitos colaterais e baixa resistência dos patógenos (ESQUENAZI et al., 2003).

1.2 Histoquímica com lectinas

A parede da célula das espécies de *Candida*, especialmente *C. albicans*, é uma significante origem de antígenos. Antígenos imunodominantes em *Candida* têm sido caracterizados como componentes da parede celular, a qual é rica em moléculas que podem desencadear uma resposta imune pela produção de anticorpos e pela ativação do complemento. Essas moléculas compreendem peptidomananos, galactomananos, glicanos, quitina e melanina (MARTÍNEZ et al., 1998; LACAZ et al., 2002).

Considerando a importância da parede celular fúngica López-Ribot et al. (2004) citam esta, como uma estrutura complexa e dinâmica de várias camadas, localizada externamente na membrana plasmática, e responsável pela manutenção da forma vegetativa do fungo. Participa da interação inicial entre o microrganismo e o ambiente, atua como

barreira permeável, possuindo funções nutricionais e protegendo o protoplasma contra injúrias físicas ou osmóticas.

Conforme Lopez-Ribot et al. (1999) os principais componentes da parede celular de leveduras, compreendendo entre 80 a 90% de sua constituição, são carboidratos: manano ou polímeros de manose covalentemente associados a proteínas, originando glicoproteínas, também referidas como mananoproteínas; β-glicanos, polímeros ramificados de glicose que possuem ligações β-1,3 e β-1,6 e quitina, um homopolímero não-ramificado de *N*-acetil-D-glicosamina contendo ligações β-1,4.

Manano, o primeiro componente identificado na parede celular das leveduras (KOCOUREK; BALLOU, 1969), é principalmente encontrado como polímeros N-ligados contendo várias centenas de resíduos de manose associadas em mananoproteínas de alto peso molecular, embora pequenos resíduos de mananoligosacarídeos N-ligados e/ou O-ligados estão associados com glicoproteínas de pequeno tamanho (CHAFIN et al., 1998).

Além de manose, todos os carboidratos representados essencialmente pelos polissacarídeos podem apresentar-se como homo ou heteropolímeros e encontram-se associados a polipeptídeos, constituindo as glicoproteínas da parede celular, que desempenham papéis enzimáticos, estruturais, homeostáticos, metabólicos e patogênicos, pois produzem estimulação antigênica no hospedeiro (SIDRIM; ROCHA, 2004).

De acordo com Coberllini et al. (1996), proteínas e lipídios estão presentes em menor quantidade na parede da célula fúngica. β-glucanos e quitina, maiores componentes da parede celular fúngica, formam uma rígida rede microfibrilar, mantêm a forma da célula e a resistência à lise pela fagocitose ou pelo complemento desencadeando reações granulomatosas, e tanto proteínas como glicomananoproteínas estão ligadas a este esqueleto, além de estarem presentes na superfície externa da célula.

López-Ribot et al. (2004) relatam que polímeros de manose (manano) representam 40% do total de carboidratos da parede celular e são o principal material dessa matrix, na qual os polímeros estruturais, β-glicano e quitina, estão imersos. Além disso, afirmam que manano não existe de maneira isolada na parede da célula, mas covalentemente associada com proteínas. Embora isso ocorra, o termo manano tem sido utilizado para referir-se ao principal componente imunodominante solúvel presente na camada externa da parede celular de *C. albicans*. Anticorpos contra esses carboidratos imunodeterminantes são

facilmente detectáveis em amostras de soro de pacientes com candidíase (CHAFIN et al., 1998; MARTÍNEZ et al., 1998). Nesse contexto, a parede celular de espécies de *Candida* não é apenas onde muitas funções biológicas essenciais ocorrem, mas também onde se origina uma significante quantidade de antígenos (LÓPEZ-RIBOT et al., 2004).

Segundo Nelson e Cox (2000) proteínas e lipídeos polares representam grande parte da massa das membranas biológicas, uma vez que os carboidratos presentes estão geralmente na forma de glicoconjugados, como glicoproteínas e glicolipídeos. As proporções relativas das proteínas e lipídeos são distintas em diferentes membranas refletindo a diversidade de suas funções biológicas (NELSON; COX, 2000).

Proteínas de origem não imunológica, como lectinas, as quais estão amplamente distribuídas em natureza, e são encontradas em seres unicelulares (YAMAGUCHI et al., 1998), pluricelulares, como fungos (KAWAGISHI et al., 1997), animais (YE; NG, 2000) e vegetais, as mais utilizadas (COELHO; SILVA, 2000), reconhecem carboidratos livres ou ligados às superfícies celulares, sem alterar a estrutura dos ligantes, através de sítios de ligação nos quais a hidrofobicidade é a principal força de interação (PEUMANS; VAN DAMME, 1998; NISHIMURA et al., 2000). Muitas lectinas são designadas de "aglutininas", utilizada nas suas abreviações, como *Ulex europaeus* agglutinin (UEA-1) e Peanut agglutinin (PNA) segundo uma lista de abreviações recomendada internacionalmente (PEUMANS; VAN DAMME, 1994).

A grande maioria das lectinas possui pelo menos dois sítios de reconhecimento a carboidratos ou derivados (aminoaçúcares, alquilácidos) a partir de uma porção limitada da molécula protéica. Este segmento é denominado de domínio de reconhecimento ao carboidrato (DRICKAMER, 1996; LORIS et al., 2000). Os carboidratos interagem com lectinas através de pontes de hidrogênio estabelecidas pela disponibilidade de um grande número de hidroxilas nos açúcares, que atuam como doadores ou receptores de hidrogênio e participam da interação lectina-carboidrato, interações hidrofóbicas e forças de Van Der Walls (SHARON; LIS, 2001).

Baseando-se na sua especificidade, cinco grupos de carboidratos podem ser identificados e estão largamente presentes em glicoproteínas e glicolipídeos das membranas celulares, como α-D-glicose/α-D-manose, α-L-fucose, ácido siálico, *N*-acetil-D-glicosamina e *N*-acetil-D-galactosamina/D-galactose (RÜDIGER, 1998; GABIUS, 2000).

Dentre as lectinas vegetais, as mais estudadas são as da família *Leguminosae,* isoladas principalmente de sementes (RÜDIGER, 1998). Essas glicoproteínas têm até 10% de carboidratos na forma de unidades N-carboidratos. Podendo ser de dois tipos: um grupo apresentando N-acetilglicosamina e manose e outro contendo uma estrutura única para plantas, com β1→2 xilose e α1→3 α-L-fucose (PEUMANS et al., 2000).

Devido à estabilidade química, metodologias de utilização bem padronizadas e sensibilidade no reconhecimento de porções de carboidratos específicos, as lectinas têm sido otimizadas e aplicadas como agentes mitogênicos e aglutinantes celulares (LOVATT et al., 2000), como determinantes de grupos sangüíneos (MORGAN; WATKINS, 2000), citotoxinas (GHOSH et al., 1999), inibidores do crescimento de células tumorais, marcadores de células transformadas (SAMES et al., 2001; BELTRÃO et al., 2003), bem como, em diversos estágios da interação parasita-hospedeiro nas doenças infecciosas (JACK et al., 2001).

Lectinas podem ser conjugadas com isotiocianato de fluroceína ou tetrametilrodamina e examinadas através da microscopia de fluorescência (REMANI et al., 1994); podem ainda ser conjugadas a enzimas como peroxidase e analisados por microscopia óptica (ZANBENEDETTI et al., 1998), ou serem biotiniladas (HONJO et al., 2000). Além disso, através da microscopia eletrônica, protocolos utilizando lectinas conjugadas a partículas de ouro coloidal ou prata, podem ser usadas como marcadores ultraestruturais prioritários para alguns tipos de células e tecidos (ROTH et al., 1998).

Segundo Shinagawa e Anderson (2000), a interação das lectinas com carboidratos no interior ou superfície das células e em fluidos fisiológicos, bem como os efeitos biológicos desta interação e facilidade de purificação fazem destas proteínas valiosos instrumentos para as diversas áreas da pesquisa biomédica.

O fenômeno natural de reconhecimento proteína-carboidrato está envolvido em diversos eventos celulares dentre os quais se destacam: a interface parasita-hospedeiro e o controle do crescimento, sinalização e motilidade das células (RÜDIGER et al., 2000). Tal reconhecimento é um evento central de inúmeros fenômenos biológicos, além de ser o primeiro passo de uma variedade de interações célula-célula, como fertilização, embriogênese, migração celular, defesa imunológica e infecção microbiana (ESQUENAZI et al., 2003).

Adicionalmente, reconhecimento específico entre fungos patogênicos e células do hospedeiro pode ser mediado pela interação de proteínas que se ligam a carboidratos, como as lectinas, presentes na superfície de uma das células e que possuirá complementaridade com açúcares na superfície da outra célula (ESQUENAZI et al., 2003).

Esta hipótese é suportada por modelos de estudo que evidenciam moléculas receptoras na interface fungo-hospedeiro (MENDES-GIANNINI et al., 2000). Diante disso, a composição qualitativa e quantitativa dos glicoconjugados das membranas celulares torna-se significativa no desenvolvimento e evolução de várias doenças (TAKANO et al., 2000; YU et al., 2001).

Pesquisas têm desenvolvido biomarcadores de fácil aplicação e alta sensibilidade no monitoramento das glicoproteínas superficiais nos mais diversos modelos biológicos (PLZAK et al., 2001; THEILER et al., 2001). Dentre os métodos de mapeamento de carboidratos estão os imunoensaios utilizando anticorpos (DANGUY et al., 2001), técnicas de biologia molecular (VILLANUEVA, 2002) e outros métodos sorológicos quantitativos (JOHANSEN et al., 2000; TAKAHASHI et al., 2000).

A descoberta de determinados carboidratos na superfície de microrganismos patogênicos tem causado impacto devido à importância destes açúcares na interação dos fungos e bactérias com seus hospedeiros (SHARON; OFEK, 2000).

A constituição da parede celular pode ser um critério taxonômico adicional de distinção entre espécies fúngicas. Ao lado de técnicas como extrações químicas, degradação enzimática seguida pela análise dos produtos hidrolisados, comparação de frações antigênicas utilizando soro imune ou microscopia eletrônica, o uso de lectinas contribui significativamente para a caracterização da natureza dos açúcares presentes na parede celular (GUILLOT et al., 1990).

Conforme Guillot et al. (1990) e Penha e Bezerra (2000), a aplicação de técnicas histoquímicas que utilizam lectinas vegetais para este tipo de abordagem, baseia-se no princípio de que a interação lectina-carboidratos favorece o entendimento sobre a composição glicídica da parede celular das espécies fúngicas, além do comportamento das micoses, tendo assim, sido testadas como ferramentas auxiliares, para a identificação de agentes etiológicos das mais variadas micoses.

Para Kwon-Chung e Bennett (1992) a dificuldade no diagnóstico de muitas micoses está relacionada a vários fatores, como a variedade de lesões, seus subtipos morfológicos e agentes etiológicos envolvidos, uma vez que, vários estudos citam lesões bastante semelhantes, embora possam ocorrer por diferentes espécies.

As lectinas, por serem altamente seletivas e específicas no reconhecimento de carboidratos, têm sido aplicadas como biomarcadores de açúcares da superfície de microrganismos, como leveduras (KORTING; OLLERT, 1994; MUÑOZ et al.; 2003) e bactérias (MUÑOZ et al., 1994; MUÑOZ et al., 1999). Muñoz et al. (2003) realizaram testes de aglutinação sobre superfície de cinco espécies de *Candida*, utilizando 14 diferentes lectinas para abranger uma larga especificidade de açúcares. Nesse estudo, foram demonstradas significativas variações interespecíficas nos padrões de aglutinação, indicando uma possível utilização desse teste para identificação e tipagem das leveduras.

De acordo com vários autores, o papel das lectinas em diagnósticos microbiológicos tem sido estudado em detalhes (DOYLE; KELLER, 1984; MUÑOZ et al. 1999; MUÑOZ et al., 2003). Está claro que nas leveduras, principalmente espécies de *Candida*, lectinas se ligam a carboidratos abundantes sobre a superfície celular, como α-manano, e tais testes não são aplicáveis, apenas quando esses açucares são removidos por processos químicos (NETH et al., 2000; MUÑOZ et al., 2003).

Bies et al (2004) relatam o uso de lectinas em sistemas de liberação controlada de drogas, as quais funcionam como proteínas que direcionam as drogas para alvos específicos, como microrganismos, células cancerosas ou com distúrbios metabólicos refletidos na superfície celular por uma expressão acentuada de carboidratos superficiais.

Atualmente, muitas questões sobre a natureza e origem da enorme heterogeneidade das glicoproteínas foram elucidadas, contudo, até o momento, sabe-se que as funções dos carboidratos nas glicoproteínas são indubitavelmente tão complexas quanto às dos aminoácidos em uma proteína, tendo sido o "glicocódigo" considerado um elemento importante correlacionado com diagnóstico e potencial patológico de diferentes microrganismos (SHARON, 2000).

Ao que tudo indica, a histoquímica de lectinas tem se tornado a técnica preferida para o entendimento de vários aspectos da biologia microbiana e que num futuro próximo

poderá tornar-se um dos instrumentos padrão na microbiologia moderna e na indústria farmacêutica.

1.3 Coleções de Culturas

A caracterização e a atualização taxonômica dos fungos são importantes para o conhecimento e diferenciação entre isolados de uma mesma espécie, bem como de espécies diferentes, tornando-se relevante o estudo de isolados depositados em Coleções de Culturas (SMITH; ONIONS, 1983).

Segundo Hawksworth e Kirsop (1998); Kirsop e Kurtzman (1998), culturas estocadas tanto de leveduras quanto de fungos filamentosos, representam elementos fundamentais em diversas pesquisas, tanto de ciência pura quanto aplicada. Desta forma, os isolados de *Candida* estocados na Coleção de Culturas – Micoteca URM do Departamento de Micologia do Centro de Ciências Biológicas (CCB), Universidade Federal de Pernambuco (UFPE), obtidos de diversos substratos, são de grande importância como um suporte no campo médico e biotecnológico.

A referida Coleção de Culturas é registrada no Commonwealt Mycological Institute (CMI) sob a sigla URM e na World Federation of Culture Collections sob o número 604, está citada em vários catálogos, destacando-se a ATCC (Washington, USA), IFO (Osaka, Japão) e WFCC (Japão).

A Micoteca URM detém o maior número de culturas no Brasil e os métodos utilizados para sua preservação incluem óleo mineral (SHERF, 1943), água destilada (CASTELLANI, 1967) e liofilização (CAVALCANTI et al., 1996). Atualmente constam na coleção aproximadamente 6500 culturas de fungos de diferentes grupos taxonômicos, incluindo leveduras e filamentosos, entre as quais se encontram os isolados de *Candida* (CAVALCANTI et al., 1996).

Na última década tem ocorrido uma maior consciência quanto ao valor das coleções de culturas de microrganismos tanto para conservação de recursos genéticos quanto para biodiversidade, assim como fornecimento de elementos essenciais para emergir projetos e indústrias baseadas na biotecnologia. Assim, os centros de recursos microbianos são vistos como elementos essenciais para a sustentação do desenvolvimento científico mundial (ATCC, 2005).

Considerando a relevância das espécies de *Candida*, estudos com culturas estocadas permitirão a ampliação do Banco de Fungos de Interesse Médico, caracterizados em diversos aspectos como: virulência, patogenicidade, determinação da variedade entre outros fatores, possibilitando ainda a realização de novas pesquisas pelo conhecimento prévio dos isolados.

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3 CAPACIDADE DE ADERÊNCIA DE *Candida albicans* E *Candida parapsilosis* ÀS CÉLULAS EPITELIAIS DA CAVIDADE ORAL CORRELACIONADO AOS CARBOIDRATOS DE SUPERFÍCIE CELULAR

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Resumo

Vários estudos estão disponíveis sobre aderência de *Candida albicans* às células epiteliais, mas pouco é conhecido sobre adesão de *Candida parapsilosis* e sua correlação com o reconhecimento de carboidratos da superfície celular. Este estudo avaliou a habilidade de aderência de 20 isolados de *C. albicans* e de 15 *C. parapsilosis* às células epiteliais da cavidade oral, além da expressão de carboidratos da superfície celular através de histoquímica com lectinas. Ensaios de aderência foram realizados incubando as células epiteliais com suspensões de leveduras (10^7 células.mL^{-1}) por 2h a 25°C. Para histoquímica, lectinas conjugadas a peroxidase (Con A, WGA, UEA I e PNA a 25 μg/mL) foram usadas com incubação por 2h a 25°C. Nossos resultados mostraram que *C. albicans* é mais aderente que *C. parapsilosis* ($P<0.01$), além de apresentar um alto conteúdo de resíduos de α-L-fucose em sua superfície celular, como evidenciado pelo padrão de marcação com UEA I. Três isolados de *C. parapsilosis* apresentaram valores similares na aderência e padrão de marcação por UEA I dos isolados de *C. albicans*, indicando que a presença de componentes fucosilados, pode representar uma forma adicional através do qual interações destas leveduras com células do hospedeiro pode ser estabelecida ($r=0.8336$, $P=0.0001$). Além disso, nossos resultados mostraram a presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico e D-galactose/*N*-acetil-D-galactosamina na parede celular fúngica.

Palavras-chave: *Candida albicans*; *Candida parapsilosis*; Aderência; Células epiteliais; Histoquímica com lectinas

1. Introdução

Aderência é o primeiro passo na colonização microbiana e um evento chave para o desencadeamento do processo patogênico [1]. *Candida albicans* (Robin) Berkhout é a mais aderente e promissora levedura na colonização da cavidade oral [2], enquanto que *Candida parapsilosis* (Ashford) Langeron et Talice tem emergido como importante patógeno nosocomial com inúmeras implicações clínicas [3,4].

Os mecanismos de aderência das espécies de *Candida* a tecidos humanos são variados, e como resultado, os sistemas de reconhecimento celular *Candida*-hospedeiro são complexos e envolvem uma variedade de componentes [5,6]. Portanto, reconhecimento específico entre parasitas fúngicos e as células de seus hospedeiros, pode ser mediado pela interação de proteínas ligadoras de carboidratos, como lectinas, sobre a superfície de uma das células, que irá reconhecer açúcares complementares sobre a superfície da outra célula [7,8].

Lectinas são proteínas de origem não imunológica que se ligam especificamente a carboidratos [9]. A seletividade das lectinas para açúcares da superfície microbiana já foi demonstrada para leveduras de *Candida* [10].

Embora muitos autores relatem que *C. albicans* e *C. parapsilosis* possuem muitas similaridades, poucos estudos comparam a capacidade de aderência destas leveduras às células epiteliais da cavidade oral, e relaciona estas observações a expressão de carboidratos de superfície celular. Além disso, a elucidação dessas interações, supostas adesinas e moléculas receptoras na interface fungo-hospedeiro, pode ajudar a desenvolver uma terapêutica mais apropriada para candidíase. O presente estudo tem o objetivo de avaliar a correlação entre aderência às células epiteliais e o perfil de carboidratos da superfície celular de *C. albicans* e *C. parapsilosis*.

2. Materiais e métodos

2.1. Leveduras e condições de crescimento

Um total de 34 isolados de *Candida*, sendo 20 *C. albicans* e 14 *C. parapsilosis*, foram adquiridos na Coleção de Culturas – Micoteca URM, do Departamento de Micologia, Centro de Ciências Biológicas da Universidade Federal de Pernambuco e estavam estocados em óleo mineral [11]. Uma linhagem de *C. parapsilosis* ATCC 22019

foi usada como referência. Testes de viabilidade e confirmação taxonômica foram realizados através de inoculação das culturas em tubos de ensaio com 5 mL de caldo glicosado a 2% [12]. Após desenvolvimento, culturas foram transferidas para tubos com ágar Sabouraud adicionado de extrato de levedura (Difco), para terem as características morfológicas, bioquímicas e fisiológicas observadas [13]. Espécie, número de registro, tempo de estocagem e substrato de isolamento estão sumarizados na Tabela 1.

2.2. Células e ensaios de aderência

Separação celular e ensaios de aderência foram realizados de acordo com os protocolos de Kimura & Pearsall [14] e Sobell et al. [15], com modificações. As células das leveduras se desenvolveram em ágar malte (Difco) por 36h a 37 °C e ressuspensas em 2mL de tampão fosfato (PBS) esterilizado a 0.1M, pH 6.8 e 4°C; lavadas duas vezes em 2 mL de PBS sob centrifugação (2.500 rpm, 5 min cada), e por fim ressuspendidas novamente em PBS a uma concentração de $2x10^7$ células.mL^{-1}, determinada por câmera de Neubauer. As células epiteliais foram coletadas de voluntário adulto saudável, através de suave escarificação da mucosa oral com "Swabs" esterilizados. Cada "Swab" foi gentilmente agitado em 5mL of PBS para liberar as células. As suspensões de células epiteliais foram lavadas duas vezes em 5 mL of PBS sob centrifugação (2.500 rpm, 5 min cada) para remover as bactérias que estivessem aderidas. As células foram ressuspensas em PBS a uma concentração de $4x10^4$ células.mL^{-1}, determinada por câmera de Neubauer. Após as lavagens, as células de leveduras epiteliais foram morfologicamente examinadas quanto a viabilidade e integridade. Ensaios de aderência foram realizados incubando a 25°C uma mistura de 1 mL de cada suspensão em um tubo de ensaio, sob suave agitação por 2h. Em seguida, realizou-se coloração com azul de metileno, com posterior observação em microscopia de luz, onde em cada lâmina, foram avaliadas 100 células epíteliais com relação à percentagem de sua área superficial aderida pelas leveduras. Os resultados foram graduados como: forte aderência (H) para adesão entre 50% a 100% da área superfície, fraca aderência (w) para adesão até 50% e sem aderência visível (0). Além disso, o número de células de leveduras aderidas a dez células epiteliais, em cada isolado, foi contado para análise estatística.

2.3. Histoquímica com lectinas

As lectinas usadas foram selecionadas para garantir uma ampla variedade de açúcares específicos. A histoquímica com lectinas foi desenvolvida de acordo com Rego et al. [16] usando Concanavalina A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA I) e peanut agglutinin (PNA), todas conjugadas a peroxidase (Sigma Chemical Co., St Louis, MO, USA). As culturas fúngicas, em ágar Sabouraud (Difco) com 15 dias de crescimento, foram suspensas em PBS (0,01M, pH 7,2) a concentração de 10^7células.mL^{-1}. Em seguida, a suspensão foi tratada com tripsina a 1% em PBS por 3 min seguida por solução metanol-H_2O_2 por 5 min e incubada com as lectinas (25 µg/mL) por 2h a 25°C. A reação com a peroxidase foi visualizada através de diaminobendizina (DAB) e peróxido de hidrogênio em PBS por 5-8min a 25°C. Entre cada protocolo, as células foram lavadas com PBS sob centrifugação (2.500 rpm por 5 min). Ensaios controles foram feitos por ligação da lectina na presença do açúcar específico corresponde: metil-α-D-manosídeo, *N*-acetil-D-glicosamina, α-L-fucose and D-galactose para Con A, WGA, UEA I e PNA, respectivamente, a concentração de 300 mM. Padrão de marcação foi avaliado por microscopia de luz e classificado qualitativamente como intenso (+++), moderado (++), fraco (+) ou negativo (-), conforme Ozer [17].

2.4. Análise estatística

Todos os ensaios foram desenvolvidos em triplicata para cada isolado e protocolos repetidos pelo menos 12 vezes. A média e o desvio padrão dos três distintos experimentos foram determinados. Análise estatística foi calculada através do programa STATISTICA 6.0 (StatSoft, Inc.). A diferença para a capacidade de aderência das duas espécies estudadas foi determinada por teste *t*. A relação entre o fator de virulência investigado e a histoquímica com lectinas foram avaliados pelo teste de correlação de Pearson. Valor de *P* igual ou abaixo de 0,05 foi considerado estatisticamente significante [18].

3. Resultados

A capacidade de aderência dos isolados de *C. albicans* e *C. parapsilosis* às células epiteliais esta sumarizada na Tabela 2. Doze (60%) dos 20 isolados de *C. albicans* aderiram

fortemente, enquanto que apenas três (20%) dos 15 de *C. parapsilosis* se comportaram similarmente (Fig. 1).

As análises da histoquímica com lectinas utilizando Con A, WGA, UEA I e PNA indicaram a presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico, α-L-fucose e D-galactose/*N*-acetil-D-galactosamina em todos os isolados estudados de *C. albicans* e *C. parapsilosis*, embora em diferentes intensidades de marcação (Tabela 2).

Isolados de *C. albicans* apresentaram fraco padrão de marcação com Con A, WGA e PNA e moderada marcação com UEA I. Isolados de *C. parapsilosis* foram fracamente marcados por Con A e PNA, enquanto que WGA mostrou variações na marcação entre moderada a intensa e UEA I entre fraca a moderada (Tabela 2).

4. Discussão

A aderência de *Candida* às células epiteliais é a principal característica patogênica do gênero. Estudos mostram variações nas diferentes espécies de *Candida*, em sua capacidade de se aderir, explicando o porquê de algumas espécies colonizarem mais freqüentemente as mucosas [5,19].

A média dos valores de aderência dos 20 isolados *C. albicans* e 15 de *C. parapsilosis* foram 49,95 e 25,60, respectivamente, apresentando diferenças estatisticamente significantes ($P < 0.01$). Em geral, a avidez de *C. albicans* às células epiteliais foi maior que *C. parapsilosis*. Estes resultados estão de acordo com as observações de Repentigny et al. [20], que investigaram a habilidade de ligação *in vitro* de várias espécies de *Candida* a mucina purificada, encontrando diferenças significantes na adesão dessas espécies, e correlacionando com sua hierarquia de virulência. Nestes experimentos *C. albicans* demonstrou maior capacidade de aderência que *C. parapsilosis*.

Histoquímica com lectinas mostrou que todos os isolados de *C. albicans* apresentaram mais resíduos de α-L-fucose, que os isolados *C parapsilosis*. Vale ressaltar que, os três isolados de *C. parapsilosis* (4984, 4889 e 4261) que foram mais aderentes, também demonstraram mais resíduos de α-L-fucose do que os outros isolados desta espécie, exceto a linhagem ATCC 22019, a qual apresentou moderado padrão de expressão para este açúcar, mas fraca capacidade de aderência. Essa linhagem vem sendo mantida por repiques

sucessivos em nosso laboratório, fato que pode ter atenuado caracteríticas fenotípicas da levedura, como fatores de virulência [21].

Esses resultados combinados nos levam a acreditar que, a presença de componentes fucosilados sobre a superfície destas leveduras, pode ser indicativo de um novo mecanismo, pelo qual as células fúngicas interagem com estruturas do hospedeiro. Quando esses dados foram correlacionados, significante correlação positiva foi observada entre adesão e expressão de α-L-fucose pela marcação específica por UEA I ($r = 0.8336$, $P = 0.0001$).

Além disso, nosso trabalho ratifica a esperada presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico e D-galactose/*N*-acetil-D-galactosamina, sugerindo que os carboidratos da parede celular contém outros monossacarídeos, além de manose, e que esta estrutura pode ter uma estrutura complexa além do pensado. A presença de oligossacarídeos isentos de manose está consistente com relatos prévios de Lopez-Ribot et al. [22] e Soares et al. [23].

Munõz et al. [10] decreveram o perfil de marcação com lectinas, para vários isolados de *Candida*, todas com especificidade para α-D-manose. Estes experimentos estão de acordo com o presente estudo, embora existam variações. Essas diferenças, que ocorrem devido às falhas no reconhecimento dos carboidratos, podem ser explicadas pelo painel de lectinas utilizado. Além disso, realizamos um pré-tratamento enzimático nas suspensões de levedura com tripsina, para garantir a acessibilidade aos resíduos dos açúcares pelas lectinas e utilizamos como único meio de crescimento ágar Sabouraud (Difco), uma vez que estudos sugerem que a expressão de carboidratos varia conforme a idade e o ambiente de desenvolvimento do fungo [24].

Os isolados de *C. albicans* que apresentaram fraca aderência, com exceção de 3716 e 3719, foram isolados de pacientes com AIDS, que receberam tratamento antifúngico, o que provavelmente diminuiu a habilidade de aderência desses isolados. *C. albicans* 3716 e 3719 foram isolados através da escarificação de dentina de paciente saudável, sem lesões clínicas, conseqüentemente da microbiota natural que é característica por ter baixa patogenicidade.

Carboidratos de superfície celular ou glicoconjugados possuem funções críticas em muitos processos celulares fundamentais, como interações célula-célula e microrganismo-hospedeiro [25,26]. Lectinas possuem importante papel nessas interações e tem

demonstrado enorme impacto histopatologia e biologia celular [27]. Essas moléculas são encontradas em microrganismos, plantas e animais [28].

Na histoquímica, lectinas com diferentes especificidades são ferramentas utilizadas para investigar mudanças na glicolisação e expressão de carboidratos, que pode ocorrer em situações normais ou patológicas [27,29].

O conhecimento do perfil sacarídico na superfície fúngica habilita o uso de lectinas, além de promissoras sondas celulares, para ser utilizada em tratamentos terapêuticos como já proposto para câncer por Mody et al. [30]. Uma vez que, estruturas que contém fucose estão presentes sobre a superfície celular de *Candida*, é possível que lectinas ligadoras de fucose, como UEA I, possa ser à base de um tratamento terapêutico para candidíase. Adicionalmente, lectinas podem servir como carreadores de agentes antifúngicos, que utiliza como alvos específicos os carboidratos existentes na superfície da célula dos microrganismos [31].

No presente estudo, relatamos o parcial perfil de carboidratos expressados na superfície celular de duas espécies de *Candida*, *C. albicans* e *C. parapsilosis*, e evidenciamos uma correlação com sua habilidade de adesão às células epiteliais da cavidade oral. Histoquímica com lectinas foi um método chave para alcançar esses resultados, se apresentando como uma potencial ferramenta para a caracterização dos glicoconjugados de superfície das leveduras. Embora muitas questões permaneçam, acreditamos que carboidratos complexos sobre a superfície de *Candida* podem representar uma maneira pela qual, interações do fungo com as células do hospedeiro possam ser estabelecidas.

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Tabela 1

Tabela 1. Isolados de *Candida* Obtidos da Micoteca URM.

Espécies	*Número de registro (URM)*	*Tempo de estocagem (anos)*	*Substrato de isolamento*
Candida albicans	4990	01	Secreção vaginal
C. albicans	4987	01	Secreção vaginal
C. albicans	4986	01	Secreção vaginal
C. albicans	4820	02	Escamas ungueais
C. albicans	4819	02	Escamas ungueais
C. albicans	4817	02	Escamas ungueais
C. albicans	4609	03	Sangue
C. albicans	4606	03	Sangue
C. albicans	4388	05	Secreção orofaríngea
C. albicans	4387	05	Secreção orofaríngea
C. albicans	4386	05	Secreção orofaríngea
C. albicans	4385	05	Secreção orofaríngea
C. albicans	4384	05	Secreção orofaríngea
C. albicans	4260	05	Secreção orofaríngea
C. albicans	4127	07	Escamas inguinais
C. albicans	4126	07	Urina
C. albicans	4125	07	Esputo
C. albicans	4124	07	Secreção orofaríngea
C. albicans	3719	10	Escarificação de dentina
C. albicans	3716	10	Escarificação de dentina
C. parapsilosis	4984	01	Secreção vaginal
C. parapsilosis	4970	01	Secreção vaginal
C. parapsilosis	4889	02	Sangue
C. parapsilosis	4818	02	Escarificação ungueal
C. parapsilosis	4804	02	IFM*
C. parapsilosis	4608	03	Sangue
C. parapsilosis	4607	03	Sangue
C. parapsilosis	4433	04	Solo de praia
C. parapsilosis	4261	05	Secreção orofaríngea
C. parapsilosis	3627	12	Esputo
C. parapsilosis	3624	12	Esputo
C. parapsilosis	3621	12	Esputo
C. parapsilosis	2633	15	**
C. parapsilosis	729	49	**

*IFM (Institute of Food Microbiology)

**Substrato não identificado

Tabela 2

Tabela 2. **A**derência a células epiteliais e perfil histoquímico dos isolados de *C. albicans* e de *C. parapsilosis* estudados.

Isolados	*Capacidade de aderência*	*Padrão de marcação com lectinas*			
		Con A	*WGA*	*UEA-1*	*PNA*
4990	H	+	+	++	+
4987	H	+	+	++	+
4986	H	+	+	++	+
4820	H	+	+	++	+
4819	H	+	+	++	+
4817	H	+	+	++	+
4609	H	+	+	++	+
4606	H	+	+	++	+
4388	H	+	+	++	+
4387	W	+	+	++	+
4386	W	+	+	++	+
4385	W	+	+	++	+
4384	W	+	+	++	+
4260	W	+	+	++	+
4127	H	+	+	++	+
4126	H	+	+	++	+
4125	W	+	+	++	+
4124	H	+	+	++	+
3719	W	+	+	++	+
3716	W	+	+	++	+
4984	H	+	++	++	+
4970	W	+	++	+	+
4889	H	+	+++	++	+
4818	W	+	++	+	+
4804	W	+	++	+	+
4608	W	+	++	+	+
4607	W	+	++	+	+
4433	W	+	++	+	+
4261	H	+	++	++	+
3627	W	+	++	+	+
3624	W	+	++	+	+
3621	W	+	++	+	+
2633	W	+	++	+	+
729	W	+	+	+	+
22019	W	+	++	++	+

Forte aderência (H) ou fraca aderência (W).
Intenso (+++), moderado (++) ou fraco (+) padrão de marcação.

Figura 1

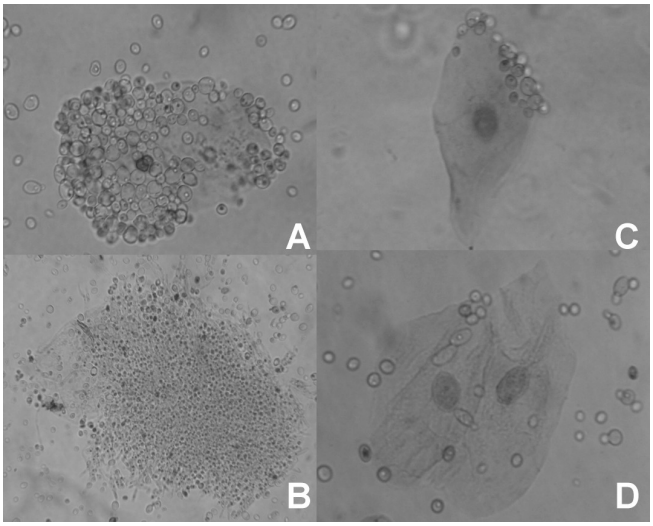

Figura 1. Forte aderência de *C. albicans* 4609 (A) e *C. parapsilosis* 4984 (B) às células epiteliais da cavidade oral. Fraca aderência de *C. albicans* 3719 (C) e *C. parapsilosis* 4818 (D). Ampliação: x 400.

4 CARBOIDRATOS DE SUPERFÍCIE CELULAR ASSOCIADOS COM ADESÃO DE ESPÉCIES DE *CANDIDA* ÀS CÉLULAS EPITELIAIS

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CARBOIDRATOS DE SUPERFÍCIE CELULAR ASSOCIADOS COM ADESÃO DE ESPÉCIES DE *CANDIDA* ÀS CÉLULAS EPITELIAIS

Candida: Adesão celular associada a glicoconjugados

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Resumo

Existem vários estudos sobre a capacidade de aderência das espécies de *Candida* às células epiteliais, mas estudos comparativos baseados neste fator de patogenicidade versus reconhecimento celular através de carboidratos de superfície celular são raros. Este estudo avaliou a habilidade de aderência de 20 isolados de *C. albicans*, cinco de *C. tropicalis* e seis de *C. krusei* às células epiteliais da cavidade oral, em relação a expressão de carboidratos da superfície celular avaliado através de histoquímica com lectinas. Ensaios de aderência foram realizados incubando as células epiteliais com suspensões de leveduras (10^7 células.mL^{-1}) por 2h a 25°C. Para histoquímica, lectinas conjugadas a peroxidase (Con A, WGA, UEA I e PNA a 25 μg/mL) foram usadas por 2h a 25°C. Nossos resultados mostraram que *C. albicans* e *C. tropicalis* foram mais aderente que *C. krusei*, além de apresentar um alto conteúdo de α-L-fucose em sua superfície celular, como evidenciado pelo padrão de marcação com UEA I. Além disso, nossos resultados mostraram a presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico e D-galactose/*N*-acetil-D-galactosamina na parede celular fúngica de todos os isolados estudados. Esses achados associam o aumento da capacidade de aderência de *Candida* com a presença de níveis significantes de compostos fucosilados. Embora muitas questões permaneçam, acreditamos que a presença de carboidratos complexos sobre a superfície de espécies de *Candida* podem representar uma maneira através da qual, interações destas leveduras com células do hospedeiro possam ser estabelecidas.

Palavras-chave: *Candida albicans*; *Candida tropicalis*; *Candida krusei*; Aderência; Células epiteliais; Histoquímica com lectinas.

1. Introducão

Aderência microbiana a superfície das mucosas é mostrada como importante evento no processo infeccioso, particularmente na cavidade oral (Sundstrom, 2002). Espécies de *Candida*, os agentes etiológicos da candidíase, são as mais aderentes e promissoras leveduras na colonização da cavidade oral (Ellepola & Samaranayake, 2001). Os mecanismos de aderência das espécies de *Candida* a tecidos humanos são variados, e como resultado, os sistemas de reconhecimento celular *Candida*-hospedeiro são complexos e envolvem uma variedade de componentes (Jabra-Rizk et al., 2001; Marchais et al., 2005).

Adesinas, proteínas envolvidas na aderência das leveduras, são capazes de reconhecer componentes (ligantes ou receptores) sobre a célula hospedeira (Sturtevant & Calderone, 1997) como carboidratos (Mendes-Giannini et al., 2000).

A histoquímica com lectinas tem sido utilizada para explorar diferenças no perfil de carboidratos de células e tecidos. Tais variações ocorrem na superfície das células durante processos fisiológicos e patológicos (Sharon & Lis, 2004). Essas interações necessitam de modelos, para elucidar as supostas adesinas e moléculas receptoras na interface fungo-hospedeiro, e então desenvolver uma terapêutica mais apropriada para candidíase (Esquenazi et al., 2003).

O presente estudo tem o objetivo de avaliar a correlação entre a patogenicidade de *Candida albicans*, *C. tropicalis* e *C. krusei*, via ensaios de aderência, e seu perfil de carboidratos de superfície celular através de histoquímica com lectinas.

2. Materiais e métodos

2.1. Leveduras e condições de crescimento

Um total de 30 isolados de *Candida* utilizados nesse estudo foi obtido da Coleção de Culturas – Micoteca URM, do Departamento de Micologia, Centro de Ciências Biológicas da Universidade Federal de Pernambuco. Estes isolados estavam estocados em óleo mineral (Sherf, 1943) e seus números de registro, tempo de estocagem, em anos, e substrato de isolamento estão sumarizados na Tabela 1. Testes de viabilidade e confirmação taxonômica foram realizados através de inoculação das culturas em tubos de ensaio com 5 mL de caldo glicosado a 2% (Fennell, 1960). Após desenvolvimento, culturas foram transferidas para tubos com ágar Sabouraud adicionado de extrato de levedura (Difco), para

terem suas características morfológicas, bioquímicas e fisiológicas observadas (de Hoog et al., 2000). Uma linhagen de *C. krusei* ATCC 6258 foi usada como referência.

2.2. Obtenção das células

Células de leveduras se desenvolveram em ágar malte (Difco) por 36h a 37 ºC e então suspendidas em 2mL de tampão fosfato (PBS) esterilizado 0,1M, pH 6,8 e 4°C; lavadas duas vezes em 2 mL de PBS sob centrifugação (2.500 rpm, 5 min cada), e por fim ressuspendidas novamente em PBS a uma concentração de $2x10^7$ células.mL^{-1}, determinada por câmera de Neubauer. As células epiteliais foram coletadas de voluntário adulto saudável, através de suave escarificação da mucosa oral com "Swabs" esterilizados. Cada "Swab" foi gentilmente agitado em 5mL of PBS para liberar as células. As suspensões de células epiteliais foram lavadas duas vezes em 5 mL of PBS sob centrifugação (2.500 rpm, 5 min cada) e ressuspendidas em PBS a uma concentração de $4x10^4$ células.mL^{-1}. Leveduras e células epiteliais foram morfologicamente examinadas em sua viabilidade e integridade.

2.3. Ensaios de aderência

Ensaios de aderência foram modificados dos protocolos estabelecidos por Kimura & Pearsall (1978) e Sobell et al. (1981). Ensaios de aderência foram avaliados incubando a 25°C uma mistura de 1 mL de cada suspensão em um tubo de ensaio, sob suave agitação por 2h. Realizou-se coloração com azul de metileno, com posterior observação em microscopia de luz, onde em cada lâmina, foram avaliadas 100 células epíteliais com relação à percentagem de sua área superficial aderida pelas leveduras. Os resultados foram graduados como: intensa aderência (H) para adesão entre 50% a 100% da área superfície, fraca aderência (w) para adesão até 50% e sem aderência visível (0). Além disso, o número de células de leveduras aderidas a dez células epiteliais, em cada isolado, foi contado para análise estatística.

2.4. Histoquímica com lectinas

As lectinas usadas foram selecionadas para garantir uma ampla variedade de açúcares específicos. A histoquímica com lectinas foi desenvolvida de acordo com Rego et al. (2005) usando Concanavalina A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA-1) e peanut agglutinin (PNA), todas conjugadas a peroxidase (Sigma Chemical Co., St Louis, MO, USA). As culturas fúngicas, em ágar Sabouraud

(Difco), foram suspensas em PBS (0,01M, pH 7,2) a concentração de 10^7células.mL^{-1}. Em seguida, a suspensão foi tratada com tripsina a 1% em PBS por 3 min seguida por solução metanol-H_2O_2 por 5 min e incubada com as lectinas (25 µg/mL) por 1h a 25°C. Reação com a peroxidase foi visualizada através de diaminobendizina (DAB) e peróxido de hidrogênio em PBS por 5-8min a 25°C. Entre cada protocolo, as células foram lavadas com PBS sob centrifugação (2.500rpm por 5 min). Ensaios controles foram feitos por ligação da lectina na presença do açúcar específico corresponde: metil-α-D-manosídeo, *N*-acetil-D-glicosamina, α-L-fucose and D-galactose para Con A, WGA, UEA I e PNA, respectivamente, a concentração de 300 mM. Padrão de marcação foi avaliado por microscopia de luz e classificado qualitativamente como intenso (+++), moderado (++), fraco (+) ou negativo (-), conforme Ozer (2000).

2.5. Análise estatística

Todos os ensaios foram desenvolvidos em triplicata para cada isolado e protocolos repetidos pelo menos 12 vezes. A média e o desvio padrão dos três distintos experimentos foram determinados. Análise estatística foi realizada através de análise de variância e calculada pelo programa STATISTICA 6.0 (StatSoft, Inc.). Valor de *P* igual ou abaixo de 0,05 foi considerado estatisticamente significante (Ferguson, 1971).

3. Resultados

Os resultados obtidos mostram que todas as espécies de *Candida* estudadas aderem às células epiteliais, mas em diferentes extensões. Doze (60%) dos 20 isolados de *C. albicans* aderiram fortemente. Quatro (80%), dos cinco isolados de *C. tropicalis* também apresentaram forte aderência às células. Entretanto, todos os isolados de *C. krusei* apresentaram fraca habilidade de aderência (Fig. 1).

As análises da histoquímica com lectinas indicaram a presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico, α-L-fucose e D-galactose/*N*-acetil-D-galactosamina em todos os isolados estudados de *C. albicans*, *C. tropicalis* e *C. krusei*, embora em diferentes concentrações, com exceção do isolado 916 que não apresentou resíduos de galactose (Tabela 2).

Os Isolados de *C. albicans* and *C. tropicalis* apresentaram fraco padrão de marcação para Con A, WGA e PNA e marcação moderada para UEA I. Isolados de *C. krusei* não

mostraram um padrão de marcação uniforme. Estes isolados demonstraram variações entre moderado a intenso padrão para WGA e fraco a intenso padrão para UEA I. Con A e PNA, reconheceu fracamente todos os isolados de *C. krusei*, exceto o 1059 que apresentou moderada marcação para Con A e intensa para PNA (Tabela 2).

4. Discussão

Aderência é o primeiro passo para o estabelecimento do processo infeccioso. Muitos estudos mostram a capacidade de aderência das espécies de *Candida* às mucosas e seu envolvimento em patologias infecciosas (Pizzo et al., 2000; Jabra-Rizk et al., 2001; Liu et al., 2006). Contudo, mudanças na prevalência de candidíase por *C. albicans* para outras espécies de *Candida*, está sendo associada com a piora dos resultados. Vários trabalhos mostram um aumento nas taxas de complicações e fatalidades com *C. tropicalis* (Pfaller, 1996; Costa et al., 2000) e *C. krusei* (Abbas et al., 2000; Viudes et al., 2002).

Análise de variância demonstrou diferenças significantes ($P < 0.05$) entre as médias de adesão de *C. albicans* e *C. tropicalis*, que exibiram alta capacidade de aderência às células epiteliais da cavidade oral, de *C. krusei*, que exibiu baixa. Estes resultados estão de acordo com Gruber et al. (2003), que investigou a habilidade de ligação de *C. albicans*, *C. tropicalis* e *C. krusei* a células imunes infectadas com HIV, encontrando diferenças significantes na adesão dessas espécies, e correlacionando com sua hierarquia de virulência. Nestes experimentos *C. krusei* foi menos aderente que *C. albicans* e *C. tropicalis*.

Ressalta-se que, além da alta habilidade de aderência, todos os Isolados de *C. albicans* e *C. tropicalis* apresentaram moderados resíduos de α-L-fucose como observado pelo padrão de marcação através da histoquímica com lectinas. Entre os isolados de *C. tropicalis*, esta observação torna-se inconsistente apenas para o isolado 4790, que mostrou-se fracamente aderente, mais apresentou moderado padrão de marcação para α-L-fucose. Esta levedura foi isolada de uma amostra não-clínica (goma de mandioca), e, portanto, apresentou uma coerente patogenicidade reduzida. Os isolados de *C. albicans* que apresentaram fraca aderência, com exceção de 3716 e 3719, foram isolados de pacientes com AIDS, que receberam tratamento antifúngico, o que provavelmente diminuiu a habilidade de aderência desses isolados. *C. albicans* 3716 e 3719 foram isolados através da

escarificação de dentina de paciente saudável, sem lesões clínicas, conseqüentemente da microbiota endógena que é característica por ter baixa patogenicidade.

Todos os isolados de *C. krusei* apresentaram fraca adesão, independente do tempo de estoque e do substrato de isolamento. Este fato aconteceu até com as linhagens 4802 and 1059 que apresentaram intenso e moderado níveis de marcação por UEA I, respectivamente, demonstrando a baixa capacidade patogênica, inerente a esta espécie. Além disso, não foi observado um padrão de marcação em comum, sugerindo variações intraespecíficas entre os isolados estudados.

Esses resultados combinados nos levam a acreditar que, a presença de componentes fucosilados sobre a superfície destas leveduras, pode ser indicativo de um novo mecanismo, pelo qual as células fúngicas interagem com estruturas do hospedeiro. Além disso, nosso trabalho ratifica a esperada presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico e D-galactose/*N*-acetil-D-galactosamina, sugerindo que os carboidratos da parede celular contém outros monossacarídeos, além de manose, e que esta estrutura pode ter uma estrutura complexa além do pensado. A presença de oligossacarídeos isentos de manose esta consistente com relatos prévios de Lopez-Ribot et al. (1999) e Soares et al. (2000).

Munõz et al. (2003) decreveram o perfil de marcação com lectinas, para vários isolados de *Candida*, todas com especificidade para α-D-manose. Estes experimentos estão de acordo com o presente estudo, embora existam variações. Essas diferenças, que ocorrem devido às falhas no reconhecimento dos carboidratos, podem ser explicadas pelo painel de lectinas utilizado. Além disso, realizamos um pré-tratamento enzimático nas suspensões de levedura com tripsina, para garantir a acessibilidade aos resíduos dos açúcares pelas lectinas e utilizamos como único meio de crescimento ágar Sabouraud (Difco), uma vez que estudos sugerem que a expressão de carboidratos varia conforme a idade e o ambiente de desenvolvimento do fungo (Alviano et al., 2004).

Quando os resultados de aderência foram avaliados de acordo com a origem da amostra clínica, não foi observada diferença significante. Bernardis et al. (1999), Panagoda et al. (2001) and Dagdeviren et al. (2005) também destacam que não existem diferenças nos valores de aderência entre isolados superficiais ou sistêmicos.

O conhecimento do perfil sacarídico na superfície fúngica habilita o uso de lectinas, além de promissoras sondas celulares, para ser utilizada em tratamentos terapêuticos como já proposto para doenças bacterianas por Sharon & Ofek (2000). Uma vez que, estruturas que contém fucose estão presentes sobre a superfície celular de *Candida*, é possível que lectinas ligadoras de fucose, como UEA I, possa ser à base de um tratamento terapêutico para candidíase. Adicionalmente, lectinas podem servir como carreadores de agentes antifúngicos, que utiliza como alvos específicos os carboidratos existentes na superfície da célula dos microrganismos (Vardar-Ünlü et al., 1998).

No presente estudo, relatamos a utilização de histoquímica com lectinas, para o mapeamento dos carboidratos encontrados em glicoconjugados na superfície celular de três espécies de *Candida*, *C. albicans*, *C. tropicalis* e *C. krusei*, e evidenciamos uma correlação com sua habilidade de adesão às células epiteliais da cavidade oral. Embora muitas questões permaneçam, acreditamos que carboidratos complexos sobre a superfície de *Candida* podem representar uma maneira pela qual, interações do fungo com as células do hospedeiro possam ser estabelecidas.

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Figura 1

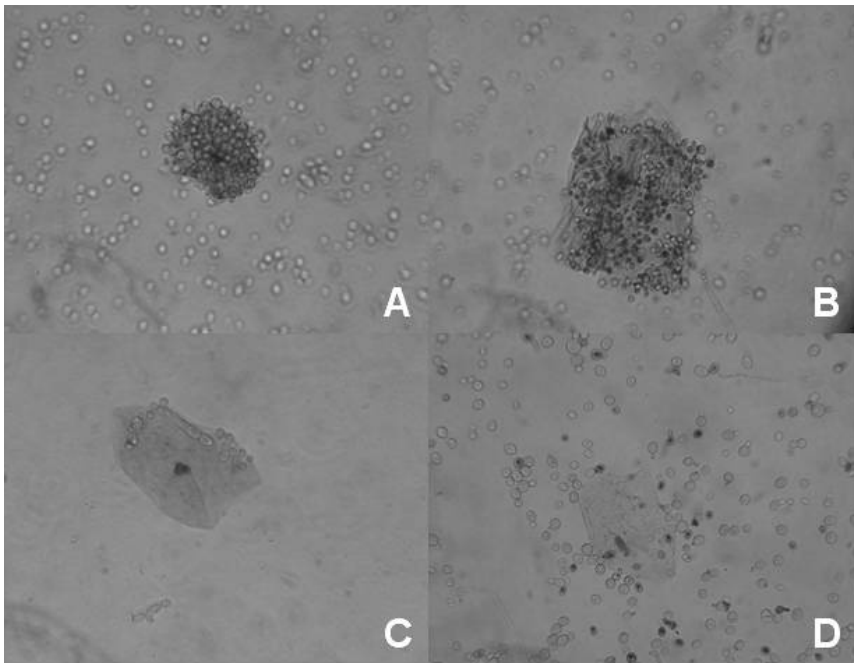

Figura 1. Forte aderência de *C. albicans* 4606 (A) e *C. tropicalis* 916 (B) às células epiteliais da cavidade oral. Fraca aderência de *C. tropicalis* 4790 (C) e *C. krusei* 934 (D). Ampliação: x 400.

Tabela 1

Tabela 1. Isolados de *Candida* Obtidos da Micoteca URM.

Espécie	Número de registro (URM)	Tempo de estocagem (anos)	Substrato de isolamento
Candida albicans	4990	01	Secreção vaginal
C. albicans	4987	01	Secreção vaginal
C. albicans	4986	01	Secreção vaginal
C. albicans	4820	02	Escamas ungueais
C. albicans	4819	02	Escamas ungueais
C. albicans	4817	02	Escamas ungueais
C. albicans	4609	03	Sangue
C. albicans	4606	03	Sangue
C. albicans	4388	05	Secreção orofaríngea
C. albicans	4387	05	Secreção orofaríngea
C. albicans	4386	05	Secreção orofaríngea
C. albicans	4385	05	Secreção orofaríngea
C. albicans	4384	05	Secreção orofaríngea
C. albicans	4260	05	Secreção orofaríngea
C. albicans	4127	07	Escamas inguinais
C. albicans	4126	07	Urina
C. albicans	4125	07	Esputo
C. albicans	4124	07	Secreção orofaríngea
C. albicans	3719	10	Escarificação de dentina
C. albicans	3716	10	Escarificação de dentina
C. tropicalis	4790	02	Goma de mandioca
C. tropicalis	4262	06	Secreção orofaríngea
C. tropicalis	1150	46	Língua
C. tropicalis	933	49	Secreção vaginal
C. tropicalis	916	49	Fezes
C. krusei	4802	02	*
C. krusei	4263	05	Secreção orofaríngea
C. krusei	1059	48	*
C. krusei	934	49	Biópsia de apêndice
C. krusei	109	52	*

*Substrato não identificado

Tabela 2

Tabela 2. Aderência a células epiteliais e perfil histoquímico dos isolados de *C. albicans*, de *C. tropicalis* e de *C. krusei* estudados.

Isolados	Capacidade de aderência	Padrão de marcação com lectinas			
		Con A	WGA	UEA-1	PNA
4990	H	+	+	++	+
4987	H	+	+	++	+
4986	H	+	+	++	+
4820	H	+	+	++	+
4819	H	+	+	++	+
4817	H	+	+	++	+
4609	H	+	+	++	+
4606	H	+	+	++	+
4388	H	+	+	++	+
4387	W	+	+	++	+
4386	W	+	+	++	+
4385	W	+	+	++	+
4384	W	+	+	++	+
4260	W	+	+	++	+
4127	H	+	+	++	+
4126	H	+	+	++	+
4125	W	+	+	++	+
4124	H	+	+	++	+
3719	W	+	+	++	+
3716	W	+	+	++	+
4790	W	+	+	++	+
4262	H	+	+	++	+
1150	H	+	+	++	+
933	H	+	+	++	+
916	H	+	+	++	-
4802	W	+	+++	+++	+
4263	W	+	++	+	+
1059	W	++	+++	++	+++
934	W	+	++	+	+
109	W	+	++	+	+
6258	W	+	++	+	+

Forte aderência (H) ou fraca aderência (W).
Intenso (+++), moderado (++) ou fraco (+) padrão de marcação.

5 ANÁLISE DA LIGAÇÃO POR LECTINAS A GLICOCONJUGADOS SOBRE A SUPERFÍCIE DE ESPÉCIES DE *CANDIDA*

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ANÁLISE DA LIGAÇÃO POR LECTINAS A GLICOCONJUGADOS SOBRE A SUPERFÍCIE DE ESPÉCIES DE *CANDIDA*

Mapeamento de glicoconjugados sobre espécies de *Candida*

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Resumo

Rápida identificação de isolados clínicos pode facilitar o diagnóstico e tratamento. Portanto, métodos rápidos e reproduzíveis, como histoquímica com lectinas, estão sendo desenvolvidos para distinção entre espécies de *Candida* de importância médica. O presente estudo tem como objetivo avaliar o padrão de aglutinação de 46 isolados *Candida* por quatro lectinas. Os isolados foram das espécies *Candida albicans* (20), *C. parapsilosis* (15), *C. tropicalis* (5) e *C. krusei* (6). As lectinas utilizadas foram Concanavalin A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA I) e peanut agglutinin (PNA) conjugadas a peroxidase (25 µg mL^{-1}). Embora em diferentes padrões de marcação, 98% dos isolados foram aglutinados pelas quatro lectinas. A única exceção foi um isolado de *C. krusei*, o qual não evidenciou aglutinação para PNA. Além disso, foram distinguidos nove diferentes painéis de marcação entre todos os isolados estudados. O mapeamento de glicoconjugados por lectinas apresenta-se como uma ferramenta auxiliar, para estudos taxonômicos e epidemiológicos de leveduras em laboratórios clínicos.

Palavras-chave: *Candida*, Lectina, Tipagem, Identificação, Diagnóstico.

1. Introducão

A incidência de infecções por *Candida* vem aumentando gradativamente nas últimas décadas, por várias razões, incluindo o uso de técnicas mais agressivas no tratamento do cancer, complicações cirúrgicas, transplante de órgãos e a pandemia da AIDS [1]. Cerca de 50% das leveduroses nosocomiais são causados por *Candida albicans* [2]. Contudo, outras espécies estão emergindo como importantes patógenos, como *C. parapsilosis*, *C. tropicalis* e *C. krusei* [3].

Numerosos procedimentos foram desenvolvidos para identificação de espécies de *Candida* [4-10], embora a maioria deles sejam demorados ou não apresentem um poder de distinção elevado.

Lectinas são proteínas de origem não imunológica que se ligam especificamente a carboidratos [9]. A seletividade das lectinas para açúcares da superfície microbiana já foi mostrada para leveduras de *Candida* [10].

No presente estudo, nós avaliamos a presença de carboidratos sobre a parede celular e a utilização de lectinas para a identificação de isolados clínicos de *Candida*.

2. Materiais e métodos

2.1. Leveduras e condições de crescimento

Um total de 44 isolados de *Candida* foi adquirido na Coleção de Culturas – Micoteca URM, do Departamento de Micologia, Centro de Ciências Biológicas da Universidade Federal de Pernambuco, os quais estavam estocados sob óleo mineral [11]. Destes, 20 isolados foram *C. albicans*, 14 *C. parapsilosis*, cinco *C. tropicalis* e cinco *C krusei*. Testes de viabilidade e confirmação taxonômica foram realizados através de inoculação das culturas em tubos de ensaio com 5 mL de caldo glicosado a 2% [12]. Após desenvolvimento, culturas foram transferidas para tubos com ágar Sabouraud adicionado de extrato de levedura (Difco), para terem suas características morfológicas, bioquímicas e fisiológicas observadas [13]. Espécies, número de registro, tempo de estocagem, em anos, e substrato de isolamento estão sumarizados na Tabela 1. Duas linhagens, *C. parapsilosis* ATCC 22019 e *C. krusei*, ATCC 6258 foram usadas como referência.

2.2. Lectinas

As lectinas foram selecionadas para garantir a marcação de uma ampla variedade de açúcares específicos. As lectinas usadas foram Concanavalin A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA I) e peanut agglutinin (PNA), todas conjugadas a peroxidase (Sigma Chemical Co., St Louis, MO, USA), a concentração de 25 $\mu g\ mL^{-1}$ em tampão fosfato (PBS), 0,01M, pH 7,2-7,4 e estocado a -20 °C em Eppendorf.

2.3. Ensaios de aglutinação

Histoquímica com lectinas foi desenvolvida de acordo com Rego et al. [16]. As culturas fúngicas cresceram em ágar Sabouraud (Difco), foram suspensas em PBS (0,01M, pH 7,2) a concentração de 10^7células.mL^{-1}. Em seguida, a suspensão foi tratada com tripsina a 1% em PBS por 3 min seguida por solução metanol-H_2O_2 por 5 min e incubada com as lectinas (25 µg/mL) por 1h a 25°C. Reação com a peroxidase foi visualizada através de diaminobendizina (DAB) e peróxido de hidrogênio em PBS por 5-8min a 25°C. Entre cada protocolo, as células foram lavadas com PBS sob centrifugação (2.500 rpm por 5 min). Ensaios controles foram feitos por ligação da lectina na presença do açúcar específico corresponde: metil-α-D-manosídeo, *N*-acetil-D-glicosamina, α-L-fucose and D-galactose para Con A, WGA, UEA I e PNA, respectivamente, a concentração de 300 mM. Padrão de marcação foi avaliado por microscopia de luz e classificado qualitativamente como intenso (+++), moderado (++), fraco (+) ou negativo (-), conforme Ozer [17]. Os ensaios foram realizados em triplicata para cada isolado e o protocolo foi repetido, pelo menos, em doze diferentes ocasiões.

3. Resultados

As analises de ligações por lectinas indicaram a presença de α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico, α-L-fucose e D-galactose/*N*-acetil-D-galactosamina em todos os isolados estudados, exceto para o isolado 916, que não foram evidenciados resíduos de galactose. Além disso, foram observados nove diferentes padrões de marcação nos isolados estudados (Tabela 2).

Os 20 isolados de *C. albicans* mostraram único padrão de aglutinação: fraco para Con A, WGA e PNA e moderada marcação para UEA I. Isolados de *C. parapsilosis*

aglutinaram as lectinas de quatro formas: fraca para Con A e PNA, enquanto que WGA mostrou mudanças na marcação que variaram entre moderado a intenso e UEA I entre fraco a moderado.

Os isolados de *C. tropicalis* foram marcados da mesma maneira que *C. albicans*, com exceção do isolado 916 que não foi aglutinado por PNA. Isolado de *C. krusei* apresentaram quatro padrões de marcação: fraco a moderado para Con A, moderado a intenso para WGA, enquanto que UEA I e PNA mostraram variações no padrão de marcação entre fraco a intenso.

4. Discussão

Identificação de espécies de *Candida* por análises morfológicas e testes de assimilação podem levar dias. Embora estudos sobre a morfologia da colônia permaneçam importantes para a identificação dessas leveduras, a insuficiência desses testes para diagnóstico preciso tem conduzido ao desenvolvimento de outros procedimentos, incluindo testes cromogênicos, kits bioquímicos e análise molecular [4-10].

As analises de ligações por lectinas a glicoconjugados sobre a superfície de espécies de *Candida*, está se apresentando como um teste alternativo para a identificação e tipagem de leveduras, sendo indicativo de um novo e não reconhecido mecanismo pelo qual a taxonomia pode ser protamente realizada na maioria dos laboratórios.

Em leveduras, as lectinas ligam-se a açúcares que são abundantes na superfície da célula, como α-D-manose and *N*-acetil-D-glicosamina [18]. A função das lectinas no diagnóstico microbiológico tem sido revisado em detalhes por vários autores desde a década de 70 [19], embora a maioria dos experimentos relatem apenas aglutinação por lectinas específicas a α- mananos, como Con A, LCA e PSA [20,21].

No presente estudo, a lectina UEA I específica para fucose, apresentou-se como bom marcador para *C. albicans* and *C. tropicalis*, enquanto que WGA aglutinou promissoramente para *C. parapsilosis*. Nos isolados de *C. krusei*, não foi observado um padrão de aglutinação em comum, sugerindo variações intraespecíficas.

Munõz et al. [12] decreveram o perfil de marcação com lectinas, para vários isolados de *Candida*, todas com especificidade para α-D-manose. Estes experimentos estão de acordo com o presente estudo, embora existam variações. Essas diferenças, que ocorrem

devido às falhas no reconhecimento dos carboidratos, podem ser explicadas pelo painel de lectinas utilizado. Além disso, nós realizamos um pré-tratamento enzimático nas suspensões de levedura com tripsina, para garantir a acessibilidade aos resíduos dos açúcares pelas lectinas e utilizamos como único meio de crescimento ágar Sabouraud (Difco), uma vez que estudos sugerem que a expressão de carboidratos varia conforme a idade e o ambiente de desenvolvimento do fungo [22].

O conhecimento do perfil sacarídico na superfície fúngica habilita o uso de lectinas, além de promissoras sondas celulares, para ser utilizada em tratamentos terapêuticos como já proposto para doenças bacterianas por Sharon & Ofek (2000). Uma vez que, estruturas que contém fucose estão presentes sobre a superfície celular de *Candida*, é possível que lectinas ligadoras de fucose, como UEA I, possa ser à base de um tratamento terapêutico para candidíase. Adicionalmente, lectinas podem servir como carreadores de agentes antifúngicos, que utiliza como alvos específicos os carboidratos existentes na superfície da célula dos microrganismos (Vardar-Ünlü et al., 1998).

Neste estudo, relatamos o parcial perfil de carboidratos expressados na superfície celular de quatro espécies de *Candida*. Histoquímica com lectinas foi um método chave para alcançar esses resultados, se apresentando como uma potencial ferramenta para a caracterização dos glicoconjugados de superfície das leveduras. Investigações adicionais são necessárias para estabelecer o real valor da tipagem por lectinas, tanto para estudos taxonômicos como epidemiológicos.

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Tabela 1

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Species	Accession number (URM)	Storage period (years)	Substratum
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C. albicans	4387	05	Secreção orofaríngea
C. albicans	4386	05	Secreção orofaríngea
C. albicans	4385	05	Secreção orofaríngea
C. albicans	4384	05	Secreção orofaríngea
C. albicans	4260	05	Secreção orofaríngea
C. albicans	4127	07	Área inguinal
C. albicans	4126	07	Urina
C. albicans	4125	07	Esputo
C. albicans	4124	07	Secreção orofaríngea
C. albicans	3719	10	Escarificação de dentina
C. albicans	3716	10	Escarificação de dentina
C. parapsilosis	4984	01	Secreção vaginal
C. parapsilosis	4970	01	Secreção vaginal
C. parapsilosis	4889	02	Sangue
C. parapsilosis	4818	02	Escamas ungueais
C. parapsilosis	4804	02	IFM
C. parapsilosis	4608	03	Sangue
C. parapsilosis	*4607*	*03*	*Sangue*
C. parapsilosis	4433	04	Solo de praia
C. parapsilosis	4261	05	Secreção orofaríngea
C. parapsilosis	3627	12	Esputo
C. parapsilosis	3624	12	Esputo
C. parapsilosis	3621	12	Esputo
C. parapsilosis	2633	15	*
C. parapsilosis	729	49	*
C. tropicalis	4790	02	Goma de mandioca
C. tropicalis	4262	06	Secreção orofaríngea
C. tropicalis	1150	46	Língua
C. tropicalis	933	49	Secreção vaginal
C. tropicalis	916	49	Fezes
C. krusei	4802	02	**
C. krusei	4263	05	Secreção orofaríngea
C. krusei	1059	48	**
C. krusei	934	49	Biópsia de apêndice
C. krusei	109	52	**

*IFM (Institute of Food Microbiology)

**Substrato não identificado

Tabela 2

Tabela 2. Padrão de aglutinação dos 46 isolados estudados com as quatro lectinas utilizadas

Isolados	*Padrão de marcação com lectinas*			
	Con A	*WGA*	*UEA-1*	*PNA*
4990	+	+	++	+
4987	+	+	++	+
4986	+	+	++	+
4820	+	+	++	+
4819	+	+	++	+
4817	+	+	++	+
4609	+	+	++	+
4606	+	+	++	+
4388	+	+	++	+
4387	+	+	++	+
4386	+	+	++	+
4385	+	+	++	+
4384	+	+	++	+
4260	+	+	++	+
4127	+	+	++	+
4126	+	+	++	+
4125	+	+	++	+
4124	+	+	++	+
3719	+	+	++	+
3716	+	+	++	+
4984	+	++	++	+
4970	+	++	+	+
4889	+	+++	++	+
4818	+	++	+	+
4804	+	++	+	+
4608	+	++	+	+
4607	+	++	+	+
4433	+	++	+	+
4261	+	++	++	+
3627	+	++	+	+
3624	+	++	+	+
3621	+	++	+	+
2633	+	++	+	+
729	+	+	+	+
22019	+	++	++	+
4790	+	+	++	+
4262	+	+	++	+
1150	+	+	++	+
933	+	+	++	+
916	+	+	++	-
4802	+	+++	+++	+
4263	+	++	+	+
1059	++	+++	++	+++
934	+	++	+	+
109	+	++	+	+
6258	+	++	+	+

Intenso (+++), moderado (++) ou fraco (+) padrão de marcação.

6 Conclusões gerais

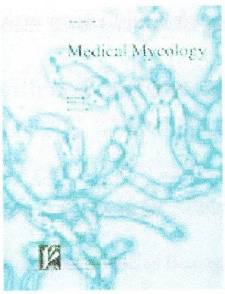
Os resultados obtidos permitem concluir que:

1. Isolados de *Candida albicans* e *C. tropicalis* apresentam maior capacidade de aderência às células epiteliais da cavidade oral, que isolados de *C. parapsilosis* e *C. krusei*;
2. Diferentes isolados de uma mesma espécie expressam variações na capacidade de aderência às células epiteliais da cavidade oral;
3. As 4 Espécies de *Candida* estudadas possuem em sua parede celular α-D-glicose/α-D-manose, *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico, α-L-fucose e D-galactose/*N*-acetil-D-galactosamina;
4. Isolado não clínico de *C. tropicalis* apresenta fraca capacidade de aderência e bom padrão de marcação com lectinas utilizadas;
5. Isolado clínico de *C. tropicalis* não evidenciou resíduos de D-galactose/*N*-acetil-D-galactosamina, observado pela ausência de marcação com PNA;
6. *C. albicans* e *C. tropicalis* apresentam na parede celular maior disponbilidade de resíduos de α-L-fucose enquanto *C. parapsilosis* e *C. krusei* resíduo de *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico;
7. Lectina UEA I, específica para α-L-fucose, apresenta-se como melhor marcador para a superfície celular de *C. albicans* e *C. tropicalis* e WGA, específica para *N*-acetil-D-glicosamina/ácido *N*-acetilneuramínico, para a superfície de *C. parapsilosis* e *C. krusei*;
8. Isolados de *C. krusei* apresentam diferenças no padrão de marcação aos glicoconjugados, sugerindo variações intraespecíficas;
9. Padrão de marcação aos glicoconjugados da supefície celular das espécies de *Candida*, através de histoquímica com lectinas, demonstra-se como uma ferramenta auxiliar na identificação de isolados clínicos facilitando o diagnóstico e a terapêutica;
10. O método de estocagem através de óleo mineral é eficaz para manutenção da viabilidade de isolados de *Candida*.

REGINALDO GONÇALVES DE LIMA NETO

ANEXOS

(ARTIGOS SUBMETIDOS E NORMAS DAS REVISTAS)



Adherence of Candida albicans and Candida parapsilosis to epithelial cells correlates with cell surface carbohydrates

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Keyword:	Candida albicans, Candida parapsilosis, Adherence, Epithelial cells, Lectin histochemistry

Adherence of *Candida albicans* and *Candida parapsilosis* to epithelial cells correlates with cell surface carbohydrates

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Abstract

Many studies are available on the adherence of *Candida albicans* to epithelial cells but little is known about *Candida parapsilosis* adhesion and its correlation with recognition of cell surface carbohydrates. This study aimed to evaluate the adherence ability of 20 *C. albicans* and 15 *C. parapsilosis* strains to human buccal epithelial cells and we evaluated the expression of cell surface carbohydrates using lectin histochemistry. Adherence assays were carried out incubating epithelial cells with the yeast suspensions (10^7 cells.mL^{-1}) for 2h at 25°C. Peroxidase conjugated lectins (Con A, WGA, UEA I and PNA at 25 μg/mL) for 2h at 25°C were used in the lectin assay. Results showed that *C. albicans* is more adherent than *C. parapsilosis* ($P<0.01$), presenting a high content of α-L-fucose moieties in the cell surface as indicated by UEA I staining pattern. Three isolates of *C. parapsilosis* presented similar adherence values with UEA I staining patterns similar to that of *C. albicans*, we leads to believe that to presence of fucosylated components may represent additional motifs through which interactions of this yeast with host cells could be established ($r=0.8336$, $P=0.0001$). In addition, our results showed the presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid and D-galactose/*N*-acetyl-D-galactosamine in fungal cell wall.

Keywords: *Candida albicans*; *Candida parapsilosis*; Adherence; Epithelial cells; Lectin histochemistry.

1. Introduction

Adherence is a first step in microbial colonization and is a key event in the initiation of the pathogenic process [1]. *Candida albicans* is the most adherent and successful yeast in colonizing the oral cavity [2], whereas *Candida parapsilosis* has emerged as an important nosocomial fungal pathogen with several clinical implications [3,4].

The mechanisms of adherence of *Candida* species to human tissues are varied and as a result the *Candida*-host cell recognition systems are extremely complex and involve a variety of components [5,6]. Therefore, specific recognition between fungal parasites and their host cell target may be mediated by the interaction of carbohydrate-binding proteins, as lectins, on the surface of one type of cell, that combine with complementary sugars on the surface of another cell [7,8].

Lectins are proteins of non-immune origin that bind specifically to carbohydrates [9]. The selectivity of lectins for microbial-surface sugars has been demonstrated for yeast of the genus *Candida* [10].

Despite the fact that many authors has stated than *C. albicans* and *C. parapsilosis* present many similarities, few studies have compared the adherence capacity of both yeast to pooled human buccal epithelial cells and related such observations to expression of cell surface carbohydrates. In addition, these interactions elucidating putative adhesins and receptor-containing molecules in the context of the fungus-host interface may help develop more appropriate therapeutics for candidal infections. The present study aims to evaluate the relationship between *C. albicans* and *C. parapsilosis*, in terms of their adherence to epithelial cell surfaces, and their cell surface carbohydrate profiles using lectin histochemistry.

2. Materials and methods

2.1. Yeast and growth conditions

A total of 34 *Candida* strains were supplied by the URM Culture Collection of Department of Mycology, Biological Sciences Center, Federal University of Pernambuco and have been stocked in mineral oil [11]. Of these, 20 strains were *C. albicans* and 14 *C. parapsilosis*. Viability test and taxonomic revision was carried out inoculating cultures in test tube containing 5 mL of 2% dextrose broth [12]. After growth, cultures were transferred to slants containing Sabouraud agar plus yeast extract (Difco), and then observed their morphological, biochemical and physiological characteristics [13]. Species, accession numbers, stock time, in years, and isolation substratum are summarized in table 1. A strain of *C. parapsilosis* ATCC 22019 was used as reference strain.

2.2. Cells and Adherence assay

Cell separation and adherence assays were modified from protocols established by Kimura and Pearsall [14] and Sobell et al. [15] as follows. The yeast cells were grown on malt agar (Difco) for 36 h at 37 °C and ressuspended in 2mL of sterile 0.1M phosphate-buffered saline (PBS) pH 6.8 at 4°C, washed twice with 2 mL of PBS under centrifugation (2,500 g, 5 min each), ressuspended in PBS ($2x10^7$ cells.mL^{-1}), determined in Neubauer chamber. The epithelial cells were collected from healthy adult volunteers through soft scrap of the cheeks mucous membrane with sterile cotton swabs. Each swab was gently stirred in 5mL of PBS to release the cells. Epithelial cells suspensions were washed twice in 5 mL of PBS (2,500g, 5 min each) to remove bacteria loosely adhered. The cells were ressuspended in PBS ($4x10^4$ cells.mL^{-1}) determined in Neubauer chamber. After washing,

the yeast and epithelial cells were separately morphological examined (viability and integrity). Adherence assays were evaluated mixing 1 mL of each suspension in test tube, followed by homogenization and incubation at 25°C under gently stirring for 2h. Following methylene blue staining. Ten microlite droplets of the suspension were covered with cover slip and examined by a light microscope, where each slide had 100 epithelial cells evaluated in accordance with the percentage of its superficial area attached by yeast cells. Results were graded as: High adherence (H) for attaching among 50% to 100% of superficial area, weak adherence (w) for attaching up to 50% and no visible adherence (0). In addition, the number of yeast cells attached to ten epithelial cells was counted for statistical analysis.

2.3. Lectin histochemistry

The lectins used were selected to cover a wide range of sugar specificities. Histochemistry was developed according to Rego et al. [16] using Concanavalin A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA-1) and peanut agglutinin (PNA) conjugated to peroxidase (Sigma Chemical Co., St Louis, MO, USA). Fungal samples grown in Sabouraud agar (Difco) were suspended in PBS and adjusted to 10^7cells.mL^{-1}. Later the suspension was treated with 1% trypsin in PBS for 3 min followed by methanol- H_2O_2 solution for 5 min and incubated with lectins (25 µg/mL) for 1h at 25°C. Peroxidase reaction was visualized using diaminobendizine (DAB) and hydrogen peroxide in PBS for 5-8min at 25°C. Between each protocol step cells were washed with PBS under centrifugation (2,500g for 5 min). Inhibition assays were done by performing the lectin binding in the presence of the corresponding lectin-specific sugar: methyl-α-D-mannoside, *N*-acetyl-D-glucosamine, α-L-fucose and D-galactose for Con A, WGA, UEA I

and PNA, respectively, at 300 mM concentration. Sample staining were evaluated by optical microscopy and classified qualitatively as intense (+++), moderate (++), weak (+) or none (-), according to Ozer [17].

2.4. Statistical analysis

Assays were developed in triplicate for each strain and protocols were repeated on at least twelve different occasions. The mean and standard deviation of the three distinct experiments were determined. Statistical analysis was calculated by using STATISTICA 6.0 (StatSoft, Inc.) computer software. The differences for adherence capability of the two study species were determined using the *t*-test. The relationship between the virulence factor investigated and lectin histochemistry were evaluated by the Pearson's correlation test. *P* values of 0.05 or less were defined as statistically significant [18].

3. Results

Adhesion values of the *C. albicans* and *C. parapsilosis* isolates to epithelial cells are summarized in Table 2. Twelve (60%) out of 20 strains of *C. albicans* adhered strongly, while only three (20%) of 15 *C. parapsilosis* strains behaved similarly (Fig. 1).

Lectin-binding analysis indicated the presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid, α-L-fucose and D-galactose/*N*-acetyl-D-galactosamine in all strains studied of *C. albicans* and *C. parapsilosis*, although in different staining pattern (Table 2).

C. albicans isolates presented a weak staining pattern for Con A, WGA and PNA and a moderate staining for UEA I. *C. parapsilosis* isolates were weakly recognized for

Con A and PNA, while WGA showed changes that varied between moderate-to-intense and UEA I between weak-to-moderate staining pattern (Table 2).

4. Discussion

The adherence of *Candida* to epithelial cells is one the main pathogenic characteristics of the genus. Studies have shown that there are variations in the adherence capabilities of different *Candida* species, which may explains why some species more frequently colonize mucosal surfaces [5,19].

The mean adherence values of the 20 *C. albicans* and 15 *C. parapsilosis* strains were found to be 49.95 and 25.60, respectively, exhibiting difference statistically significant ($P < 0.01$). In general, the avidity of *C. albicans* to the epithelial cells was more than that of *C. parapsilosis* isolates. These findings are in accordance with observations of Repentigny et al. [20], who investigated the *in vitro* binding abilities of several *Candida* species to purified mucin, and showed significant differences in adhesion, closely correlating with their hierarchy of virulence. In their experiments *C. albicans* was found more adherent that *C. parapsilosis*.

Histochemical analysis showed that all isolates of *C. albicans* presented more α-L-fucose moieties, than *C parapsilosis*. Interestingly, the three isolates of *C. parapsilosis* (4984, 4889 and 4261) that were the most adherent, displayed more α-L-fucose moieties than other strains of the same species, except for the isolate 22019, which showed moderate staining pattern for this sugar, but weak adherence capacity. This strain has been maintained in our laboratory for many years and therefore undergone multiples successive subcultures. Such conditions may have attenuated the phenotypic characteristics of the

yeast leading to the current observation. It is known that repeated laboratory subculture attenuates many virulence attributes of yeasts [21].

This combined findings we leads to believe that, presence of several fucosylated components on the surface of the yeast, may be indicative of a new, yet unrecognized mechanism by which the fungal cells interact with host structures. When this data were correlated, a significant positive correlation was noted between adhesion and α-L-fucose expression by specific staining UEA I ($r = 0.8336$, $P = 0.0001$).

Furthermore, was ratified for our work the expected presence of glucose/mannose, *N*-acetylglucosamine/*N*-acetylneuraminic acid and galactose/*N*-acetylgalactosamine, suggesting that at least some cell wall carbohydrates contain monosaccharides other than mannose and that these may to have a much more complex structure than initially thought. The presence of non-mannan oligosaccharides is consistent with previous reports of Lopez-Ribot et al. [22] and Soares et al. [23].

Munõz et al. [10] describe lectin profiling for a large number of *Candida* strains, all of them with α-D-mannose specificity. This result is in accordance with our findings, although there are variances. Such differences, than occur due the failure in recognizing of carbohydrates, may be explained by panel of lectin used. In addition, we carry out an enzyme pretreatment in the yeast suspensions with tripsin to warrant accessibility to the sugar moieties by lectins and used like single grown media Sabouraud agar (Difco), since several studies suggest that carbohydrate expression varies according to the age/environment of the fungus [24].

The strains of *C. albicans* that presented weak adherence, with exception of 3716 and 3719, were isolated of patients with AIDS interned at the clinical hospital. Such patients got antifungal treatment, which likely may to have decreased the ability to adhere

of these strains. *C. albicans* 3716 and 3719 were isolated of healthy tooth scrap, without clinical lesions, hence in indigenous microbiota that is feature for to have weak pathogenicity.

Cell surface carbohydrates or glycoconjugates play critical roles in many fundamental cellular processes, such as cell-cell and microorganism-host cell interactions [25,26]. Lectins, proteins that bind to specific carbohydrate structures and play an important role in cell recognition, have had enormous impact in cell biology and histopathology [27]. They are found in microorganisms, plants and animals [28].

In histochemistry, lectins with different carbohydrate specificity are useful tools for the investigation of changes in glycosylation and carbohydrate expression that may occur in normal or pathological conditions [27,29].

The knowledge of the saccharide profile in fungal surface enables the use of lectins, besides promising cell probes, to be used in therapeutic treatments as already proposed for cancer by Mody et al. [30] in the nineties. Since fucose-containing structures are present on *Candida* cell surface, it is possible that fucose-binding lectin, such UEA I, could be the basis of a therapeutic treatment of candidosis. As an extension of this, lectins with drug substituents might serve as carriers of antifungal agents, than use as targets their specific carbohydrates found in the cell surface of microorganisms [31].

We here reported the partial profile of carbohydrates expressed in the cell surface of two species of *Candida* (*C. albicans and C. parapsilosis*) and showed a correlation with their ability of adhesion to buccal epithelial cells. Lectin histochemistry was the key method to reach the results showing to be once more a potential auxiliary tool for the characterization of cell surface glycoconjugates of yeasts. Although many questions remain, we believe that complex carbohydrates on the surface of *Candida* species may

represent additional motifs through which interactions of this fungus with host cells could be established.

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Table 1

Table 1. *Candida* strains used for the study (Obtained form the URM Culture Collection)

Species	Accession number (URM)	Storage period (years)	Substratum
Candida albicans	4990	01	Vaginal secretion
C. albicans	4987	01	Vaginal secretion
C. albicans	4986	01	Vaginal secretion
C. albicans	4820	02	Ungual scrap
C. albicans	4819	02	Ungual scrap
C. albicans	4817	02	Ungual scrap
C. albicans	4609	03	Blood
C. albicans	4606	03	Blood
C. albicans	4388	05	Oropharyngeal secretion
C. albicans	4387	05	Oropharyngeal secretion
C. albicans	4386	05	Oropharyngeal secretion
C. albicans	4385	05	Oropharyngeal secretion
C. albicans	4384	05	Oropharyngeal secretion
C. albicans	4260	05	Oropharyngeal secretion
C. albicans	4127	07	Inguinal area
C. albicans	4126	07	Urine
C. albicans	4125	07	Spittle
C. albicans	4124	07	Oropharyngeal secretion
C. albicans	3719	10	Tooth scrap
C. albicans	3716	10	Tooth scrap
C. parapsilosis	4984	01	Vaginal secretion
C. parapsilosis	4970	01	Vaginal secretion
C. parapsilosis	4889	02	Blood
C. parapsilosis	4818	02	Ungual scrap
C. parapsilosis	4804	02	IFM
C. parapsilosis	4608	03	Blood
C. parapsilosis	4607	03	Blood
C. parapsilosis	4433	04	Beach soil
C. parapsilosis	4261	05	Oropharyngeal secretion
C. parapsilosis	3627	12	Spittle
C. parapsilosis	3624	12	Spittle
C. parapsilosis	3621	12	Spittle
C. parapsilosis	2633	15	*
C. parapsilosis	729	49	*

*Substratum no identified

Table 2

Table 2. Adherence assay and lectin histochemistry of the strains of *Candida albicans* and *Candida parapsilosis* studied

Strains	Adherence capacity	Lectin staining patterns			
		Con A	WGA	UEA-1	PNA
4990	H	+	+	++	+
4987	H	+	+	++	+
4986	H	+	+	++	+
4820	H	+	+	++	+
4819	H	+	+	++	+
4817	H	+	+	++	+
4609	H	+	+	++	+
4606	H	+	+	++	+
4388	H	+	+	++	+
4387	W	+	+	++	+
4386	W	+	+	++	+
4385	W	+	+	++	+
4384	W	+	+	++	+
4260	W	+	+	++	+
4127	H	+	+	++	+
4126	H	+	+	++	+
4125	W	+	+	++	+
4124	H	+	+	++	+
3719	W	+	+	++	+
3716	W	+	+	++	+
4984	H	+	++	++	+
4970	W	+	++	+	+
4889	H	+	+++	++	+
4818	W	+	++	+	+
4804	W	+	++	+	+
4608	W	+	++	+	+
4607	W	+	++	+	+
4433	W	+	++	+	+
4261	H	+	++	++	+
3627	W	+	++	+	+
3624	W	+	++	+	+
3621	W	+	++	+	+
2633	W	+	++	+	+
729	W	+	+	+	+
22019	W	+	++	++	+

High adherence (H) or weak adherence (W).
Intense (+++), moderate (++) or weak (+) staining pattern.

Figure 1

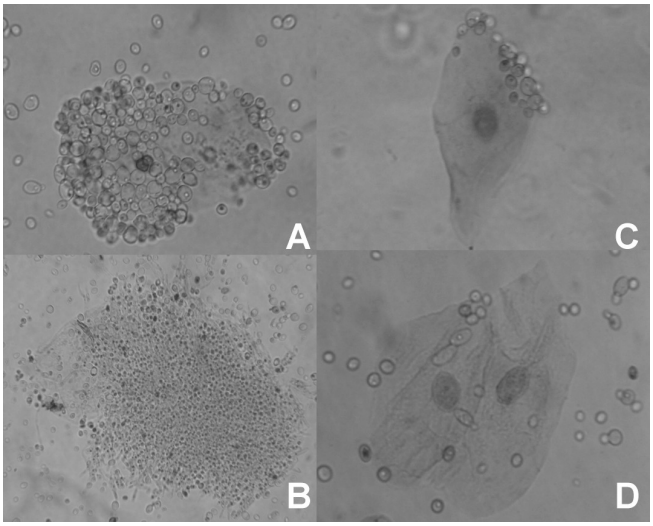

Figure 1. A: High adherence of *C. albicans* 4609 (A) and *C. parapsilosis* 4984 (B) to human buccal epithelial cells. Weak adherence of *C. albicans* 3719 (C) and *C. parapsilosis* 4818 (D). Magnification: x 400.

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12. Part of a database on the Internet MeSH Browser [database on the Internet]. Bethesda (MD): National Library of Medicine (US); 2002 - [cited 2003 Jun 10]. Meta-analysis; unique ID: D015201; [about 3 p.]. Available from: <http://www.nlm.nih.gov/mesh/MBrowser>www.www.nlm.nih.gov/mesh/MBrowser> Files updated weekly.

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Cell surface carbohydrates associated with adhesion of *Candida* species to epithelial cells

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Abstract

Several studies exist on the adherence capacity of *Candida* spp. to epithelial cells, but comparative studies based in this pathogenicity factor versus cellular recognition via cell surface carbohydrates are scarce. This study aimed to evaluate the adherence ability of 20 *Candida albicans*, five *C. tropicalis* and six *C. krusei* strains to human buccal epithelial cells in relation to the expression of cell surface carbohydrates evaluated via lectin histochemistry. Adherence assays were carried out incubating epithelial cells with the yeast suspensions (10^7 cells.mL^{-1}) under stirring for 2h at 25°C. Lectin histochemistry was examined using peroxidase conjugated lectins (Con A, WGA, UEA I and PNA at 25 μg/mL) for 2h at 25°C. Results showed that *C. albicans* and *C. tropicalis* were more adherents to epithelial cells than *C. krusei*, besides to present high α-L-fucose moieties in the cell surface as displayed by UEA I staining pattern. In addition, our results showed the presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid and D-galactose/*N*-acetyl-D-galactosamine in fungal cell wall. Our findings associate the increase in *Candida* adherence capacity with the presence of significant levels of fucosylated compounds. Although many questions remain, we believe that complex carbohydrates on the surface of *Candida* species may represent additional motifs through which interactions of this fungus with host cells could be established.

Keywords: *Candida albicans*; *Candida tropicalis*; *Candida krusei*; Adherence; Epithelial cells; Lectin histochemistry.

1. Introduction

Microbial attachment to mucosal surfaces has been shown to be an important step in infectious disease processes, particularly in the oral cavity (Sundstrom, 2002). *Candida* species, the etiologic agents of candidosis, are the most adherent and successful yeast in colonizing the oral cavity (Ellepola & Samaranayake, 2001). The mechanisms of adherence to human tissues are varied and as a result the *Candida*-host cell recognition systems are extremely complex and involve a variety of components (Jabra-Rizk et al., 2001; Marchais et al., 2005).

Adhesins, proteins involved in yeast adherence, are able to recognize components (ligants or receptors) on the host cell (Sturtevant & Calderone, 1997) as carbohydrates (Mendes-Giannini et al., 2000).

Lectins in histochemistry have been used to exploit the differences in the carbohydrate profile of cells and tissues. Such changes occur on cell surfaces during physiological and pathological processes (Sharon & Lis, 2004). These interactions supply precise models to study putative recognition processes where adhesins, carbohydrates and receptor-containing molecules play important roles in the fungus-host interface, and thus to develop a more appropriate therapeutics (Esquenazi et al., 2003).

The present study aims to evaluate the possible relationship between the pathogenicity of *Candida albicans*, *C. tropicalis* and *C. krusei,* via adherence assays, and their cell surface carbohydrate profile using lectin histochemistry.

2. Materials and methods

2.1. Yeast and growth conditions

A total of 30 *Candida* strains used in this study were obtained from the URM Culture Collection of Department of Mycology, Biological Sciences Center, Federal University of Pernambuco. The strains have been stocked in mineral oil (Sherf, 1943) and their accession numbers, stock time, in years, and isolation substratum are summarized in table 1. Viability test and taxonomic revision was carried out inoculating cultures in test tube containing 5 mL of 2% dextrose broth (Fennell, 1960). After growth, cultures were transferred to slants containing Sabouraud agar plus yeast extract (Difco), and then observed their morphological, biochemical and physiological characteristics (de Hoog et al., 2000). An isolate of *C. krusei* ATCC 6258 was used as reference strain.

2.2. Cells

Epithelial cells were collected from healthy adult volunteer through mucosal swabbing. Each swab was gently stirred in 5mL of PBS to release the cells. Epithelial cells suspensions were washed twice in 5 mL of PBS, centrifuged (2,500 g; 5 min each) and ressuspended in PBS ($4x10^4$ cells.mL^{-1}). Yeast and epithelial cells were morphologically examined for viability and integrity.

Yeast cells were grown on malt agar (Difco) for 36 h at 37 °C and ressuspended in 2mL of sterile 0.1M phosphate-buffered saline (PBS) pH 6.8 at 4 °C, washed twice with 2 mL of PBS under centrifugation (2,500 g, 5 min each), ressuspended in PBS ($2x10^7$ cells.mL^{-1}), determined in Neubauer chamber. Epithelial cells were collected from healthy adult volunteer through soft scrap of the cheeks mucous membrane with sterile cotton swabs. Each swab was gently stirred in 5mL of PBS to release the cells. Epithelial cells suspensions were washed twice in 5 mL of PBS (2,500g, 5 min each) to remove bacteria loosely adhered and ressuspended in PBS ($4x10^4$ cells.mL^{-1}) determined in Neubauer

chamber. Yeast and epithelial cells were morphologically examined for viability and integrity by microscopy.

2.3. Adherence assay

Assays were modified from protocols established by Kimura and Pearsall (1978), Sobell et al. (1981) and Kearns et al. (1983) as follows.

Adherence assays were evaluated mixing 1 mL of each suspension in test tube, followed by homogenization and incubation at 25°C under gently stirring for 2h. Following methylene blue staining. Ten microlite droplets of the suspension were covered with cover slip and examined by a light microscope, where each slide had 100 epithelial cells evaluated in accordance with the percentage of its superficial area attached by yeast cells. Results were graded as: High adherence (H) for attaching among 50% to 100% of superficial area, weak adherence (w) for attaching up to 50% and no visible adherence (0). Assays were developed in triplicate for each strain and protocols were repeated on at least twelve different occasions. Results were blind evaluated by multiple readers.

2.4. Lectin histochemistry

The lectins used were selected to cover a wide range of sugar specificities. Histochemistry was developed according to Rêgo et al. (2005) using Concanavalin A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA I) and peanut agglutinin (PNA) conjugated to peroxidase (Sigma Chemical Co., St Louis, MO, USA). Fungal samples grown in Sabouraud agar (Difco) were suspended in PBS and adjusted to 10^7cells.mL^{-1}. Later the suspension was treated with 1% trypsin in PBS for 3 min followed by methanol- H_2O_2 solution for 5 min and incubated with lectins (25 µg/mL) for 1h at 25

°C. Peroxidase reaction was visualized using diaminobendizine (DAB) and hydrogen peroxide in PBS for 5-8 min at 25 °C. Among each protocol step cells were washed with PBS under centrifugation (2,500g for 5 min). Inhibition assays were done by performing the lectin binding in the presence of the corresponding lectin-specific sugar: methyl-α-D-mannoside, *N*-acetyl-D-glucosamine, α-L-fucose and D-galactose for Con A, WGA, UEA I and PNA, respectively, at 300 mM concentration. . Sample staining were evaluated by optical microscopy and classified qualitatively as intense (+++), moderate (++), weak (+) or none (-), according to Ozer [17].

3. Results

The results obtained showed that all three *Candida* species studied adhere to epithelial cells, although at different extents (Table 2). Twelve (60%) out of 20 *C. albicans* strains adhered strongly to epithelial cells. Four (80%) out of five *C. tropicalis* strains also showed strong adherence to cells. However, all *C. krusei* strains presented weak adhesion ability (Fig. 1).

Lectin-binding analysis indicated the presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid, α-L-fucose and D-galactose/*N*-acetyl-D-galactosamine in all strains studied, except for strain 916, which were not evidenced galactose moieties (Table 2).

C. albicans and *C. tropicalis* strains presented a weak staining pattern for Con A, WGA and PNA and a moderate staining for UEA I. The *C. krusei* strains did not show a staining profile uniform (Table 2). Those strains presented stainings varying from moderate to intense pattern for WGA and weak to intense pattern for UEA I. Con A and PNA,

recognized weakly all *C. krusei* strains, unless 1059 that presented moderate staining to Con A and intense staining to PNA.

4. Discussion

Adherence is the first step in the infectious process and many studies have shown the adherence capacity of *Candida* spp. to mucosal surfaces and their involvement in infectious pathologies (Pizzo et al., 2000; Jabra-Rizk et al., 2001; Liu et al., 2006). However the change from *C. albicans* to others *Candida* species is also associated with a worse outcome. Several works have shown an increase in fatality and complication rates with *C. tropicalis* (Pfaller, 1996; Costa et al., 2000) and *C. krusei* (Abbas et al., 2000; Viudes et al., 2002)

In the present study when adherence was evaluated, *C. albicans* and *C. tropicalis* exhibited the higher capacity of adhesion to human buccal epithelial cells while *C. krusei* exhibited the lowest. These findings are in accordance with Gruber et al. (2003), which investigated the binding abilities of *C. albicans*, *C. tropicalis* and *C. krusei* to HIV-infected immune cells, showed significant differences, closely related to their hierarchy of virulence. In yours experiments *C. krusei* was less adherent that *C. albicans* and *C. tropicalis*.

Interestingly, beyond high adherence ability, all *C. albicans* and *C. tropicalis* strains presented a moderate content of α-L-fucose residues as observed by the staining pattern using lectin histochemistry. Among *C. tropicalis* strains, such observations become inconsistent only for isolate 4790, which showed weak adherence capacity, meanwhile moderate staining pattern for α-L-fucose. This strain has been isolated from non-clinical samples (cassava powdery) and therefore presented a coherent expected reduced pathogenicity. The strains of *C. albicans* that presented weak adherence, exceptionally

3716 and 3719, were isolated from patients with AIDS treated with antifungal drugs, which likely may to have decreased the ability to adhere of these strains. *C. albicans* 3716 and 3719 were isolated of healthy tooth scrap, without clinical lesions, hence in indigenous microbiota that is feature for to have weak pathogenicity.

All strains of *C. krusei* adhered weakly, independent of the stock time and isolation substratum. This fact happened even with the strains 4802 and 1059 that presented intense and moderate level of staining by the fucose-specific UEA, respectively, demonstrating the low pathogenic capacity, inherent to this species. In addition, was not observed a lectin histochemistry staining pattern in common, suggesting interspecies variations among the strains studied.

These combined findings lead to the belief that presence of several fucosyl residues in glycoconjugates on the surface of the yeast may be an indicative of a mechanism of anchoring by which the fungal cells interact with host structures. Furthermore, was ratified for our work the expected presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid and D-galactose/*N*-acetyl-D-galactosamine, suggesting that at least some cell wall carbohydrates contain monosaccharides other than mannose and that these may to have a much more complex structure than initially thought . The presence of non-mannan oligosaccharides is consistent with previous reports of Lopez-Ribot et al. (1999) and Soares et al. (2000).

Munõz et al. (2003) describe lectin profiling for a large number of *Candida* strains, all of them with α-D-mannose specificity. This result is in accordance with our findings, although there are variances. Such differences, than occur due the failure in recognizing of carbohydrates, may be explained by panel of lectin used. In addition, we carry out an enzyme pretreatment in the yeast suspensions with tripsin to warrant accessibility to the

sugar moieties by lectins and used like single grown media Sabouraud agar (Difco), since several studies suggest that carbohydrate expression varies according to the age/environment of the fungus (Alviano et al., 2004).

When the adherence results were evaluated in terms of source of the clinical samples, it was no observed significant difference. Bernardis et al. (1999), Panagoda et al. (2001) and Dagdeviren et al. (2005) also stated that there were no differences in the adherence values between the systemic and superficial isolates.

The knowledge of the saccharide profile in fungal surface enables the use of lectins, besides promising cell probes, to be used in therapeutic treatments as already proposed for bacterial diseases by Sharon & Ofek (2000). Since fucose-containing structures are present on *Candida* cell surface, it is possible that fucose-binding lectin, such UEA I, could be the basis of a therapeutic treatment of candidosis. As an extension of this, lectins with drug substituents might serve as carriers of antifungal agents, using as targets their specific carbohydrates found in the cell surface of microorganisms (Vardar-Ünlü et al., 1998).

We here reported the use of lectin histochemistry for the study of the carbohydrate profile found in glycoconjugates of the cell surface of three species of *Candida* (*C. albicans*, *C. tropicalis* and *C. krusei*) and a correlation with their ability of adhesion to buccal epithelial cells. Although many questions remain, we believe that complex carbohydrates on the surface of *Candida* species may represent additional motifs through which interactions of this fungus are established with host cells.

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Figure 1

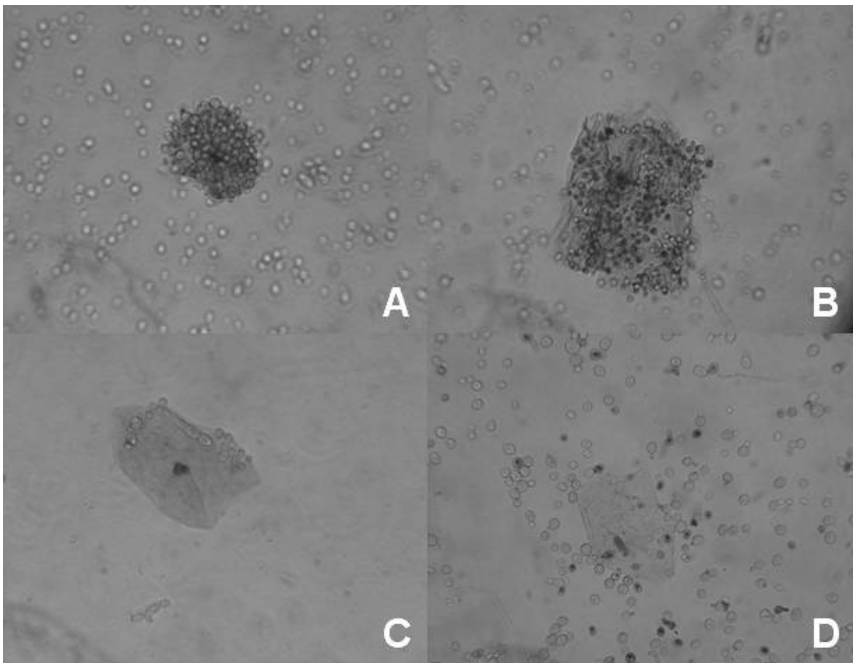

Figure 1. High adherence of *C. albicans* 4606 (A) and *C. tropicalis* 916 (B) to human buccal epithelial cells. Weak adherence of *C. tropicalis* 4790 (C) and *C. krusei* 934 (D). Magnification: x 400.

Table 1

Table 1. *Candida* strains used for the study (Obtained form the URM Culture Collection)

Species	Accession number (URM)	Storage period (years)	Substratum
Candida albicans	4990	01	Vaginal secretion
C. albicans	4987	01	Vaginal secretion
C. albicans	4986	01	Vaginal secretion
C. albicans	4820	02	Ungual scrap
C. albicans	4819	02	Ungual scrap
C. albicans	4817	02	Ungual scrap
C. albicans	4609	03	Blood
C. albicans	4606	03	Blood
C. albicans	4388	05	Oropharyngeal secretion
C. albicans	4387	05	Oropharyngeal secretion
C. albicans	4386	05	Oropharyngeal secretion
C. albicans	4385	05	Oropharyngeal secretion
C. albicans	4384	05	Oropharyngeal secretion
C. albicans	4260	05	Oropharyngeal secretion
C. albicans	4127	07	Inguinal area
C. albicans	4126	07	Urine
C. albicans	4125	07	Spittle
C. albicans	4124	07	Oropharyngeal secretion
C. albicans	3719	10	Tooth scrap
C. albicans	3716	10	Tooth scrap
C. tropicalis	4790	02	Cassava powdery
C. tropicalis	4262	06	Oropharyngeal secretion
C. tropicalis	1150	46	Tongue
C. tropicalis	933	49	Vaginal secretion
C. tropicalis	916	49	Feces
C. krusei	4802	02	*
C. krusei	4263	05	Oropharyngeal secretion
C. krusei	1059	48	*
C. krusei	934	49	Appendix biopsy
C. krusei	109	52	*

*Substratum no identified

Table 2

Table 2. Adherence assay and lectin histochemistry of the strains of *C. albicans*, *C. tropicalis* and *C. krusei* studied

Strains	Adherence capacity	Lectin staining patterns			
		Con A	WGA	UEA-1	PNA
4990	H	+	+	++	+
4987	H	+	+	++	+
4986	H	+	+	++	+
4820	H	+	+	++	+
4819	H	+	+	++	+
4817	H	+	+	++	+
4609	H	+	+	++	+
4606	H	+	+	++	+
4388	H	+	+	++	+
4387	W	+	+	++	+
4386	W	+	+	++	+
4385	W	+	+	++	+
4384	W	+	+	++	+
4260	W	+	+	++	+
4127	H	+	+	++	+
4126	H	+	+	++	+
4125	W	+	+	++	+
4124	H	+	+	++	+
3719	W	+	+	++	+
3716	W	+	+	++	+
4790	W	+	+	++	+
4262	H	+	+	++	+
1150	H	+	+	++	+
933	H	+	+	++	+
916	H	+	+	++	-
4802	W	+	+++	+++	+
4263	W	+	++	+	+
1059	W	++	+++	++	+++
934	W	+	++	+	+
109	W	+	++	+	+
6258	W	+	++	+	+

High adherence (H) or weak adherence (W).
Intense (+++), moderate (++) or weak (+) staining pattern.

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Lectin-binding analysis the glycoconjugates on surface of *Candida* species

Short title: Glycoconjugates Mapping on *Candida* species

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Abstract

Rapid identification of clinical isolates could facilitate diagnosis and treatment. Therefore, lectins in histochemistry have been used to develop fast and reliable methods for discriminating between medically important *Candida* species. This study aims to evaluate the patterns of agglutination of 46 *Candida* isolates by four lectins. The clinical isolates and type culture collection were of the species *Candida albicans* (20), *C. parapsilosis* (15), *C. tropicalis* (5) and *C. krusei* (6). The lectins used were Concanavalin A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA-1) and peanut agglutinin (PNA) conjugated to peroxidase (25 $\mu g\ mL^{-1}$). Nineteen eight percent of isolates were agglutinated by all lectins, although in different staining pattern. The only exception was a *C. krusei* isolate, which not evidenced agglutination for PNA. In addition, nine staining panels could be distinguished among the isolates. Glycoconjugates mapping by lectins may be of potential value for taxonomic and epidemiological studies of yeasts in clinical laboratories.

Keywords: *Candida*, Lectin, Typing, Identification, Diagnosis

1. Introduction

The incidence of *Candida* infections has increased greatly over the last decade, for various reasons including the use of more intensive cancer therapy procedures, complications of abdominal or cardiothoracic surgery, organ transplantation and the increased incidence of AIDS [1]. About 50% of nosocomial yeast infections are caused by *Candida albicans* [2]. However, other species are emerging as important pathogens, as *C. parapsilosis*, *C. tropicalis* and *C. krusei* [3].

Numerous procedures have been described for identification of *Candida* species [4-10], but most are time-consuming or have suboptimal discriminatory power.

Lectins are proteins of non-immune origin that bind specifically to carbohydrates [11]. The selectivity of lectins for microbial-surface sugars has been demonstrated for yeast of the genus *Candida* [12].

In the present study we evaluated the presence of carbohydrates on candidal cell wall and the use of lectins for the typing of clinical *Candida* isolates.

2. Materials and methods

2.1. Yeast and growth conditions

A total of 44 *Candida* strains were supplied by the URM Culture Collection of Department of Mycology, Biological Sciences Center, Federal University of Pernambuco and have been stocked in mineral oil [13]. Of these, 20 strains were *C. albicans,* 14 *C. parapsilosis*, five *C. tropicalis* and five *C krusei*. Viability test and taxonomic revision was carried out inoculating cultures in test tube containing 5 mL of 2% dextrose broth [14]. After growth, cultures were transferred to slants containing Sabouraud agar plus yeast

extract (Difco), and then observed their morphological, biochemical and physiological characteristics [15]. Species, accession numbers, stock time, in years, and isolation substratum are summarized in table 1. Two strains, *C. parapsilosis* ATCC 22019 and C. krusei ATCC 6258 were used as reference.

2.2. Lectins

Lectins were selected to cover a wide range of sugar specificities. The lectins used were Concanavalin A (Con A), Wheat-germ agglutinin (WGA), *Ulex europeus* agglutinin (UEA-1) and peanut agglutinin (PNA) conjugated to peroxidase (Sigma Chemical Co., St Louis, MO, USA), at concentration of 25 µg mL^{-1} in phosphate-buffered saline (PBS), pH 7.2-7.4, and stored at -20 °C in Eppendorf tubes.

2.3. Agglutination assay

Histochemistry was developed according to Rego et al. [16]. Fungal samples grown in Sabouraud agar (Difco) were suspended in PBS and adjusted to 10^7cells.mL^{-1}. Later the suspension was treated with 1% trypsin in PBS for 3 min followed by methanol- H_2O_2 solution for 5 min and incubated with lectins for 1h at 25 °C. Peroxidase reaction was visualized using diaminobendizine (DAB) and hydrogen peroxide in PBS for 5-8min at 25°C. Between each protocol step cells were washed with PBS under centrifugation (2,500g for 5 min). Inhibition assays were done by performing the lectin binding in the presence of the corresponding lectin-specific sugar: methyl-α-D-mannoside, *N*-acetyl-D-glucosamine, α-L-fucose and D-galactose for Con A, WGA, UEA I and PNA, respectively, at 300 mM concentration. Sample staining were evaluated by optical microscopy and classified qualitatively as intense (+++), moderate (++), weak (+) or none (-), according to Ozer [17]. Assays were developed in triplicate for each isolate and protocol was repeated on at least twelve different occasions.

3. Results

Lectin-binding analysis indicated the presence of α-D-glucose/α-D-mannose, *N*-acetyl-D-glucosamine/*N*-acetylneuraminic acid, α-L-fucose and D-galactose/*N*-acetyl-D-galactosamine in all isolates studied, except for isolate 916, which were not evidenced galactose moieties (Table 2). In addition, were observed nine different agglutination patterns for all isolates investigated.

All twenty *C. albicans* isolates showed a single agglutination pattern: weak for Con A, WGA and PNA and a moderate staining for UEA I. *C. parapsilosis* were agglutinated from four different ways: weak for Con A and PNA, while WGA showed changes that varied between moderate-to-intense and UEA I between weak-to-moderate staining pattern.

The *C. tropicalis* isolates were stained of the same way that *C. albicans*, with exception of the isolate 916 that was not agglutinated for PNA. *C. krusei* presented four staining patterns: weak-to-moderate for Con A, moderate-to-intense for WGA, while UEA-1 and PNA showed changes that varied between weak-to-intense staining pattern.

4. Discussion

Identification of *Candida* species by conventional morphology and assimilation tests can require 3-5 days or even longer for more difficult or unusual species. Although studies of colony morphology in solid medium remain important for the detection and presumptive identification of *Candida* yeasts, the inadequacy of such tests for accurate diagnosis has led to the use of other procedures, including differential medium tests, chromogenic tests, biochemical test kits and molecular analysis [4-10].

Lectin-binding analysis the glycoconjugates on surface of *Candida* species should be another approach for the identification and typing of yeasts, and it is an indicative of a new and unrecognized mechanism by which the taxonomy may be readily performed in the majority of the laboratories.

In yeasts, lectins bind to sugars that are abundant on the cell surface, as α-D-mannose and *N*-acetyl-D-glucosamine [18]. The role of lectins in microbiological diagnosis has been reviewed in detail by several authors since 1970 [19], although most of the experiments reports the agglutination by specifics lectins to α- mannans, as Con A, LCA and PSA [20,21].

In the present study, staining by the fucose-specific UEA is evidently a good marker for *C. albicans* and *C. tropicalis*, while WGA agglutinate successfully for *C. parapsilosis*. In *C. krusei*, was not observed an agglutination pattern in common, suggesting interspecies variations among the studied isolates.

Munõz et al. [12] describe lectin profiling for a large number of *Candida* strains, all of them with α-D-mannose specificity. This result is in accordance with our findings, although there are variances. Such differences, than occur due the failure in recognizing of carbohydrates, may be explained by panel of lectin used. In addition, we carry out an enzyme pretreatment in the yeast suspensions with tripsin to warrant accessibility to the sugar moieties by lectins and used like single grown media Sabouraud agar (Difco), since several studies suggest that carbohydrate expression varies according to the age/environment of the fungus [22].

In addition, the knowledge of the saccharide profile in fungal surface enables the use of lectins, besides promising cell probes, to be used in therapeutic treatments as already proposed for bacterial diseases by Sharon & Ofek [23]. Since fucose-containing structures

are present on *Candida* cell surface, it is possible that fucose-binding lectin, such UEA I, could be the basis of a therapeutic treatment of candidosis. As an extension of this, lectins with drug substituents might serve as carriers of antifungal agents, using as targets their specific carbohydrates found in the cell surface of microorganisms [24].

We here reported the partial profile of carbohydrates expressed in the cell surface of four species of *Candida*. Lectin histochemistry was the key method to reach the results showing to be once more a potential auxiliary tool for the characterization of cell surface glycoconjugates of yeasts. Further investigations are needed to establish the true value of lectin typing, both for taxonomic and epidemiological studies on yeasts.

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Table 1

Table 1. *Candida* isolates used for the study (Obtained from the URM Culture Collection).

Species	Accession number (URM)	Storage period (years)	Substratum
Candida albicans	4990	01	Vaginal secretion
C. albicans	4987	01	Vaginal secretion
C. albicans	4986	01	Vaginal secretion
C. albicans	4820	02	Ungual scrap
C. albicans	4819	02	Ungual scrap
C. albicans	4817	02	Ungual scrap
C. albicans	4609	03	Blood
C. albicans	4606	03	Blood
C. albicans	4388	05	Oropharygeal secretion
C. albicans	4387	05	Oropharygeal secretion
C. albicans	4386	05	Oropharygeal secretion
C. albicans	4385	05	Oropharygeal secretion
C. albicans	4384	05	Oropharygeal secretion
C. albicans	4260	05	Oropharygeal secretion
C. albicans	4127	07	Inguinal area
C. albicans	4126	07	Urine
C. albicans	4125	07	Spittle
C. albicans	4124	07	Oropharygeal secretion
C. albicans	3719	10	Tooth scrap
C. albicans	3716	10	Tooth scrap
C. parapsilosis	4984	01	Vaginal secretion
C. parapsilosis	4970	01	Vaginal secretion
C. parapsilosis	4889	02	Blood
C. parapsilosis	4818	02	Ungual scrap
C. parapsilosis	4804	02	IFM
C. parapsilosis	4608	03	Blood
C. parapsilosis	4607	03	Blood
C. parapsilosis	4433	04	Beach soil
C. parapsilosis	4261	05	Oropharygeal secretion
C. parapsilosis	3627	12	Spittle
C. parapsilosis	3624	12	Spittle
C. parapsilosis	3621	12	Spittle
C. parapsilosis	2633	15	*
C. parapsilosis	729	49	*
C. tropicalis	4790	02	Cassava powdery
C. tropicalis	4262	06	Oropharygeal secretion
C. tropicalis	1150	46	Tongue
C. tropicalis	933	49	Vaginal secretion
C. tropicalis	916	49	Feces
C. krusei	4802	02	*
C. krusei	4263	05	Oropharygeal secretion
C. krusei	1059	48	*
C. krusei	934	49	Appendix biopsy
C. krusei	109	52	*

**Substratum no identified

Table 2

Table 2. Agglutination pattern obtained for the 46 isolates with the 4 lectins.

Isolates	Lectin staining patterns			
	Con A	WGA	UEA-1	PNA
4990	+	+	++	+
4987	+	+	++	+
4986	+	+	++	+
4820	+	+	++	+
4819	+	+	++	+
4817	+	+	++	+
4609	+	+	++	+
4606	+	+	++	+
4388	+	+	++	+
4387	+	+	++	+
4386	+	+	++	+
4385	+	+	++	+
4384	+	+	++	+
4260	+	+	++	+
4127	+	+	++	+
4126	+	+	++	+
4125	+	+	++	+
4124	+	+	++	+
3719	+	+	++	+
3716	+	+	++	+
4984	+	++	++	+
4970	+	++	+	+
4889	+	+++	++	+
4818	+	++	+	+
4804	+	++	+	+
4608	+	++	+	+
4607	+	++	+	+
4433	+	++	+	+
4261	+	++	++	+
3627	+	++	+	+
3624	+	++	+	+
3621	+	++	+	+
2633	+	++	+	+
729	+	+	+	+
22019	+	++	++	+
4790	+	+	++	+
4262	+	+	++	+
1150	+	+	++	+
933	+	+	++	+
916	+	+	++	-
4802	+	+++	+++	+
4263	+	++	+	+
1059	++	+++	++	+++
934	+	++	+	+
109	+	++	+	+
6258	+	++	+	+

Intense (+++), moderate (++) or weak (+) staining pattern.

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