

Activitat in vitro de nous antifúngics i epidemiologia molecular de les infeccions per *Candida albicans*

Francesc Marco Reverté

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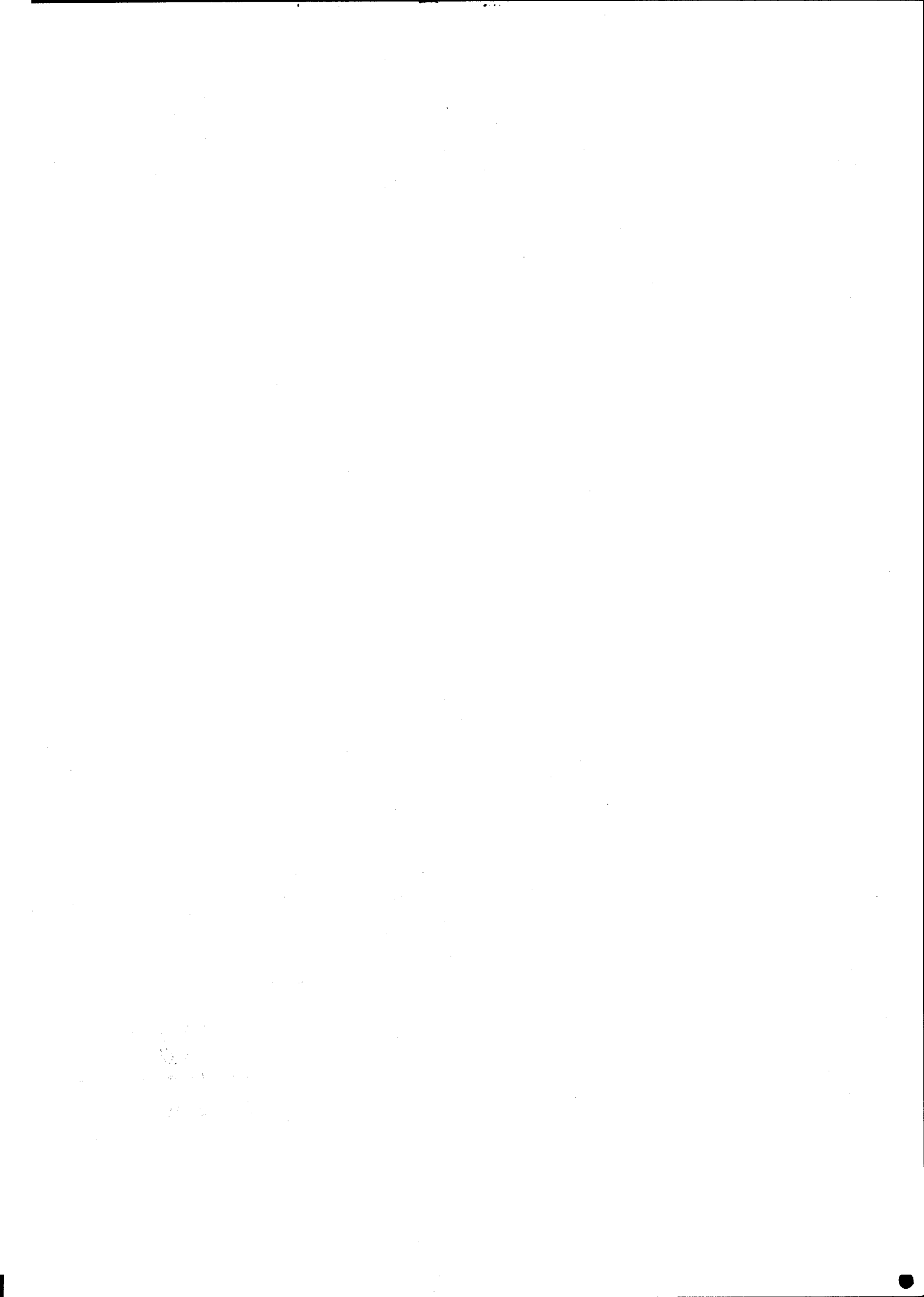
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**Activitat in vitro de nous antifúngics i epidemiologia
molecular de les infeccions per *Candida albicans*.**

**Memòria presentada per FRANCESC MARCO REVERTÉ
per a optar al grau de Doctor en Medicina i Cirurgia.**

Director: M. Teresa Jiménez de Anta Losada

SETEMBRE 2002



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I.- JUSTIFICACIÓ

I. JUSTIFICACIÓ.

Els avenços que han experimentat tots els camps de la medicina en els darrers 20 anys han influït de forma notable en els tipus de malalts que són atesos als centres hospitalaris, sobre tot en hospitals de tercer nivell. L'aplicació de noves tecnologies o actituds terapèutiques com el trasplantament de medulla òssia o d'òrgan sòlid i la utilització d'agents quimioteràpics han esdevingut cada cop més freqüents. A més a més, factors com la millora en l'atenció dels malalts ingressats en unitats de cures intensives, la nutrició parenteral, l'hemodiàlisi o els antibiòtics d'ampli espectre han contribuït de forma clara i favorable al tractament dels pacients en situacions crítiques. Això però, té una contrapartida negativa que es tradueix en la presència d'un major nombre de malalts hospitalitzats amb un compromís immunitari evident o amb patologies de base molt greus. Considerats com un conjunt, aquests tipus de pacients constitueixen una població altament susceptible a patir una infecció nosocomial que pot estar causada per diversos microorganismes entre els que cal incloure els fongs. Les infeccions fúngiques en aquests malalts són sovint severes, ràpidament progressives i generalment, hi ha certes dificultats per arribar al seu diagnòstic o realitzar un tractament adequat.

En la dècada dels 80 ja es va fer evident que la freqüència amb la que els fongs estaven implicats com agents etiològics de diverses infeccions intrahospitalàries era cada cop més important (Harvey and Myers, 1987; Bodey, 1988; Anaissie and Bodey, 1989). Entre el 1980 i 1990, les dades proporcionades pels CDC (Centers for Disease Control) a través del sistema NNIS (National Nosocomial Infections Surveillance) indicaven un increment en la proporció d'infeccions nosocomials fúngiques de 2 a 3.8 per 1000 altes (Beck-Sague et al, 1993). Aquest augment era evident no tant sols en fungèmies (5.4% a 9.9%) sino també en pneumònies, infeccions urinàries o de la ferida quirúrgica. En una anàlisi sobre l'increment dels

agents patògens implicats en diversos hospitals dels EEUU, es va apreciar un augment en la proporció d'infeccions nosocomials degudes a *Candida* spp que va passar d'un 2% el 1980 a un 5% el 1986, xifra que es va mantenir fins el 1989 (Schaberg et al). Dades de diversos hospitals del NNIS dels anys 1990 a 1992 van situar a *Candida albicans* en el seté lloc entre els agents patògens responsables d'infeccions nosocomials (Emori and Gaynes, 1993). En diversos estudis de candidèmia realitzats en aquelles dates, la mortalitat crua estimada atribuïble a aquesta infecció se situava en un 50-60% i aproximadament un terç dels malalts morien com a conseqüència de la candidèmia (Karabinis et al, 1988; Wey et al, 1988; Bross et al, 1989; Kosmian et al, 1989).

Les infeccions nosocomials per *Aspergillus* spp, sobre tot *A. fumigatus* i *A. flavus*, s'han convertit en una complicació terrible en els malalts amb immunodepressió severa ja sigui com a conseqüència d'un trasplantament de medul·la òssia o d'òrgan sòlid o per rebre corticoteràpia (Anaissie and Bodey, 1989; Bodey, 1988; Rhame, 1991). Tot i que les dades del sistema NNIS indicaven que només un 1.3% de les infeccions nosocomials fúngiques comunicades entre 1980 i 1990 eren atribuïbles a *Aspergillus* spp (Beck-Sague et al, 1993), la incidència era probablement més alta en àrees especialitzades. Així, en un estudi realitzat en una unitat de trasplantament de medul·la òssia es va aïllar *Aspergillus* spp en el 36% dels malalts amb pneumònia nosocomial (Pannuti et al, 1991)

Treballs publicats en els darrers 10 anys també constaten una tendència creixent a l'augment de les infeccions fúngiques invasives (Fridkin and Jarvis, 1996; Kao et al, 1999; Rees et al, 1999). En un estudi realitzat als EEUU on s'analitzen les causes de mort declarades en els certificats de defunció entre 1980 i 1997 es comunica un augment (3.7 vegades superior) en el nombre de morts directament relacionades o que va precipitar, l'existència d'una micosi invasiva (McNeil et al, 2001). El nombre de candidiasis invasiva, si no considerem els malalts amb la SIDA, va augmentar de

forma progressiva des de 1980 fins al 1989, i a partir d'aquest any va anar disminuint de forma paulatina, probablement relacionat amb la utilització de pautes de profilaxi i/o tractament de fàrmacs antifúngics de la família dels azols. En una comunicació recent (Blumberg et al, 2001) en la que s'analitzaven els factors de risc per desenvolupar una candidemia en els malalts ingressats en unitats de cures intensives quirúrgiques, es va registrar una incidència de candidemia de 9.82 per 1000 admissions amb una mortalitat del 41%. En els malalts que no van patir aquesta complicació, la mortalitat va ser del 8%. En el treball de de McNeil i cols (McNeil et al, 2001), el nombre de morts atribuïbles a infeccions per *Aspergillus* spp i altres fongs (si s'exceptua *Candida* spp) va créixer de forma quasi exponencial assolint valors del 375% i 329% respectivament, sobre les xifres inicials. En un estudi epidemiològic realitzat en un grup ampli de malalts que van rebre un trasplantament al·logènic de progenitors de medul·la òssia (TPH) també es va constatar una incidència creixent d'infecció per *Aspergillus* spp (Wald et al, 1997). Aquest tipus d'infeccions tenen una elevada mortalitat, entre un 99% i un 86% en cas d'infecció pulmonar i cerebral, respectivament (Denning, 1996). Una publicació més recent (Lin et al, 2001) realitzada amb casos diagnosticats entre 1995 i 1999 comunica una mortalitat del 88.1% en l'aspergil·losi disseminada o amb afectació del sistema nerviós central i del 86.7% en els malalts amb un TPH.

El 90% de totes les infeccions fúngiques nosocomials estan produïdes per espècies dels gèneres *Candida* i *Aspergillus* (Warnock, 1998). Hi ha però diversos fets que cal tenir present en el moment de considerar aquests tipus d'infeccions. La mortalitat atribuïble a una candidiasi invasiva, malgrat el tractament, segueix sent elevada. Tot i que *Candida albicans* és l'espècie que amb més freqüència està implicada en les infeccions nosocomials, hi ha evidències d'un canvi cap a altres espècies com *C. tropicalis*, *C. parapsilosis*, *C. glabrata* i *C. krusei* (Wingard, 1995; Pfaller, 1996; Coleman et al, 1998). Algunes d'aquestes espècies són menys sensibles als antifúngics habituals i per tant, en dificulten el tractament. La mortalitat per

aspergil·losi en malalts immunodeprimits encara és més elevada que en la candidiasi sistèmica i el pronòstic de la malaltia en aquests pacients és dolent, probablement més per la situació immunitària del malalt que no pas pel tractament amb amfotericina B (Lin et al, 2001). Finalment, diversos tipus de fongs menys habituals fins ara, s'aïllen cada cop amb més freqüència en infeccions fúngiques invasives: *Rhizopus* spp, *Fusarium* spp, *Scedosporium* spp, *Acremonium* spp i fongs dematiacis (Gamis et al, 1991; Annaissie, 1992; Van den Saffele and Boelaert, 1996; Perfect and Schell, 1996).

L'increment experimentat en el nombre d'infeccions nosocomials fúngiques ha comportat, de forma paral·lela, un augment en el nombre de comunicacions científiques que descriuen diferents brots epidèmics nosocomials causats per fongs (Fridkin and Jarvis, 1996). Es obvi que el focus d'origen o el mecanisme de transmissió pot ser molt diferent d'un brot a l'altre segons les característiques de l'agent implicat. A més a més, per determinar la causa d'un brot epidèmic concret i poder adoptar les mesures de control més adients per aturar-lo, és fonamental conèixer la fisiopatologia del fong implicat. Però, tal com ha passat amb les infeccions bacterianes, a mesura que aprofundim en l'epidemiologia d'una determinada infecció fúngica, i per extensió, la dels brots epidèmics, es fa cada cop més evident la necessitat d'utilitzar una determinada metodologia que ens ajudi a avaluar les troballes obtingudes. L'aplicació de mètodes moleculars per comprendre millor l'epidemiologia de les infeccions fúngiques i la consegüent tipificació dels fongs implicats ha experimentat un impuls notable en els darrers 10 anys. Tot i els diversos mètodes existents, no hi ha però, un mètode considerat "estandard" i l'elecció d'un o altre dependrà de les preguntes plantejades i les possibilitats de cada centre (Soll, 2000).

Per tot el comentat anteriorment, és obvi que la millora en el pronòstic de les infeccions fúngiques, sobre tot en malalts amb immunosupressió, és un objectiu que cal assolir el més aviat possible. Aquest objectiu es pot abordar des de diferents punts de vista. Així, en la pràctica diària hi ha dues opcions que semblen clares: avançar en les tècniques de diagnòstic precoç d'aquestes infeccions i la introducció en el tractament de nous antifúngics que millorin l'activitat, tolerància i seguretat dels antifúngics actuals. Hi ha però altres possibilitats que també cal estudiar per aconseguir l'objectiu que hem comentat. Algunes són necessàries com a pas previ a les opcions anteriors i altres simplement les complementen. En aquesta tesi hem centrat el nostre treball en un aspecte més bàsic i inicial com és la valoració de l'activitat *in vitro* de nous antifúngics sobre fongs patògens prevalents amb la finalitat d'esbrinar les seves possibilitats com a fàrmacs potencialment útils en el tractament de les infeccions fúngiques. A més a més, hi ha un segon aspecte que també ha estat objecte d'anàlisi com és l'aplicació de la sonda semirepetitiva *Ca3* a l'estudi de les infeccions nosocomials per *Candida albicans*.



II.- INTRODUCCIÓ



II. INTRODUCCIÓ.

1. Característiques generals dels fongs.

Els fongs son organismes eucariotes, uni o pluricel.lulars, dotats d'una paret cel.lular amb quitina, heteròtrofs, que es nutreixen per absorció i amb una reproducció sexual o asexual que dóna lloc a la formació d'espores. Una forma clàssica i senzilla d'estudiar els fongs d'interés mèdic és dividir-los segons la seva morfologia en fongs filamentosos i llevats. Els primers són microorganismes pluricel.lulars constituïts per unes estructures tubulars o hifes que creixen per prolongació apical fins a formar el miceli. Els llevats són unicel.lulars amb una morfologia ovoïdea, esfèrica o cilíndrica. En realitat, els llevats no son tant diferents de les hifes i no representen més que una forma de creixement. De fet, hi ha fongs que segons les condicions ambientals canvien de la forma d'hifa a llevat i viceversa (fongs dimòrfics).

Des d'un punt de vista taxonòmic, els fongs constitueixen el regne Fungi (*Eumycota*) completament diferenciat dels regnes animal, vegetal, protista i procariota (*Monera*). L'estudi i classificació taxonòmica dels fongs és un camp en continua evolució. Fins no fa gaire els fongs es dividien en sis grups o Filum segons les seves característiques: *Chytridiomycota*, *Zygomycota*, *Ascomycota*, *Deuteromycota*, *Basidiomycota* i *Oomycota* (Alexopoulos et al, 1996). Recentment, estudis filogenètics moleculars han reduït el nombre de grups del regne *Eumycota* a quatre: *Chytridiomycota*, *Zygomycota*, *Ascomycota* i *Basidiomycota* (Guarro et al, 1999). Es creu que hi ha descrites més de 100.000 espècies de fongs, però poc més d'unes 100 poden produir algun tipus de patologia en humans. La taula 1 (modificada de Alexopoulos et al, 1996 i Guarro et al, 1999) conté de forma resumida els grups més importants en patologia humana i alguns dels gèneres més representatius.

formació d'esferoplasts. Es comporta com una interfase que protegeix al fong del medi ambient, evita la lisi osmòtica i actúa de filtre de molècules. A més a més, intervé en diverses funcions fisiològiques i pot manifestar propietats antigèniques. L'anàlisi química de la paret cel.lular revela un predomini de polisacàrids, quantitats menors de proteïnes i encara menys lípids. Tot i que la composició de la paret pot variar segons el tipus de fong i fins i tot, la fase del cicle de creixement, els polisacàrids més importants són la quitina, glucans (polímers de glucosa) i manans (manoproteïnes). La quitina està formada per homopolímers de N-acetilglucosamina que son sintetitzats per quitina sintases a partir de UDP-N-acetilglucosamida afegint dos sucres. Forma unes microfibrilles que actuen com un esquelet que utilitzaran altres components per unir-se i formar la paret. En molts fongs, els glucans més importants són α -(1,3)-, β (1,3)- i β (1,6)- glucà. El β (1,3)-D-glucà es sintetitza a partir d'UDP-glucosa per l'enzim β (1,3)-D-glucà sintasa (lligat a la membrana plasmàtica com la quitina sintasa) i que s'uneix a la cèl.lula a través d'un component fibrillar de quitina. Els manans són glicoproteïnes que constitueixen el major component de la capa externa de la paret. Estan formats per complexes cadenes de polisacàrids de manosa units a proteïnes per residus de N-acetilglucosamida (Debono, 1994; Gooday, 1995). En alguns llevats, com *Cryptococcus neoformans*, la paret pot estar envoltada per una càpsula de polisacàrids que actúa enmascarant components antigènics i evita la fagocitosi per cèl.lules nucleades.

Síntesi de proteïnes. La síntesi de proteïnes en els fongs no difereix en gran mesura de la que es produeix en les cèl.lules eucariotes. Hi ha tres factors d'elongació, però cal citar l'existència de dues diferències importants que poden ser d'utilitat. Una primera radica en el fet que el factor d'elongació 3 (EF3) és propi dels fongs i no es troba en les cèl.lules dels mamífers. La segona consisteix en l'observació que el factor d'elongació 2 (EF2) és funcionalment diferent del seu equivalent en les cèl.lules humanes.

2. Fàrmacs antifúngics d'ús sistèmic.

Amb l'excepció de la fluorocitosina, els antifúngics que fins recentment podem utilitzar per a tractar una micosi sistèmica actuen sobre l'ergosterol ja sigui de forma directa com l'amfotericina B o bé inhibint-ne la seva síntesi, com els compostos azòlics (Kerridge, 1988; Geogopapadakou and Walsh, 1998; Ghannoum and Rice, 1999). L'ergosterol, l'esterol més important de la membrana plasmàtica fúngica, intervé en una varietat important de funcions cel·lulars. El seu paper és fonamental per mantenir l'integritat de la membrana i el funcionament correcte de molts enzims lligats a la membrana com la quitina sintasa, la qual és imprescindible per a la divisió i creixement cel·lular. Hi ha altres antifúngics, com les alilamines, les bencilamines i els tiocarbamats, que també interfereixen la síntesi de l'ergosterol si bé ho fan en un lloc diferent d'on actuen els azols. La fluorocitosina és un anàleg de la pirimidina que un cop a l'interior de la cèl·lula actua interferint-ne la síntesi de l'ARN i ADN (Ghannoum and Rice, 1999; Andriole, 1999).

En els últims anys s'han dedicat grans esforços al desenvolupament de nous antifúngics. La indústria farmacèutica ha intentat, per una part, millorar els compostos existents (per ex, amfotericina B lipídica, itraconazol amb ciclodextrina) i per una altra, sintetitzar nous fàrmacs derivats de mol·lècules existents (per ex, nous derivats azòlics). A aquestes línies de recerca s'han sumat en els darrers anys, dues noves estratègies completament diferents. Una d'elles es fonamenta en la recerca de fàrmacs inhibidors de la síntesi proteica, com les sordarines (Dominguez et al, 1998). Una segona línia, "a priori" més prometedora, s'ha dirigit a la recerca de compostos que interfereixen la formació o funció dels diferents polímers que constitueixen la paret fúngica. Dintre d'aquest grup, si bé s'han descobert diverses molècules com pradimicines, benanomícines, polioxines i nikkomicines, el grup de fàrmacs més

rellevants és el constituït per les equinocandines (Debono and Gordee, 1994; Geogopapadakou, 2001; Walsh and Giri, 1997).

2.1. Antifúngics de la família dels poliens.

Les dues molècules més importants que formen part de la família dels poliens són la nistatina i l'amfotericina B. Des de la seva introducció fa més de 40 anys fins a l'actualitat, l'amfotericina B segueix sent l'antifúngic estandard per a tractar moltes infeccions fúngiques invasores. En la seva versió clàssica (desoxicolat) manifesta una relativa afinitat pel colesterol de les cèl·lules humanes, que explicaria la toxicitat sobre el túbul renal i és un potent inductor de la síntesi del factor de necrosi tumoral i interleucina-1 per les cèl·lules mononuclears, a la que caldria atribuir l'aparició de febre i escalfrets relacionats amb l'administració intravenosa (Goodwin et al, 1995). Per intentar minimitzar o reduir aquests efectes indesitjables s'han desenvolupat en els últims anys les formulacions que incorporen l'amfotericina B a liposomes unilamelars o a complexos lipídics (Wong-Beringer et al, 1998). En fase d'avaluació clínica hi ha una preparació liposòmica de nistatina que ha demostrat ser útil en models animals experimentals (Wallace et al, 1997; Johnson et al, 1998).

Mecanisme d'acció.

Els antifúngics de la família dels poliens, com l'amfotericina B i la nistatina, són compostos amfipàtics que actuen a nivell de les membranes cel·lulars que contenen ergosterol. Es creu que aquests fàrmacs s'intercalen en la membrana formant uns canals o porus que utilitzen diversos components cel·lulars, sobre tot ions potasi, per sortir a l'exterior amb la qual cosa s'altera el gradient de protons de la membrana que condueix a la mort cel·lular (Ghannoum and Rice, 1999). En el cas concret de l'amfotericina B, un poliè de gran tamany, s'ha proposat que la interacció amb la membrana cel·lular es deguda a canals o porus aquosos formats per una

estructura anular de 8 molècules de l'antifúngic unides amb enllaços hidrofòbics a l'ergosterol (Holz, 1974; Ghannoum and Rice, 1999). No es coneix molt bé perquè els poliens tenen aquesta especificitat per les membranes amb ergosterol. S'ha suggerit que podria estar relacionada amb la composició d'àcids greixosos dels fosfolípids i la relació esterol-fosfolípids (Vanden Bossche et al, 1994).

Un altre possible mecanisme d'acció de l'amfotericina B seria que el fàrmac fóra el responsable d'un efecte oxidatiu sobre la membrana plasmàtica fúngica (Vanden Bossche et al, 1987; Vanden Bossche et al, 1994), encara que també s'ha suggerit que in vivo produiria l'efecte contrari, antioxidant (Osaka et al, 1997).

L'amfotericina B té un espectre d'activitat ampli que abarca la major part dels agents implicats en micosis sistèmiques, incloent-hi les espècies que integren els tres gèneres més freqüents: *Candida* spp, *Cryptococcus* spp i *Aspergillus* spp. Tot i que, tal com es comentarà després, la resistència a l'amfotericina B és rara cal tenir en compte aquesta possibilitat davant de l'aïllament de determinats tipus de fongs. S'ha trobat resistència a aquest antifúngic en *Pseudallescheria boydii*, *Scedosporium* spp, *Fusarium* spp, *Aspergillus terreus*, alguns aïllats d'*Aspergillus flavus*, *Trichosporum beigelli*, *Candida lusitaniae* i *Candida guilliermondii*. En altres fongs com, *C. glabrata*, *C. krusei* o *C. neoformans*, la detecció de resistència és encara menys freqüent (Dick et al, 1980; Walsh et al, 1990; Wingard et al, 1991; Karytakakis et al 1993; Nguyen et al, 1998; Vanden Bossche et al, 1998; Ghannoum and Rice, 1999; Kontoyiannis and Lewis, 2002).

Mecanisme de resistència.

Els mecanismes de resistència als poliens no han estat estudiats en detall perquè, malgrat la seva utilització durant prop de 40 anys, la resistència a aquests compostos és rara (White et al, 1998; Ghannoum and Rice, 1999). S'ha proposat una possible hipòtesi bioquímica (Hamilton-Miller, 1972) segons la qual, la resistència es

deuria a canvis, quantitativs o qualitativs, en el contingut d'ergosterol de la cèl.lula. Això faria que la unió dels poliens a les cèl.lules resistents (alteració de l'esterol) fóra menor que a les cèl.lules sensibles. En *C. albicans* podria atribuirse a tres supòsits (Ghannoum and Rice, 1999): 1) descens en el contingut total d'ergosterol sense canvis concomitants en la composició de l'esterol, 2) substitució de tots o d'alguns esterols als que s'uneixen els poliens per uns altres als que no s'unirien bé, per exemple: substitució de l'ergosterol, colesterol o estigmasterol per un 3-hidroxi o 3-oxoesterol i 3) reorientació o enmascarament de l'ergosterol de tal forma que la unió als poliens sigui menys favorable. Les alteracions en el contingut d'ergosterol de la membrana secundaris a mutacions en la via de la biosíntesi de l'ergosterol sembla ser el mecanisme de resistència als poliens més probable en *Candida* spp (Dick et al, 1980). Per una altra banda, la deficiència d'ergosterol en les cèl.lules mutants no sembla que comporti cap avantatge evolutiu i podria explicar la manca de selecció in vivo (Polak and Hartman, 1991). En *Cryptococcus* spp la resistència (infreqüent) a l'amfotericina B es deuria a mutacions en la via de la biosíntesi de l'ergosterol amb una deficiència Δ -8-7-isomerasa (Perfect and Cox, 1999).

2.2. Antifúngics Azòlics.

En l'actualitat, els compostos azòlics constitueixen una família d'antifúngics àmpliament utilitzada en el tractament tant de micosis superficials com sistèmiques. La primera molècula de la família (clormidazol), d'aplicació tòpica, es va comercialitzar als EEUU l'any 1958. Onze anys després van començar a utilitzar-se el miconazol i el clotrimazol. Poc després (1974) va introduir-se el econazol i, a finals de la dècada dels 70, es va disposar d'una formulació parenteral de miconazol. Als anys 80, l'únic antifúngic nou va ser el ketoconazol i, uns deu anys després, el fluconazol i l'itraconazol van sumar-se als pocs antifúngics disponibles per tractar una micosi sistèmica (Sheehan et al, 1999; Andriole, 1999).

El fluconazol i l'itraconazol són fàrmacs generalment ben tolerats. El primer està disponible per via oral i parenteral i el segon presenta problemes de biodisponibilitat oral amb una absorció erràtica. Aquest inconvenient s'ha intentat evitar solubilitzant-ne el fàrmac en ciclodextrina amb la qual cosa s'ha aconseguit una formulació per ús intravenos i una sol.lució oral que millora l'absorció del fàrmac (De Beule and Gestel, 2001). És freqüent referir-se als compostos azòlics segons el nombre de nitrògens que conté l'anell azòlic. Els imidazols (ketoconazol, miconazol, clotrimazol) en tenen dos i els triazols com fluconazol i itraconazol, tres. Els nous compostos triazòlics com voriconazol (UK-109,496), ravuconazol (BMS-207147, ER-30346) i posaconazol (SCH 56592) són derivats del fluconazol, els dos primers, i de l'itraconazol, el tercer.

Mecanisme d'acció.

Els antifúngics azòlics interrompeixen la síntesi de l'ergosterol a l'inhibir l'enzim P-450 14- α esterol demetilasa (Hitchcock et al, 1990). La inhibició d'aquest enzim condueix a una disminució de la quantitat d'ergosterol i a una acumulació dels seus precursors amb la formació d'una membrana plasmàtica alterada, tant pel que fa a la seva estructura com per la seva funció. En la figura 1 es resumeix la via metabòlica de síntesi de l'ergosterol. En *C. neoformans* els triazols també afecten la reducció de obtusifoliona a obtusifoliol que produeix una acumulació de precursors d'esterol metilats (Vanden Bossche et al, 1993; Ghannoum et al, 1994).

El fluconazol i l'itraconazol són antifúngics actius enfront de *Candida* spp (*C. krusei* és intrínscament resistent al fluconazol i un percentatge important dels aïllats de *C. glabrata* tenen CMIs elevades), *C. neoformans* i *Aspergillus* spp (fluconazol és resistent). El nous triazols tenen un espectre d'activitat més ampli que el dels seus predecessors i, en línies generals, una activitat intrínseca superior.

Mecanismes de resistència.

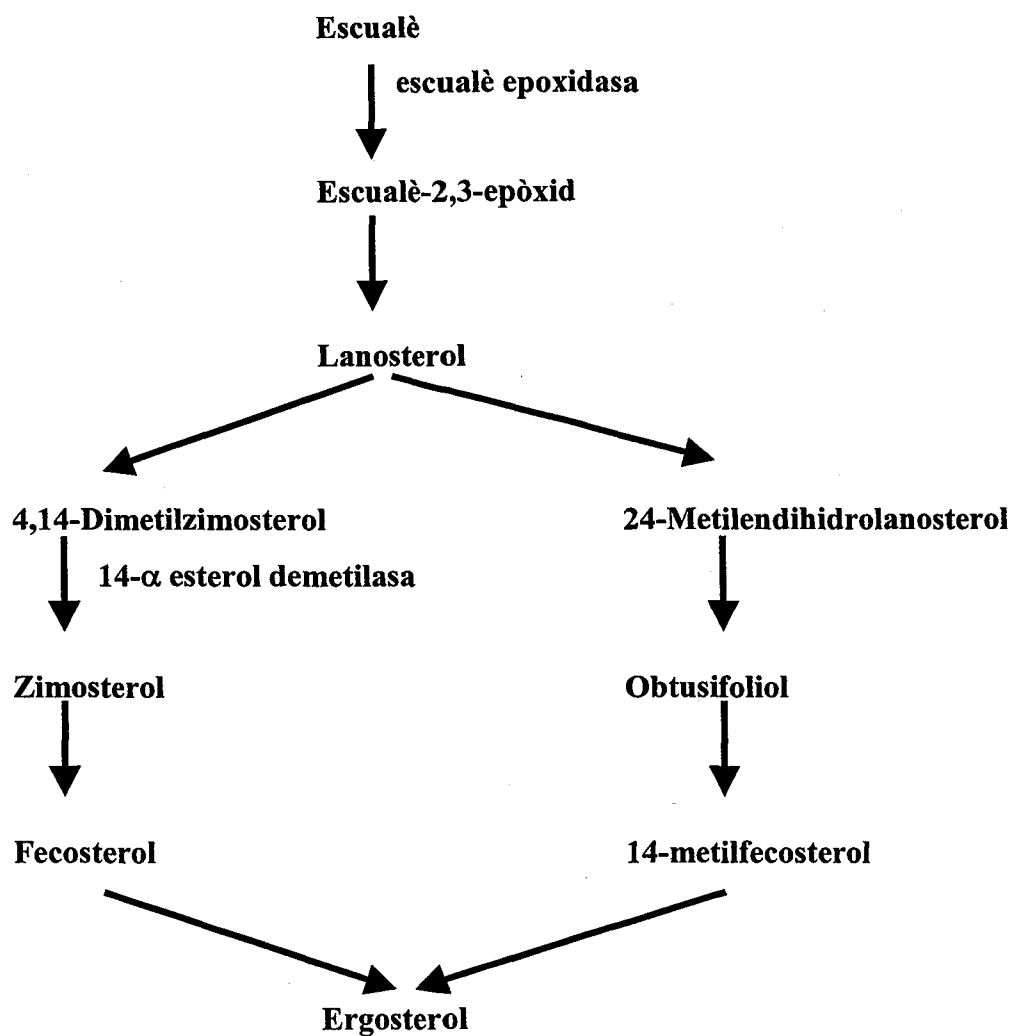
Hi ha diversos mecanismes que poden estar involucrats en la detecció d'un fenotip de resistència als antifúngics azòlics. Els més rellevants es podem agrupar en dos grans grups.

1.- Modificacions en la via de la síntesi de l'ergosterol. Aquest mecanisme de resistència es fonamentaria bàsicament en la modificació de la diana sobre la que actuen els azols. La síntesi de l'enzim 14- α esterol demetilasa està codificada per l'enzim *ERG11* el qual podria experimentar diverses alteracions genètiques com una mutació puntual o una sobreexpressió (White al al, 1998; Ghannoum and Rice, 1999). Això es traduiria en una alteració de la 14- α esterol demetilasa que no seria reconeguda com a tal per l'antifúngic o bé hi hauria una quantitat excessiva de l'enzim (White, 1997; Sanglard et al, 1995; Marichal et al, 1997). Cal destacar que l'enzim, tot i la seva modificació, segueix sent activa i catalitza la demetilació. Les alteracions en altres gens *ERG*, sobre tot el gen *ERG3*, que intervenen en la biosíntesi de l'ergosterol també podrien contribuir a la resistència als azols. La modificació de l'enzim Δ -5-6-desaturasa (codificat pel gen *ERG3*) es traduiria en l'acumulació de 14 α -metil fecosterol en lloc d'ergosterol (Kelly et al, 1996; Kelly et al, 1997).

2.- Reducció de l'acumulació de l'antifúngic. La disminució de l'acumulació dels azols a l'interior de les cèl.lules s'ha estudiat emprant fàrmacs, com el fluconazol, marcats amb isòtops radioactius. Aquests estudis han demostrat l'importància de la resistència als antifúngics azòlics per un mecanisme de bombes d'expulsió. Els fongs poden tenir dos tipus de bombes d'expulsió: a) proteïnes de la superfamília MF ("major facilitator") i b) proteïnes de la superfamília ABC ("ATP binding cassette"). Els dos sistemes intervenen fisiològicament expulsant de l'interior de la cèl.lula diversos tipus de molècules (White al al, 1998; Ghannoum and Rice, 1999). Quan es produeix una sobreexpressió d'algunes de les proteïnes que en formen part d'aquests sistemes es pot produir un augment de la resistència als antifúngics

azòlics. Dintre de la família MF s'ha identificat el gen *MDR1* el qual codifica la síntesi d'una proteïna, MDR ("multidrug resistant") o BEN-R, implicada en la resistència a fluconazol, benomil i metotrexate. Les proteïnes de la família ABC implicades en la resistència als azols són les codificades pels gens *CDR* ("Candida drug resistance"). Una sobreexpressió d'aquestes proteïnes contribuiria a la resistència de tots els antifúngics azòlics (Parkinson et al, 1995; Sanglard et al, 1995).

Figura 1. Esquema de la biosíntesi de l'ergosterol.



2.3. Alilamines.

La naftilina i la terbinafina, constitueixen la família de les alilamines, una classe de fàrmacs antifúngics que inhibeixen la síntesi de l'ergosterol a un nivell diferent d'on actuen els compostos azòlics. S'utilitzen generalment per via tòpica en el tractament de les dermatofitosis tot i que la terbinafina, disponible per via oral, podria emprar-se en el tractament d'infeccions per *Candida* spp resistents als azols o per fongs filamentosos amb dificultats terapèutiques (Ryder, 1999).

Mecanisme d'acció.

Les alilamines actuen en una etapa inicial de la síntesi de l'ergosterol a l'inhibir l'enzim escualè epoxidasa que catalitza la epoxidació de l'escualè (Fig 1). La mort cel.lular es produeix més per l'acumulació d'escualè que per la manca d'ergosterol ja que nivells elevats d'escualè incrementarien la permeabilitat de la membrana amb la consegüent disrupció de la organització cel.lular (Ryder and Favre, 1997).

Mecanismes de resistència.

El mecanisme intrínsec de resistència a les alilamines no és conegut molt bé però podria estar relacionat amb l'activitat de bombes d'expulsió com CDR1 (Sanglard et al, 1996).

2.4. Fluoropirimidines.

L'únic component d'aquesta família utilitzat en el tractament d'infeccions fúngiques és la fluorocitosina.

Mecanisme d'acció.

La fluorocitosina és un anàleg de la pirimidina que penetra a l'interior de la cèl.lula amb l'ajut d'una citosina permeasa i que posteriorment es converteix per desaminació (citosina desaminasa) en 5-fluorouracil. En últim terme, el fluorouracil actuarà interferint-ne la síntesi de l'ARN, ADN i proteïnes (Diasio et al, 1978; Polak and Scholer, 1975).

L'espectre d'activitat de la fluorocitosina es reduït, limitat a *Candida* spp i *Cryptococcus* spp. Té resistència primària a pràcticament tots els fongs filamentosos i hi ha un risc elevat de desenvolupar resistències secundàries si s'utilitza com a monoteràpia.

Mecanismes de resistència.

La resistència a la fluorocitosina pot produir-se per disminució de la penetració de l'antifúngic a l'interior de la cèl.lula (pèrdua de l'activitat de l'enzim permeasa) o pèrdua de l'activitat enzimàtica responsable de la conversió en les molècules que interfereixen en la síntesi dels àcids nucleics. Aquest segon mecanisme es probablement el més important (Whelan, 1987; Whelan and Kerridge, 1984).

2.5. Antifúngics que actuen sobre la paret cel.lular.

La paret fúngica és una estructura essencial per a la supervivència dels fongs que està intimament lligada a les fases de creixement i divisió cel.lular. Aquesta paret està composta per un complex de proteïnes i polícarbohidrats com glucans, manoproteïnes i quitina que no estan representats en les cèl.lules humanes. Els components estructurals més importants són els glucans, sobre tot el (1,3)- β -D-glucà sintetitzat a partir de UDP-glucosa per l'enzim (1,3)- β -D-glucà sintasa (Debono and Gordee, 1994). Tot i que s'han desenvolupat fàrmacs amb activitat sobre D-manòsids (pradimicines i benanomicines) o quitina (polioxines i nikkomicines), el grup

farmacològic més prometedor és el dels inhibidors de la (1,3)- β -D-glucà sintasa, com les equinocandines i les papulacandines (Debono and Gordee, 1994; Gooday, 1995; Denning, 1997; Kurtz and Douglas, 1997; Georgopapadakou, 2001). Aquest últim grup probablement no continuarà el seu desenvolupament perquè el seu espectre d'activitat in vitro està limitat a *Candida* spp i no s'ha observat una bona correlació entre les dades obtingudes in vitro i resposta in vivo.

2.5.1. Equinocandines

Les equinocandines són lipopèptids cíclics units a través d'un nitrògen a una cadena lateral lipídica. El primer membre d'aquest grups de fàrmacs, la cilofungina, només tenia activitat sobre *Candida* spp. Modificacions posteriors de l'estructura han conduït a una nova generació d'equinocandines semisintètiques amb un increment de la potència i el espectre d'activitat així com unes característiques farmacocinètiques més favorables. De les tres molècules més desenvolupades, la caspofungina (MK-0991, L-743-872) s'ha comercialitzat recentment. Les altres dues, l'anidulofungina (LY-303366) i la micafungina (FK 463) estan en fase d'avaluació. L'espectre d'activitat d'aquests antifúngics abarca les diferents espècies de *Candida* (*C. parapsilosis* presenta CMI més elevades), *Aspergillus* spp, altres fongs filamentosos i *Pneumocystis carinii*. No són actius sobre *Cryptococcus neoformans*.

Mecanisme d'acció.

Les equinocandines actuen de forma no competitiva inhibint l'enzim (1,3)- β -D-glucà sintasa, localitzat a la membrana plasmàtica i codificat pel gen *FKSI*. El (1,3)- β -D-glucà és un polímer estructural de la paret fúngica que estableix enllaços amb el (1,6)- β -D-glucà i la quitina i això resulta essencial per conferir a la paret rigidesa i elasticitat.

Mecanismes de resistència.

Les dades que es disposen sobre resistència a les equinocandines procedeixen d'estudis realitzats amb mutants obtinguts in vitro (Kurtz et al, 1996). Aquests aïllats manifesten un nivell de resistència 10 cops superior a la CMI de la població sensible i presenten mutacions en un dels al·lels del gen *FKSI*. No es coneix l'existència d'aïllats mutants in vivo.

2.5.2. Pradimicines i benanomicines.

Les pradimicines i benanomicines tenen una estructura similar i constitueixen una classe única de fàrmacs antifúngics. Aquests compostos s'uneixen al fragment terminal D-mannòsid de la paret fúngica formant un complex ternari (D-mannòsid-pradimicina/benanomicina-calci) que produeix una disrupció en la integritat de la membrana cel·lular (Walsh and Giri, 1997). Totes dues tenen un espectre d'activitat ampli que inclou *Candida* spp, *C. neoformans*, *Aspergillus* spp, zygomycets i fongs dematiacis (Fung-Tomc et al, 1995; Watanabe et al, 1996; Walsh and Giri, 1997). Desafortunadament, la detecció de problemes de toxicitat hepàtica en les avaluacions inicials han motivat la interrupció del seu desenvolupament.

2.5.3. Polioxines i Nikkomicines.

La quitina és un polímer lineal constituït per la unió de residus de (1-4)- β -N acetilglucosamida que es sintetitza a la membrana plasmàtica per la quitina sintasa (Gooday, 1995). Les polioxines (utilitzades a l'agricultura) i nikkomicines són potents inhibidors competitiu de la quitina sintasa. L'activitat de la nikkomicina Z està limitada a determinats fongs endèmics com *C. immitis* i *B. dermatitidis* tot i que s'ha observat un efecte sinèrgic en determinats aïllats de *Candida* spp si es

combinava amb azols (Milewski et al, 1991; Hector and Schaller K, 1992; Li and Rinaldi, 1999)

2.6.Sordarines

La síntesi proteica sempre ha estat considerada una diana molt atractiva en el desenvolupament d'agents antimicrobians. L'aplicació d'aquesta idea en el camp de la terapia antifúngica és arriscada ja que té l'inconvenient del grau de similitud existent entre les cèl.lules eucariotes. Malgrat això, les sordarines, tenen una activitat inhibidora de la síntesi proteica altament específica (Dominguez et al, 1998). El mecanisme d'acció seria la inhibició del factor d'elongació 2, que en *C. albicans* té una homologia del 85% amb la corresponent proteïna humana (Dominguez and Martin, 1998). Hi ha diverses molècules que han demostrat ser actives en *Candida* spp (a excepció de *C. parapsilosis* i *C. krusei*), fongs dimòrfics i *Pneumocystis carinii*. L'activitat sobre *Aspergillus* spp i altres fongs filamentosos problemàtics es escassa o nul.la (Herreros et al, 1998). Recentment s'ha comunicat la síntesi de sis noves molècules derivades de les sordarines, les azasordarines (Herreros et al, 2001). El seu espectre d'activitat és similar al de les sordarines i han demostrat ser efectives en un estudi experimental de candidiasi oral i vulvovaginal per *C. albicans* en rates immunodeprimides (Martinez et al, 2001).

3. Determinació de l'activitat in vitro dels antifúngics.

A començament dels anys 80 les dades que s'obtenien a l'estudiar l'activitat in vitro dels antifúngics tenien com a problema més important una manca evident de reproductibilitat interlaboratori. A més a més, la rellevància clínica que podien tenir aquests estudis era realment incerta. En un treball publicat per Galgiani i cols. (Galgiani et al, 1987), es va posar de manifest que la utilització de diversos mètodes per part dels laboratoris que participaven en un estudi multicèntric donava lloc a una variació molt ampla en els resultats de les CMI's obtingudes amb un determinat antifúngic i emprant en tots els casos els mateixos aïllats. L'any 1982, el NCCLS, conscient d'aquesta situació, va crear un subcomité per establir unes directrius que permetessin determinar l'activitat in vitro dels antifúngics ja que en aquell moment, era evident que aquests estudis estaven molt endarrerits, sobre tot, si es comparava amb tota la tasca realitzada amb l'estudi de l'activitat in vitro dels antibacterians.

Aquesta situació era comprensible si tenim en conte que les infeccions fúngiques, abans dels anys 80, eren relativament infreqüents i a més a més, el nombre d'agents terapèutics era escàs. En aquell moment, tampoc es contemplava com un problema la possibilitat de l'aparició de resistències als antifúngics. En l'actualitat el panorama és clarament diferent i les infeccions fúngiques han adquirit un clar protagonisme motivat per diversos factors (malalts greus amb immunodeficiències, neoplàsies, trasplantaments, utilització de procediments invasius, administració d'antibiòtics d'ampli espectre, hemodiàlisi o diàlisi peritoneal). Un altre fet a tenir en compte és que la resistència als antifúngics és una realitat, afortunadament poc freqüent, però amb clares repercussions clíniques i epidemiològiques. Per una altra banda, la indústria farmacèutica, conscient d'aquesta situació està dedicant grans esforços al desenvolupament de nous antifúngics tant pel tractament d'infeccions sistèmiques com superficials. Tots aquests factors han contribuït a que sigui cada cop

més necessari disposar d'una metodologia per determinar l'activitat in vitro dels antifúngics que ajudi a decidir el millor tractament, que contribueixi al desenvolupament de nous fàrmacs i que a més a més permeti, a través d'estudis epidemiològics, vigilar l'aparició de resistències als antifúngics.

3.1. Mètode de referència. Documents del NCCLS.

La necessitat de disposar d'unes normes per determinar l'activitat in vitro dels antifúngics va fer que molts investigadors col·laboresin amb el NCCLS per desenvolupar una metodologia estandarditzada i al mateix temps investigar les variables que poden influir més en el resultat final, com per exemple, la preparació i tamany de l'inòcul, el medi de cultiu, la temperatura i duració de la incubació i com determinar la CMI ("end-point"). Com a conseqüència d'aquest treball el subcomitè del NCCLS ha anat elaborant al llarg dels anys unes recomanacions per determinar l'activitat in vitro dels antifúngics en *Candida* spp i *Cryptococcus neoformans* les quals han quedat reflectides en tres documents: M27P (1992); M27T (1995) i M27A (1997) en els seus formats succesius: P, proposta; T, tentatiu i A, aprovat(NCCLS, 1992; NCCLS, 1995; NCCLS, 1997). L'any 1998 el NCCLS va proposar el document M28P (NCCLS, 1998) per determinar l'activitat in vitro dels antifúngics en fongs filamentosos (*Aspergillus* spp, *Fusarium* spp, *Rhizopus* spp, *Pseudallescheria boydii*, *Sporotrix schenckii*). Tots aquests documents fan especial èmfasi en aquelles variables que es consideren mes importants i que més poden influir en el resultat final obtingut. En les taules 2 i 3 es resumeixen aquestes variables.

Taula 2. Principals variables contemplades en el document M27A del NCCLS.

Medi de cultiu	RPMI 1640, a pH7.0
Tampó	MOPS 0.165 M
Inòcul	$0.5 - 2.5 \times 10^3$ UFC/ml
Estandardització de l'inocul	McFarland 0.5 utilitzant un espectrofotòmetre
Format	Microdilució o macrodilució
Temperatura d'incubació	35°C
Temps d'incubació	48 h per <i>Candida</i> spp, 72 h per <i>C. neoformans</i>
Definició de CMI "end point"	Anfotericina B: no creixement visible Fluorocitosina, antifúngics azòlics: 80% de reducció en el creixement o una disminució prominent de la terbolesa comparat amb el control

Taula 3. Principals variables contemplades en el document M28P del NCCLS.

Medi de cultiu	RPMI 1640, a pH7.0
Tampó	MOPS 0.165 M
Inòcul	$0.4 - 5 \times 10^4$ UFC/ml
Estandardització de l'inocul	Densitat òptica segons gènere i amb espectrofotòmetre
Format	Microdilució
Temperatura d'incubació	35°C
Temps d'incubació	24 h per <i>Rhizopus</i> spp, 48 h per <i>Aspergillus</i> spp, <i>Fusarium</i> spp, <i>S. schenckii</i> , 72 h per <i>P. boydii</i> .
Definició de CMI "end point"	Amfotericina B: no creixement visible Fluorocitosina, compostos azòlics: disminució prominent de la terbolesa (50%) comparat amb el control

3.2. Document M27A, 1997, NCCLS. Metodologia.

Els aspectes més fonamentals del document M27A es comenten a continuació.

1. Antifúngics.

1.1 Obtenció dels antifúngics. Cal aconseguir-los dels respectius laboratoris farmacèutics en forma de substància pura valorada (generalment expressat en µg) o bé adquirir-los a cases comercials (per ex, Sigma).

1.2 Preparació de la solució mare (“stock”) inicial. La solució d’antifúngic inicial es prepararà a una concentració com a mínim de 1280 µg/ml o deu vegades la concentració més alta que s’estudïi. Per preparar-la es poden emprar les següents formules segons convingui:

$$\text{Pes (mg)} = \frac{\text{Volum (ml)} \times \text{Concentració (}\mu\text{g/ml)}}{\text{Potència (}\mu\text{g/mg)}}$$

o bé

$$\text{Volum (ml)} = \frac{\text{Pes (mg)} \times \text{Potència (}\mu\text{g/mg)}}{\text{Concentració (}\mu\text{g/ml)}}$$

A les fòrmules, Pes, representa la quantitat d’antifúngic que pesarem; Volum, la quantitat de solvent necessari; Concentració, la concentració de l’antifúngic que volem (1280 µg/ml o superior); Potència, el grau de puressa de l’antifúngic (expressat en µg).

Els antifúngics cal disoldre’ls en el seu solvent adequat. Generalment es fa servir aigua per fluconazol i fluorocitosina. Per altres antifúngics com amfotericina B, itraconazol o ketoconazol s’utilitza dimetil sulfòxid (DMSO). Com que aquest solvent pot afectar el creixement dels fongs, cal fer les dilucions necessàries per aconseguir que la concentració final no superi l’1%.

Normalment les concentracions mare “stock” preparades no permeten el creixement de microorganismes contaminants i cal assumir que son estèrils. Es poden guardar en tubs estèrils (preferible tubs amb taps de rosca) a temperatures inferiors a -20°C (aconsellable a -60°C o inferior ja que conserven la seva activitat sis o més messos). Cal tenir present que els tubs, un cop descongelats, s’han de fer servir el mateix dia i no es poden tornar a congelar.

1.3 Concentracions a estudiar. Es recomana fer servir les següents concentracions: amfotericina B, ketoconazol i itraconazol: 0.03 – 16 µg/ml, fluconazol i fluorocitosina: 0.125 – 64 µg/ml.

1. Procediment.

2.1 Medi de cultiu. Cal utilitzar un medi sintètic: RPMI 1640 amb glutamina, sense bicarbonat i amb un indicador de canvi de pH. La seva composició i com preparar-lo es comenta al final de la descripció del document.

2.2 Tampó. El medi de cultiu ha de tenir un pH de 7.0 a 25°C. Es recomana utilitzar un tampó “zwiteriònic”, com el MOPS (0.165 M), el qual no travessa ràpidament la membrana cel.lular com els tampons fosfat i teòricament, no interacciona amb els fàrmacs antifúngics.

2.3 Preparació de les concentracions dels antifúngics. Les concentracions dels diferents antifúngics es preparen seguint el model de les taules 4 (antifúngics disolts amb aigua) i 5 (antifúngics no solubles amb aigua).

2.3.1 Macrodilució. En el mètode de macrodilució es treballa en tubs que contenen un volum total de 1 ml, del qual 0.9 ml corresponent a l’inòcul i 0.1 ml a l’antifúngic. Per tant, cal preparar cada antifúngic a una concentració 10x ja que al realitzar una dilució 1:10 ens quedarà la concentració desitjada. Es destinarà un tub a control de creixement amb medi RPMI, inòcul i solvent (en cas de no ser aigua).

2.3.2 Microdilució. En aquest mètode s'utilitzen plaques estèrils de microdilució (96 pouets) que contenen un volum final de 200 µl, del qual 100 µl corresponen a l'antifúngic i 100 µl a l'inòcul. Els antifúngics es preparen a una concentració 2x que es dispensen als pouets corresponents. Generalment, el número 1 conté la concentració més elevada i el número 10 la més baixa. Tambè cal fer servir pouets com control de creixement i es poden utilitzar pouets com a control d'esterilitat.

2.4 Preparació de l'inòcul. Els microorganismes es cultiven en un medi adequat (agar Sabouraud dextrosa o agar peptona dextrosa) al menys dos cops amb la finalitat d'assegurar la seva viabilitat i puresa. L'inòcul es prepara agafant 4-5 colònies d'un cultiu de 24 h (*Candida* spp) o 48 h (*C. neoformans*) que es resuspenen en 5 ml de SF i s'ajusta a un McFarland 0.5 emprant un espectrofotòmetre (530 nm). La suspensió obtinguda conté $1 - 5 \times 10^6$ UFC/ml.

2.4.1 Macrodilució. Si utilitzem aquest mètode cal diluir l'inòcul 1:100 seguit d'una nova dilució 1:20 amb RPMI 1640, que permet aconseguir l'inòcul final desitjat ($0.5 - 2.5 \times 10^3$ UFC/ml).

2.4.2 Microdilució. En aquest cas la dilució inicial ha de ser 1:50 seguida d'una dilució 1:20 amb RPMI 1640 per obtenir una concentració doble de l'inòcul ($1 - 5 \times 10^3$ UFC/ml). Aquest inòcul es diluirà 1:1 a les plaques i aconseguirem l'inòcul final.

2.5 Inoculació.

2.5.1 Macrodilució. Dispensar 0.1 ml de les diferents concentracions d'antifúngic als tubs corresponents als quals hi posarem 0.9 ml de l'inòcul final. El tub control contindrà el volum corresponent a l'inòcul i 0.1 ml del diluent de l'antifúngic.

2.5.2 Microdilució. Un cop hem dispensat l'antifúngic (100 µl, concentració 2x), hi posarem els 100 µl corresponents a l'inòcul. El pouet control contrindrà aquest inòcul i 100 µl de medi de cultiu sense antifúngic.

2.6 Incubació. Tant els tubs com les plaques de microtítol s'incuben a 35°C per un temps de 48 h (*Candida spp*) o 72 h (*C. neoformans*).

2.7 Lectura dels resultats. La CMI es defineix com la concentració d'antifúngic més petita que, macroscòpicament, produeix una inhibició substancial del creixement del fong. A l'hora de realitzar la lectura cal comparar el creixement del tub o pouet control amb l'obtingut als tubs o pouets amb l'antifúngic. Per l'amfotericina B, la CMI serà aquella concentració en la que no s'observa creixement visible. Per la fluorocitosina i antifúngics azòlics, al ser fàrmacs fungistàtics, la inhibició del creixement no és tant clara com passa amb l'amfotericina B. Amb el mètode de macrodilució es pot definir com aquella concentració en la que es produeix una reducció del creixement igual o superior al 80% si es compara amb el tub control. Això ho podem estimar si diluïm 200 µl del tub control amb 800 µl de medi sense inocular. Amb el mètode de microdilució s'estableix una puntuació numèrica del 0 al 4, comparat amb el control, segons la següent escala: 0, òpticament clar, no creixement; 1, lleuger creixement; 2, disminució marcada o prominent de la terbolesa; 3, lleugera disminució o reducció de la terbolesa; i 4, cap canvi en la terbolesa. La CMI per la fluorocitosina i antifúngics azòlics serà aquella concentració en la que es produeix una disminució prominent de la terbolesa (número 2).

2.8 Modificacions. El document comtempla la possibilitat de realitzar algunes modificacions a la metodologia descrita en un intent de millorar determinats problemes concrets d'aquest mètode. Per determinar l'activitat de l'amfotericina B en *Candida spp*, la utilització d'Antibiotic Medium 3 podria millorar la detecció de soques resistents. Hi ha l'inconvenient que aquest medi no està estandaritzat i es poden produir variacions importants del resultat segons el lot. En *C. neoformans* la utilització com medi de cultiu de Yeast Nitrogen Base pot traduir-se en un augment del creixement del fong i una millora en la correlació de les CMIs i resposta clínica (Witt, 1996; Ghannoum, 1992). Una tercera possible modificació suggereix

suplementar el medi RPMI 1640 amb glucosa fins aconseguir una concentració final de 20 g/L amb la finalitat de millorar o facilitar la determinació de la CMI. (Rodríguez-Tudela, 1994)

2.9 Soques control. El document M27A proposa utilitzar com soques control per a l'amfotericina B, fluconazol, itraconazol, ketoconazol i fluorocitosina dues soques de referència ATCC: *Candida parapsilosis* ATCC 22019 i *Candida krusei* ATCC 6258. Els intervals de CMIs para cada antifúngic i soca s'especifiquen a la taula 6. Recentment s'han proposat els intervals de CMIs per a cinc nous antifúngics: voriconazol, ravuconazol, posaconazol, caspofungina i anidulafungina (Barry AL, et al. 2000).

3. Interpretació dels resultats.

El document proposa la creació dels següents punts de tall per només tres antifúngics i *Candida* spp:

ANTIFÚNGIC	SENSIBLE	SENSIBLE DOSI DEPENDENT	INTERMEDI	RESISTENT
Fluconazol	≤ 8	16 – 32	-	≥ 64
Itraconazol	≤ 0.125	0.25 – 0.5	-	≥ 1
Fluorocitosina	≤ 4	-	8 – 16	≥ 32

El desenvolupament dels punts de tall de fluconazol i itraconazol, aixó com la seva interpretació basant-se amb una anàlisi acurada de les dades obtingudes in vitro i la seva correlació in vivo es va publicar l'any 1997 pel subcomité d'estudi de la sensibilitat als antifúngics del NCCLS (Rex et al, 1997). Els valors proposats per fluconazol i itraconazol són aplicables a infeccions de mucoses. Per fluconazol probablement també ho són en cas d'infeccions sistèmiques per *Candida* spp. Els aïllats de *C. krusei* es consideren intrinsecament resistents a fluconazol. La categoria

sensible dosi depenent fa referència a la necessitat d'utilitzar dosi elevades per aconseguir els nivells plasmàtics màxims. Els valors proposats per fluorocitosina es fonamenten en la informació històrica disponible del fàrmac i les seves característiques farmacocinètiques. El document no recomana punts de tall per amfotericina B i ketoconazol. En el cas de l'amfotericina B, l'experiència demostra que la majoria de CMIs tenen un valor que se situa entre 0.25 i 1 µg/ml, i que el mètode no permet detectar de forma consistent les soques amb resistència a aquest antifúngic. Pel que fa referència al ketoconazol no hi han dades de correlació entre els valors de CMI obtinguts i resposta clínica al tractament amb aquest antifúngic que permetin establir un valors de referència.

Preparació del medi RPMI 1640 amb MOPS, 0.165 M (1 litre)

Dissoldre 10.4 g de medi en 900 ml d'aigua destil.lada. Afegir 34.53 g de MOPS i agitar fins que es dissolgui. Mentre estem agitant cal ajustar el pH a 7.0 (25°C) amb hidròxid sòdic 1 M. Afegir aigua fins arribar a un volum final d'1 L. Esterilitzar per filtració i guardar a 4°C.

Components del medi RPMI 1640

COMPONENT	G/L	COMPONENT	G/L
L-arginina	0.200	Biotina	0.0002
L-asparagina	0.050	Àcid pantotenic	0.00025
Àcid L-aspartic	0.020	Clorur de colina	0.003
L-cistina · 2HCl	0.0652	Àcid fòlic	0.001
Àcid L-glutàmic	0.020	Mioinositol	0.035
L-glutamina	0.300	Niacinamida	0.001
Glicina	0.010	PABA	0.001
L-histidina	0.015	Pirodoxina HCl	0.001
L-hidroxi prolina	0.020	Riboflavina	0.0002
L-isoleucina	0.050	Tiamina HCl	0.001
L-leucina	0.050	Vitamina B ₁₂	0.000005
L-lisina · HCl	0.040	Nitrat de calci · H ₂ O	0.100
L-metionina	0.015	Clorur de potasi	0.400
L-fenilalanina	0.015	Sulfat de magnesi	0.04884
L-prolina	0.020	Clorur de sodi	6.000
L-serina	0.030	Fosfat de sodi, dibàsic	0.800
L-treonina	0.020	D-glucosa	2.000
L-triptofan	0.005	Glutationa, reduïda	0.001
L-tirosina · 2Na	0.02883	Vermell de fenol, Na	0.0053
L-valina	0.020		

Taula 4. Esquema proposat per diluir el fluconazol i fluorocitosina (solvent: aigua)

Dilució	Concentració (µg/ml)	Procedència	Volum (ml)	Medi (ml)	Concentració (µg/ml)	Concentració Final 1:10 (µg/ml)
1	5120	Solució mare "stock"	1	7	640	64
2	640	Dilució 1	1	1	320	32
3	640	Dilució 1	1	3	160	16
4	160	Dilució 3	1	1	80	8
5	160	Dilució 3	0.5	1.5	40	4
6	160	Dilució 3	0.5	3.5	20	2
7	20	Dilució 6	1	1	10	1
8	20	Dilució 6	0.5	1.5	5	0.5
9	20	Dilució 6	0.5	3.5	2.5	0.25
10	2.5	Dilució 9	1	1	1.25	0.125
11	2.5	Dilució 9	0.5	1.5	0.625	0.06
12	2.5	Dilució 9	0.5	3.5	0.3125	0.03

Taula 5. Esquema proposat per diluir l'amfotericina B, itraconazol, ketoconazol (solvent: DMSO)

Dilució	Concentració (µg/ml)	Procedència	Volum (ml)	Solvent (ml)	Concentració (µg/ml)	Concentració Final 1:100 (µg/ml)
1	1.600	Sol mare "stock"			1600	16
2	1.600	Sol mare "stock"	0.5	0.5	800	8
3	1.600	Sol mare "stock"	0.5	1.5	400	4
4	1.600	Sol mare "stock"	0.5	3.5	200	2
5	200	Dilució 4	0.5	0.5	100	1
6	160	Dilució 4	0.5	1.5	50	0.5
7	20	Dilució 4	0.5	3.5	25	0.25
8	20	Dilució 7	0.5	0.5	12.5	0.125
9	20	Dilució 7	0.5	1.5	6.25	0.06
10	2.5	Dilució 7	0.5	3.5	3.13	0.03

Taula 6. Interval de CMI's de les dues soques control recomanades i deu antifúngics.

Soca	Antifúngic	Interval de CMI's ($\mu\text{g/ml}$)
<i>Candida parapsilosis</i> ATCC 22019	Amfotericina B	0.25 – 1
	Fluconazol	2 – 8
	Itraconazol	0.06 – 0.25
	Ketoconazol	0.06 – 0.25
	Fluorocitosina	0.12 – 0.25
	Voriconazol	0.03 – 0.25
	Ravuconazol	0.03 – 0.25
	Posaconazol	0.06 – 0.25
	Caspofungina	0.5 – 4
	Anidulafungina	1 – 8
<i>Candida krusei</i> ATCC 6258	Amfotericina B	0.5 – 2
	Fluconazol	16 – 64
	Itraconazol	0.12 – 0.5
	Ketoconazol	0.12 – 0.5
	Fluorocitosina	4 – 16
	Voriconazol	0.12 – 1
	Ravuconazol	0.25 – 1
	Posaconazol	0.12 – 1
	Caspofungina	0.25 – 1
	Anidulafungina	0.06 – 0.5

3.3 Document M28-P, 1998, NCCLS. Metodologia.

En aquest document es descriu la metodologia per estudiar l'activitat in vitro dels antifúngics enfront de fongs filamentosos formadors de conidis i esporangioespores. Encara que en el document només es contempla la seva utilització en *Aspergillus* spp, *Pseudallescheria boydii*, *Fusarium* spp, *Rhizopus* spp i *Sporotrix schenckii*, també s'ha emprat en altres fongs filamentosos.

La metodologia recomanada és molt similar a la descrita en el document M27-A i només en comentarem les modificacions.

1. Antifúngics, preparació, concentracions, dilucions, medi de cultiu, tampó.

Cal seguir les mateixes recomanacions del document M27-A

2. Preparació de l'inòcul.

Segons estudis preliminars, la suspensió de conidis o esporangioespores pot mesurar-se amb un espectrofotòmetre i la reproductibilitat en les CMI's amb un inòcul de $0.4 - 5 \times 10^4$ UFC/ml és bona. La formació de conidis o esporangioespores s'aconsegueix sembrant els aïllats en agar inclinat dextrosa patata a 35°C per un període de 7 dies (*Aspergillus* spp, *P. boydii*, *Rhizopus* spp i *S. schenckii*) o a 35°C durant 48 - 72h i després a 25 - 28°C fins al setè dia (*Fusarium* spp). Als 7 dies les colònies es cobreixen amb 1 ml de SF i amb l'ajut d'una pipeta Pasteur es frega la superfície inclinada. En *Aspergillus* spp podem facilitar aquesta operació si hi posem una gota de Tween 20. La suspensió obtinguda (conté conidis o esporangioespores i fragments d'hifes) es traspasa a un tub estèril, es deixa sedimentar 3-5 minuts i el sobrenadant s'homogenitza en un altre tub amb l'ajut d'un agitador. La densitat dels conidis o esporangioespores es llegeix i ajusta amb un espectrofotòmetre (530 nm) a una densitat òptica que per *Aspergillus* spp i *S. schenckii* és de 0.09 a 0.11 (80-82% de transmitància) i per *P. boydii*, *Rhizopus* spp i *Fusarium* spp és de 0.15 a 0.17 (68-

70% de transmitància). La preparació de l'inòcul final varia segons el mètode utilitzat.

2.1 Macrodilució. Les suspensions obtingudes es dilueixen 1:100 en el medi RPMI 1640 per aconseguir l'inòcul final.

2.2 Microdilució. En aquest cas cal fer una dilució 1:50 per aconseguir una concentració 2x de l'inòcul final.

3. Inoculació.

3.1 Macrodilució. Es dispensa 0.9 ml de l'inòcul final als tubs que ja contenen 0.1 ml de les diferents concentracions d'antifúngic. El tub control contindrà el volum corresponent a l'inòcul i 0.1 ml del diluent de l'antifúngic.

3.2 Microdilució. Dispensem 100 µl de l'inòcul 2x a cada pouet que ja conté l'antifúngic (concentració 2x, 100 µl). El pouet control contrindrà aquest inòcul i 100 µl de medi de cultiu sense antifúngic.

2. Incubació.

Tant les plaques de microdilució com els tubs s'incuben a 35°C i es llegeixen els resultats a les 24 h (*Rhizopus* spp), 48 h (*Aspergillus* spp, *Fusarium* spp i *S. schencki*) i 72 h (*P. boydii*).

3. Lectura dels resultats.

Per determinar la CMI s'estableix una puntuació numérica al comparar el creixement en els tubs o pouets amb antiúngic i el que es produeix al control: 4, cap disminució del creixement; 3, lleugera reducció del creixement o una disminució aproximada del 75% comparat amb el control; 2, reducció prominent del creixement o una disminució aproximada del 50% comparat amb el control; 1, lleuger creixement o una disminució aproximada del 25% comparat amb el control; i 0, òpticament clar o ausencia de creixement. La CMI de l'amfotericina B serà aquella concentració en la que no s'observa creixement visible (puntuació 0). Per la fluorocitosina i especialment els antifúngics azòlics la CMI correspondrà a aquella concentració en

la que es produeix una inhibició del 50% o més del creixement al comparar-ho amb el control (puntuació 2).

4. Intrepretació dels resultats.

Fins ara, la relevància clínica dels resultats obtinguts determinant la CMI dels antifúngics en fongs filamentosos és realment poc clara degut, entre altres factors, a la manca d'estudis que intenten correlacionar-ho.

5. Soques control.

A part de les soques control recomanades en el document M27-A (*Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258), cal utilitzar dos aïllats d'*Aspergillus* (*A. fumigatus*, *A. flavus*) de referència per amfotericina B i itraconazol. Les dues soques que va utilitzar el subcomité del NCCLS encara no tenien número d'ATCC quan es va publicar el document M28P. Recentment, a una d'elles (*A. flavus*) se li ha assignat el número ATCC 204304.

3.4. Altres mètodes.

La dificultat que comporta determinar l'activitat in vitro dels antifúngics i la tardança en el·laborar un mètode de referència són dos dels factors que han afavorit que s'explorés la possibilitat d'utilitzar altres metodologies per estudiar l'activitat in vitro dels antiúngics. Alguns dels mètodes emprats no són certament habituals en els laboratoris de microbiologia clínica.

3.4.1 Mètode E-test.

El mètode E-test desenvolupat per la companyia sueca AB Biodisk, (Solna, Suècia) permet determinar l'activitat in vitro dels antimicrobians i ha estat adaptat a diversos antifúngics. Consta d'una tira de plàstic impregnada d'un gradient de diferents concentracions de l'antimicrobià a estudiar que es diposita a sobre d'una placa d'agar on previament s'ha sembrat el microorganisme. La CMI es determina en el punt d'intersecció on coincideix la inhibició del creixement i la tira de plàstic. La seva utilitat en el camp de les infeccions bacterianes ha estat àmpliament contrastada i és de suposar que, a priori, l'aplicació d'aquest mètode en infeccions fúngiques pot tenir un enorme potencial. Tot i que l'aplicació de l'E-test per determinar la CMI dels antifúngics en *Candida* spp, *C. neoformans* i fongs filamentosos requereix certa experiència, diversos estudis han tobat una bona correlació entre aquest mètode i el considerat de referència (NCCLS) amb *Candida* spp i antifúngics azòlics (Chen et al, 1995; Colombo et al, 1995; Espinel-Ingroff et al, 1996; Pfaller et al, 1996; Wanger et al, 1995; Warnock et al, 1998). En *Aspergillus* spp i altres fongs filamentosos també s'ha observat una bona correlació entre les CMIs d'amfotericina B i itraconazol obtingudes amb E-test i el document M28P (Szekely et al, 1999; Pfaller et al, 2000).

3.4.2 Mètodes de microdilució.

La complexitat del mètode de referència i el consum de temps que comporta la metodologia proposada fa que sigui poc probable la seva realització en la rutina diària d'un laboratori de microbiologia. Això ha motivat a la indústria a desenvolupar diversos sistemes per determinar la sensibilitat als antifúngics que puguin ser utilitzats en els laboratoris. Entre els mètodes que utilitzen reactius colorimètrics cal citar-ne dos. El sistema Sensititre Yeast One (Trek Diagnostic Systems Inc, EEUU) és un mètode de microdilució que fa servir com medi de cultiu RPMI 1640 més glucosa i que permet determinar l'activitat *in vitro* de cinc antifúngics (amfotericina B, ketoconazol, itraconazol, fluconazol i fluorocitosina). Els antifúngics, en forma deshidratada, estan inclosos en una placa de microdilució juntament amb un indicador de canvi de pH (blau Alamar). La placa s'inocula amb el medi RPMI que conté l'inòcul i s'incuba a 35°C. La determinació de la CMI es realitza en funció del canvi de color que es produeix amb el creixement del microorganisme. És un mètode estandarditzat que té un grau de concordància elevat amb el mètode de referència (Messer and Pfaller, 1996; Arikan et al, 1997; Poseraro et al, 2000; Espinel-Ingroff et al, 1999). El ASTY colorimetric panel (Kyokuto Pharmaceutical Industrial Co, Japó) permet determinar la sensibilitat a quatre antifúngics (amfotericina B, itraconazol, fluconazol i fluorocitosina) que es troben deshidratats en una placa de microdilució a concentracions similars a les del document M27A i, com en el cas anterior, incorpora un indicador colorimètric de canvi de pH. En el treball publicat per Pfaller i cols (Pfaller et al, 1998) el grau de correlació amb el mètode de referència va ser excelent.

Altres mètodes comercialitzats que fan servir una metodologia de dilució són: Candifast (International Microbio/Stago Group, Milan, Italia), Integral Systems Yeasts (Liofilchem Diagnostics, L'Aquila, Italia), Fungitest (Bio-Rad SDP, Paris, França) ATB fungus (API-bioMerieux, Marcy l'Etoile, França), Mycostandard

(Institut Pasteur, Paris, França) y Mycototal (Behring Diagnostic, Rueil-Malmaison, França). Aquests sistemes tenen com a principals inconvenients el estudiar un interval de concentracions limitat (format tipus “break-point”) que en ocasions són poc adequades o bé, que els resultats obtinguts no tenen una correlació acceptable al comparar-ho amb el mètode de referència (Davey KG et al, 1998; Druetta A, 1993; Guinet et al, 1988; Posteraro et al, 2000; Quindós et al, 1994; Schmalreck et al, 1995; Swinne et al, 1999; Willinger et al, 2000; Witthuhn et al, 1999).

3.4.3 Mètode Disc-Difusió.

El mètode de disc-difusió s’ha intentat adaptar a la determinació in vitro de l’activitat del fluconazol amb una bona correlació amb el mètode de referència (Barry et al, 1996; Meis et al, 2000; Sandven P, 1999). Per millorar la lectura, s’ha proposat mullar la superfície de l’agar amb blau de metilè a una concentració de 0.5 µg/ml (Meis et al, 2000). Hi ha dos sistemes comercialitzats, Neo-Sensitabs (Rosco Diagnostica, Taastrup, Dinamarca) i Diff Test (Diagnostics Pasteur, Paris, França) que en la seva utilització no s’ha trobat una bona correlació amb el mètode de referència o bé no s’ha fet la comparació (Cantón et al, 1999; Swinne et al, 1999; Schmalreck et al, 1995).

3.4.4 Altres metodologies.

La utilització de la citometria de fluxe per determinar la CMI en fongs, sobre tot en *Candida* spp, ha estat l’objecte d’estudi en diversos treballs (Favel et al, 1999; Green et al, 1994; Pore RS, 1990; Pore RS, 1994). La combinació d’aquesta metodologia amb la utilització de diversos colorants permetria detectar l’existència de lesions en el fong estudiat. Diversos treballs han trobat una bona correlació amb el mètode de referència (Ramani et al, 1997; Ramani et al, 2000; Wenisch et al, 1996) i

s'ha suggerit que podria ser una metodologia potencialment útil per detectar la resistència a l'amfotericina B. (Favel et al, 1999).

Els colorants fluorescents també han servit per valorar l'efecte dels antifúngics sobre els fongs fent una estimació del grau de lesió que produeixen o com afectaven la seva viabilitat i com es traduïa a l'hora de determinar la CMI i la CMF (Liao et al, 1999; Lass-Flörl et al, 2001).

La quantificació del contingut d'ergosterol directament en soques exposades a l'acció de fàrmacs azòlics ha estat desenvolupat per Arthington-Skaggs i cols (Arthington-Skaggs et al, 1999). En aquest estudi es va trobar una bona correlació amb la metodologia del document M27A i en un model animal ha demostrat la seva utilitat a l'hora de correlacionar els estudis *in vitro* amb la resposta *in vivo* (Arthington-Skaggs et al, 2000).

La mesura de la concentració intracel·lular d'ATP (Kertschmar et al, 1996) o el consum de glucosa en el medi (Riesselman et al, 2000) han estat dues metodologies també proposades per determinar la CMI i que han demostrat una correlació molt bona amb el mètode de referència.

Una altra metodologia proposada ha estat suplementar el medi amb acetat. Amb això s'aconsegueix que la lectura de la CMI s'apropi a la concentració crítica en la que s'inhibeix la síntesi d'ergosterol (Shimokawa and Nakayama, 1999; Shimokawa and Nakayama, 2000).

La incorporació al medi de cultiu de dues substàncies colorimètriques com a marcadors del potencial redox, el MTT [3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-bromur de tetrazoli] i el XTT (2,3-bis(2-metoxi-4-nitro-5-dulfofenil)-5-[(fenilamino) carbonil]-2H-hidroxid de tetrazoli) ha demostrat la seva utilitat obtenint-se unes

CMI's similars a les del mètode de referència (Clancy and Nguyen, 1997; Hawser et al 1998; Yang et al, 1998; Meletiadis et al, 2000)

La utilització del medi cromogènic CHROMagar suplementat amb una concentració fixa (8 µg/ml) de fluconazol s'ha emprat per determinar la sensibilitat o resistència a aquest antifúngic amb bons resultats (Patterson et al, 1996a; Patterson et al, 1996b), tot i que la seva utilitat requereix més avaluacions, sobre tot amb soques amb resistència coneguda al fluconazol.

3.5. Modificacions del document M27A.

3.5.1 Amfotericina B.

Al determinar l'activitat de l'amfotericina B enfront de *Candida* spp fent servir la metodologia del document M27-A, l'interval de CMI's que s'obté queda restringit generalment a unes quatre dilucions (0.12 – 1 µg/ml). Això ha fet pensar que aquesta metodologia potser no és la més adequada ja que no permetria discriminar correctament les soques sensibles i resistents. En un treball realitzat per Rex i cols, (Rex et al, 1995) en el que es van estudiar una serie d'aïllats de *Candida* spp amb resistència ben caracteritzada a l'amfotericina B es va comprovar que, si bé era cert que els aïllats resistents tenien una tendència a donar CMI's més altes que els aïllats sensibles, les diferències se situaven en no més d'una o dues dilucions (log) i per tant, dintre del límit de variació del mètode. Una modificació del mètode consistent en utilitzar com a medi de cultiu Antibiotic Medium 3 a pH 7.0 (0.1 M fosfat), suplementat amb glucosa (20 g/L) i realitzant la lectura de les CMI's a les 24 h semblava que permetia la diferenciació dels aïllats en sensibles i resistents de forma més evident (Rex et al, 1995, Lozano-Chiu et al, 1997). Però els estudis realitzats també van posar en evidència l'existència de factors tècnics no del tot resolts. Si bé és cert que en el treball inicial amb Antibiotic Medium 3, la CMI de l'amfotericina B dels aïllats resistents era ≥ 1 µg/ml (Rex et al, 1995), en un treball posterior en el que

es van utilitzar diversos lots del mateix medi la CMI obtinguda era de $\geq 0.25 \mu\text{g/ml}$ (Lozano-Chiu et al, 1997). Aquestes dades eren similars a les comunicades per Law i cols (Law et al, 1997) però no coincideixen amb les obtingudes per Nguyen i cols (Nguyen et al, 1998) on en un estudi prospectiu s'aval.luava la posible correlació entre les CMIs de l'amfotericina B i resposta al tractament en un grup de malalts amb candidemia que rebien aquest antifúngic. La millor correlació clínica es trobava si el medi no es suplementava amb glucosa, la lectura de les CMIs es realitzava a les 48 h i, el resultat era óptim si es determinava la CFM. Els valors que van recomanar aquests autors per considerar una soca resistent a l'amfotericina B van ser: CMI $\geq 1 \mu\text{g/ml}$ i CFM $> 1 \mu\text{g/ml}$.

Una altra possibilitat proposada per millorar la detecció de la resistència a l'amfotericina B seria determinar la CMI utilitzant el mètode del E-test ja que fa servir agar i s'obté un interval de CMIs més ampli (Clancy et al, 1999; Pfaller et al, 1998). Fent servir aquesta metodologia seria possible la separació entre soques sensibles i resistents (Wanger et al, 1995). Fins i tot s'han suggerit punts de tall per considerar una soca com resistent segons el medi utilitzat: $\geq 0.5 \mu\text{g/ml}$ en AM3 (Law et al, 1997), $> 1 \mu\text{g/ml}$ en AM3 (Wanger et al, 1995) i $\geq 0.38 \mu\text{g/ml}$ en agar RPMI 1640 (Clancy et al, 1999). Des d'un punt de vista pràctic cap d'aquests possibles punts de tall es pot fer servir ja que ens fan falta treballs addicionals utilitzant soques control i aïllats de *Candida* amb resistència confirmada a l'amfotericina B.

Altres intents que s'han realitzat per millorar la detecció dels aïllats resistents a l'amfotericina B, com la d'utilitzar un reactiu colorimètric (Alamar Blue), han estat infructuosos (Lozano-Chiu et al, 1998).

3.5.2 Determinació de la CMI.

El determinar la CMI de forma visual segons recomana el document M27A del NCCLS és en ocasions difícil, en especial en el cas de fàrmacs fungistàtics com el derivats azòlics. La raó fonamental és la presència de l'efecte anomenat "trailing" o creixement residual que manifesten alguns aïllats. Aquesta característica no representa cap forma de resistència a l'antifúngic tal com ho demostren estudis experimentals (Arthington-Skaggs et al, 2000; Rex et al, 1998) i probablement es tracta d'un fenomen inherent a la metodologia ja que, modificacions en les condicions, com el canvi de pH, el fan desaparèixer (Marr et al, 1999). Dades clíniques de malalts amb candidiasi orofaríngea produïda per aquests aïllats que responien amb dosis baixes de fluconazol com les utilitzades en els aïllats sensibles també ho corroboren (Revankar et al, 1998). Una alternativa per evitar aquesta dificultat és la de determinar la CMI de forma automatitzada amb un espectrofotòmetre. Això, en teoria, permetria efectuar una determinació de la inhibició del creixement a diferents nivells (Odds et al, 1995). S'han efectuat diversos estudis per avaluar el grau d'inhibició del creixement que es produeix en presència de l'antifúngic en comparació amb el creixement obtingut al control. El nivell de concordància assolit entre els resultats espectrofotomètrics i els del mètode de referència han estat excel·lents (Pfaller et al, 1995; Nguyen and Yu, 1999; Espinel-Ingroff et al, 1995; Rodríguez-Tudela et al, 1996). La major coincidència es produeix quan considerem els valors obtinguts amb una inhibició del 50% pels antifúngics azòlics i del 80-90% amb l'amfotericina B (Pfaller et al, 1995; Odds et al, 1995; Espinel-Ingroff, 1998)

3.5.3. *Cryptococcus neoformans*.

Un cop desenvolupada la metodologia descrita en els diferents documents M27 s'ha fet evident que potser no és la millor forma de determinar la sensibilitat en *C. neoformans*. Encara que els resultats de les CMIs son reproduïbles i perfectament vàlids, el creixement del fong en el medi es lent, requereix una incubació de 72 hores

i algunes soques, simplement, no creixen. Ghannoum i cols (Ghannoum et al, 1992) van proposar unes modificacions que semblen ser una alternativa vàlida al document del NCCLS. En aquesta nova metodologia es proposa utilitzar yeast nitrogen base (YNB) a pH 7.0, un inòcul de 10^4 UFC/ml, incubació a 35°C, 48 h i microdilució. La lectura de la CMI per fluconazol es fa amb un espectrofotòmetre emprant un filtre de 420 nm i es defineix com una inhibició del creixement del 50% comparat amb el creixement del control. Els avantatges d'aquesta proposta son: un millor creixement del microorganisme, una bona reproducibilitat i la capacitat de proporcionar un interval de CMIs de fluconazol que generalment es reflecteixen amb la resposta clínica a aquest agent (Witt et al, 1996). En un estudi on es va aval.luar aquesta modificació del mètode de referència en malalts HIV+ amb criptococosi meníngea es va observar una bona correlació amb l'evolució clínica (Jessup et al, 1998). Tant en aquest estudi com el de Witt i cols es suggereix que el risc de fracàs del tractament es elevat quan la CMI se situa en 2 – 4 µg/ml (Witt et al, 1996). En un tercer estudi publicat recentment fent servir el mateix medi, el fracàs clinic era uniforme quan la CMI era ≥ 16 µg/ml (Aller et al, 2000).

Com passa amb *Candida* spp, la detecció de la resistència a l'amfotericina B en *C. neoformans* és difícil. Lozano-Chiu i cols van demostrar que el mètode de referència no permetia diferenciar correctament les soques sensibles i resistents a l'amfotericina B al estudiar una selecció d'aïllats recuperats en malalts amb meningitis criptocòcica i resposta coneguda a l'amfotericina B (Lozano-Chiu et al, 1998). La identificació d'aquestes soques podria efectuar-se per repetició de les CMIs però això es inviable a la pràctica diària per l'estret marge d'interval de CMIs. Utilitzant AM3 s'obtenia una bona separació entre aïllats resistents i sensibles, però els millors resultats s'obtenien amb E-test ja sigui en RPMI 1640 o AM3.

Altres modificacions proposades per afavorir el creixement de *C. neoformans* són el efectuar un inòcul més elevat (10^5 UFC/ml) amb agitació constant a 35°C (Rodríguez-Tudela et al, 2000) o efectuar la incubació a 30°C (Davey et al, 1998).

3.5.4. Medi de cultiu i inòcul. La modificació proposada de suplementar el medi RPMI amb glucosa al 2% (Rodríguez-Tudela et al, 1994) té com a finalitat la de facilitar la determinació de la CMI, ja que s'obté comparativament, una major terbolesa en el control de creixement (Odds et al, 1995; Lozano-Chiu et al, 1999). Un altre avantatge d'aquest medi podria ser el d'escurçar el temps d'incubació a 24 h, fet que no podem tenir en conte amb el medi RPMI sense suplementar ja que hi ha aïllats que no tenen suficient creixement a les 24 h d'incubació (Rodríguez-Tudela and Martínez-Suárez, 1995). Un fet interessant és que la utilització de RPMI amb 2% de glucosa i un inòcul més elevat ($10^4 - 10^5$ UFC/ml) que el recomanat en el document del NCCLS no es tradueix en un augment de les CMIs (Cuenca-Estrella et al, 1999; Cantón et al, 1999). Aquests resultats han servit per a que el subcomité per a l'estudi de la sensibilitat als antifúngics de l'EUCAST (European Committee on Antibiotic Susceptibility Testing) iniciés un estudi comparatiu de la metodologia del document M27A amb un nou mètode que fa servir RPMI-2% de glucosa i un inòcul de 10^5 UFC/ml. Dades preliminars indiquen que l'interval de CMIs obtingut amb les soques control són pràcticament idèntics als del NCCLS i la cinètica de creixement comparativa dels dos mètodes suggereix que la incubació de 24 h és el període òptim per determinar la CMI (Cuenca-Estrella and Rodríguez-Tudela, 2001).

3.6 Modificacions del document M28P.

3.6.1 Preparació de l'inòcul.

El document M28P és la proposta del NCCLS per determinar la CMI en determinats fongs filamentosos. Una possible modificació suggerida per als propers documents fa énfasi en la forma de preparació de l'inòcul. Per fer-ho, el document

recomana utilitzar un espectrofotòmetre i això podria ser una font d'errors o d'inexactitud pel simple fet que els conidis tenen diferent tamany segons l'espècie i a més a més, poden tenir color amb la conseqüent repercussió en la lectura de l'espectrofotòmetre (Gehrt et al, 1995; Guarro et al, 1997; Llop et al, 2000). L'alternativa que es podria fer servir seria la de contar els conidis amb una càmera de recompte d'eritròcits (Denning et al, 1992; Moore et al, 2000). Sigui quina sigui la metodologia utilitzada, quantificar l'inòcul utilitzat sembla una actitud assenyada.

2) Determinació de la CMI.

La determinació de la CMI quan es valora l'activitat in vitro dels fàrmacs azòlics també planteja alguns dubtes com passa amb el document M27A. Hi ha dades recents que suggereixen que la determinació de la CMI per a l'itraconazol i els nous triazols caldria realitzar-la considerant una inhibició total del creixement (100%) i no del 50% com recomana el document M28P, amb la qual cosa es detectaria millor la resistència a aquests antifúngics en *Aspergillus* spp (Denning et al, 1997; Espinel-Ingroff et al, 1999).

4. Epidemiologia molecular de les infeccions fúngiques.

L'epidemiologia molecular aplicada a l'estudi de les infeccions fúngiques en general i a la tipificació de possibles brots d'origen nosocomial en particular, com ha passat amb els estudis de sensibilitat als antifúngics, sempre ha anat uns anys endarrerida respecte a l'ús que se n'ha fet dels estudis moleculars aplicats a les infeccions bacterianes. En els últims anys però, els avenços realitzats són enormes (Hunter, 1991; Soll, 2000). Si bé es cert que un gruix important d'els estudis s'han realtzat en *C. albicans* per ser l'agent patògen prevalent, això ha servit per a que aquests esforços inicials permetessin després l'estudi d'altres espècies o fongs.

4.1 Mètodes fenotípics.

Abans de la introducció en micologia mèdica dels diferents mètodes que es fonamentent en l'anàlisi de l'ADN, els estudis epidemiològics s'adressaven a estudiar el fenotip del fong assumint que les diferències observades en aquest fenotip reflexaven diferències genètiques entre els aïllats. Un dels primers esforços per intentar discriminar les soques de *C. albicans* va ser la seroaglutinació. Hasenclever i cols van descriure en aquesta espècie l'existència de dos serotips, A i B (Hasenclever et al, 1961a; Hasenclever et al, 1961b). És obvi però, que la separació d'una espècie en només dos grups no aporta una informació rellevant davant de la majoria dels problemes epidemiològics. A més a més, hi ha un inconvenient que questiona tot el mètode, com és el fet que la expressió antigènica es pot veure afectada per la fase de creixement i les cèl.lules del serotip B poden produir l'antígen del serotip A (Poulain et al, 1985). Uns anys més tard es va constatar que la comparació de tres mètodes de serotipificació no agrupava de igual forma els diferents aïllats (Brawner, 1991).

Al començament dels anys 80 es va desenvolupar un sistema complex de biotipificació que pretenia diferenciar les diferents espècies de *Candida* i els aïllats de cada espècie (Odds and Abbot, 1980; Odds and Abbot, 1983). El mètode valorava el creixement del microorganisme en un agar que variava la seva composició i condicions. Tot i que aquest mètode s'ha utilitzat en molts estudis epidemiològics, una avaluació realitzada en 5 laboratoris va evidenciar una pobra reproductibilitat interlaboratori (Odds et al, 1989).

Hi han descrits altres mètodes fenotípics per discriminar les soques de *C. albicans* com la morfotipificació que es fonamenta en la variació de la morfologia de les colònies (Phongpaichit et al, 1987), la resistotipificació que valora la capacitat de creixement en un medi sòlid que conté concentracions conegudes de diferents inhibidors químics (McCreight et al, 1985), la tipificació en funció de la capacitat de 9 substàncies "letals" (Killer Yeast Typing) d'inhibir selectivament el creixement de les diferents soques de *C.albicans* (Polonelli et al, 1983), tipificació enzimàtica (Casal and Linares, 1983), tipificació per assimilació de sucres (Buesching et al, 1979) o tipificació segons la sensibilitat a diferents antifúngics (Quindós et al, 1996). A les limitacions pròpies de tots aquests mètodes cal sumar-hi un altre problema. Tots els aïllats de *C. albicans* tenen la capacitat d'experimentar canvis en el seu fenotip ("phenotypic switching"), alguns d'ells, una minoria, amb una freqüència elevada. Això significa que una mateixa soca pot adoptar dos fenotips diferents al mateix temps, tot i créixer sota les mateixes condicions (Soll et al, 1987; Soll, 1992). L'adopció d'un determinat fenotip pot comportar canvis antigènics, en la sensibilitat als antifúngics o en l'assimilació de carbohidrats (Anderson et al, 1990; Soll, 1990; Soll et al, 1991).

"Multilocus Enzyme Electrophoresis". Amb aquest mètode els microorganismes es poden diferenciar a partir de l'anàlisi dels perfils electroforètics obtinguts amb un nombre variable d'enzims metabòlics (isoenzims i aloenzims) solubles. Si l'elecció

dels enzims és acurada es poden discriminar els productes de diferents al·lels per a un determinat nombre de loci. El mètode consisteix en fer una extracció que ha de conservar l'estructura i funció de les proteïnes. Després d'una electroforesi (generalment en gels de midó) els enzims són visualitzats prèvia exposició a uns substrats específics. Aquest mètode s'ha utilitzat per a la tipificació de *C. albicans* (Le Guennec et al, 1995; Boerlin et al, 1996; Arnavielhe et al, 1997; Pujol et al 1997), *C. tropicalis* (Doebbeling et al, 1993), *C. lusitaniae* (Merz et al, 1992), *C. parapsilosis* (Lin et al, 1995), *Cryptococcus neoformans* (Brandt et al, 1993; Brandt et al, 1995; Brandt et al, 1996) i *A. fumigatus* (Rodríguez et al, 1996). És un excel·lent mètode de tipificació però té com a principal inconvenient la necessitat de convinar les dades d'almenys 10-15 enzims per poder obtenir la suficient variabilitat entre els aïllats.

4.2 Mètodes genotípics.

4.2.1 Anàlisi de l'ADN sense hibridació. Conegut amb el nom d'anàlisi dels fragments de restricció (RFA), anàlisi amb endonucleases de restricció (REA) o RFLP (polimorfisme del tamany dels fragments de restricció) és segurament el primer mètode d'anàlisi de l'ADN utilitzat per averiguar el grau de relació entre diferents aïllats. Després de l'extracció de l'ADN a partir dels esferoplasts, aquest es digereix amb una endonucleasa de restricció (generalment *EcoRI*), els fragments se separen per electroforesi en un gel d'agarosa i la visualització de les bandes generades es realitza després d'una tinció amb bromur d'etidi. S'ha utilitzat en multitud d'estudis incloent-hi els patògens més habituals com *C. albicans* (Scherer and Stevens, 1987; Pfaller et al, 1990; Clemons et al, 1991; Clemons et al, 1997), *C. tropicalis* (Doebbeling et al, 1991), *C. parapsilosis* (Branchini et al, 1994; Pfaller et al, 1995), *C. lusitaniae* (Pfaller et al, 1994; King et al, 1995), *C. neoformans* (Currie et al, 1994), i *A. fumigatus* (Burnie et al, 1992; Lin et al, 1995). El principal inconvenient d'aquest mètode radica en la complexitat del genoma fúngic, ja que això

fa que el nombre de bandes obtingudes amb endonucleases convencionals sigui molt elevat i la resolució obtinguda deficient.

4.2.2 Anàlisi de l'ADN amb sondes d'hibridació. Aquest mètode permet visualitzar un nombre limitat de fragments de l'ADN i obtenir uns perfils més vàlids per a analitzar-los. Per a visualitzar uns fragments particulars cal realitzar un Southern blot amb el gel de RFLP i després hibridar amb una sonda determinada que reconeix fragments específics. Tot i que s'han descrit sondes d'ADN ribosòmic (Stein et al, 1991) i ADN mitocondrial (Wills et al, 1984; Olivo et al, 1987), no s'ha generalitzat el seu ús, perquè el nombre de bandes obtingudes (màxim 5) no permetia obtenir uns perfils el suficientment complexos per discriminar els aïllats analitzats.

Les sondes més utilitzades són les de fragments obtinguts per clonació que contenen seqüències genòmiques repetitives. En *C. albicans* les sondes més conegudes són la 27A (Scherer and Stevens, 1988) i Ca3 (Soll et al, 1987), que van ser clonades a finals dels anys 80 i posteriorment s'ha comprovat que estan relacionades (Cindamporn et al, 1998; Pujol et al, 1999). La utilització d'aquestes sondes es fonamenta en el fet que, en un Southern blot d'ADN genòmic digerit amb endonucleases de restricció, una sonda d'aquestes característiques hibriditzarà amb seqüències repetitives disperses pel genoma, identificant d'aquesta forma la variabilitat dels aïllats. També hibriditzaria amb seqüències addicionals menys variables i amb seqüències hipervariables que indicarien petits canvis evolutius ("microevolutius") en una soca. Una sonda d'aquestes característiques ha de ser capaç de generar un perfil suficientment complexe que al mesurar-lo permeti averiguar la relació existent entre els aïllats. Una altra característica addicional és que la sonda ha de contenir una o més seqüències que hibriditzen amb fragments monomòrfics (fragments que tenen el mateix tamany en totes o la majoria de soques d'una espècie). Aquests fragments són d'enorme utilitat ja que faciliten la normalització posterior amb programes d'anàlisi de la imatge. Tant la sonda 27A com

la Ca3 s'han fet servir en diversos estudis epidemiològics de *C. albicans* (Schmid et al, 1990; Schmid et al, 1992; Hellstein et al, 1993; Lockhart et al, 1995; Lockhart et al, 1996; Kleinegger et al, 1996; Ruiz-Diez et al, 1997; Kuehnert et al, 1998). Per *C. albicans* també s'han utilitzat sondes d'oligo o polinucleòtids (Sullivan et al, 1993; Wilkinson et al, 1992) pero no ha estat aval.luada la seva eficàcia com a mètode de tipificació.

La utilització d'aquestes sondes complexes, com la sonda Ca3 de *C. albicans*, té com a principal virtut que en un sol gel s'obté molta informació. Aquesta enorme ventatja ha servit d'al.licient per disenyar sondes específiques per altres espècies com *C. tropicalis*, *C. glabrata* i *C. dubblinensis* (Joly et al, 1996; Lockhart et al, 1997; Joly et al, 1999). Diversos investigadors han dirigit els seus esforços ha desenvolupar sondes específiques per tipificar altres fongs com *C. neoformans* (Spitzer and Spitzer,1992; Franzot el al, 1997), *A. fumigatus* (Girardin et al, 1993; Girardin et al, 1994), i *A. flavus* (McAlpin and Mannarelli, 1995) les quals han demostrat la seva utilitat.

4.2.3 Reacció en cadena de la polimerasa (PCR). Posiblement, la metodologia fonamentada en la reacció en cadena de la polimerasa més utilitzada per a la tipificació dels fongs implicats en infeccions nosocomials sigui el RAPD (random amplified polymorphic DNA). Aquest mètode utilitza 'primers' o iniciadors aleatoris d'aproximadament 10 bases que amplifiquen els amplicons o seqüències complementàries distribuïdes al llarg del genoma. La visualització es realitza després d'una eletroforesi i tinció amb bromur d'etidi (Williams et al, 1990). Quan es desenvolupa aquest mètode per primer cop en una espècie en particular cal provar diferents iniciadors i seleccionar aquells que proporcionen la major variabilitat entre aïllats independents. Un cop seleccionats, s'han de provar en cada aïllat per separat i combinar el resultats obtinguts (Pujol et al,1997). Aquesta metodologia ha esdevingut molt popular i s'ha utilitzat amb èxit en diversos estudis moleculars així com en la

tipificació de *C. albicans* (Bostok et al, 1993; Robert et al, 1995; Clemons et al, 1997; Pujol et al, 1997), *C. tropicalis* (Lin and Lehmann, 1995), *C. glabrata* (Scwab et al, 1997), *C. parapsilosis* (Lott et al, 1993), *C. dubliniensis* (Coleman et al, 1997), *C. lusitaniae* (Lehman et al, 1992; King et al, 1995), *A. fumigatus* (Anderson et al, 1996; Aufavre-Brown et al, 1992), *A. flavus* (Bufflington et al, 1994), *C. neoformans* (Brandt et al, 1995; Brandt et al, 1996; Boekhout et al, 1997; Franzot et al) i *H. capsulatum* (Kersulyte et al, 1992; Woods et al, 1993).

Hi ha que tenir en compte que en aquest mètode existeix el problema subjacent de la reproductibilitat. Pràcticament, qualsevol aspecte metodològic poc afectar-la, fins i tot el lot o procedència de la *Taq* polimerasa. Això explicaria les dificultats de reproductibilitat observades entre diferents laboratoris i fins hi tot, en un mateix laboratori (Ellsworth et al, 1993; Loudon et al, 1995).

4.2.4 Anàlisi del cariotip. El descobriment de l'electroforesi en camp pulsant (Pulsed Field Gel Electrophoresis) i les seves variants, va fer possible estudiar fragments d'ADN de gran tamany. Com l'ADN està protegit a l'interior d'un bloc d'agarosa no hi ha perill de ruptura al manipular-lo. L'ADN cromosòmic dels llevats es separa, segons el seu tamany, al sotmetre'l a una electroforesi en un camp elèctric altern i es visualitza amb bromur d'etidi (Schwartz and Cantor, 1984). L'electroforesi del cariotip s'ha utilitzat en múltiples estudis per a la tipificació de diferents espècies de *Candida* com *C. albicans* (Asakura et al, 1991; Barchesi et al, 1995a), *C. parapsilosis* (Branchini et al, 1994; Pfaller et al, 1995), *C. tropicalis* (Doebbeling et al, 1993), *C. glabrata* (Vazquez et al, 1993; Cormican et al, 1996), *C. lusitaniae* (King et al, 1995) o *C. krusei* (Essayag et al, 1996). També s'ha emprat, entre altres, en *C. neoformans* (Ingram et al, 1993; Barchiesi et al, 1995b; Fries et al, 1996; Franzot et al, 1997), *H. capsulatum* (Steele et al, 1989) i *C. immitis* (Pan and Cole, 1992). Un inconvenient d'aquest mètode es que *C. albicans* pot presentar reorganitzacions del seu cromosoma amb dos tipus de freqüència: alta i baixa (Suzuki

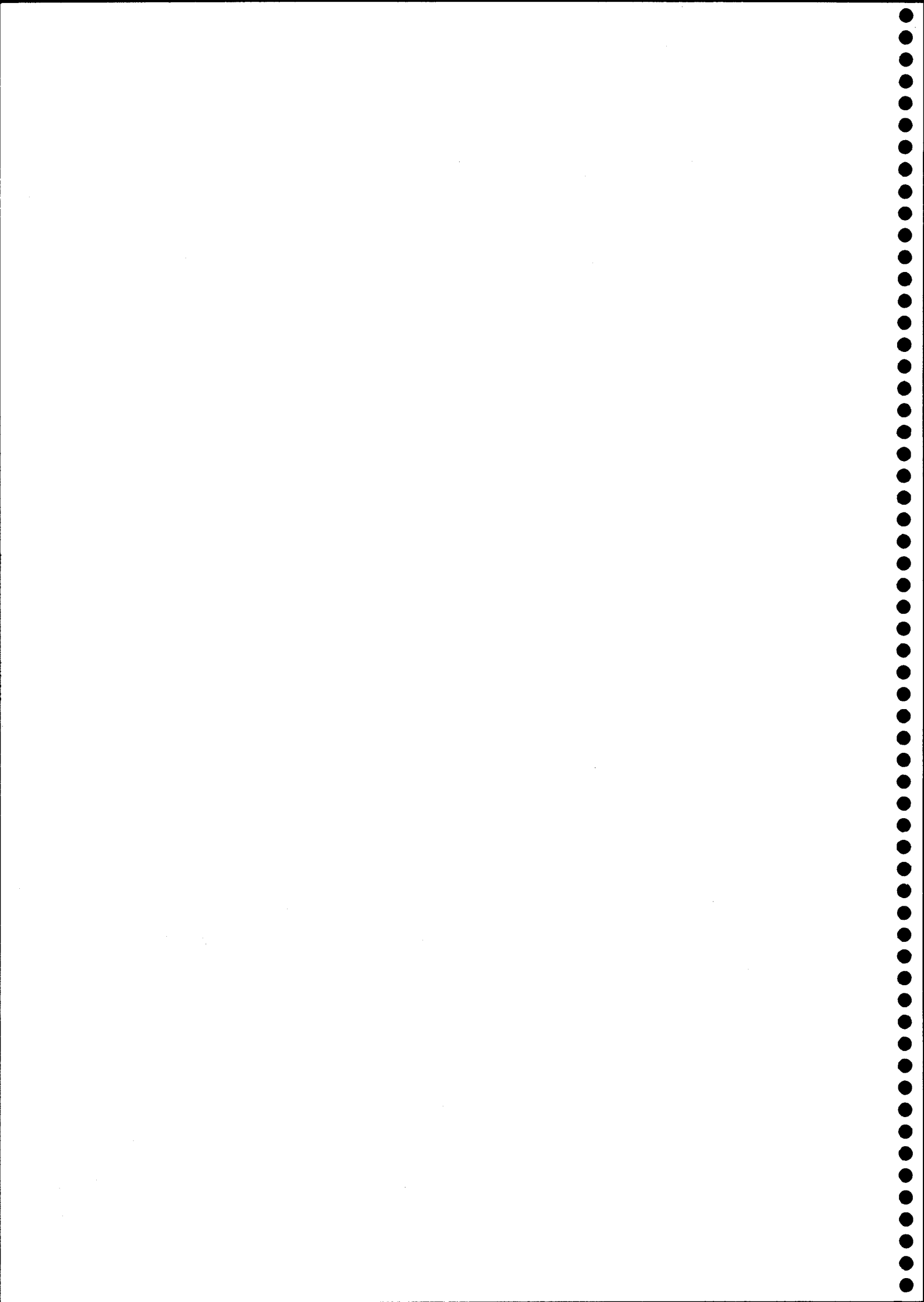
et al, 1989; Ramsey et al, 1995) que produirien canvis en el cariotip. Una modificació d'aquests mètode consistent en digerir el cromosoma amb endonucleases de restricció de baixa freqüència abans de l'electroferesi incrementa la complexitat del perfil obtingut i l'efectivitat del mètode (Cormican et al, 1996; Dikema et al, 1997).

III.- OBJECTIUS

III. OBJECTIUS.

1. Avaluar l'activitat in vitro d'un nou antifúngic azòlic (voriconazol) i dues equinocandines (caspofungina, anidulafungina) enfront de diferents espècies de *Candida* aïllades en hemocultius
2. Conèixer la freqüència de les diferents espècies de *Candida* responsables de candidèmia en el nostre medi (1996-2001) i la seva sensibilitat als antifúngics, incloent-hi els nous compostos voriconazol i anidulafungina.
3. Avaluar l'activitat in vitro de dos nous antifúngics azòlics (voriconazol, posaconazol) i dues equinocandines (caspofungina, anidulafungina) enfront de diferents espècies de fongs filamentosos aïllades en mostres clíniques.
4. Avaluar l'aplicació de la sonda semirepetitiva *Ca3* en l'estudi del grau de similitud dels aïllats de *Candida albicans* en pacients amb candidèmia i ingressats en Unitats de Cures Intensives.

IV.- ARTICLES



Article 1.

In vitro activities of voriconazole (UK-109,496) and four other antifungal agents against 394 clinical isolates of *Candida* spp. *Antimicrob Agents Chemother* 1998; 42:161-163. (FI: 3,503 / 2000)

In Vitro Activities of Voriconazole (UK-109,496) and Four Other Antifungal Agents against 394 Clinical Isolates of *Candida* spp.

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Voriconazole (formerly UK-109,496) is a new monotriazole antifungal agent which has potent activity against *Candida*, *Cryptococcus*, and *Aspergillus* species. We investigated the in vitro activity of voriconazole compared to those of fluconazole, itraconazole, amphotericin B, and flucytosine (5FC) against 394 bloodstream isolates of *Candida* (five species) obtained from more than 30 different medical centers. MICs of all antifungal drugs were determined by the method recommended by the National Committee for Clinical Laboratory Standards using RPMI 1640 test medium. Overall, voriconazole was quite active against all the yeast isolates (MIC at which 90% of the isolates are inhibited [MIC₉₀], ≤0.5 µg/ml). *Candida albicans* was the most susceptible species (MIC₉₀, 0.06 µg/ml) and *Candida glabrata* and *Candida krusei* were the least (MIC₉₀, 1 µg/ml). Voriconazole was more active than amphotericin B and 5FC against all species except *C. glabrata* and was also more active than itraconazole and fluconazole. For isolates of *Candida* spp. with decreased susceptibility to fluconazole and itraconazole MICs of voriconazole were also higher. Based on these results, voriconazole has promising antifungal activity and further in vitro and in vivo investigations are warranted.

Newer azoles such as fluconazole and itraconazole are frequently used in the treatment of fungal infections due to *Candida* spp. They offer potential advantages over amphotericin B, including reduced toxicity and versatility of oral or intravenous (fluconazole only) administration. However, acquired or intrinsic resistance to these compounds is well known, and failure of azole therapy has been reported (11, 12). There is, therefore, a clear need for new drugs to improve the treatment of fungal infections.

Voriconazole (UK-109,496) is a new monotriazole antifungal agent obtained by modification of the structure of fluconazole (14). It exhibits dose-dependent pharmacokinetics and is usually well tolerated after oral or intravenous administration (10). Early clinical studies have suggested that voriconazole may be effective in the treatment of oropharyngeal candidiasis and of acute or chronic pulmonary aspergillosis (3, 4, 17). The efficacy of voriconazole in experimental models of invasive aspergillosis and in the treatment of *Aspergillus fumigatus* endocarditis has also been documented (5, 7, 8). Previous in vitro investigations have shown activity against several fungal pathogens, including *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. (1, 6, 16). Barry and Brown (2) found that voriconazole had better in vitro activity than fluconazole against six *Candida* species. Ruhnke et al. (15) also reported good activity of voriconazole against *Candida albicans* strains isolated from patients with human immunodeficiency virus infection. However, the number of clinical isolates of *Candida* spp. included in these studies is limited, and there is a lack of comparative data for other antifungal agents.

In this study, we evaluated the in vitro activities of voriconazole and four other antifungal agents against 394 clinical isolates of *Candida* spp. The comparison agents tested were fluconazole, itraconazole, amphotericin B, and flucytosine (5FC).

The in vitro susceptibility testing method employed was a microdilution adaptation of the guidelines set forth by the National Committee for Clinical Laboratory Standards (NCCLS) (9).

MATERIALS AND METHODS

Yeast isolates. A total of 394 recent bloodstream isolates of *Candida* spp. obtained from 31 different medical centers were selected for this study. The isolates included were *C. albicans* (206 strains), *Candida glabrata* (77 strains), *Candida tropicalis* (54 strains), *Candida parapsilosis* (40 strains), and *Candida krusei* (17 strains). All isolates were stored as suspensions in sterile distilled water at room temperature until the study was performed. Prior to testing, each isolate was subcultured at least twice on potato dextrose agar plates (Remel, Lenexa, Kans.) to ensure purity and optimal growth.

Antifungal agents. Standard antifungal powders of voriconazole and fluconazole were supplied by Pfizer Inc., Central Research Division (Groton, Conn.). Amphotericin B, 5FC, and itraconazole were obtained from their respective manufacturers. Stock solutions were prepared in water (fluconazole and 5FC), dimethyl sulfoxide (amphotericin B), and polyethylene glycol (voriconazole and itraconazole). Antifungal agents were diluted with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) which had been buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma), and the mixtures were dispensed into 96-well microdilution trays. Trays containing an aliquot of 0.1 ml in each well were sealed and frozen at -70°C until they were used in the study. The NCCLS recommendations (9) were followed for the dilution of each antifungal agent.

Antifungal susceptibility testing. Broth microdilution MICs were determined by the NCCLS method (9). The final concentrations of the antifungal agents ranged from 0.015 to 16 µg/ml for voriconazole, 0.125 to 128 µg/ml for fluconazole, 0.007 to 8 µg/ml for itraconazole, 0.015 to 8 µg/ml for amphotericin B, and 0.06 to 128 µg/ml for 5FC. The yeast inoculum was adjusted to a concentration of 0.5×10^3 to 2.5×10^3 CFU/ml in RPMI 1640 medium, and an aliquot of 0.1 ml was added to each well of the microdilution tray. In each case, the inoculum size was verified by colony counting. MIC endpoints were determined after incubation for 48 h in ambient air at 35°C. For amphotericin B this endpoint was defined as the lowest concentration that completely inhibited growth. For the azole compounds and 5FC the MIC was defined as the lowest concentration that produced an 80% reduction of growth compared with that of the drug-free growth control.

Quality control. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control organisms and were included each time a set of isolates was tested.

RESULTS AND DISCUSSION

Table 1 summarizes the MICs of voriconazole, itraconazole, fluconazole, amphotericin B, and 5FC for the 394 clinical iso-

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TABLE 1. In vitro susceptibilities of 394 clinical yeast isolates to voriconazole and other antifungal agents

Organism (no. of isolates)	Antifungal agent	MIC ($\mu\text{g/ml}$)		
		Range	50%	90%
<i>C. albicans</i> (206)	Voriconazole	≤ 0.015 ->16	0.03	0.06
	Itraconazole	0.03->8	0.125	0.25
	Fluconazole	≤ 0.125 ->128	0.25	2
	Amphotericin B	0.5-2	1	1
	5FC	≤ 0.06 ->128	0.25	4
<i>C. glabrata</i> (77)	Voriconazole	0.03-8	0.5	1
	Itraconazole	0.06->8	1	4
	Fluconazole	0.25->128	16	64
	Amphotericin B	1-2	1	2
	5FC	≤ 0.06 -16	0.125	0.25
<i>C. tropicalis</i> (54)	Voriconazole	≤ 0.015 ->16	0.06	0.125
	Itraconazole	0.03->8	0.25	0.5
	Fluconazole	0.25->128	0.5	2
	Amphotericin B	0.5-2	1	2
	5FC	≤ 0.06 ->128	0.25	1
<i>C. parapsilosis</i> (40)	Voriconazole	≤ 0.015 -1	0.06	0.25
	Itraconazole	0.125-2	0.25	0.5
	Fluconazole	0.25-16	1	8
	Amphotericin B	0.5-2	1	2
	5FC	≤ 0.06 ->128	0.125	1
<i>C. krusei</i> (17)	Voriconazole	0.25-1	0.5	1
	Itraconazole	0.5-2	1	2
	Fluconazole	32-128	64	128
	Amphotericin B	1-2	2	2
	5FC	16-64	32	64
All organisms (394)	Voriconazole	≤ 0.015 ->16	0.06	0.5
	Itraconazole	0.03->8	0.25	2
	Fluconazole	≤ 0.125 ->128	0.5	16
	Amphotericin B	0.5-2	1	2
	5FC	≤ 0.06 ->128	0.25	8

lates of *Candida* spp. Overall, voriconazole was highly active (MIC at which 90% of the isolates are inhibited [MIC_{90}], ≤ 0.5 $\mu\text{g/ml}$) against all isolates, and *C. albicans* was the most susceptible species (MIC_{90} , 0.06 $\mu\text{g/ml}$). *C. glabrata* and *C. krusei* were the least susceptible to voriconazole (MIC_{90} , 1 $\mu\text{g/ml}$); the most highly resistant strains were *C. albicans* and *C. tropicalis* strains (MIC, >16 $\mu\text{g/ml}$). Voriconazole was more active than amphotericin B and 5FC against all species except *C. glabrata* and was also more active than itraconazole and fluconazole.

Among the 394 isolates studied, a total of 18 strains (7 *C. albicans*, 8 *C. glabrata*, and 3 *C. tropicalis* strains) from eight different medical centers were resistant to both fluconazole (MIC, ≥ 64 $\mu\text{g/ml}$) and itraconazole (MIC, ≥ 1 $\mu\text{g/ml}$) (13). MICs of voriconazole for these strains were >16 $\mu\text{g/ml}$ (seven *C. albicans* and two *C. tropicalis* strains), 8 $\mu\text{g/ml}$ (three *C. glabrata* strains), 4 $\mu\text{g/ml}$ (three *C. glabrata* and one *C. tropicalis* strain), and 2 $\mu\text{g/ml}$ (two *C. glabrata* strains).

These results support and extend findings reported previously (1, 2, 12). Like Barry and Brown (2), we found that voriconazole was more active than fluconazole against all *Candida* isolates tested. In addition, the spectrum of activity was better than that of itraconazole. This enhanced in vitro activity against two species frequently considered refractory to azoles, *C. krusei* and *C. glabrata* (MIC_{90} , 1 $\mu\text{g/ml}$), is remarkable. Although pharmacokinetic studies with animal models have

found that levels of voriconazole in serum could range from 1 to 5 $\mu\text{g/ml}$ (5, 7, 8) and similar levels are expected to be achieved in humans, clinical trials are clearly required to prove the utility of voriconazole in infections due to these two species.

Ruhnke et al. (15) used a broth microdilution test following the NCCLS guidelines (9) to determine the in vitro activities of voriconazole and fluconazole against 105 isolates of *C. albicans* recovered from the oral cavities of human immunodeficiency virus-infected patients. They also observed that voriconazole was more potent in vitro than fluconazole.

The data from standardized and reference in vitro susceptibility testing indicate that voriconazole is more potent than either itraconazole or fluconazole against all clinical isolates tested. Although others have reported that voriconazole could be active against fluconazole-resistant *C. albicans* isolates (2, 15), we were unable to demonstrate this finding, and in our study the MICs of voriconazole (>16 $\mu\text{g/ml}$) for fluconazole-resistant *C. albicans* isolates tested were high. Even though greater voriconazole activity was observed with eight *C. glabrata* isolates and one *C. tropicalis* isolate that were resistant to fluconazole, MICs for these isolates were ≥ 2 $\mu\text{g/ml}$. These data suggest a possible cross-resistance mechanism among highly azole-resistant strains.

The translation of this in vitro activity into clinical efficacy still needs to be established; however, Troke et al. (17) have shown in a guinea pig model of systemic candidiasis that voriconazole efficacy was similar to that of fluconazole or itraconazole in *C. albicans* infections but that voriconazole was more active when the animal was infected with *C. krusei*, *C. glabrata*, or azole-resistant strains of *C. albicans*. Preliminary clinical data also suggest that voriconazole is efficacious in the treatment of oropharyngeal candidiasis, even that caused by fluconazole-resistant strains (15, 17). In view of the potent in vitro activity demonstrated here as well as the promising early in vivo information, voriconazole warrants further investigation.

ACKNOWLEDGMENTS

Francesco Marco is partially supported by a grant from Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC-Hoechst96) and a Permiso de Ampliación de Estudios from Hospital Clínic, Barcelona, Spain. This study was supported by a grant from Pfizer Pharmaceuticals-Roerig Division.

REFERENCES

- Barchiesi, F., M. Restrepo, D. A. McGough, and M. G. Rinaldi. 1995. In vitro activity of a new antifungal triazole: UK-109,496, abstr. F71, p. 125. In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
- Barry, A. L., and S. D. Brown. 1996. In vitro studies of two triazole antifungal agents (voriconazole [UK-109,496] and fluconazole) against *Candida* species. Antimicrob. Agents Chemother. 40:1948-1949.
- Denning, D., A. del Favero, E. Gluckman, D. Norfolk, M. Ruhnke, S. Youren, P. Troke, and N. Sarantis. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: clinical efficacy in acute invasive aspergillosis, abstr. F80, p. 126. In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
- Dupont, B., D. Denning, H. Lode, S. Youren, P. Troke, and N. Sarantis. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: clinical efficacy in chronic invasive aspergillosis, abstr. F81, p. 127. In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
- George, D., P. Minitier, and V. T. Andriole. 1996. Efficacy of UK-109,496, a new azole antifungal agent, in an experimental model of invasive aspergillosis. Antimicrob. Agents Chemother. 40:86-91.
- Hitchcock, C. A., G. W. Fye, G. P. Oliver, and P. F. Troke. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: antifungal activity and selectivity in vitro, abstr. F72, p. 125. In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and

- Chemotherapy. American Society for Microbiology, Washington, D.C.
7. Martin, M. V., J. Yates, and C. A. Hitchcock. 1997. Comparison of voriconazole (UK-109,496) and itraconazole in prevention and treatment of *Aspergillus fumigatus* endocarditis in guinea pigs. *Antimicrob. Agents Chemother.* 41:13-16.
 8. Murphy, M., E. M. Bernard, T. Ishimaru, and D. Armstrong. 1997. Activity of voriconazole (UK-109,496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. *Antimicrob. Agents Chemother.* 41:696-698.
 9. National Committee for Clinical Laboratory Standards. 1997. Reference method for broth dilution antifungal susceptibility testing of yeast. Approved standard M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
 10. Patterson, B. E., and P. E. Coates. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: pharmacokinetics in man, abstr. F78, p. 126. *In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Washington, D.C.
 11. Pfaller, M. A., J. Rhine-Chalberg, S. W. Redding, J. Smith, G. Farinacci, A. W. Fothergill, and M. G. Rinaldi. 1994. Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. *J. Clin. Microbiol.* 32: 59-64.
 12. Rex, J. H., M. G. Rinaldi, and M. A. Pfaller. 1995. Resistance of *Candida* species to fluconazole. *Antimicrob. Agents Chemother.* 39:1-8.
 13. Rex, J. H., M. A. Pfaller, J. N. Galgiani, M. S. Bartlett, A. Espinel-Ingroff, M. A. Ghannoum, M. Lancaster, F. C. Odds, M. G. Rinaldi, T. J. Walsh, and A. L. Barry for the Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. 1997. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. *Clin. Infect. Dis.* 24:235-247.
 14. Richardson, K., A. S. Bell, R. P. Dickinson, S. Narayanaswami, and S. J. Ray. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: synthesis and SAR, abstr. F69, p. 125. *In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Washington, D.C.
 15. Ruhnke, M., A. Schmidt-Westhausen, and M. Trautmann. 1997. In vitro activities of voriconazole (UK-109,496) against fluconazole-susceptible and -resistant *Candida albicans* isolates from oral cavities of patients with human immunodeficiency virus infection. *Antimicrob. Agents Chemother.* 41:575-577.
 16. Troke, P. F., A. S. Bell, R. P. Dickinson, C. A. Hitchcock, S. Jezequel, S. Narayanaswami, S. J. Ray, and K. Richardson. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of systemic fungal infections: discovery and antifungal properties, abstr. F70, p. 125. *In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Washington, D.C.
 17. Troke, P. F., K. W. Brammer, C. A. Hitchcock, S. Yonren, and N. Sarantis. 1995. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: activity in systemic candidiasis models and early clinical efficacy in oropharyngeal candidiasis (OPC), abstr. F73, p. 125. *In Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Washington, D.C.

Article 2.

Activity of MK-0991 (L-743,872), a new echinocandin, compared with those of LY303366 and four other antifungal agents tested against blood stream isolates of *Candida* spp. *Diagn Microbiol Infect Dis.* 1998;31:33-37 (FI: 1,932 / 2000)

Activity of MK-0991 (L-743,872), a New Echinocandin, Compared with Those of LY303366 and Four Other Antifungal Agents Tested Against Blood Stream Isolates of *Candida* spp.

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MK-0991 (formerly L-743,872) is a water soluble semisynthetic echinocandin that possess potent, broad-spectrum antifungal activity. We evaluated the *in vitro* activity of MK-0991 and an echinocandin derivative LY303366, compared with that of itraconazole, fluconazole, amphotericin B and 5-flucytosine against 400 blood stream isolates of *Candida* spp. (nine species) obtained from more than 30 different medical centers. MICs for all antifungal drugs were determined by the NCCLS method using RPMI 1640 test medium. Both MK-0991 and LY303366 were very active against all *Candida* spp. isolates (MIC₉₀, 0.25 and 1 µg/mL, respectively). MK-0991 was two-fold to 256-fold more active than amphotericin B, fluconazole,

itraconazole (except against *C. parapsilosis*), and 5-flucytosine (except against *C. glabrata* and *C. parapsilosis*). LY303366 was comparable to MK-0991, but was fourfold less active against *C. tropicalis* (MIC₉₀, 0.5 versus 0.12 µg/mL) and *C. parapsilosis* (MIC₉₀, >2 versus 1 µg/mL). All of the isolates for which fluconazole and itraconazole had elevated MICs (≥64 µg/mL and ≥1 µg/mL, respectively) were inhibited by ≤0.5 µg/mL of MK-0991 and LY303366. These results suggest both MK-0991 and LY303366 possess promising antifungal activity and further *in vitro* and *in vivo* investigations are warranted. © 1998 Elsevier Science Inc.

INTRODUCTION

In the last few years, considerable efforts have been dedicated to developing new antifungal agents that interact with a fungal structure not found in other eukaryotic cells (Debono and Gordee 1994). In theory, the fungal cell wall is a potential target that fulfills this principle and compounds that inhibit its biosynthesis should have lower side effects when

administered to humans. Echinocandins are cyclic hexapeptides that disrupt cell wall glucan formation by a noncompetitive inhibition of β-(1-3)-glucan synthase (Debono and Gordee 1994). This enzyme catalyzes the polymerization of UDP-glucose into β-(1-3)-D-glucan, an essential cell wall component for fungi. Echinocandins exhibit fungicidal activity against *Candida* spp. (Bartizal et al. 1995) and their potential role as therapeutic agents for disseminated candidiasis and aspergillosis has been demonstrated in animal models (Abruzzo et al. 1995).

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Received 11 February 1998; revised and accepted 5 April 1998.

MK-0991 (L-743,872) is a new water-soluble echinocandin derivative that has been shown to have potent *in vitro* activity against *Candida* spp. (Bartizal et al. 1997; Espinel-Ingroff 1996; Vazquez et al. 1997), *Aspergillus* spp. (del Poeta et al. 1996; Espinel-Ingroff 1996), and *Histoplasma capsulatum* (Espinel-Ingroff

1996). The efficacy of MK-0991 in animal models of disseminated candidiasis (Abruzzo et al. 1997; Graybill et al. 1997), aspergillosis (Abruzzo et al. 1997), and histoplasmosis (Graybill et al. 1998) has also been reported. In addition, MK-0991 has been found to be useful in the treatment and prevention of *Pneumocystis carinii* pneumonia in murine models (Powles et al. 1996).

In this report we compared the in vitro activity of MK-0991 with that of an echinocandin derivative, LY303366, and four established antifungal agents against 400 recent blood stream isolates of *Candida* spp.. The in vitro susceptibility testing method used was the broth microdilution procedure outlined in the NCCLS M27-A document (NCCLS 1997).

MATERIALS AND METHODS

Organisms

A total of 400 recent blood stream isolates of *Candida* spp. obtained from 31 different medical centers between 1993 and 1996 were tested in this study. The isolates included were *C. albicans* (206 isolates), *C. glabrata* (77 isolates), *C. tropicalis* (54 isolates), *C. parapsilosis* (40 isolates), *C. krusei* (17 isolates), *C. lusitanae* (three isolates), and one isolate each of *C. famata*, *C. rugosa*, and *C. guilliermondii*. All isolates were stored as suspensions in sterile distilled water at room temperature until the study was performed. Before testing, each isolate was subcultured at least twice on potato dextrose agar plates (Remel, Lenexa, KS, USA) to ensure purity and optimal growth.

Antifungal Agents

MK-0991 and LY303366 were obtained as standard antifungal powders from Merck Research Laboratories (Rahway, NJ, USA) and Eli Lilly and Company (Indianapolis, IN, USA), respectively. Amphotericin B, flucytosine, fluconazole, and itraconazole were supplied by their respective manufacturers. Stock solutions were prepared in water (MK-0991, fluconazole, and flucytosine), dimethyl sulfoxide (amphotericin B and LY303366), or polyethylene glycol (itraconazole). Plastic microdilution trays were prepared with RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and containing the appropriate dilution of each antifungal drug. Trays containing an aliquot of 0.1 mL in each well were sealed and frozen at -70°C until they were used in the study. The recommendations stated in the NCCLS document M27-A (NCCLS 1997) were followed for the dilution of each antifungal agent. The final concentration of the antifungal

agents ranged from 0.007 to 8 $\mu\text{g}/\text{mL}$ for MK-0991, 0.001 to 2 $\mu\text{g}/\text{mL}$ for LY303366, 0.125 to 128 $\mu\text{g}/\text{mL}$ for fluconazole, 0.007 to 8 $\mu\text{g}/\text{mL}$ for itraconazole, 0.015 to 8 $\mu\text{g}/\text{mL}$ for amphotericin B, and 0.06 to 128 $\mu\text{g}/\text{mL}$ for 5-flucytosine.

Antifungal Susceptibility Testing

Broth microdilution MICs were determined by the NCCLS M27-A methodology (NCCLS 1997). Yeast inoculum was adjusted to a concentration of 0.5 to 2.5×10^3 CFU/mL and an aliquot of 0.1 mL was added to each well of the microdilution tray. In each case, the inoculum size was verified by colony counts. MIC endpoints were determined after an incubation of 48 h in air at 35°C with the aid of a reading mirror. For MK-0991, LY303366, and amphotericin B this endpoint was the lowest concentration of antifungal agent that completely inhibited growth. MICs of itraconazole, fluconazole, and flucytosine were defined as the lowest concentration that produced a prominent decrease in turbidity ($\sim 50\%$ inhibition) when compared with that of the drug-free growth control (NCCLS 1997).

Quality Control

The two quality control strains recommended in the NCCLS document M27-A (NCCLS 1997), *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were used as quality control organisms and included each time a set of isolates was tested.

RESULTS AND DISCUSSION

The in vitro activities of MK-0991 and LY303366 compared with those of itraconazole, fluconazole, amphotericin B, and flucytosine are summarized in Table 1. Both MK-0991 and LY303366 were very active against all *Candida* isolates (MIC_{90} , 0.25 and 1 $\mu\text{g}/\text{mL}$, respectively). MK-0991 was twofold to 16-fold more active than amphotericin B against all isolates tested and eightfold to 64-fold more active than flucytosine against all isolates except for *C. glabrata* and *C. parapsilosis*. By comparison with the two triazoles, MK-0991 displayed more potent in vitro activity than fluconazole (eightfold to 256-fold more active) and itraconazole (twofold to 16-fold more active) against all isolates, except *C. parapsilosis*. LY303366 was generally comparable to MK-0991, but was fourfold less active against *C. tropicalis* (MIC_{90} , 0.5 versus 0.12 $\mu\text{g}/\text{mL}$) and *C. parapsilosis* (MIC_{90} , >2 versus 1 $\mu\text{g}/\text{mL}$).

The in vitro activity of MK-0991 and LY303366 against 18 isolates (seven *C. albicans*, eight *C. glabrata* and three *C. tropicalis*) from eight different medical

TABLE 1 In Vitro Susceptibilities of 400 Clinical Yeast Isolates to MK-0991, LY 303366, Itraconazole, Fluconazole, Amphotericin B, and Flucytosine

Organism (no. of isolates)	Antifungal Agent	MIC ($\mu\text{g}/\text{ml}$)		
		Range	50%	90%
<i>C. albicans</i> (206)	MK-0991	0.015-0.5	0.06	0.125
	LY303366	0.06-1	0.25	0.25
	Itraconazole	0.03->8	0.125	0.25
	Fluconazole	≤ 0.125 ->128	0.25	2
	Amphotericin B	0.5-2	1	1
	Flucytosine	≤ 0.06 ->128	0.25	4
<i>C. glabrata</i> (77)	MK-0991	0.03-0.5	0.06	0.25
	LY303366	0.125-2	0.25	0.25
	Itraconazole	0.03->8	1	4
	Fluconazole	0.25->128	16	64
	Amphotericin B	1-2	1	2
	Flucytosine	≤ 0.06 -16	0.125	0.25
<i>C. tropicalis</i> (54)	MK-0991	0.03-0.25	0.06	0.125
	LY303366	0.06->2	0.25	0.5
	Itraconazole	0.03->8	0.25	0.5
	Fluconazole	0.25->128	0.5	2
	Amphotericin B	0.5-2	1	2
	Flucytosine	≤ 0.06 ->128	0.25	1
<i>C. parapsilosis</i> (40)	MK-0991	0.03-2	0.5	1
	LY303366	0.125->2	>2	>2
	Itraconazole	0.125-2	0.25	0.5
	Fluconazole	0.25-16	1	8
	Amphotericin B	0.5-2	1	2
	Flucytosine	≤ 0.06 ->128	0.125	1
<i>C. krusei</i> (17)	MK-0991	0.125-1	0.5	1
	LY303366	0.125-1	0.25	0.5
	Itraconazole	0.5-2	1	2
	Fluconazole	32-128	64	128
	Amphotericin B	1-2	2	2
	Flucytosine	16-64	32	64
<i>Candida</i> spp. (6) ^a	MK-0991	0.06->8	0.125	—
	LY303366	0.125->2	0.5	—
	Itraconazole	0.06-2	0.25	—
	Fluconazole	0.25-16	2	—
	Amphotericin B	1-2	1	—
	Flucytosine	0.06-2	0.5	—
All organisms (400)	MK-0991	0.015->8	0.06	0.25
	LY303366	0.06->2	0.25	1
	Itraconazole	0.03->8	0.25	2
	Fluconazole	≤ 0.125 ->128	0.5	16
	Amphotericin B	0.5-2	1	2
	Flucytosine	≤ 0.06 ->128	0.25	8

^a Includes *C. lusitanae* (three isolates), *C. rugosa* (one isolate), *C. guilliermondii* (one isolate) and *C. famata* (one isolate).

centers that were resistant to fluconazole (MIC ≥ 64 $\mu\text{g}/\text{mL}$) and itraconazole (MIC ≥ 1 $\mu\text{g}/\text{mL}$) (Rex et al. 1997) was also examined. All of these isolates were inhibited by ≤ 0.5 $\mu\text{g}/\text{mL}$ of both echinocandins (data not shown), but MK-0991 showed more potent in vitro activity than LY303366 (MIC₅₀/MIC₉₀, 0.06/0.25 $\mu\text{g}/\text{mL}$ versus 0.25/0.5 $\mu\text{g}/\text{mL}$, respectively).

These results extend the previous observations of the in vitro activity of the echinocandin antifungal

agents (Bartizal et al. 1997; Espinel-Ingroff 1996; Pfaller et al. 1997; Vazquez et al. 1997). We found MK-0991 to be very active in vitro against most species of *Candida* spp. All isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis* were inhibited by ≤ 0.5 μg of MK-0991 per milliliter. Although *C. krusei* (range 0.125 to 1 $\mu\text{g}/\text{mL}$) and *C. parapsilosis* (range 0.03 to 2 $\mu\text{g}/\text{mL}$) isolates showed higher MICs than the other species, the only strain with a MIC > 2 $\mu\text{g}/\text{mL}$ was an isolate of *C. rugosa* (MIC > 8 $\mu\text{g}/\text{mL}$). In agreement

with Nelson et al. (1997) and Vazquez et al. (1997), we also observed that MK-0991 displayed similar in vitro activity against both azole-resistant and azole-susceptible strains. LY303366 results were comparable to those reported previously by Pfaller et al. (1997) against clinical isolates of *Candida* spp. and other yeast species.

Kurtz et al. (1994) have reported that the echinocandins, as cell wall synthesis inhibitors, confer susceptibility to lysis in the absence of osmotic support. It should be noted that in our study we have used a hyperosmotic medium, buffered RPMI 1640, as described in the NCCLS document M27-A (NCCLS 1997). The correlation of these results into clinical efficacy may therefore be underestimated; however, the efficacy of MK-0991 in an animal model of disseminated candidiasis has recently been demonstrated by Abruzzo et al. (1997).

In summary, MK-0991 is a promising new antifungal agent to add to the current series of investigational echinocandins. Like LY303366, it exhibits

potent in vitro activity against both fluconazole-susceptible and fluconazole-resistant *Candida* strains and results against several medically important moulds are encouraging (del Poeta et al. 1996; Espinel-Ingroff 1996). As expected, little in vitro activity has been observed against *Cryptococcus neoformans* (Abruzzo et al. 1997; Espinel-Ingroff 1996); however, Franzot et al. (1997) have found synergistic activity when MK-0991 was combined with amphotericin B or fluconazole. Based on the available data, both MK-0991 and LY303366 deserve further investigation. The potential role of these compounds in the treatment of clinical infections should be considered.

Francesc Marco is partially supported by a grant from Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC-Hoechst96) and a Permiso de Ampliación de Estudios from Hospital Clínic, Barcelona, Spain.

REFERENCES

- Abruzzo GK, Flattery AM, Gill CJ, Kong L, Smith JG, Krupa D, Pikounis VB, Kropp H, Bartizal K (1995) Evaluation of water-soluble pneumocandin analogs L-733560, L-705589 and L-731373 with mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob Agents Chemother* 39:1077-1081.
- Abruzzo GK, Flattery AM, Gill CJ, Kong L, Smith JG, Pikounis VB, Kropp H, Bartizal K (1997) Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob Agents Chemother* 41:2333-2338.
- Bartizal K, Gill CJ, Abruzzo GK, Flattery AM, Kong L, Scott PM (1997) In vitro preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). *Antimicrob Agents Chemother* 41:2326-2332.
- Bartizal K, Scott T, Abruzzo GK, Gill CJ, Pacholok C, Lynch L, Kropp H (1995) In vitro evaluation of the pneumocandin antifungal agent L-733560, a new water-soluble hybrid of L-705589 and L-731373. *Antimicrob Agents Chemother* 39:1070-1076.
- Debono M, Gordee RS (1994) Antibiotics that inhibit fungal cell wall development. *Annu Rev Microbiol* 48:471-497.
- del Poeta M, Schell WA, Perfect JR (1996) In vitro antifungal activity of L-743,872 against a variety of clinically important moulds. *Antimicrob Agents Chemother* 41:1835-1836.
- Espinel-Ingroff A (1996) In vitro studies with L-743,872, a water soluble pneumocandin: a comparative study (Abstr. F31). *Programs and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, LA, 1996*. Washington, DC: American Society for Microbiology, p. 105.
- Franzot SP, Casadevall A (1997) Pneumocandin L-743,872 enhances the activities of amphotericin B and fluconazole against *Cryptococcus neoformans* in vitro. *Antimicrob Agents Chemother* 41:331-336.
- Graybill JR, Najvar LK, Luther MF, Fothergill AW (1997) Treatment of murine disseminated candidiasis with L-743,872. *Antimicrob Agents Chemother* 41:1775-1777.
- Graybill JR, Najvar LK, Montalbo EM, Barchiesi FJ, Luther MF, Rinaldi MG (1998) Treatment of histoplasmosis with MK-991 (L-743,872). *Antimicrob Agents Chemother* 42:151-153.
- Kurtz MB, Douglas C, Marrinan J, Nollstadt K, Onishi J, Dreikorn S, Milligan J, Mandala S, Thompson J, Balkovec JM (1994) Increased antifungal activity of L-733,560, a water-soluble, semisynthetic pneumocandin, is due to enhanced inhibition of cell wall synthesis. *Antimicrob Agents Chemother* 38:2750-2757.
- National Committee for Clinical Laboratory Standards (NCCLS) (1997) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast: Approved Standard M27-A*. Wayne, PA: NCCLS.
- Nelson PW, Lozano-Chiu M, Rex JH (1997) In vitro growth-inhibitory activity of pneumocandins L-733,560 and L-743,872 against putatively amphotericin-B and fluconazole-resistant *Candida* isolates: influence of assay conditions. *J Med Vet Mycol* 35:285-287.
- Pfaller MA, Messer SA, Coffman S (1997) In vitro susceptibilities of clinical yeast isolates to a new echinocandin derivative, LY303366, and other antifungal agents. *Antimicrob Agents Chemother* 41:763-766.
- Powles MA, Anderson J, Liberator P, Schmatz DM (1996) Efficacy of semisynthetic pneumocandin analog L-743,872 against *Pneumocystis carinii* in murine models (Abstr. F42). *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy*,

Article 3.

Trends in frequency and in vitro susceptibilities to antifungal agents, including voriconazole and anidulafungin, of *Candida* bloodstream isolates. Results from a six years study (1996-2001). *Diagn Microbiol Infect Dis.* (enviat a publicar)

Trends in frequency and in vitro susceptibilities to antifungal agents, including voriconazole and anidulafungin, of *Candida* bloodstream isolates. Results from a six-year study (1996-2001).

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Abstract.

The frequency of isolation and antifungal susceptibility patterns to established and two new antifungal agents were determined for 218 *Candida* spp isolates causing bloodstream infection from 1996 to 2001. Overall, 41.7% of the candidemias were due to *C. albicans*, followed by *C. parapsilosis* (22%), *C. tropicalis* (16.1%), *C. glabrata* (11.9%), *C. krusei* (6%) and miscellaneous *Candida* spp (2.3%). Isolates of *C. albicans*, *C. parapsilosis* and *C. tropicalis* (80% of isolates) were highly susceptible to fluconazole (94 to 100% at ≤ 8 $\mu\text{g/ml}$) and voriconazole (97 to 100 % at ≤ 1 $\mu\text{g/ml}$). However, against these isolates, itraconazole displayed a lower in vitro activity (77 to 97 % at ≤ 0.12 $\mu\text{g/ml}$). Only 77% and 11% of *C. glabrata* isolates were inhibited by fluconazole at ≤ 8 $\mu\text{g/ml}$ and itraconazole at ≤ 0.12 $\mu\text{g/ml}$, respectively. Voriconazole showed a remarkable in vitro potency against *C. glabrata* as well as *C. krusei* isolates (100% at ≤ 1 $\mu\text{g/ml}$). Anidulafungin demonstrated to be extremely active against *Candida* spp isolates (MIC₉₀: ≤ 0.5 $\mu\text{g/ml}$), except *C. parapsilosis* (MIC₉₀: 4 $\mu\text{g/ml}$) and two *C. guilliermondii* isolates (MIC: ≥ 32 $\mu\text{g/ml}$).

Introduction.

The incidence of systemic, often life-threatening fungal infections has increased steadily over the past two decades (Fridkin et al, 1996; McNeil et al, 2001; Rees et al, 1999). Several factors like the proliferation of patients who are severely immunocompromised by cancer chemotherapy, organ transplantation or HIV infection and patients who have been subjected to systemic glucocorticosteroids, broad spectrum antibacterials, or invasive medical procedures have contributed to this fact (Fridkin et al, 1996; Walsh et al, 1994). Although *Candida albicans* and *Aspergillus* spp account for most of the invasive infections in these patients, non-*albicans* *Candida* spp. and previously uncommon opportunistic fungal pathogens such as *Fusarium* spp., zygomycetes, and dematiaceous molds are observed with increasing frequency in these patients (Annaissie, 1992; Kao et al, 1999; Perfect et al, 1996).

In recent years, it seems that there has been a shift in the spectrum of *Candida* infections. In a multicenter, prospective study of candidemia conducted between 1990 and 1994, the incidence of candidemia due to non- *albicans* spp. surpassed that due to *C. albicans* in the latter half of the study (Nguyen et al, 1996). In more recent studies, although *C. albicans* was still the most common cause of fungemia in USA, Canada, Europe, and Latin America, an expanding incidence of other species was observed (Pfaller et al. 2000; Pfaller et al, 2001). *C. glabrata*, a species associated with the increased use of azole antifungal agents (Abi-Said, et al, 1997, Wingard et al, 1993), was the leading cause of candidemia after *C. albicans* in the USA and the second in Canada and Europe (Pfaller et al, 2001). In the last two geographic areas and Latin America, the species that ranked second behind *C. albicans* was *C. parapsilosis* (Pfaller et al, 2001).

In the past decade, pharmaceutical companies have devoted great efforts to improve old antifungal agents and develop new compounds that can help to the treatment of fungal infections. Among them, new triazoles like voriconazole and

anidulafungin, a member of the echinocandin family, have demonstrated a broad spectrum of in vitro activity against commonly fungal pathogens (Krishnarao et al, 1997; Marco et al, 1998a and 1998b; Pfaller et al 1998 and 1999)

The aim of this study was to determine the frequency of occurrence and species distribution of *Candida* spp isolated in blood cultures within the last six years in our institution. In addition, the activity and potency of established antifungal agents as well as the new azole derivative voriconazole and anidulafungin, is also described.

Materials and Methods.

Setting: The Hospital Clinic of Barcelona is a university teaching hospital with 700 beds that serves a direct population close to 500.000 inhabitants. It has a median number of 35.000 admissions/year and provides universal health care coverage. The hospital has 40 ICU beds and several active transplant programs including bone marrow and solid organ transplantation.

Organisms. All *Candida* isolates recovered from blood cultures (Bactec 9240, Becton Dickinson) between 1996 and 2001 were identified by standard methods including CHROMagar *Candida* Medium (CHROMagar, France), API C (bioMerieux, France) or Auxacolor (BioRad, France). Isolates were stored in sterile distilled water at room temperature until susceptibility testing was performed.

Antifungal agents. The antifungal drugs tested in this study were obtained as standard powders of known potency from the following manufacturers: voriconazole and fluconazole (Pfizer Central Research, Sandwich, UK), itraconazole (Janssen, Beerse, Belgium) amphotericin B (Squibb, Princeton, NJ, USA), 5-flucytosine (Sigma, Madrid, SPAIN) and anidulafungin, previously LY303366, (Eli Lilly and Company, Indianapolis, IN, USA). Stocks solutions were prepared in dimethyl sulfoxide

(voriconazole, itraconazole, amphotericin B, and anidulafungin) or sterile distilled water (fluconazole and 5-flucytosine).

Antifungal susceptibility testing. Broth microdilution minimal inhibitory concentrations (MICs) were determined by the NCCLS M27-A methodology using RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid (MOPS) buffer. Before to add the inoculum, trays were prepared containing an aliquot of 0.1 ml in each well of appropriate drug solution (2x final concentration). The final concentration of the antifungal agents ranged from 0.03 to 32 $\mu\text{g/ml}$ for voriconazole, itraconazole, amphotericin B and anidulafungin, and 0.12 to 128 $\mu\text{g/ml}$ for fluconazole and 5-flucytosine. Before to test, isolates were cultured at least twice in Sabouraud dextrose agar to assure their viability. Yeast inocula were adjusted to a concentration of 0.5 to 2.5 $\times 10^3$ UFC/ml. MIC endpoints were determined after an incubation of 48 h in air at 35°C with the aid of a reading mirror. For amphotericin B and anidulafungin the MIC endpoint was the lowest concentration of antifungal agent that completely inhibited growth. MICs of azole compounds and 5-flucytosine were defined as the lowest concentration that produced an 80% reduction of growth or prominent decrease in turbidity when compared with that of growth control well.

Susceptibility results were analyzed using the criteria published in the NCCLS document M27A for fluconazole (S: ≤ 8 $\mu\text{g/ml}$; SDD: 16-32 $\mu\text{g/ml}$, and R: ≥ 64 $\mu\text{g/ml}$), itraconazole (S: ≤ 0.12 $\mu\text{g/ml}$; SDD: 0.25-0.5 $\mu\text{g/ml}$, and R: ≥ 1 $\mu\text{g/ml}$) and 5-flucytosine (S: ≤ 4 $\mu\text{g/ml}$; I: 8-16 $\mu\text{g/ml}$, and R: ≥ 32 $\mu\text{g/ml}$). For voriconazole, a breakpoint of ≤ 1 $\mu\text{g/ml}$ was used based on previous pharmacokinetic studies (Sheehan et al, 1999).

Quality control. The two QC strains recommended in the NCCLS document M27-A, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were used as quality control organisms and included each time any testing was performed.

Results and Discussion

Species isolated. During the six-year period, a total of 218 *Candida* isolates were recovered from blood cultures submitted to the microbiology laboratory. Along the period study, these isolates accounted for 3 to 5% of nosocomial blood stream infections and they represented the seventh to ninth-most-common cause of this infections in our institution (Blood cultures study group, personal communication).

Table 1 summarizes the species distribution, number of isolates and the corresponding percentage of *Candida* bloodstream isolates from 1996 to 2001. *C. albicans* was the most commonly species identified each year accounting for 29.3 to 59.3% of all *Candida* isolates. However, in the last five years (1997 to 2001) the frequency of isolation of this species was lower than 50% and was surpassed by non-*albicans* *Candida* species isolates. *C. parapsilosis* (22%) was the second-most-common species the six years except in 1997. The overall rank order of *C. tropicalis* (35 strains, 16.1%) and *C. glabrata* (26 strains, 11.9%) was third and fourth. *C. krusei* (6%) was the less common species isolated and miscellaneous *Candida* spp (2.3%) was encountered occasionally. The rank order of species and their distribution over the years in our hospital is quite similar to the recently described by Pfaller et al (Pfaller et al, 2001) in Latin America with data collected by the Sentry antimicrobial surveillance program, from 1997 to 1999. In the same report, data from European hospitals showed a higher number of *C. albicans* isolates (58%) that in our center (41.7%). The differences are still more pronounced if we compare the same period of time (1997 to 1999): 58% vs. 39.4%. As Pfaller et al have previously noted (Pfaller et al, 1999; Pfaller et al, 2001), the proportion of species isolated varies considerably among medical centers and probably, the reasons for such differences can be attributed to many facts. Several reports have postulated that the widespread use of azole drugs in prophylaxis regimens to prevent fungal infections could be linked to a higher number of *C. glabrata* and *C. krusei* infections (Abi-Said et al, 1997; Wingard et al, 1991; Wingard et al, 1993). In our

institution, azole compounds (fluconazole and itraconazole) are extensively used since they were commercialized, predominantly in patients with bone marrow or organ solid transplant (Harousseau et al, 2000). The overall percentage of both, *C. glabrata* and *C. krusei*, recovered along the study period was 17.9%. Most of *C. glabrata* and all *C. krusei* isolates were recovered in patients with azole prophylaxis regimen (Blood cultures study group, personal communication) but, the low number of isolates identified each year and the high percentage of these species isolated in 1998 clearly conditioned the final results. Poor care of intravascular catheters or a gap in infection control practice seems to be involved in *C. parapsilosis* bloodstream infections (Levin et al, 1998). Because of we have detected a high percentage of infections due to this species (22%), a review of our catheter care guidelines is ongoing. Finally, another reason that could contribute to the differences observed among medical centers is the low number of isolates recovered in some locations. A clear example is the data from the three Spanish hospitals participating in the Sentry program, that are very similar to our institution, but they have reported only 21, 14 and less than 10 isolates in a three-year period study (Pfaller et al, 2001).

Susceptibility results. The antifungal activities of voriconazole, fluconazole, itraconazole, amphotericin B, 5-flucytosine and anidulafungin against the 218 *Candida* spp isolated during the study period are shown in Table 2. Among azole compounds, voriconazole was the most active drug overall with a MIC₉₀ of 0.25 µg/ml. For these particular drugs, the rank order of activity (based on MIC₉₀ results) was voriconazole (MIC₉₀, 0.25 µg/ml) > itraconazole (MIC₉₀, 0.5 µg/ml) > fluconazole (MIC₉₀, 8 µg/ml). Against *C. albicans*, *C. parapsilosis* and *C. tropicalis* isolates, voriconazole (MIC₉₀, ≤0.03-0.25 µg/ml) was four to eight-fold and 16 to 32-fold more active than itraconazole (MIC₉₀, 0.12-1 µg/ml) and fluconazole (MIC₉₀, 0.5-8 µg/ml), respectively. Voriconazole and itraconazole displayed similar in vitro activity against *C. glabrata* (MIC₉₀, 0.5 and 1 µg/ml, respectively) and both drugs showed lower MICs than fluconazole.

Table 3 summarizes the percentages of strains inhibited by the three azoles compounds using the categories suggested by the NCCLS M27A document (NCCLS, 1997) for fluconazole and itraconazole. Voriconazole breakpoint of ≤ 1 $\mu\text{g/ml}$ is also included for comparison purposes. Fluconazole showed a high activity and inhibited, at a MIC of ≤ 8 $\mu\text{g/ml}$ (susceptible category), 99%, 100% and 94% of *C. albicans*, *C. parapsilosis* and *C. tropicalis* isolates, respectively. The number of isolates of these species categorized as susceptible to itraconazole (≤ 0.12 $\mu\text{g/ml}$) were lower than fluconazole and 11% of *C. tropicalis* isolates were resistant (≥ 1 $\mu\text{g/ml}$). *C. glabrata* was less susceptible to both triazole compounds. Twenty-three percent and 39% of isolates were susceptible dose-dependent to fluconazole (16-32 $\mu\text{g/ml}$) and itraconazole (0.25-0.5 $\mu\text{g/ml}$), respectively. Interestingly, 46% of isolates were found to be resistant to itraconazole (≥ 1 $\mu\text{g/ml}$) and none to fluconazole (≥ 64 $\mu\text{g/ml}$). Among the three-azole drugs compared, voriconazole displayed the better activity and inhibited the majority of isolates at the breakpoint proposed (≤ 1 $\mu\text{g/ml}$). Only two isolates (one *C. albicans* and one *C. tropicalis*), also resistant to fluconazole (MIC ≥ 128 $\mu\text{g/ml}$), showed a MIC of 8 $\mu\text{g/ml}$. Our voriconazole results are in agreement with the findings reported by other authors (refs) and they support the promising future of this compound for the treatment of *Candida* spp infections.

The echinocandin drug tested, anidulafungin, inhibited all *Candida* spp isolates with a MIC of ≤ 0.5 $\mu\text{g/ml}$, except *C. parapsilosis* (MIC₉₀, 4 $\mu\text{g/ml}$) and two *C. guilliermondii* (MIC ≥ 32 $\mu\text{g/ml}$) isolates. These results are also in concordance with the previously encountered by other investigators (Krishnarao et al, 1997; Marco et al, 1998; Pfaller et al, 1997). It should be mentioned that higher MICs against *C. parapsilosis* in comparison with other *Candida* spp isolates is also observed with the other two currently developed echinocandin compounds, caspofungin and micafungin (Krishnarao et al, 1997; Marco et al, 1998; Tawara et al, 2000). Pharmacokinetic studies suggest that a plasmatic concentration near to *C. parapsilosis* MICs can be achieved when anidulafungin is administrated (Brown et al, 2000). For that reason, in locations

where the incidence of infections due to this species is high, empirical treatment with this antifungal drug should be cautious until *Candida* isolate is speciated.

The pyrimidine 5-flucytosine showed a remarkable in vitro activity against all species, except *C. krusei*. Resistance to this drug in species potentially susceptible was detected in only one *C. parapsilosis* and one *C. tropicalis* isolates with a MIC > 128 µg/ml. As expected, MICs of amphotericin B were very narrow and although they ranged from 0.06 to 1 µg/ml, 92% of isolates had a MIC between 0.25 and 1 µg/ml (data not shown).

In summary, our results show that *C. albicans* remains the *Candida* species most frequently implicated in nosocomial candidemia in our institution followed by *C. parapsilosis* and *C. tropicalis*. However, the frequency of candidemia attributable to *C. albicans* accounted for less than 50% in the last 5 years. Currently, resistance to fluconazole in the 80% of isolates recovered (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*) remains rare. Isolates intrinsically resistant to fluconazole (*C. krusei*, 6%), or with decreased susceptibility to this drug, like *C. glabrata* (11.9%), were clearly inhibited by the new triazole compound voriconazole. Anidulafungin also demonstrated excellent in vitro activity against commonly isolated species, except *C. parapsilosis*.

TABLE 1. Species distribution of 218 *Candida* strains isolated from blood cultures from 1996 to 2001.

Species	Number of isolates (%) by year						Total
	1996	1997	1998	1999	2000	2001	
<i>C. albicans</i>	16 (59.3)	12 (42.9)	12 (29.3)	18 (46.1)	15 (42.9)	18 (37.5)	91 (41.7)
<i>C. parapsilosis</i>	6 (22.2)	3 (10.7)	11 (26.8)	9 (23.1)	8 (22.9)	11 (22.9)	48 (22)
<i>C. tropicalis</i>	2 (7.4)	8 (28.6)	5 (12.2)	7 (18)	6 (17.1)	7 (14.6)	35 (16.1)
<i>C. glabrata</i>	2 (7.4)	3 (10.8)	9 (21.9)	3 (7.7)	4 (11.4)	5 (10.4)	26 (11.9)
<i>C. krusei</i>	1 (3.7)	1 (3.6)	4 (9.8)	2 (5.1)	2 (5.7)	3 (6.3)	13 (6)
<i>Candida</i> spp	-	1 ^a (3.6)	-	-	-	4 ^b (8.3)	5 (2.3)
All species	27	28	41	39	35	48	218

a) *C. guilliermondii*; b) *C. guilliermondii* and *C. famata* (one isolate each), *Candida* spp (two isolates).

TABLE 2. In vitro susceptibilities of 218 *Candida spp* bloodstream isolates to voriconazole, fluconazole, itraconazole, amphotericin B, 5-flucytosine and anidulafungin

Organisms (No. of isolates)	Antifungal agent	Range	MIC ($\mu\text{g/ml}$)	
			50%	90%
<i>C. albicans</i> (91)	voriconazole	≤ 0.03 -8	≤ 0.03	≤ 0.03
	fluconazole	≤ 0.12 ->128	0.25	0.5
	itraconazole	≤ 0.03 -8	≤ 0.03	0.12
	amphotericin B	0.06-1	0.25	0.5
	5-flucytosine	≤ 0.12 -1	≤ 0.12	0.5
	anidulafungin	≤ 0.03 -0.25	0.06	0.12
<i>C. parapsilosis</i> (48)	voriconazole	≤ 0.03 -0.06	≤ 0.03	≤ 0.03
	fluconazole	0.12-8	0.5	1
	itraconazole	≤ 0.03 -1	0.06	0.25
	amphotericin B	0.25-1	0.5	1
	5-flucytosine	≤ 0.12 ->128	≤ 0.12	0.25
	anidulafungin	2-8	2	4
<i>C. tropicalis</i> (35)	voriconazole	≤ 0.03 -8	≤ 0.03	0.25
	fluconazole	0.25->128	1	8
	itraconazole	≤ 0.03 -1	0.06	1
	amphotericin B	0.25-1	0.5	1
	5-flucytosine	≤ 0.12 ->128	≤ 0.12	0.5
	anidulafungin	≤ 0.03 -0.5	0.12	0.25
<i>C. glabrata</i> (26)	voriconazole	≤ 0.03 -1	0.12	0.5
	fluconazole	0.5-32	4	32
	itraconazole	0.06-4	0.5	1
	amphotericin B	0.12-1	0.5	1
	5-flucytosine	≤ 0.12 -4	≤ 0.12	0.25
	anidulafungin	0.12-0.25	0.12	0.12
<i>C. krusei</i> (13)	voriconazole	0.03-0.5	0.5	0.5
	fluconazole	32-64	64	64
	itraconazole	0.12-1	0.5	1
	amphotericin B	0.5-1	1	1
	5-flucytosine	8-32	16	32
	anidulafungin	0.12-0.5	0.25	0.5

Table 2, continued.

<i>Candida</i> spp (5)*	voriconazole	0.03-0.06	0.06	
	fluconazole	1-4	2	
	itraconazole	0.12-1	0.5	
	amphotericin B	0.06-0.25	0.12	
	5-flucytosine	≤0.12-1	≤0.12	
	anidulafungin	≤0.03->32	0.12	
All strains (218)	voriconazole	≤0.03-8	≤0.03	0.25
	fluconazole	≤0.12->128	0.5	8
	itraconazole	≤0.03-8	0.06	0.5
	amphotericin B	0.06-1	0.5	1
	5-flucytosine	≤0.12->128	≤0.12	1
	anidulafungin	≤0.03->32	0.12	4

* *Candida famata* (1 isolate), *C. guilliermondii* (2 isolates) and *Candida* spp (2 isolates)

Table 3. Isolates inhibited (%) at each MIC (µg/ml) for fluconazole, itraconazole and voriconazole.

Species	FLU			ITR			VOR
	≤8	16-32	≥64	≤0.12	0.25-0.5	≥1	≤1
<i>C. albicans</i>	99	-	1	97	2	1	99
<i>C. parapsilosis</i>	100	-	-	83	5	2	100
<i>C. tropicalis</i>	94	3	3	77	12	11	97
<i>C. glabrata</i>	77	23	-	15	39	46	100
<i>C. krusei</i>	-	38	62	8	69	23	100

FLU: fluconazole, ITR: itraconazole, VOR: voriconazole.

REFERENCES

Abi-Said D, Anaissie E, Uzum O, Raad I, Pinzcowski H, Vartivarian S. The epidemiology of hematogeneous candidiasis caused by different *Candida* species. *Clin Infect Dis*. 1997;24:1122-1128.

Anaissie E. (1992) Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. *Clin Infect Dis*. 14(Suppl 1):43-53.

Brown GL, White RJ, Taubel J. (2000) Phase I dose optimization study for V-echinocandin (Abstr 1105). *Programs and Abstracts of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, 2000*. American Society for Microbiology: Washington DC: American Society for Microbiology. p 371.

Fridkin SK, Jarvis WR. (1996) Epidemiology of nosocomial fungal infections. *Clin Microbiol Rev*. 9:499-511.

Harousseau JL, Dekker AW, Stamatoullas-Bastad, Fassas A, Linkesch W, Gouvela J, De Bock R, Rovira M, Seifert WF, Joosen H, Peeters M, De Beule K. (2000) Itraconazole oral solutions for primary prophylaxis of fungal infections in patients with hematological malignancy and profound neutropenia: a randomized, double-blind, double-placebo, multicenter trial comparing itraconazole and amphotericin B. *Antimicrob Agents Chemoter*. 44:1887-1893.

Kao AS, Brandt ME, Pruitt WR, et al. (1999) The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin Infect Dis*. 29:1164-1170.

Krishnarao TV, Galgiani JN. (1997) Comparison of the in vitro activities of the echinocandin LY303366, the pneumocandin MK-0991, and fluconazole against *Candida* species and *Cryptococcus neoformans*. *Antimicrob Agents Chemoter*. 41:1957-1960.

Levin AS, Costa SF, Mussi NS, Basso M, Sinto SI, Machado C, Geiger DC, Villres MCB; Schreiber AZ, Baraone AA, Branchini MLM. (1998) *Candida parapsilosis* fungemia associated with implantable and semi-implantable central venous catheters and hands of health care workers. *Diagn Microbiol Infect Dis* 30:243-249.

McNeil M, Nash SL, Hajjeh R, et al (2001) Trends in mortality due to invasive myotic diseases in the United States, 1980-1997. *Clin Infect Dis* 33:641-647.

Marco F, Pfaller MA, Messer S, Jones RN. (1998a) In vitro activities of voriconazole (UK-109,496) and four other antifungal agents against 394 clinical isolates of *Candida* spp. *Antimicrob Agents Chemother* 42:161-163.

Marco F, Pfaller MA, Messer S, Jones RN. (1998b) Activity of MK-0991 (L-743,872), a new echinocandin, compared with those of LY303366 and four other antifungal agents tested against blood stream isolates of *Candida* spp. *Diagn Microbiol Infect Dis* 31:33-37.

National Committee for Clinical Laboratory Standards (NCCLS) (1997) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast: Approved Standard M27-A*. Wayne, Pa:NCCLS.

Nguyen MH, Peacock Jr JE, Morris AJ, Tanner DC, Nguyen ML, Snyderman DR, Wagener MM, Rinaldi MG, Yu VL. (1996) The changing face of candidemia: emergence of non-*Candida albicans* and antifungal resistance. *Am J Med* 100:617-623.

Perfect JR, Schell WA. (1996) The new fungal opportunists are coming. *Clin Infect Dis*. 22(Suppl 2):112-118.

Pfaller MA, Messer SA, Coffman S (1997) In vitro susceptibilities of clinical yeast isolates to a new echinocandin derivative, LY303366, and other antifungal agents. *Antimicrob Agents Chemother* 41:763-766.

Pfaller MA, Messer SA, Hollis RJ, Jones RN, Doern GV, Brandt ME, Hajjeh RA (1998) In vitro susceptibilities of *Candida* bloodstream isolates to the new triazole antifungal agents BMS-207147, Sch 56592, and voriconazole. *Antimicrob Agents Chemother* 42:3242-3244.

Pfaller MA, Jones RN, Doern GV, Fluit AC, Verhoef J, Sader HS, Messer SA, Houston A, Coffman S, Hollis RJ for the SENTRY participant group (Europe) (1999) International surveillance of bloodstream infections due to *Candida* species in the European SENTRY program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. *Diagn Microbiol Infect Dis* 35:19-25.

Pfaller MA, Diekema DJ, Jones RN, Sader HS, Fluit AC, Hollis RJ, Messer SA, and the SENTRY participant group (2001) International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program. *J Clin Microbiol*. 39:3254-3259.

Rees J, Pinner R, Hajjeh R, Brandt M, Reingold A. (1999) The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992-1993; results of the population-based laboratory active surveillance. *Clin Infect Dis* 27:1138-1147.

Sheehan DJ, Hitchcock CA, Sibley CM. (1999) Current and emerging azole antifungal agents. *Clin Microbiol Rev* 12:40-79.

Tawara S, Ikeda F, Maki K, Morishita Y, Otomo K, Teratani N, Goto T, Tomishima M, Ohki H, Yamada A, Kawabata K, Takasugi H, Sakane K, Tanaka H, Matsumoto F, Kuwahara S. (2000) In vitro activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. *Antimicrob Agents Chemother.* 44:57-62.

Walsh TJ, Hiemenz J, Pizzo PA. (1994) Evolving risk factors for invasive fungal infections: all neutropenic patients are not the same. *Clin Infect Dis* 18:793-798.

Wingard JR, Merz WG, Rinaldi MG, Johnson TR, Karp JE, Saral R. (1991) Increase in *Candida krusei* infection among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. *N Engl J Med* 325:1274-1277.

Wingard JR, Merz WG, Rinaldi MG et al, (1993) Association of *Torulopsis glabrata* infections with fluconazole prophylaxis in neutropenic bone marrow transplant patients. *Antimicrob Agents Chemother.* 37:1847-1849.

Article 4.

Antifungal activity of a new triazole, voriconazole (UK-109,496), compared with three other antifungal agents tested against clinical isolates of filamentous fungi. *Med Mycology*. 1998;36:433-436. (FI: 1,494 / 2000)

Short communication

Antifungal activity of a new triazole, voriconazole (UK-109,496), compared with three other antifungal agents tested against clinical isolates of filamentous fungi

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Voriconazole is a new triazole antifungal agent with potent activity against yeast and moulds. We investigated the *in vitro* activity of voriconazole, itraconazole, amphotericin B and 5-flucytosine against 51 clinical isolates of filamentous fungi. Overall, voriconazole was active (MIC_{50} , 0.5 mg l^{-1} and MIC_{90} , 8 mg l^{-1}) against these mould isolates. Voriconazole was most active against *P. boydii* (MIC_{50} , 0.12 mg l^{-1}) and *Aspergillus* spp. (MIC_{90} , 0.5 mg l^{-1}) and least active against *Fusarium* spp. (MIC_{90} , 8 mg l^{-1}) and *Rhizopus* spp. (MIC_{50} , 8 mg l^{-1}). Voriconazole was more active than amphotericin B against *Aspergillus* spp. and *P. boydii*. By comparison with itraconazole, voriconazole was more active against all isolates except *Rhizopus* spp. Based on these results, voriconazole has promising activity against commonly encountered isolates of filamentous fungi and its clinical usefulness should be established by further studies.

Keywords triazole, voriconazole, moulds

Introduction

Voriconazole (UK-109,496) is a novel wide-spectrum triazole which has potent *in vitro* activity against *Candida* spp. and *Aspergillus* spp. [1-3]. Pharmacokinetic studies have shown a good bioavailability after oral or intravenous administration and reduced toxicity [4]. Early clinical studies have documented its promising usefulness in the treatment of oropharyngeal candidosis and invasive aspergillosis [5,6]. Voriconazole has also been found to be effective in experimental models of pulmonary aspergillosis and in prevention and treatment of *Aspergillus fumigatus* endocarditis in guinea pigs [7,8].

In this report we describe the *in vitro* activity of voriconazole tested against several filamentous fungi isolated from recent clinical infections. The comparative antifungal drugs tested were itraconazole, amphotericin B and flucytosine. The *in vitro* susceptibility testing was performed using the broth microdilution version of the NCCLS reference method described in the M27-A document [9,10].

Materials and methods

The following 51 recent clinical isolates were included in the study: *A. fumigatus* (12 isolates), *A. flavus* (10 isolates), *Fusarium oxysporum* (five isolates), *F. solani* (five isolates), *Fusarium* spp. (three isolates), *Pseudallescheria boydii* (five isolates) *Rhizopus* spp. (six isolates) and one isolate each of *A. niger*, *A. terreus*, *Acremonium* spp., *Paecilomyces* spp. and *Trichoderma* spp. The isolates were obtained from a variety of respiratory sources including sputum, bronchoscopy and tissue biopsy specimens. All isolates were stored as conidia or spore suspensions in sterile distilled water at room temperature until they were used in the study.

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Table 1 *In vitro* susceptibilities of 51 clinical filamentous fungi to voriconazole and other antifungal agents

Organism (no. of isolates)	Antifungal drug	MIC (mg/l)		
		Range	50%	90%
<i>Acremonium</i> spp. (1)	Voriconazole	0.25	—	—
	Itraconazole	1	—	—
	Amphotericin B	2	—	—
	Flucytosine	>128	—	—
<i>Aspergillus flavus</i> (10)	Voriconazole	0.12–0.5	0.25	0.5
	Itraconazole	0.25–1	0.5	1
	Amphotericin B	1–2	2	2
	Flucytosine	32–>128	>128	>128
<i>Aspergillus fumigatus</i> (12)	Voriconazole	0.25–0.5	0.25	0.5
	Itraconazole	0.5–1	1	1
	Amphotericin B	1–2	2	2
	Flucytosine	128–>128	>128	>128
<i>Aspergillus</i> spp. (2)*	Voriconazole	0.25–0.5	—	—
	Itraconazole	0.5–1	—	—
	Amphotericin B	2	—	—
	Flucytosine	>128	—	—
<i>Fusarium</i> spp. (13)†	Voriconazole	2–8	2	8
	Itraconazole	4–>16	>16	>16
	Amphotericin B	1–2	2	2
	Flucytosine	>128	>128	>128
<i>Paecilomyces</i> spp. (1)	Voriconazole	0.5	—	—
	Itraconazole	4	—	—
	Amphotericin B	4	—	—
	Flucytosine	>128	—	—
<i>Pseudallescheria boydii</i> (5)	Voriconazole	0.12–0.25	0.12	—
	Itraconazole	1	1	—
	Amphotericin B	2–4	4	—
	Flucytosine	128	>128	—
<i>Rhizopus</i> spp. (6)	Voriconazole	8–32	8	—
	Itraconazole	0.5–2	1	—
	Amphotericin B	1–2	1	—
	Flucytosine	>128	>128	—
<i>Trichoderma</i> spp. (1)	Voriconazole	0.25	—	—
	Itraconazole	2	—	—
	Amphotericin B	1	—	—
	Flucytosine	>128	—	—

*Includes one isolate each of *Aspergillus niger* and *A. terreus*.

†Includes *Fusarium oxysporum* (five isolates), *F. solani* (five isolates) and *Fusarium* spp. (three isolates).

Before testing, each isolate was subcultured at least twice on potato dextrose agar slants (Remel, Lenexa, KS, USA) to ensure its viability and purity.

The four antifungal drugs used in this study were provided as standard powders of known potency. Voriconazole was obtained from Pfizer Inc., Central Research Division (Groton, CT, USA). Itraconazole (Janssen, Beerse, Belgium), amphotericin B (Sigma, St Louis, MO, USA) and flucytosine (Sigma) were supplied by their respective manufacturers. Stock solutions were prepared in polyethylene glycol (voriconazole, itraconazole), dimethyl sulphoxide (amphotericin B)

and water (flucytosine). All drugs were diluted in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS) buffer (Sigma) and dispensed into 96-well microdilution trays. The final ranges of tested drug concentrations were as follows: voriconazole from 0.015 to 16 mg l⁻¹; itraconazole from 0.008 to 8 mg l⁻¹; amphotericin B from 0.015 to 8 mg l⁻¹ and flucytosine from 0.06 to 128 mg l⁻¹.

MICs were determined by a broth microdilution method using the NCCLS guidelines [9,10]. A spectrophotometric method previously described was used

for the inoculum preparation [10]. The turbidity of conidial suspensions was adjusted to obtain a final inoculum of 0.4×10^4 – 5×10^4 cfu/ml. The trays were incubated at 35°C and read at 48 h and 72 h. Only wells that showed no growth (optically clear) or approximately 75% reduction in growth compared with drug-free controls were recorded as the MIC [10].

The two QC strains recommended in the NCCLS document M27-A [9], *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were included each time any testing was performed. The MIC limits for each antifungal agent and the two QC strains are as follows: amphotericin B, 0.25–1 µg ml⁻¹ (*C. parapsilosis*) and 0.5–2.0 µg ml⁻¹ (*C. krusei*); fluconazole, 2.0–8.0 µg ml⁻¹ (*C. parapsilosis*) and 16–64 µg ml⁻¹ (*C. krusei*); itraconazole, 0.06–0.25 µg ml⁻¹ (*C. parapsilosis*) and 0.12–0.5 µg ml⁻¹ (*C. krusei*); flucytosine, 0.12–0.5 µg ml⁻¹ (*C. parapsilosis*) and 4.0–16 µg ml⁻¹ (*C. krusei*).

Results

The antifungal activities of voriconazole, itraconazole, amphotericin B and flucytosine at 72 h are summarized in Table 1. Overall, voriconazole was active (MIC₅₀, 0.5 mg l⁻¹ and MIC₉₀, 8 mg l⁻¹) against these mould isolates. Voriconazole showed greater activity against *P. boydii* (MIC₅₀, 0.12 mg l⁻¹) and *Aspergillus* spp. (MIC₅₀, 0.5 mg l⁻¹) than against *Fusarium* spp. (MIC₅₀, 8 mg l⁻¹) and *Rhizopus* spp. (MIC₅₀, 8 mg l⁻¹). By comparison with itraconazole, voriconazole was two- to eightfold more active against all isolates except *Rhizopus* spp. Itraconazole was eightfold more active than voriconazole against isolates of this genera. Voriconazole was four- to eightfold more active than amphotericin B against *A. fumigatus*, *A. flavus* and *P. boydii* and was 32- to >2000-fold more active than flucytosine against all species tested.

Discussion

Following the publication of standardized procedures for testing of yeast [9], the NCCLS subcommittee on antifungal susceptibility testing focused its efforts toward the development of standard guidelines for the antifungal susceptibility testing of filamentous fungi [10]. Using these procedures, Espinel-Ingróff [2] and McGinnis *et al.* [11] have documented a potent *in vitro* activity of voriconazole against *Aspergillus* spp. and *P. boydii*. Similarly, Radford *et al.* [12] have shown good activity against a broad array of emerging and less common mould pathogens. We have also observed that voriconazole was highly active against *P. boydii* (MIC

range 0.12–0.25 mg l⁻¹) and *Aspergillus* spp. (MIC range 0.12–0.5 mg l⁻¹). These promising *in vitro* data seem to be corroborated with encouraging *in vivo* results from early clinical studies and several experimental models [6–8]. Our findings for voriconazole activity against *Fusarium* spp. and *Rhizopus* spp. generally agreed with the values reported by others [2,12].

We also observed that the guidelines suggested by the NCCLS subcommittee on antifungal susceptibility testing [10] appear suitable for testing filamentous fungi and that RPMI 1640 medium sustained adequate growth of all isolates tested at 48 h (except one *Trichoderma* isolate that needed 72 h). Overall, voriconazole MICs at 72 h remained the same in 34 isolates or increased by 1 log₂ dilution (16 isolates) when compared to MICs at 48 h (data not shown). Itraconazole MICs showed similar changes to those of voriconazole. MICs of amphotericin B remained unchanged in 41 isolates, increased by 1 log₂ dilution in eight isolates or by 2 log₂ dilution in one isolate.

Finally, these findings should be interpreted cautiously as with any other *in vitro* susceptibility testing results. Although preliminary pharmacokinetic data suggest that serum levels of voriconazole are expected to be higher than those of itraconazole [3–6], the clinical significance of the potency of voriconazole against filamentous fungi awaits the results of comparative clinical trials.

Acknowledgements

Francisc Marco is partially supported by a grant from Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC-Hoescht96) and a Permiso de Ampliación de Estudios from Hospital Clínic, Barcelona, Spain. This study was also supported by grant from Pfizer Pharmaceuticals-Roerig Division.

References

- 1 Barry AL, Brown SD. *In vitro* studies of two antifungal agents (voriconazole [UK-109,496] and fluconazole) against *Candida* species. *Antimicrob Agents Chemother* 1996; 40: 1948–9.
- 2 Espinel-Ingróff A. *In vitro* activity of the new triazole voriconazole (UK-109,496) against opportunistic and dimorphic fungi and common and emerging yeast pathogens. *J Clin Microbiol* 1998; 36: 198–202.
- 3 Marco F, Pfaller MA, Messer S, Jones RN. *In vitro* activities of voriconazole (UK-109,496) and four other antifungal agents against 394 clinical isolates of *Candida* spp. *Antimicrob Agents Chemother* 1998; 42: 161–3.
- 4 Patterson BE, Coates PE. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: pharmacokinetics in man. In: *Program and Abstracts of the Thirty-sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco CA*, Abstract F78. Washington DC: American Society for Microbiology, 1995: 126.

- 5 Ruhnke M, Schmidt-Westhausen A, Trautmann M. *In vitro* activities of voriconazole (UK-109,496) against fluconazole-susceptible and -resistant *Candida albicans* isolates from oral cavities of patients with human immunodeficiency virus infection. *Antimicrob Agents Chemother* 1997; 41: 575-7.
- 6 Dupont B, Denning D, Lode H, Yonren S, Troke PF, Sarantis N. UK-109,496, a novel, wide-spectrum triazole derivative for the treatment of fungal infections: clinical efficacy in chronic invasive aspergillosis. In: *Program and Abstracts of the Thirty-sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco CA*, Abstract F81. Washington DC: American Society for Microbiology, 1995: 127.
- 7 Murphy M, Bernard EM, Ishimaru T, Armstrong D. Activity of voriconazole (UK-109,496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. *Antimicrob Agents Chemother* 1997; 41: 696-8.
- 8 Martin MV, Yates J, Hitchcock CA. Comparison of voriconazole (UK-109,496) and itraconazole in prevention and treatment of *Aspergillus fumigatus* endocarditis in guinea pigs. *Antimicrob Agents Chemother* 1997; 41: 13-16.
- 9 National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast. Approved Standard M27-A. Wayne, PA: NCCLS, 1997.
- 10 Espinel-Ingroff A, Bartlett M, Bowden R, et al. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *J Clin Microbiol* 1997; 35: 139-43.
- 11 McGinnis MR, Pasarell L, Sutton DA, Fothergill AW, Cooper Jr CR, Rinaldi MG. *In vitro* evaluation of voriconazole against some clinically important fungi. *Antimicrob Agents Chemother* 1997; 41: 1832-4.
- 12 Radford SA, Johnson EM, Warnock DW. *In vitro* studies of activity of voriconazole (UK-109,496), a new triazole antifungal agent, against emerging and less-common mold pathogens. *Antimicrob Agents Chemother* 1997; 41: 841-3.

Article 5.

In vitro activity of a new triazole antifungal agent, Sch 56592, against clinical isolates of filamentous fungi. *Mycopathologia*. 1998;141:73-77. (FI: 0.267 / 2000)



***In vitro* activity of a new triazole antifungal agent, Sch 56592, against clinical isolates of filamentous fungi**

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Received 1 April 1998; accepted in final form 5 May 1998

Abstract

Sch 56592 is a new triazole derivative that possesses potent, broad-spectrum antifungal activity. We evaluated the *in vitro* activity of Sch 56592 compared with that of itraconazole, amphotericin B and 5-fluorocytosine against 51 clinical isolates of filamentous fungi, including *Aspergillus flavus* (10), *A. fumigatus* (12), *Fusarium* spp. (13), *Rhizopus* spp. (6), *Pseudallescheria boydii* (5), and one isolate each of *Acremonium* spp., *A. niger*, *A. terreus*, *Paecilomyces* spp., and *Trichoderma* spp. *In vitro* susceptibility testing was performed using the microdilution broth method outlined in the NCCLS 27-A document. Sch 56592 was highly active against *A. flavus* (MIC₉₀, 0.25 µg/ml), *A. fumigatus* (MIC₉₀, 0.12 µg/ml), *P. boydii* (MIC₅₀, 1 µ/ml) and *Rhizopus* spp (MIC₅₀, 1 µg/ml). By comparison with itraconazole, Sch 56592 was four- to eight-fold more active against isolates of *Aspergillus* and both compounds showed equipotent *in vitro* activity against *P. boydii* and *Rhizopus* spp. Sch 56592 was four- to 16-fold more active than amphotericin B against *Aspergillus* spp. and *P. boydii* and both antifungal drugs displayed similar activity against *Rhizopus* spp. Overall, Sch 56592 showed good *in vitro* activity against all isolates tested (MIC, ≤ 2 µg/ml) except isolates of *Fusarium* (MIC range, 1-→4 µg/ml). On the basis of these data Sch 56592 has promising activity against *Aspergillus* spp. and other species of filamentous fungi that are likely to be encountered clinically. Additional *in vitro* and *in vivo* studies are warranted.

Introduction

In the last few years, opportunistic fungal infections have increased in frequency as a result of the increasing numbers of immunocompromised patients due to cancer chemotherapy, organ transplantation or AIDS. Among the opportunistic mycoses, invasive disease caused by filamentous fungi is commonly associated with a high morbidity and mortality [1]. Of the currently available antifungal agents, only amphotericin B, and possibly itraconazole, have documented efficacy in the treatment of these infections. Because none of these agents is ideal and toxicity or antifungal resistance could develop during treatment, newer antifungal compounds are clearly required.

Sch 56592 is a novel triazole analog of Sch 51048 [2] with a broad spectrum of potent activity against

a wide range of fungi. Pfaller et al. [3] found Sch 56592 to be very active against clinical isolates of *Candida* spp and *Saccharomyces cerevisiae*. Sch 56592 has also been reported to have good *in vitro* and *in vivo* activity against *Cryptococcus neoformans* [4, 5] and *Aspergillus* spp. [6, 7]. Recent pharmacokinetic studies in healthy volunteers have demonstrated that Sch 56592 is well tolerated and the mean elimination half-life is between 19 h and 30 h depending on the dose administered [8]. This study also found that serum levels were well above the MIC for most fungal pathogens.

In this report we describe the *in vitro* activity of Sch 56592 tested against a spectrum of filamentous fungi that are likely to be encountered clinically and compare it with those of itraconazole, amphotericin B and 5-fluorocytosine. The *in vitro* susceptibility testing was performed using the broth microdilution version

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of the NCCLS reference method recently described in the M27-A document [9].

Materials and methods

Organisms The 51 organisms tested in this study were recent clinical strains isolated at the University of Iowa Hospitals and Clinics. The distribution of species included were as follows: *Aspergillus fumigatus* (12 isolates), *Aspergillus flavus* (10 isolates), *Fusarium oxysporum* (5 isolates), *Fusarium solani* (5 isolates), *Fusarium* spp. (3 isolates), *Pseudallescheria boydii* (5 isolates), *Rhizopus* spp. (6 isolates), and one isolate each of *Aspergillus niger*, *Aspergillus terreus*, *Acremonium* spp., *Paecilomyces* spp. and *Trichoderma* spp. All isolates were stored as spore suspensions in sterile distilled water at room temperature until they were used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar slants (Remel, Lenexa, KS) to ensure its viability and purity.

Antifungal drugs. All antifungal drugs used in this study were provided as standard powders of known potency. Sch 56592 was obtained from Schering-Plough Research Institute (Kenilworth, NJ). Amphotericin B, 5-fluorocytosine and itraconazole were supplied by their respective manufacturers. Stock solutions were prepared in polyethylene glycol (Sch 56592 and itraconazole), dimethyl sulfoxide (amphotericin B) or water (5-fluorocytosine). All drugs were diluted in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and dispensed into 96-well microdilution trays. The recommendations stated in the NCCLS document M27-A were followed for the dilution of each antifungal agent. Trays containing an aliquot of 0.1 ml in each well of appropriate drug solution (2× final concentration) were sealed and stored at -70°C until they were used. The final ranges of drug concentrations tested were as follows: Sch 56592 from 0.003 to 4 µg/ml; itraconazole from 0.008 to 8 µg/ml; amphotericin B from 0.015 to 8 µg/ml and 5-fluorocytosine from 0.06 to 128 µg/ml.

Susceptibility testing MICs were determined by a broth microdilution method using the NCCLS guidelines [9]. A spectrophotometric method recommended by the NCCLS subcommittee on antifungal susceptibility testing [10] was used for the inoculum prepa-

ration. Briefly, each isolate was grown on potato dextrose agar slants at 35°C for a period of 7 days. The fungal colonies were then covered with 1 ml of sterile 0.85% saline and gently scraped with a sterile pipette. The resulting suspensions were transferred to sterile tubes and heavy particles were allowed to settle. The turbidity of the conidial suspensions was measured at 530 nm and was adjusted to obtain a final inoculum of 0.4×10^4 cfu/ml. To determine the final inoculum, appropriate dilution was performed and an aliquot (0.01 ml) was plated on potato dextrose agar (Remel, Lenexa, KS). Plates were incubated at 30°C and were examined daily for the presence of fungal colonies. The microdilution trays were incubated at 35°C and MICs were read at 24 h, 48 h and 72 h. Drug-free controls were included in each tray. Following incubation, MIC endpoints were interpreted with the aid of a reading mirror. Only wells that showed no growth (optically clear) or approximately 75% reduction in growth compared with drug free controls were recorded as the MIC.

Quality control Quality control was ensured by testing the following strains recommended in the NCCLS document M27-A: *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258. For these particular strains an inoculum concentration of $0.5-2.5 \times 10^3$ cfu/ml prepared from a 24 h old culture was used.

Results and discussion

The antifungal activities of Sch 56592, itraconazole, amphotericin B and 5-fluorocytosine against 51 clinical mould isolates (72 h incubation) are shown in Table 1. Sch 56592 was highly active against *A. flavus* (MIC₉₀, 0.25 µg/ml), *A. fumigatus* (MIC₉₀, 0.12 µg/ml), *P. boydii* (MIC₅₀, 1 µg/ml) and *Rhizopus* spp (MIC₅₀, 1 µg/ml). By comparison with itraconazole, Sch 56592 was four- to eight-fold more active against isolates of *Aspergillus*. Both compounds showed equipotent *in vitro* activity against *P. boydii* and *Rhizopus* spp. Sch 56592 was four- to 16-fold more active than amphotericin B against *Aspergillus* spp and *P. boydii* and both antifungal drugs exhibited similar *in vitro* activity against *Rhizopus* spp. Sch 56592 was also active (MIC, 1 µg/ml) against *Acremonium* spp. (one isolate), *Paecilomyces* spp. (one isolate) and *Trichoderma* spp. (one isolate). Overall, Sch 56592 displayed good *in vitro* activity against all filamentous fungi tested

Table 1. *In vitro* susceptibilities of 51 clinical filamentous fungi to Sch 56592 and other antifungal agents

Organism (No. of isolates)	Antifungal drug	MIC ($\mu\text{g/ml}$)		
		Range	50%	90%
<i>Acremonium</i> sp. (1)	Sch 56592	1	-	-
	Itraconazole	1	-	-
	Amphotericin B	2	-	-
	5-fluorocytosine	>128	-	-
<i>Aspergillus flavus</i> (10)	Sch 56592	0.06-0.25	0.12	0.25
	Itraconazole	0.25-1	0.5	1
	Amphotericin B	1-2	2	2
	5-fluorocytosine	32->128	>128	>128
<i>Aspergillus fumigatus</i> (12)	Sch 56592	0.12-0.25	0.12	0.12
	Itraconazole	0.5-1	1	1
	Amphotericin B	1-2	2	2
	5-fluorocytosine	128->128	>128	>128
<i>Aspergillus</i> spp. (2) ^a	Sch 56592	0.12	-	-
	Itraconazole	0.5-1	-	-
	Amphotericin B	2	-	-
	5-fluorocytosine	>128	-	-
<i>Fusarium</i> spp (13) ^b	Sch 56592	1->4	2	>4
	Itraconazole	4->8	>8	>8
	Amphotericin B	1-2	2	2
	5-fluorocytosine	>128	>128	>128
<i>Paecilomyces</i> sp. (1)	Sch 56592	1	-	-
	Itraconazole	4	-	-
	Amphotericin B	4	-	-
	5-fluorocytosine	>128	-	-
<i>Pseudallescheria boydii</i> (5)	Sch 56592	1	1	-
	Itraconazole	1	1	-
	Amphotericin B	2-4	4	-
	5-fluorocytosine	>128	>128	-
<i>Rhizopus</i> spp. (6)	Sch 56592	0.5-2	1	-
	Itraconazole	0.5-2	1	-
	Amphotericin B	1-2	1	-
	5-fluorocytosine	>128	>128	-
<i>Trichoderma</i> sp. (1)	Sch 56592	1	-	-
	Itraconazole	2	-	-
	Amphotericin B	1	-	-
	5-fluorocytosine	>128	-	-
All organisms (51)	Sch 56592	0.06->4	0.5	>4
	Itraconazole	0.25->8	1	>8
	Amphotericin B	1-4	2	4
	5-fluorocytosine	32->128	>128	>128

^aIncludes one isolate each of *Aspergillus niger* and *Aspergillus terreus*.

^bIncludes *Fusarium oxysporum* (five isolates), *Fusarium solani* (five isolates) and *Fusarium* spp. (three isolates).

(MIC, $\leq 2 \mu\text{g/ml}$) with the exception of *Fusarium* (MIC₉₀, $> 4 \mu\text{g/ml}$).

The potent *in vitro* activity found in this study against isolates of *Aspergillus* agrees with the results reported by Espinel-Ingroff [11] and Oakley et al. [7]. This *in vitro* activity seems to correlate with *in vivo* efficacy observed in animal models of invasive and pulmonary aspergillosis [6, 12]. In a neutropenic murine model of invasive aspergillosis, Sch 56592 was highly effective and a correlation between MICs of Sch 56592 and quantitative organ culture results was observed [12]. Although Sch 56592 prolonged survival when an itraconazole-resistant strain of *Aspergillus fumigatus* was used in the model, the number of CFU/organs recovered was 100-fold higher than that recovered when the challenge isolate was itraconazole-susceptible. This *in vivo* result suggests the possibility of cross-resistance between Sch 56592 and itraconazole. Cross-resistance to Sch 56592 has also been observed among fluconazole- and itraconazole-resistant isolates of *Candida* [3].

Sch 56592 was active against five isolates of *Pseudallescheria boydii* ($1 \mu\text{g/ml}$) and six isolates of *Rhizopus* spp (MIC range, $0.5 \rightarrow 2 \mu\text{g/ml}$). Using the same methodology, Espinel-Ingroff [11] recently reported higher MICs against *Rhizopus arrhizus* (MIC range, $2 \rightarrow 16 \mu\text{g/ml}$). The low number of strains tested in both studies could probably explain this apparent discrepancy.

The methodology published by the NCCLS subcommittee on antifungal susceptibility testing [10] appears suitable for testing filamentous fungi. The buffered RPMI 1640 medium recommended in these guidelines sustained adequate growth of all isolates tested at 48 h (except one *Trichoderma* isolate that needed 72 h). Overall, few variations were observed when Sch 56592 MICs at 48 h and at 72 h were compared (data not shown). The MIC values for six isolates increased by one log₂ dilution (five *Aspergillus* spp, and one *Fusarium* spp) and one (*P. boydii*) by two log₂ dilutions.

In conclusion, our data confirm and extend the promising activity of Sch 56592 against *Aspergillus* spp., *P. boydii*, *Rhizopus* spp. and other species of filamentous fungi that are likely to be encountered clinically. Based on these findings, the favorable results from animal models and recent pharmacokinetic studies in healthy volunteers, Sch 56592 warrants further clinical evaluation.

Acknowledgments

Francesc Marco is partially supported by a grant from Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC-Hoechst96) and a Permiso de Ampliación de Estudios from Hospital Clínic, Barcelona, Spain.

References

- Groll AH, Shah PM, Mentzel C, Schneider M, Just-Nuebling G, Huebner K. Trends in the post-mortem epidemiology of invasive fungal infections at a university hospital. *J Infect* 1996; 33: 23-32.
- Saksena AK, Girijavallabhan VM, Lovey RO, Bennet F, Pike RB, Wang H, Pinto P, Liu YT, Patel N, Ganguly AK. Novel analogs of Sch 51048: synthesis and preliminary structure activity relationships. Abstract F83, 1995; 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA.
- Pfaller MA, Messer SA, Jones RN. Activity of a new triazole, Sch 56592, compared with those of four other antifungal agents tested against clinical isolates of *Candida* spp. and *Saccharomyces cerevisiae*. *Antimicrob Agents Chemother* 1997; 41: 233-235.
- Galgiani JH, Lewis ML. In vitro studies of activities of the antifungal triazoles Sch 56592 and itraconazole against *Candida albicans*, *Cryptococcus neoformans*, and other pathogenic yeasts. *Antimicrob Agents Chemother* 1997; 41: 180-183.
- Perfect JR, Cox GM, Dodge RK, Schell WA. In vitro and in vivo efficacies of the azole Sch 56592 against *Cryptococcus neoformans*. *Antimicrob Agents Chemother* 1996; 40: 1910-1913.
- Moss Jr EL, Menzel Jr F, Norris C, Michalski M, Cacciapuoti AF, Nomeir A, Loebenberg D, Hare RS, Miller GH. Efficacy of Sch 56592 against fungal infections in guinea pigs. Abstract B 17, 1997; 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada.
- Oakley KL, Moore CB, Denning DW. In vitro activity of Sch 56592 and comparison with activities of amphotericin B and itraconazole against *Aspergillus* spp. *Antimicrob Agents Chemother* 1997; 41: 1124-1126.
- Laughlin M, Pai S, Menon S, Nomeir A, Colucci R, Affrime M, Kosoglou T. Rising multiple-dose safety, tolerance, and pharmacokinetic evaluation in healthy volunteers. Abstract A87, 1997; 37th Interscience Conference on Antimicrobial Agents and Chemotherapy Toronto, Ontario, Canada.
- National Committee for Clinical Laboratory Standards (NCCLS) Reference method for broth dilution antifungal susceptibility testing of yeast. Approved standard M27-A. Wayne, PA: NCCLS, 1997.
- Espinel-Ingroff A, Bartlett M, Bowden R, Chin NX, Cooper C, Fothergill A, McGinnis MR, Menezes P, Messer SA, Nelson PW, Odds FC, Pasarell L, Peter J, Pfaller MA, Rex JH, Rinaldi MG, Shankland GS, Walsh TJ, Weitzman I. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *J Clin Microbiology* 1997; 35: 139-143.
- Espinel-Ingroff A. Evaluation of the in vitro activity of three new antifungal agents against opportunistic filamentous and

dimorphic fungi. Abstract F68, 1997; 37th Interscience Conference on Antimicrobial Agents and Chemotherapy Toronto, Ontario, Canada.

Oakley KL, Morrissey G, Denning DW. Efficacy of Sch 56592 in a temporarily neutropenic murine model of invasive aspergillosis with an itraconazole-susceptible and an itraconazole-resistant isolate of *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 1997; 41: 1504-1507.

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Article 6.

In vitro activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus*, *Fusarium*, *Rhizopus*, and other filamentous fungi. *Diag Microbiol Infect Dis.* 1998;30:251-255 (FI: 1,932 / 2000)

In Vitro Activity of Two Echinocandin Derivatives, LY303366 and MK-0991 (L-743,792), Against Clinical Isolates of *Aspergillus*, *Fusarium*, *Rhizopus*, and Other Filamentous Fungi

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R. N. Jones

LY303366 and MK-0991 (previously L-743,792) are new echinocandin derivatives with excellent broad-spectrum antifungal activity. We investigated the in vitro activity of LY303366, MK-0991, itraconazole, amphotericin B, and 5-flucytosine against 51 clinical isolates of filamentous fungi, including *Aspergillus flavus* (10), *A. fumigatus* (12), *Fusarium* spp. (13), *Rhizopus* spp. (6), *Pseudallescheria boydii* (5), and one isolate each of *Acremonium* spp., *A. niger*, *A. terreus*, *Paecilomyces* spp., and *Trichoderma* spp. In vitro susceptibility testing was performed using a microdilution broth method performed according to National Committee for Clinical Laboratory Standards guidelines. LY303366 was two- to fourfold more active than MK-0991 against *A. flavus*, *A. fu-*

migatus, and *Trichoderma* spp. Both LY303366 and MK-0991 were considerably more active (MIC₉₀ of 0.03–0.12 µg/mL) than itraconazole, amphotericin B, and 5-flucytosine against *Aspergillus* spp., but were less active than itraconazole and amphotericin B against *Rhizopus* spp. MK-0991 was more active than either LY303366 or itraconazole against *Acremonium* spp., *Paecilomyces* spp., and *P. boydii*. These data demonstrate promising activity of both LY303366 and MK-0991 against *Aspergillus* spp. and other species of filamentous fungi that are likely to be encountered clinically. Further in vitro and in vivo investigation is indicated. © 1998 Elsevier Science Inc.

INTRODUCTION

Echinocandins are antifungal cyclic lipopeptides that inhibit the biosynthesis of the fungal cell wall. The mechanism of action of these agents is thought to involve noncompetitive inhibition of the enzyme β -(1,3)-glucan-synthase, which results in a weakened cell wall and lysis (Debono and Gordee 1994). In the last few years, several semisynthetic amine deriva-

tives of the natural product pneumocandin Bo have been developed (Abruzzo et al. 1995). These antifungal drugs have been shown to have potent in vitro and in vivo activity against isolates of *Candida* and *Aspergillus* (Abruzzo et al. 1995; Bartizal et al. 1995). LY303366 and MK-0991 (L-743,792), a water soluble compound, are new echinocandin derivatives with potent in vitro and in vivo activity against *Candida* spp. (Graybill et al. 1997; Pfaller et al. 1997; Vazquez et al. 1997; Zeckner et al. 1993b), *Aspergillus fumigatus* (Abruzzo et al. 1997; Zeckner et al. 1993a), *Histoplasma capsulatum* (Fothergill et al. 1996; Najvar et al. 1996; Zeckner et al. 1993a), and *Pneumocystis carinii* (Current et al. 1993; Powles et al. 1993). Although their activity against several molds has been evaluated, the number of clinical isolates of filamentous fungi included in these studies is limited, and there is

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Received 11 November 1997; revised and accepted 10 December 1997.

a lack of comparative data with other antifungal agents.

In this study, we describe the *in vitro* activity of LY303366 and MK-0991 tested against a broad spectrum of filamentous fungi isolated from recent clinical infections. The comparative antifungal drugs tested were itraconazole, amphotericin B, and 5-flucytosine. The *in vitro* susceptibility testing was performed using the broth microdilution version of the National Committee for Clinical Laboratory Standards (NCCLS) reference method recently described in the M27-A document (NCCLS 1997).

MATERIALS AND METHODS

Organisms

A total of 51 recent clinical isolates were selected for testing. The collection included the following isolates: *A. fumigatus* (12 isolates), *A. flavus* (10 isolates), *Fusarium oxysporum* (5 isolates), *F. solani* (5 isolates), *Fusarium* spp. (3 isolates), *Pseudallescheria boydii* (5 isolates), *Rhizopus* spp. (6 isolates), and one isolate each of *A. niger*, *A. terreus*, *Acremonium* spp., *Paecilomyces* spp., and *Trichoderma* spp. All isolates were stored as spore suspensions in sterile distilled water at room temperature until they were used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar slants (Remel, Lenexa, KS) to ensure its viability and purity.

Antifungal Drugs

The five antifungal drugs used in this study were provided as standard powders of known potency. LY303366 and MK-0991 were supplied by Eli Lilly and Company (Indianapolis, IN) and Merck Research Laboratories (Rahway, NJ), respectively. Amphotericin B, 5-flucytosine, and itraconazole were obtained from their respective manufacturers. Stock solutions were prepared in dimethyl sulfoxide (amphotericin B and LY303366), water (MK-0991 and 5-flucytosine), or polyethylene glycol (itraconazole). All drugs were diluted in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and dispensed into 96-well microdilution trays. The recommendations stated in the NCCLS document M27-A were followed for the dilution of each antifungal agent. Trays containing an aliquot of 0.1 mL in each well of appropriate drug solution (2× final concentration) were sealed and stored at -70°C until they were used. The final ranges of drug concentrations tested were as follows: LY303366 from 0.001 to 2 µg/mL; MK-0991 from 0.008 to 8 µg/mL; itraconazole from 0.008 to 8 µg/

mL; amphotericin B from 0.015 to 8 µg/mL; and 5-flucytosine from 0.06 to 128 µg/mL.

Susceptibility Testing

MICs were determined by a broth microdilution method using the NCCLS guidelines (NCCLS 1997). A spectrophotometric method previously described by Espinel-Ingroff et al. (1997) was used for the inoculum preparation. Briefly, each isolate was grown on potato dextrose agar slants at 35°C for a period of 7 days. The fungal colonies were then covered with 1 mL of sterile 0.85% saline and gently scraped with a sterile pipette. The resulting suspensions were transferred to sterile tubes and heavy particles were allowed to settle. The turbidity of the conidial suspensions was measured at 530 nm and was adjusted to obtain a final inoculum of 0.4×10^4 to 5×10^4 CFU/mL. To determine the final inoculum, appropriate dilution was performed and an aliquot (0.01 mL) was plated on potato dextrose agar (Remel, Lenexa, KS), incubated at 30°C, and examined daily for the presence of fungal colonies. The microdilution trays were incubated at 35°C and read at 24, 48, and 72 h. Drug-free controls were included in each tray. After incubation, MIC endpoints were interpreted with the aid of a reading mirror. Only wells that showed no growth (optically clear) (amphotericin B) or approximately 75% reduction in growth (LY303366, MK-0991, 5-flucytosine, and itraconazole) compared with drug-free controls were recorded as the MIC.

Quality Control

The two QC strains recommended in the NCCLS document M27-A, *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were included each time any testing was performed. For these particular strains, an inoculum concentration of $0.5\text{--}2.5 \times 10^3$ CFU/mL prepared from a 24-h-old culture was used.

RESULTS AND DISCUSSION

The antifungal activities of LY303366, MK-0991, itraconazole, amphotericin B, and 5-flucytosine at 72 h are summarized in Table 1. LY303366 was two- to fourfold more active than MK-0991 against *A. flavus* and *A. fumigatus*, and both antifungal drugs were considerably more active (MIC₉₀ of 0.03–0.12 µg/mL) than itraconazole, amphotericin B, and 5-flucytosine against these isolates. MK-0991 (MIC₅₀, 0.5 µg/mL) was slightly more active than either LY303366 or itraconazole (MIC₅₀, 1 µg/mL) against

TABLE 1 In Vitro Susceptibilities of 51 Clinical Filamentous Fungi to LY303666, MK-0991, and Other Antifungal Agents

Organism (no. of isolates)	Antifungal Drug	MIC ($\mu\text{g}/\text{mL}$)		
		Range	50%	90%
<i>Acromonium</i> sp. (1)	LY303666	0.5	—	—
	MK-0991	0.03	—	—
	Itraconazole	1	—	—
	Amphotericin B	2	—	—
	5-Flucytosine	>128	—	—
<i>Aspergillus flavus</i> (10)	LY303666	0.015–0.03	0.015	0.03
	MK-0991	0.03–0.5	0.06	0.12
	Itraconazole	0.25–1	0.5	1
	Amphotericin B	1–2	2	2
	5-Flucytosine	32–>128	>128	>128
<i>Aspergillus fumigatus</i> (12)	LY303666	0.004–0.12	0.03	0.06
	MK-0991	0.06–0.12	0.06	0.12
	Itraconazole	0.5–1	1	1
	Amphotericin B	1–2	2	2
	5-Flucytosine	128–>128	>128	>128
<i>Aspergillus</i> spp. (2) ^a	LY303666	0.06	—	—
	MK-0991	0.03–0.06	—	—
	Itraconazole	0.5–1	—	—
	Amphotericin B	2	—	—
	5-Flucytosine	>128	—	—
<i>Fusarium</i> spp. (13) ^b	LY303666	>2	>2	>2
	MK-0991	>8	>8	>8
	Itraconazole	4–>8	>8	>8
	Amphotericin B	1–2	2	2
	5-Flucytosine	>128	>128	>128
<i>Paecilomyces</i> sp. (1)	LY303666	2	—	—
	MK-0991	0.5	—	—
	Itraconazole	4	—	—
	Amphotericin B	4	—	—
	5-Flucytosine	>128	—	—
<i>Pseudallescheria boydii</i> (5)	LY303666	1	1	—
	MK-0991	0.25–2	0.5	—
	Itraconazole	1	1	—
	Amphotericin B	2–4	4	—
	5-Flucytosine	>128	>128	—
<i>Rhizopus</i> spp. (6)	LY303666	>2	>2	—
	MK-0991	>8	>8	—
	Itraconazole	0.5–2	1	—
	Amphotericin B	1–2	1	—
	5-Flucytosine	>128	>128	—
<i>Trichoderma</i> sp. (1)	LY303666	0.06	—	—
	MK-0991	0.25	—	—
	Itraconazole	2	—	—
	Amphotericin B	1	—	—
	5-Flucytosine	>128	—	—
All organisms (51)	LY303666	0.004–>2	0.5	>2
	MK-0991	0.03–>8	0.25	>8
	Itraconazole	0.25–>8	1	>8
	Amphotericin B	1–4	2	4
	5-Flucytosine	32–>128	>128	>128

^a Includes one isolate each of *Aspergillus niger* and *Aspergillus terreus*.

^b Includes *Fusarium oxysporum* (five isolates), *Fusarium solani* (five isolates), and *Fusarium* spp. (three isolates).

Pseudallescheria boydii and eightfold more active than amphotericin B (MIC₅₀, 4 $\mu\text{g}/\text{mL}$). Itraconazole and amphotericin B (MIC₉₀, 1 $\mu\text{g}/\text{mL}$) were more active

than both echinocandins against *Rhizopus* spp. Amphotericin B was the most active agent against isolates of *Fusarium*.

In this study, we have used the methodology recently published by the NCCLS subcommittee on antifungal susceptibility testing (Espinel-Ingroff et al. 1997). Using a high inoculum (0.4×10^4 to 5×10^4 CFU/mL), we observed potent in vitro activity of LY303366 and MK-0991 against all isolates of *Aspergillus* tested. Both compounds were also active (MIC $\leq 2 \mu\text{g/mL}$) against *Acremonium* spp. (one isolate), *Paecilomyces* spp. (one isolate), *Trichoderma* spp. (one isolate), and *P. boydii* (five isolates). Neither LY303366 nor MK-0991 appeared to be active against *Rhizopus* spp. and *Fusarium* spp. These results generally agreed with the findings reported by others (Chin et al. 1996; Espinel-Ingroff 1996; Fothergill et al. 1996; del Poeta et al. 1997).

We found that the guidelines suggested by the NCCLS subcommittee on antifungal susceptibility testing (Espinel-Ingroff et al. 1997) appear to be suitable for testing filamentous fungi. The medium recommended sustained adequate growth of all isolates tested at 48 h (except one *Trichoderma* isolate that needed 72 h). Overall, MK-0991 and LY303366 MICs at 72 h remained the same in 49 and 45 isolates, respectively, when compared to MICs at 48 h (data not shown). One and five isolates, respectively, increased the MIC by 1 log₂ dilution.

The translation of in vitro susceptibility testing results into in vivo activity is especially troublesome

with filamentous fungi. Although standardization of antifungal susceptibility testing of filamentous fungi is at a relatively early stage, the multicenter study published by Espinel-Ingroff et al. (1997) showed good intra- and interlaboratory agreement for amphotericin B and itraconazole and is, therefore, encouraging. Although studies of the in vitro activity of echinocandins against *Aspergillus* spp., as well as in vivo animal models, have demonstrated their utility (Abruzzo et al. 1995, 1997; Bartizal et al. 1995; Bartizal et al. 1997), further investigation of variables that impact on in vitro susceptibility results and prospective studies of clinical outcome are clearly needed.

In conclusion, our data demonstrate promising activity of both LY303366 and MK-0991 against *Aspergillus* spp. and other species of filamentous fungi that are likely to be encountered clinically. Based on these findings and the favorable results from animal models in the treatment of systemic aspergillosis, both compounds deserve further in vitro and in vivo investigation.

Francesc Marco is partially supported by a grant from Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC-Hoescht96) and a Permiso de Ampliación de Estudios from Hospital Clínic, Barcelona, Spain.

REFERENCES

- Abruzzo GK, Flattery AM, Gill CJ, Kong L, Smith JG, Krupa D, Pikounis VB, Kropp H, Bartizal K (1995) Evaluation of water-soluble pneumocandin analogs L-733560, L-705589 and L-731373 with mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob Agents Chemother* 39:1077-1081.
- Abruzzo GK, Flattery AM, Gill CJ, Kong L, Smith JG, Pikounis VB, Balkovec JM, Bouffard AF, Dropinski JF, Rosen H, Kropp H, Bartizal K (1997) Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob Agents Chemother* 41:2333-2338.
- Bartizal K, Gill CJ, Abruzzo GK, Flattery AM, Kong L, Scott PM, Smith JG, Leighton CE, Bouffard A, Dropinski JF, Balkovec J (1997) In vitro preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). *Antimicrob Agents Chemother* 41:2326-2332.
- Bartizal K, Scott T, Abruzzo GK, Gill CJ, Pacholok C, Lynch L, Kropp H (1995) In vitro evaluation of the pneumocandin antifungal agent L-733,560, a new water-soluble hybrid of L-705589 and L-731,373. *Antimicrob Agents Chemother* 39:1070-1076.
- Chin NX, Weitzman I, Della-Latta P (1996) A study of in vitro activity of L-743,792 against *Fusarium*, *Rhizopus* and *Trichosporon* species. In: *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA)*, Abstract F34. Washington, DC: American Society for Microbiology, p. 105.
- Current WL, Boylan CJ, Raab PP (1993) Anti-*Pneumocystis* activity of LY303366 and other echinocandin B analogs. In: *Program and Abstracts of the 33th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA)*, Abstract 368. Washington, DC: American Society for Microbiology, p. 186.
- Debono M, Gordee RS (1994) Antibiotics that inhibit fungal cell wall development. *Annu Rev Microbiol* 48:471-497.
- Espinel-Ingroff A (1996) In vitro studies with L-743,792, a water soluble pneumocandin: a comparative study. In: *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA)*, Abstract F31. Washington, DC: American Society for Microbiology, p. 105.
- Espinel-Ingroff A, Bartlett M, Bowden R, et al. (1997) Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *J Clin Microbiol* 35:139-143.
- Fothergill AW, Sutton DA, Rinaldi MG (1996) Antifungal susceptibility testing of Merck L-743,792 against a broad range of fungi. In: *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA)*, Abstract F29. Washington, DC: American Society for Microbiology, p. 105.
- Graybill JR, Najvar LK, Luther MF, Fothergill AW (1997)

- Treatment of murine disseminated candidiasis with L-743,792. *Antimicrob Agents Chemother* 41:1775-1777.
- Najvar L, Graybill J, Montalbo E, Barchiesi F, Luther M (1996) Evaluation of L-743,792 in the treatment of murine histoplasmosis. In: *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA), Abstract F43*. Washington, DC: American Society for Microbiology, p. 107.
- National Committee for Clinical Laboratory Standards (1997) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast. Approved Standard M27-A*. Wayne, PA: NCCLS.
- Pfaller MA, Messer SA, Coffman S (1997) In vitro susceptibilities of clinical yeast isolates to a new echinocandin derivative, LY303366, and other antifungal agents. *Antimicrob Agents Chemother* 41:763-766.
- del Poeta M, Schell WA, Perfect JR (1997) In vitro antifungal activity of L-743,792 against a variety of clinically important moulds. *Antimicrob Agents Chemother* 41:1835-1836.
- Powles MA, Anderson J, Liberator P, Schmatz DM (1996) Efficacy of semisynthetic pneumocandin analog L-743,792 against *Pneumocystis carinii* in murine models. In: *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA), Abstract F42*. Washington, DC: American Society for Microbiology, p. 107.
- Vazquez JA, Lynch M, Boikov D, Sobel JD (1997) In vitro activity of a new pneumocandin antifungal, L-743,792, against azole-susceptible and -resistant *Candida* species. *Antimicrob Agents Chemother* 41:1612-1614.
- Zeckner D, Butler T, Boylan C, Boyll B, Lin Y, Raab P, Schmidtke J, Current W (1993a) LY303366, activity against systemic aspergillosis and histoplasmosis in murine models. In: *Program and Abstracts of the 33th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA), Abstract 364*. Washington, DC: American Society for Microbiology, p. 186.
- Zeckner D, Butler T, Boylan C, Boyll B, Lin Y, Raab P, Schmidtke J, Current W (1993b) LY303366, activity in a murine systemic candidiasis model. In: *Program and Abstracts of the 33th Interscience Conference on Antimicrobial Agents and Chemotherapy (New Orleans, LA), Abstract 365*. Washington, DC: American Society for Microbiology, p. 186.

Article 7.

Elucidating the origins of nosocomial infections with *Candida albicans* by DNA fingerprinting with the complex probe Ca3. J Clin Microbiol 1999;37:2817-2828.

(FI: 3,503 / 2000)

Elucidating the Origins of Nosocomial Infections with *Candida albicans* by DNA Fingerprinting with the Complex Probe Ca3

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Received 10 February 1999/Returned for modification 6 April 1999/Accepted 14 May 1999

Computer-assisted DNA fingerprinting with the complex probe Ca3 has been used to analyze the relatedness of isolates collected from individuals with nosocomial bloodstream infections (BSIs) and hospital care workers (HCWs) in the surgical and neonatal intensive care units (ICUs) of four hospitals. The results demonstrate that for the majority of patients (90%), isolates collected from commensal sites before and after collection of a BSI isolate were highly similar or identical to the BSI isolate. In addition, the average similarity coefficient for BSI isolates was similar to that for unrelated control isolates. However, the cluster characteristics of BSI isolates in dendrograms generated for each hospital compared to those of unrelated control isolates in a dendrogram demonstrated a higher degree of clustering of the former. In addition, a higher degree of clustering was observed in mixed dendrograms for HCV isolates and BSI isolates for each of the four test hospitals. In most cases, HCW isolates from an ICU were collected after the related BSI isolate, but in a few cases, the reverse was true. Although the results demonstrate that single, dominant endemic strains are not responsible for nosocomial BSIs in neonatal ICUs and surgical ICUs, they suggest that multiple endemic strains may be responsible for a significant number of cases. The results also suggest that cross-contamination occurs between patients and HCWs and between HCWs in the same ICU and in different ICUs. The temporal sequence of isolation also suggests that in the majority of cases HCWs are contaminated by isolates from colonized patients, but in a significant minority, the reverse is true. The results of this study provide the framework for a strategy for more definitive testing of the origins of *Candida albicans* strains responsible for nosocomial infections.

Immunosuppression and other compromising conditions can result in life-threatening fungal bloodstream infections (BSIs). When such infections arise during hospitalization, they are referred to as nosocomial infections (31). There is an inclination to assume that because these infections arise in a hospital setting, the origin of the infecting strain is the hospital staff or environment. However, because *Candida albicans* and related species, which are responsible for the majority of nosocomial fungal infections (31), reside in the natural microflora of a majority of immunocompetent individuals as relatively benign commensal organisms (55), a nosocomial infection may also originate from the commensal strain carried into the hospital by the patient. If a nosocomial infection arises from an endogenous commensal strain, prior mucosal colonization has been implicated as an independent risk factor (9, 18, 41, 63) and the gastrointestinal tract has been implicated as the most likely reservoir (1, 28, 30, 38, 41, 60, 61, 63). In the case of exogenous transmission, contaminated infusates, biomedical devices, and the hands of health care workers (HCWs) represent docu-

mented sources (11, 12, 17, 26, 39, 46, 47, 51). In cases of nosocomial infections in newborns, the infection must originate in the hospital setting (40) since we can assume that the fetus is sterile in utero.

The epidemiology of nosocomial infections of *Candida* spp. has been investigated by a variety of DNA fingerprinting methods, including restriction fragment length polymorphism analysis (3, 6, 19, 39), electrophoretic karyotyping (8, 14, 59), randomly amplified polymorphic DNA analysis (13, 21, 42), and Southern blot hybridization with discriminating probes (27, 34, 44, 48, 56). Although the majority of these methods hold the potential for use in strain discrimination and valid cluster analyses, in most studies that have used them there has been no attempt to validate the methods used, no attempt to quantitate the levels of similarity or dissimilarity of isolates, and no attempt to perform cluster analyses of moderately related isolates. Instead, there has been complete reliance on subjective interpretations. Even more disturbing is the absence in most studies of a collection of unrelated isolates analyzed by the same fingerprinting method for comparison. Straightforward methods have been developed to assess the efficacy of a DNA fingerprinting method and to test whether the method possesses the necessary attributes for broad epidemiological studies (36, 53, 58). These attributes include the capacity to (i) identify the same strain in different isolates, (ii) distinguish between completely unrelated strains, (iii) cluster moderately related isolates, and (iv) distinguish microevolution in highly similar but nonidentical isolates. Recently, the use of Southern

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blot hybridization with the complex species-specific probe Ca3 was validated for DNA fingerprinting of *C. albicans* by demonstrating parity between it and both the method of randomly amplified polymorphic DNA analysis and the method of multilocus enzyme electrophoresis (36). This characterization of Ca3 fingerprinting provided quantitative measures of (i) identicalness, (ii) microevolution and high levels of relatedness, (iii) thresholds for clustering of moderately related isolates, and (iv) unrelatedness. It also provided cluster characteristics for a set of unrelated isolates that can be used to assess the relatedness of other sets of *C. albicans* isolates, such as collections of nosocomial isolates (36).

In the present study, we have used this validated DNA fingerprinting method to examine the relatedness of isolates of *C. albicans* from candidemia patients in the neonatal and surgical intensive care units (NICUs and SICUs, respectively) of four medical centers participating in the National Epidemiology of Mycoses Survey (NEMIS) (29, 35). The collection included 35 isolates primarily from the blood of 30 patients with candidemia and 75 isolates from stool, urine, respiratory, and/or gastric specimens from 28 of these patients collected before, during, and/or after collection of the isolates that caused candidemia. Infections did not occur in close temporal association in any of the four hospitals. In addition, 42 isolates were obtained from the hands of HCWs in the same intensive care units (ICUs) at the time of or very close to the time of infection.

MATERIALS AND METHODS

Collection of isolates. The NEMIS study was established under the auspices of Pfizer Inc. (New York, N.Y.) to define the spectrum of pathogens in seven hospitals causing nosocomial fungal infections in SICUs and NICUs, to characterize the organisms with respect to their susceptibilities to commonly used antifungal agents, and to define the molecular epidemiology of these infections with respect to endogenous sources and cross-infection (29, 35). The four hospitals in the study reported here were located in New York, N.Y. (hospital A), Iowa City, Iowa (hospital B), San Antonio, Tex. (hospital C), and Atlanta, Ga. (hospital D). Prospective surveillance was conducted over a 2-year period (1993 to 1995) for all patients who were hospitalized for at least 72 h in the SICU and NICU of each study site. ICU-acquired candidemia was defined as the occurrence of a new episode after a minimum of 72 h of hospitalization in the respective ICU. Microbiologic studies included weekly surveillance cultures of stool and urine for *Candida* spp. (rectal swab only for NICU patients), as described previously (28, 35). Thirty-five isolates were obtained from 30 ICU patients with infections, and for 28 of these patients, 75 additional isolates were collected from other body locations that can normally be colonized by commensal strains. Because 31 of the 35 isolates from ICU patients with infections were derived from patients with BSIs, all such isolates will be referred to as BSI isolates for convenience. The remaining four isolates from ICU patients with infections were obtained from peritoneal fluid, ascitic fluid, a tissue biopsy specimen, and an abscess. Specimens were also obtained from the hands of HCWs by the broth-bag method (57). Specimens from hands were obtained on a monthly basis and whenever an episode of candidemia was recognized in an ICU. Forty-two *C. albicans* isolates were obtained. Because of privacy rules at the respective institutions, HCWs could be identified only by their professional role, and therefore, specific HCWs could not be tracked over time, although in most cases HCWs were distinguishable from one another by professional role, ICU, and time of sampling. Isolates from patients were initially labeled according to patient, day of isolation, and body location. For example, an isolate obtained from the stool of patient 4 on day 106 was labeled P4(106)st. In dendrograms developed exclusively for BSI isolates, the isolates were also labeled according to ICU and date of isolation. For example, an isolate from patient 2 in the NICU collected on 29 March 1995 was labeled P2 N 3/29/95. HCW isolates were labeled according to HCW title, ICU, and date of collection. For example, an isolate from HCW5, a registered nurse in the NICU, collected on 20 March 1995 was labeled HWSRN N 3/20/95. The following HCW titles and abbreviations are used: registered nurse, RN; supervisor, SU; medical doctor, MD; X-ray technician, XT; nurse's assistant, NA; technician, TE; respiratory therapist, RT; nurse's orderly, NU; clerk, CL; and other, OT.

Organism identification. All isolates of *Candida* spp. were initially identified to the species level by routine procedures established at each participating institution and were then sent to the University of Iowa Hospitals and Clinics for banking and further analysis (35). Upon receipt at the University of Iowa, isolates were subcultured onto potato dextrose agar (Remel, Lenexa, Kan.) and

CHROMagar (Hardy Diagnostics, Santa Maria, Calif.) to assess viability and species homogeneity. Species were then identified with Vitek and API products (bio Merieux, St. Louis, Mo.) and by other conventional methods as required (62). All *Candida* isolates were stored as water suspensions or on agar slants at ambient temperature.

DNA fingerprinting. The complex DNA probe Ca3 (2, 22, 45) was used to assess the genetic relatedness and microevolution of the *C. albicans* isolates (22, 23, 55). The methods for DNA preparation and electrophoresis have been presented in detail elsewhere (24). DNA from reference strain 3153A was run in the outer two lanes of each gel in order to normalize the gel image.

DNA fingerprint analysis. To compare the fingerprints of isolates, the DENDRON software package (version 2.0; Solltech Inc., Oakdale, Iowa), based in a Macintosh computer, was used. The methods for analysis of fingerprint patterns have been described in detail elsewhere (53). Autoradiogram images were digitized and processed for distortions. Lanes and bands were automatically identified, and the similarity coefficient (S_{AB}) between patterns for every pair of isolates A and B was computed by the formula $S_{AB} = 2E/(2E + a + b)$, where E is the number of common bands in the patterns of A and B, a is the number of bands in pattern A with no correlates in pattern B, and b is the number of bands in pattern B with no correlates in pattern A. Dendrograms based on S_{AB} values were generated by the unweighted pair-group method with arithmetic averages (UPGMA) (52). To test the stability of clusters generated by UPGMA, the Test Dendrogram Stability option of the DENDRON, version 2.0, software package was used. In this assessment, the order of data entry was randomized 20 times, and members of the major clusters at an S_{AB} threshold of 0.80 were assessed.

Statistical tests. A two-sample t test for independent samples with unequal variances was used to compare the average S_{AB} s between defined collections (43). The distributions of S_{AB} s were verified to be normal enough for the t test. A chi-square test was used to compare proportions of isolates in clusters generated at a particular S_{AB} threshold (43).

RESULTS

DNA fingerprinting with the Ca3 probe. Representative Ca3 Southern blot hybridization patterns obtained with probe Ca3 are presented in Fig. 1A through D for collections from patients P5, P17, P4, and P28, respectively. Ca3 hybridized to between 15 and 20 bands in each Southern blot under the conditions used, but only the 10 to 15 bands above 2.3 kb were used to compute S_{AB} s (48). With the Ca3 probe, *EcoRI*-digested genomic DNA, and an S_{AB} based on band position alone, it has been empirically demonstrated that (i) an S_{AB} of 1.00 is achieved with multiple samples of the same clone; (ii) S_{AB} s ranging between 0.90 and 0.99 represent highly similar but nonidentical patterns and usually reflect microevolution of a single strain when isolates are obtained from the same patient; (iii) S_{AB} s ranging between 0.80 and 0.89 represent patterns for less related isolates that can still be clustered in a reproducible fashion; and (iv) S_{AB} s below 0.75 represent patterns for unrelated isolates (22, 23, 36, 37, 53).

In Fig. 2, a dendrogram has been generated from the S_{AB} s computed for 29 unrelated BSI isolates, each collected in a different hospital in the continental United States (34). The average S_{AB} for this control collection is 0.72 ± 0.10 , which represents an estimate of unrelatedness for BSI isolates that will be used in this study. The dendrogram generated for this collection also provides a measure of clustering among unrelated isolates at selected thresholds (53).

Comparison of BSI and commensal isolates from the same patients. In Table 1, the sequence of isolates, their anatomical origins, and the times of isolation are presented for isolates from each of the 30 infected patients. In addition, the average S_{AB} , the S_{AB} between the BSI isolate and the commensal isolate obtained immediately preceding collection of the BSI isolate, and the S_{AB} between the BSI isolate and the commensal isolate obtained immediately succeeding collection of the BSI isolate are presented for each patient. For 19 patients, an isolate was obtained from a site of commensal carriage prior to collection of the first BSI isolate (Table 1). In 17 of these patients (89%), the average S_{AB} between the commensal isolates and the subsequent BSI isolate ranged between 0.91 and

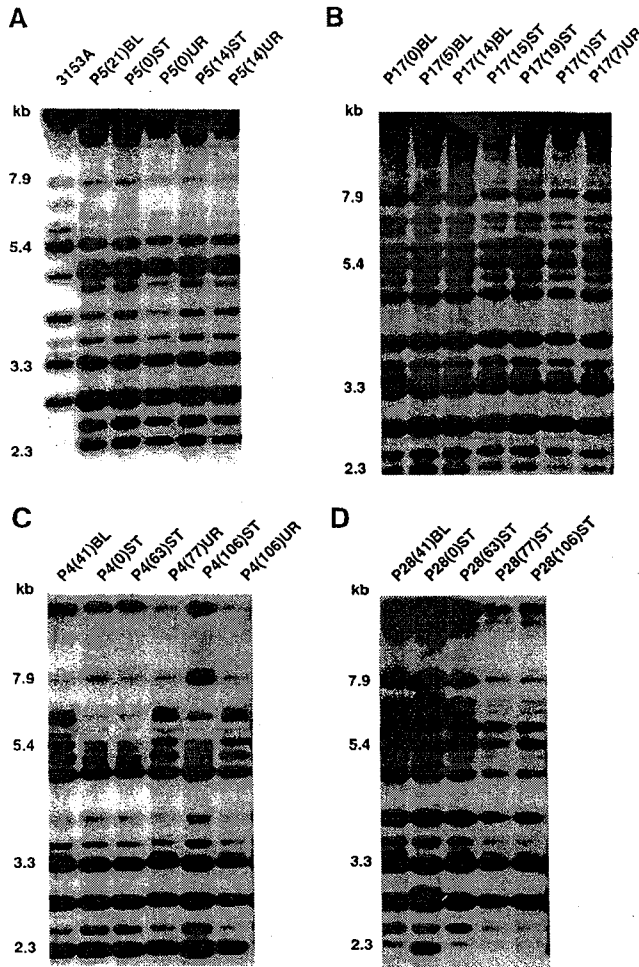


FIG. 1. Examples of the Southern blot hybridization patterns obtained with the complex probe Ca3. (A) Isolates from patient P5 and the reference strain 3153A; (B) isolates from patient P17; (C) isolates from patient P4; (D) isolates from patient P28. Isolate labels are explained in Materials and Methods. Molecular weights (in kilobases) are noted to the left of each Southern blot hybridization pattern.

1.00, a range of values considered to reflect high levels of relatedness (22, 23, 34, 53). The patterns of the isolates from patient P5 obtained by hybridization with the Ca3 probe (Fig. 1A) provide an example of the high level of relatedness observed between the BSI and preceding isolates in a majority of patients. Isolate P5(21)BL and the isolates from two prior urine samples, isolates P5(0)UR and P5(14)UR, differed by only one high-molecular-mass band, while isolate P5(21)BL and the two isolates from prior stool samples, isolates P5(0)ST and P5(14)ST, were identical. For only 2 of the 17 patients, patients P4 and P28, did the initial commensal isolates differ markedly from the subsequent BSI isolates (Fig. 1C and D, respectively). Interestingly, the times between collection of the commensal isolates and the subsequent BSI isolates in these two patients were the most extensive in the collection: 41 days for each patient (Table 1).

For 18 patients an isolate was collected from a site of commensal carriage after the BSI isolate was collected (Table 1). For 16 of these patients (89%), the S_{AB} between the BSI and subsequent commensal isolates ranged between 0.91 and 1.00 (Table 1). In two patients (11%), patients P4 and P28, a sub-

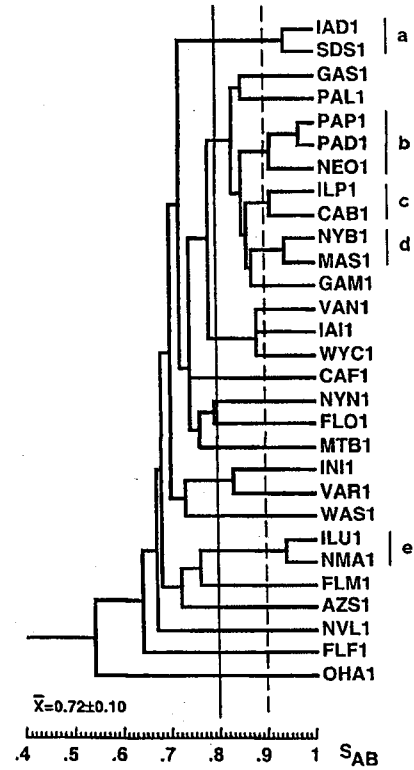


FIG. 2. Dendrogram of a control collection of 29 unrelated BSI isolates each collected from a different hospital across the continental United States. a through e, clusters of two or more isolates with S_{AB} s of ≥ 0.90 . S_{AB} thresholds for cluster analysis are drawn at 0.80 (straight line) and 0.90 (dashed line).

sequent commensal isolate was unrelated to the BSI isolate (Table 1; Fig. 1C and D, respectively). In the case of patient P4, isolate P4(106)ST was unrelated to P4(41)BL (Fig. 1C). Interestingly, the stool isolate, isolate P4(0)ST, was also unrelated to the BSI isolate but was identical to subsequent stool isolates P4(63)ST and P4(106)ST (Fig. 1C). These results suggest that patient P4 was initially colonized by two unrelated strains, one in the stool and one in the urine, and that the strain in the urine emerged as the cause of the BSI. In the case of patient P28, preceding stool isolate P28(0)ST was unrelated to P28(41)BL, but subsequent stool isolates P28(63)ST, P28(77)ST, and P28(106)ST were identical to P28(41)BL (Fig. 1D; Table 1), suggesting strain replacement. In a third scenario, the BSI isolate was similar but nonidentical to the commensal isolates, suggesting significant microevolution. In patient P21, the BSI isolate and both the succeeding commensal isolates were similar but nonidentical, with S_{AB} s of 0.91 and 0.88, respectively (Table 1).

For the 18 collections of isolates with an average S_{AB} of 1.00 (Table 1), the dendrogram that was generated was composed of a single cluster at an S_{AB} of 1.00. The collection of isolates from patient P17 provides an example of such a dendrogram (Fig. 3A). For dendrograms of collections with average S_{AB} below 1.00, the complexity (degree of branching) of the dendrogram increased. For example, the dendrogram generated for the collection of isolates from patient P5, which had an average S_{AB} of 0.98 (Table 1), contained two clusters separated by a node at an S_{AB} of 0.97. One cluster contained two identical stool isolates collected at days 0 and 14, and the second cluster contained three identical isolates, two from

TABLE 1. Description of collections of *C. albicans* isolates from individuals with BSIs or other candidemias^a

Hospital	ICU	Patient no.	No. of isolates	Anatomic origins of isolates ^b	Time of isolation (days) ^c	Time (days) between first isolate and Candidemia	Average S_{AB}	S_{AB} for ST and UR isolates and subsequent blood isolates	S_{AB} for blood isolate and subsequent ST and UR isolates
A	SICU	P1	5	ST-ST-UR, UR-BL	0-7-15, 15-17	17	0.99	1.00	
	NICU	P2	4	UR-BL-UR-ST	0-4-6-12	4	1.00	1.00	1.00
	NICU	P3	3	ST-ST-BL	0-7-13	13	0.94	0.91	
	SICU	P16	4	ST, TR-AB-ST	0, 0-7-8	7	1.00	1.00	1.00
	NICU	P17	7	BL-ST-BL-UR-BL-ST-ST	0-1-5-7-14-15-19	0	1.00		1.00
	NICU	P18	5	BL-GA-ST-BL-ST	0-1-2-3-21	0	0.96		0.93
B	SICU	P10	6	ST, UR-ST, UR-BL-ST	0, 0-4, 4-5-7	5	1.00	1.00	1.00
	SICU	P11	3	ST-BL-UR	0-9-10	9	0.98	0.97	0.97
	NICU	P12	2	ST-BL	0-2	2	0.97	0.97	
	NICU	P13	2	ST-BL	0-21	21	1.00	1.00	
	SICU	P14	4	ST-ST-BL-PR	0-20-27-28	27	1.00	1.00	1.00
	SICU	P21	5	TB-ST-AS-ST, UR	0-6-10-11, 11	10	0.93	0.91	0.88
	SICU	P22	2	BL-UR	0-2	0	1.00		1.00
	NICU	P24	2	TR-UR-BL-TR, UR	0-8-10-11, 11	10	1.00	1.00	1.00
C	SICU	P15	4	UR-UR, SP, BL	0-12, 12, 12	12	1.00	1.00	
	SICU	P27	2	BL-ST	0-1	0	1.00		1.00
	NICU	P31	1	BL	0				
D	SICU	P4	6	ST-BL-ST-LR-ST, UR	0-41-63-77-106, 106	41	0.83	0.73	0.73
	SICU	P5	5	ST, UR-ST, UR-BL	0, 0-14, 14-21	21	0.98	0.97	
	NICU	P6	4	ST, BL, ST, ST	0-2-7-14	2	1.00	1.00	1.00
	NICU	P7	4	ST, BL-ST-ST	0-6-7-14	6	1.00	1.00	1.00
	NICU	P8	4	ST-ST-ST, BL	0-7-13, 13	13	1.00	1.00	
	NICU	P9	4	ST-ST-ST-BL	0-7-14-17	17	1.00	1.00	
	NICU	P19	4	ST-ST-BL-ST	0-8-11-15	11	1.00	1.00	1.00
	NICU	P20	3	BL-ST-ST	0-5-20	0	1.00		1.00
	NICU	P26	2	BL-ST	0-60	0	1.00		1.00
	SICU	P28	6	ST-BL-ST-ST-ST, UR	0-41-63-77-106, 106	41	0.92	0.79	1.00
	NICU	P29	1	BL	0				
	NICU	P30	1	BL	0				

^a Abbreviations: ST, stool; UR, urine; BL, blood; TR, tracheal lavage; AB, abscess; GA, gastric aspirate; PR, peritoneal fluid; TB, tissue biopsy specimen; AS, ascitic fluid; SP, sputum. For simplicity, isolates from blood, peritoneal fluid, tissue biopsy specimens, abscesses, and ascitic fluid are referred to as BSI isolates in the text, since 28 of the 30 infected patients (93%) had BSIs. Sequences of anatomic origin and dates of collection are noted.

urine collected on days 0 and 14, suggesting that substrains resulting from microevolution had established themselves in alternative body locations. For this patient the blood isolate clustered with the stool isolates (Figure 3B). In Fig. 3C, a dendrogram is presented for the collection of isolates from patient P4; the average S_{AB} for these isolates was 0.83 (Table 1). The dendrogram contained two clusters with a node at an S_{AB} of 0.79. Just as in the case of the dendrogram for the collection of isolates from patient P5, the isolates from stool and urine samples separated into respective clusters, but in this case the separated clusters appeared to represent two unrelated strains. The blood isolate from this patient clustered with the urine isolates, not the stool isolates (Fig. 3C). In Fig. 3D, a dendrogram is presented for the collection of isolates from patient P28; the average S_{AB} for these isolates was 0.92 (Table 1). A node at an S_{AB} of 0.76 separated the first stool isolate from a cluster containing the subsequent blood, stool, and urine isolates. The combined results summarized in Table 1 demonstrate that for the majority of patients, commensal isolates and the subsequent BSI isolate from the same patient are highly similar, and for the majority of patients, a BSI isolate and subsequent commensal isolates from the same patient are

highly similar. The results also suggest that in one-third of the patients, microevolution occurs in the colonizing strain.

Genetic relatedness of BSI isolates obtained from the same hospital. To obtain a measure of the genetic diversity of BSI isolates in each test hospital, dendrograms that included only one BSI isolate from each patient were generated for each hospital (Fig. 4). The average S_{AB} s for these restricted collections from hospitals A, B, C, and D were 0.76 ± 0.10 ($n = 6$), 0.71 ± 0.10 ($n = 9$), 0.75 ± 0.13 ($n = 3$), and 0.69 ± 0.11 ($n = 12$), respectively (Table 2). The average S_{AB} for the combined collection of isolates from patients with candidemia from the four hospitals was 0.72 ± 0.10 ($n = 30$), which was identical to the value obtained for the 29 unrelated BSI isolates described previously (34) (Table 2). While the average S_{AB} s for the collections from hospitals B and D were lower than that for the random control collection of BSI isolates ($P = 0.358$ and 0.616 , respectively), the average S_{AB} s for the collections from hospitals A and C were slightly higher ($P = 0.265$ and 0.615 , respectively).

The average S_{AB} s computed for each hospital collection suggested that in each hospital the BSI isolates were approximately as diverse as the control collection of unrelated isolates

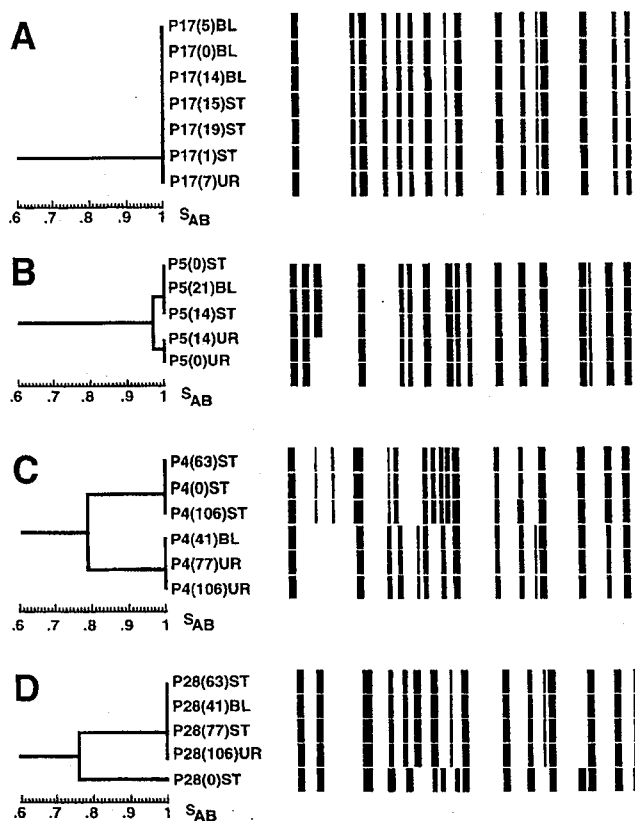


FIG. 3. Examples of dendrograms for collections of isolates from patients in which all isolates are identical (A), isolates show some variability reflecting microevolution (B), and isolates separate into unrelated clusters of isolates (C and D). Horizontal models of the Ca3 hybridization patterns are displayed next to the respective isolates in the dendrograms.

(Table 2). However, a simple comparison of the average S_{AB} s can be misleading, since one very unrelated isolate in a small collection can have an inordinately strong influence (52). The cluster characteristics of the dendrogram for BSI isolates from each of the four hospitals (Fig. 4A to D) were therefore individually compared to the cluster characteristics of the dendrogram generated for the control collection of unrelated BSI isolates (Fig. 2). The dendrogram for the collection of six BSI isolates from hospital A (Fig. 4A) included two (from patients P18 and P16) with identical patterns and four (from patients P18, P16, P17, P3), which represented 67% of the collection, in a cluster defined at an S_{AB} threshold of 0.86. In the dendrogram for the control collection (Fig. 2), no isolates exhibited identical patterns and only 52% of the isolates in the control collection were members of clusters defined at an S_{AB} threshold of 0.86 (Fig. 2) ($P = 0.03$). These results suggest a significantly higher than expected level of clustering of highly and moderately related isolates in hospital A ($P = 0.03$). The dendrogram for the collection of nine BSI isolates from hospital B (Fig. 4B) included five in clusters defined at an S_{AB} threshold of 0.86, which represented 56% of the collection, compared to 52% for the control collection. The dendrogram for the three BSI isolates from hospital C (Fig. 4C) included two in a cluster defined at an S_{AB} threshold of 0.86, which represented 67% of the collection, compared to 52% for the control collection. Finally, the dendrogram for 12 BSI isolates from hospital D (Fig. 4D) contained 6 isolates in clusters defined at an S_{AB} threshold of 0.86, which represented 50% of the collection,

which is roughly the same proportion as that for the control collection (Fig. 2). However, the hospital D collection contained a cluster of four isolates that were defined at an S_{AB} threshold of 0.90 and that were collected from patients in the same NICU (Fig. 4D). This cluster represented 33% of the hospital D collection. In the control collection of unrelated BSI isolates (Fig. 2), the largest cluster defined at a threshold of 0.90 contained three isolates, which represented only 10% of the collection. The difference was significant, with a P value of 0.0002.

In the dendrograms generated for BSI isolates in each of the four respective hospitals (Fig. 4), there was no indication of temporal associations of highly related BSI isolates. In the hospital A collection, none of the isolates in the major cluster were collected within 2 months of one another, and even the identical pair of isolates, from patients P18 and P6, were collected 5 months apart and from different ICUs (Fig. 4A). In the hospital B collection, the two most closely related isolates, from patients P12 and P10, were collected 9 months apart, and two isolates, from patients P11 and P23, were collected within 3 days of one another in the same ICU and were unrelated (Fig. 4B). In the hospital D collection, two of the three most related isolates, from patients P8 and P9, were collected from the same ICU within 4 days of each other (Fig. 4D). However, four isolates, collected from patients P28, P19, P20, and P26 within 12 days of each other, were not highly related (Fig. 4D). These results together demonstrate the absence of single dominant endemic strains responsible for BSIs that occur in close temporal proximity in both the NICUs and the SICUs of the four hospitals in this study. However, even though related isolates did not exhibit temporal clustering, the proportion of clusters in the dendrogram for each hospital suggested a greater number of groups of related isolates than would be expected on the basis of comparisons with the control collection of unrelated BSI isolates.

Genetic relatedness of isolates from HCWs in individual hospitals. The average S_{AB} s for isolates collected from the hands of HCWs from hospitals A, B, C, and D were 0.84 ± 0.05 ($n = 7$), 0.71 ± 0.11 ($n = 11$), 0.77 ± 0.06 ($n = 4$), and 0.72 ± 0.10 ($n = 20$), respectively (Table 2). The average S_{AB} s for HCW isolates from hospitals A and C were higher than the S_{AB} for the 29 unrelated BSI isolates in the control collection, as was the case for the S_{AB} s for BSI isolates from the same hospitals. Only in the case of the HCW isolates in hospital A was the difference with the control collection significant ($P = 0.004$). Dendrograms for the HCW isolates from each hospital (Fig. 5) had some of the same characteristics as those generated for BSI isolates from the respective hospitals (Fig. 4). For instance, the dendrogram for the HCW isolate collection from hospital A was dominated by a cluster defined by an S_{AB} threshold of 0.83 that contained six of the seven isolates (86%) (Fig. 5A). A similar cluster of four of the six BSI isolates (67%) from hospital A defined by an S_{AB} threshold of 0.86 dominated the dendrogram for isolates from that hospital (Fig. 4D). Several additional characteristics of the dendrograms for HCW isolates are noteworthy. At an S_{AB} threshold of 0.89, 38% of the control collection of BSI isolates formed clusters (Fig. 2). Except for one cluster of three isolates, which represented 10% of the control collection, all other clusters contained two isolates (Fig. 2). In addition, there were no clusters in the control collection defined by an S_{AB} threshold of 0.96 (Fig. 2). In contrast, the proportion of HCW isolates in clusters that were defined at an S_{AB} threshold of 0.89 and that contained three or more isolates for hospitals A, B, and D were 43, 27, and 50% respectively; all of these values are higher than the proportion of 10% for the control BSI isolate collection ($P = 0.0001$).

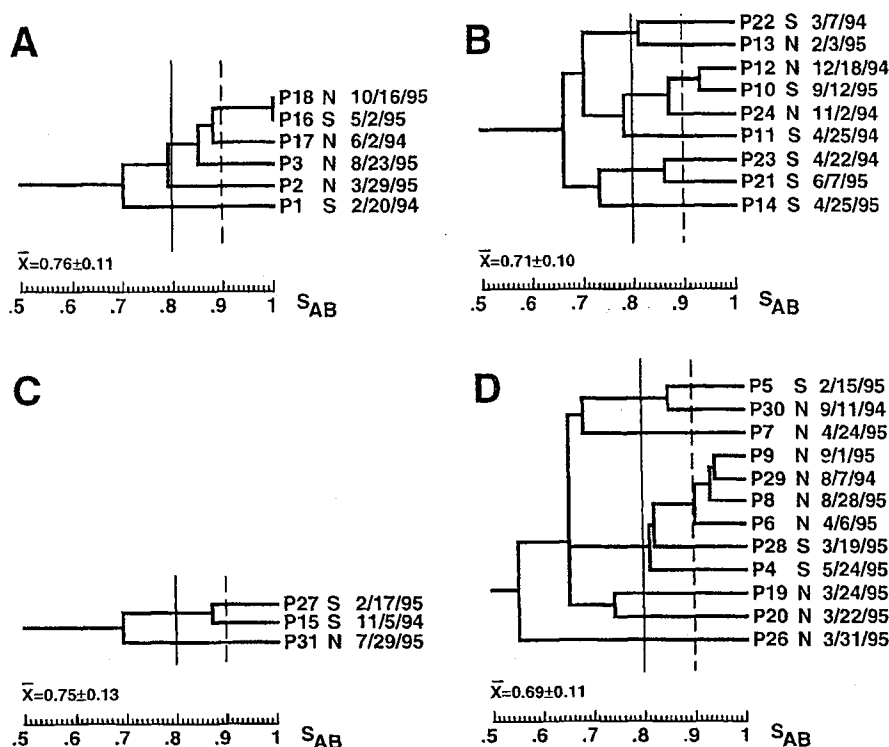


FIG. 4. Dendrograms of the BSI isolates from the patients in hospitals A (A), B (B), C (C), and D (D). Only one BSI isolate from each patient was incorporated. The type of ICU and the date of collection are noted to the right of each isolate. Arbitrary S_{AB} thresholds are drawn at 0.80 (straight line) and 0.90 (dashed line).

0.0005 and 0.0001, respectively). In addition, the dendrograms for HCW isolates from hospitals B and D each contained a pair of identical isolates (Fig. 5B and D). Finally, at a threshold of 0.91 the dendrogram for HCW isolates from hospital D contained a cluster of six isolates (Fig. 5D), which was twice as large as the largest cluster in the dendrogram for control BSI isolates at that threshold (Fig. 2).

In contrast to the relative lack of temporal associations observed among BSI isolates from individual hospitals, there were several examples of this among HCW isolates. In the hospital A collection, two of the three isolates (isolates HW4SU and HW3RN) in the most highly related cluster were collected from different individuals in the NICU on the same day (Fig. 5A). In the hospital B collection, three isolates (isolates HW34RN, HW32XT, and HW33NA) in the most related cluster were collected from three different individuals in the SICU on the same day (Fig. 5B). Finally, in the hospital D collection, three of the seven isolates (isolates HW21RN, HW19RT, and HW20RN) in the largest cluster were collected from different individuals in the NICU on the same day, and an additional isolate in the cluster (isolate HW13RN) had been collected 15 days earlier (Fig. 5D). Three of the four isolates (isolates HW9CL, HW8RN, and HW10RN) in the second largest cluster were collected from individuals in the SICU on the same day, and the fourth isolate (isolate HW27RN) in this cluster had been collected 15 days earlier from an individual in the NICU (Fig. 5D). These results demonstrate transfer of strains among HCWs or from patients to several HCWs in the same ICUs.

Genetic relatedness of BSI and HCW isolates from the same hospitals. To assess the relatedness between BSI and HCW isolates collected in the same hospitals, mixed dendrograms were generated (Fig. 6). One BSI isolate from each patient and

from each HCW was included. The average S_{AB} for the combined collection of hospital A isolates was 0.83 ± 0.10 , which was significantly higher than that for the control collection of unrelated BSI isolates (Table 2) ($P = 0.002$). The proportion of isolates in clusters defined at an S_{AB} of 0.85 was 85% (Fig. 6A), which was higher than the 59% value for the control BSI isolate population ($P = 0.07$). The combined collection of isolates from hospital A formed two clusters at an S_{AB} threshold of 0.89 (Fig. 6A). Cluster a included two isolates, one from patient P2 and one from HW5RN; these were collected 9 days apart. The HCW isolate was collected 9 days before collection of the BSI isolate (Fig. 6A) and 5 days before collection of the first commensal isolate from this patient. Cluster b included five isolates. Three of the five (from P18, P16, and HW4SU) were identical ($S_{ABs} = 1.00$). Interestingly, the three isolates were collected over a 1-year period. None were collected within a month of each other. This observation is one of the strongest supporting the establishment of an endemic strain in this study. Again, the HCW isolate was collected before the BSI isolates, 1 year prior to collection of the isolate from patient P18, and 7 months before collection of the isolate from patient P16. The remaining two isolates in cluster b included one from P3 and one from HW7RN, and these were collected 1 day apart.

The average S_{AB} for the combined collection of isolates from hospital B was similar to that for the control collection of BSI isolates (Table 2). However, while 59% of the control collection of unrelated BSI isolates formed clusters at an S_{AB} threshold of 0.85, 80% of the combined collection from hospital B formed clusters at this threshold (Fig. 6B) ($P = 0.002$). The combined collection from hospital B formed five clusters of two to three isolates each at an S_{AB} threshold of 0.90 (Fig. 6B). The first cluster, cluster a, contained three HCW isolates

TABLE 2. Average S_{AB} s for patients and staff of select hospitals compared with that of control isolates

Hospital(s)	Group	No. of isolates	Avg S_{AB}
A, B, C, D	PAT ^a	30	0.72 ± 0.10
	HCW	42	0.73 ± 0.11
A	PAT, HCW	13	0.83 ± 0.08
	PAT	6	0.76 ± 0.11
	HCW	7	0.84 ± 0.05
	HCW (NICU)	5	0.85 ± 0.05
	HCW (SICU)	2	0.80 ± 0.00
B	PAT, HCW	19	0.71 ± 0.10
	PAT	8	0.71 ± 0.10
	HCW	11	0.71 ± 0.11
	HCW (NICU)	3	0.79 ± 0.01
	HCW (SICU)	8	0.71 ± 0.12
C	PAT, HCW	8	0.73 ± 0.11
	PAT	3	0.75 ± 0.13
	HCW	6	0.77 ± 0.06
	HCW (NICU)	4	0.65 ± 0.10
	HCW (SICU)	2	0.79 ± 0.00
D	PAT, HCW	31	0.73 ± 0.12
	PAT	11	0.69 ± 0.11
	HCW	20	0.72 ± 0.10
	HCW (NICU)	12	0.71 ± 0.11
	HCW (SICU)	8	0.74 ± 0.11
29 hospitals (control)	PAT	29	0.72 ± 0.10

^a PAT, patients.

(from HW34RN, HW32XT, and HW33NA), all collected on the same day. The next three clusters, clusters b, c, and d, each contained one patient isolate and one HCW isolate (from P11 and HW35RN, P21, and HW29RN, and P24 and HW37TE, respectively) collected 1, 5, and 27 days apart, respectively. The fifth cluster, cluster e, contained two patient isolates and one HCW isolate (from P10, P12, and HW38RN). None of the latter three isolates were collected within a month of each other, even though the three were highly related or identical. The HCW isolate in this cluster was collected 2 months after collection of the first BSI isolate but 7 months prior to collection of the second BSI isolate. The final cluster, cluster f, defined at an S_{AB} threshold of 0.86, included isolates from P22 and HW31RT, collected within 2 days of each other.

The average S_{AB} for the combined collection from hospital C was 0.77 ± 0.09 , which was somewhat higher than that for the control collection (Table 2). The dendrogram for the hospital C collection contained a cluster of two highly related isolates, one from HW43NU and one from P27 (Fig. 6C). In this case, the HCW isolate was collected a year prior to the collection of the patient isolate.

The average S_{AB} for the combined collection from hospital D was also similar to that for the control collection of unrelated BSI isolates (Table 2). However, the proportion of isolates in clusters defined at an S_{AB} threshold of 0.85 was 74% (Fig. 6D), compared to 59% for the control collection ($P = 0.214$). The combined collection from hospital D contained six clusters defined at an S_{AB} threshold of 0.88 (Fig. 6D). Clusters a, b, c, and d each contained a patient isolate and an HCW isolate (from P5 and HW14RN, P30 and HW12RT, P20 and HW24RN, and P7 and HW23RT, respectively) collected 2 months, 6 months, 1 month, and 1 day apart, respectively. In

each of these instances, the patient isolate was collected before collection of the HCW isolate. Cluster e contained isolates from three patients and four HCWs. Five of the seven isolates in this cluster (from HW10RN, P9, HW9CL, HW8RN, and HW27RN) were collected within 19 days of each other. Finally, cluster f contained eight isolates, one from a patient and seven from HCWs. Seven of the eight isolates were collected in a 6-month period. Among the isolates in the latter cluster, the isolates from P8 and HW19RT were connected at an S_{AB} node of 0.98. In this case, the HCW isolate was collected more than 1 year prior to collection of the patient isolate.

In 11 cases, a BSI and an HCW isolate collected from the same hospitals within a 2-month period were highly related ($S_{AB} \geq 0.88$) (Table 3). In all but one of the cases, the BSI isolate was collected in the same ICU as the HCW isolate. In the one exceptional case, isolates from three HCWs in the SICU of hospital D were highly similar to the isolate from patient P9 in the NICU (Table 3). The isolates from the SICU in this case were collected 19 days after collection of the BSI isolate from patient P9 in the NICU and 15 days after collection of a related isolate from an HCW in the NICU. Although in the preceding analysis, cases were noted of HCW isolates collected prior to related BSI isolates, in the majority of cases, BSI isolates were collected prior to related HCW isolates from the same hospital (Table 3). In all but one of these cases, isolates were also obtained from HCWs in the same ICU prior to collection of the BSI isolate that were unrelated to the BSI isolates (Fig. 6).

Stability of clusters. In this analysis, we have considered unrelatedness to be reflected by an S_{AB} of 0.72, on average, and a high degree of relatedness to be reflected by S_{AB} s above 0.90. In analyzing the cluster characteristics of collections, we used thresholds of 0.85 or 0.86 to define clusters. Since the order of data input by the UPGMA method can affect branching and, thus, the stability of clusters in a dendrogram (5), we randomized data input for the largest dendrogram in the study, the combination dendrogram for patients and HCWs from hospital D (Fig. 6D). The input was randomized 20 times. At the S_{AB} threshold of 0.80, 100% of the isolates that separated into the two major clusters x and y remained in those two clusters, demonstrating that the intermediately rooted branches that defined those clusters were stable. In addition, all clusters above the S_{AB} threshold of 0.90 remained intact, demonstrating that the highly related clusters were also stable.

DISCUSSION

The National Nosocomial Infections Surveillance System conducted by the Centers for Disease Control and Prevention reported an increase from 2.0 nosocomial fungal infections per 1,000 discharges in 1980 to 3.8 per 1,000 discharges in 1990, an approximately twofold increase (7). In that survey, significant increases were observed in medical, surgical, and newborn services, as well as in subspecialty services such as burn and trauma, cardiac surgery, and high-risk nursery services, in a 10-year period (7). The rates of nosocomial fungal infections, therefore, have increased in all types of hospitals, for all types of specialty services, and at all sites of infection (32).

Candida spp. account for approximately 8% of all nosocomial BSIs (31–33), and of these, *C. albicans* accounts for the majority (50 to 70%) (7, 30, 31–33). Because *Candida* spp. can be carried as commensal organisms, several possible origins of nosocomial infections must be considered. First, it has been demonstrated that at least two-thirds of healthy individuals carry a *Candida* sp. in their natural microflora (55). In a significant number of these cases of *Candida* carriage, individual

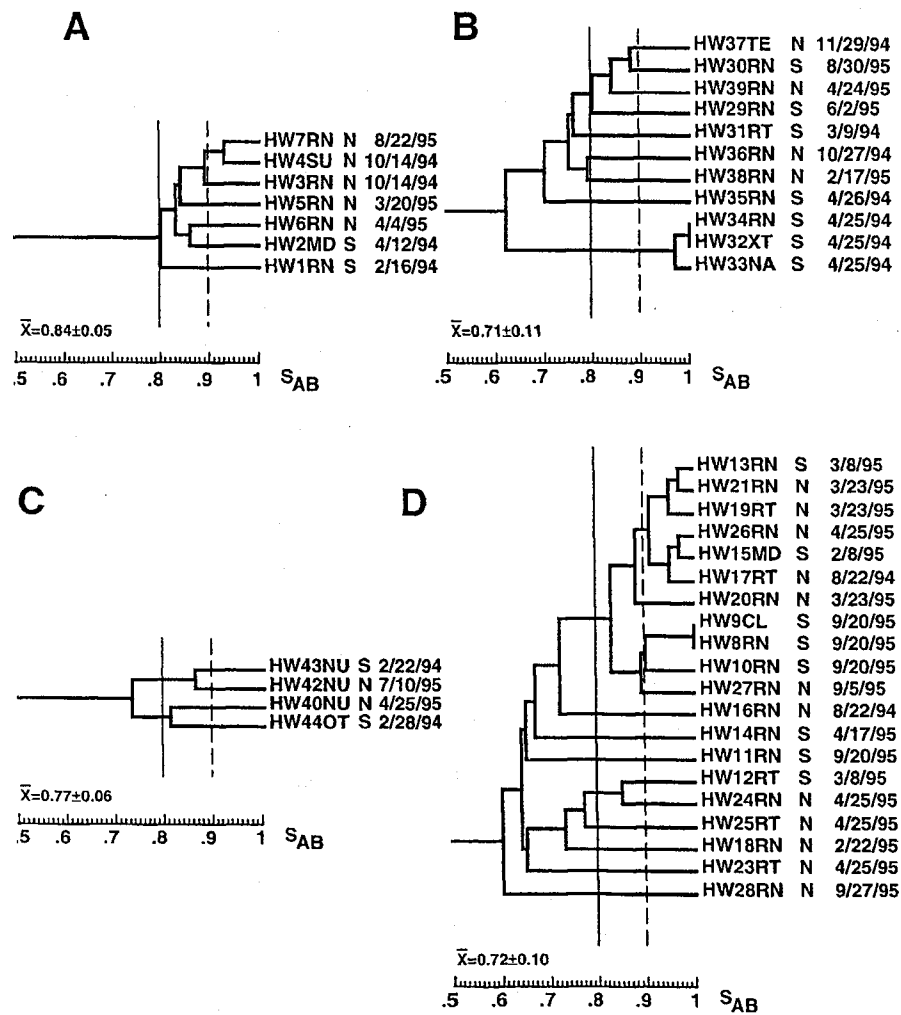


FIG. 5. Dendrograms of the isolates from HCWs of hospitals A (A), B (B), C (C), and D (D). The type of HCW is noted immediately to the right of health care worker number. The type of ICU and the date of collection are noted to the right of each isolate. Arbitrary S_{AB} thresholds are drawn at 0.80 (straight line) and 0.90 (dashed line). Abbreviations for HCWs are provided in Materials and Methods.

carry *Candida* spp. in at least two anatomical niches, most notably the vaginal canal and the oral cavity. In approximately two-thirds of such individuals, unrelated *C. albicans* strains or different species colonize the alternative anatomical locales, and in the remaining third, substrains that are highly related but nonidentical colonize the alternative locales (55). Since there is growing genetic evidence suggesting that in the majority of patients commensal organisms are the source of subsequent infection (38, 60), commensal organisms established in the patient at the time of hospitalization should represent the major source of nosocomial yeast infections. However, just as the majority of patients carry commensal organisms prior to infection, so do the HCWs who interact with patients and so do individuals who visit patients. Therefore, there is also the possibility that infectious yeasts can be transferred from the latter individuals to susceptible patients (4, 16, 41, 43, 44, 55). In addition, there is growing concern, especially in the case of aspergillosis, that the physical environment of the hospital can harbor endemic strains of infectious fungi that may be responsible for a portion of nosocomial infections (10, 20, 25). A recent analysis of the genetic diversity of BSI isolates by the same fingerprinting methods used here suggested that partic-

ular BSI strains are more highly concentrated in particular geographical locales, that established BSI strains may be endemic in some hospitals, and that these endemic strains may adapt through microevolution to those hospital settings (34). Molecular genetic studies have also demonstrated that single strains have been responsible for a number of temporally associated outbreaks of candidemia in the same hospital or ICU (15, 26, 39, 46, 47, 51). In some cases, the isolates cultured from the hands of HCWs have been found to be genetically similar or identical to nosocomial strains (11, 12, 15, 35), although the direction of transfer in these cases was usually not apparent.

Isolates from the same patient. There is compelling genetic evidence from a variety of studies that have used a variety of DNA fingerprinting methods that individuals usually harbor the same commensal or infecting strain of *C. albicans* over extended periods of time (23, 38, 42, 46, 47, 50, 54, 56, 60) and that over time colonizing strains undergo microevolution that can be monitored through reorganization of the hypervariable regions identified by the C1 fragment of the Ca3 probe, which contains a cluster of the repeat element RPS (23, 55). Here, we have compared isolates from urine, stool, and other sites of

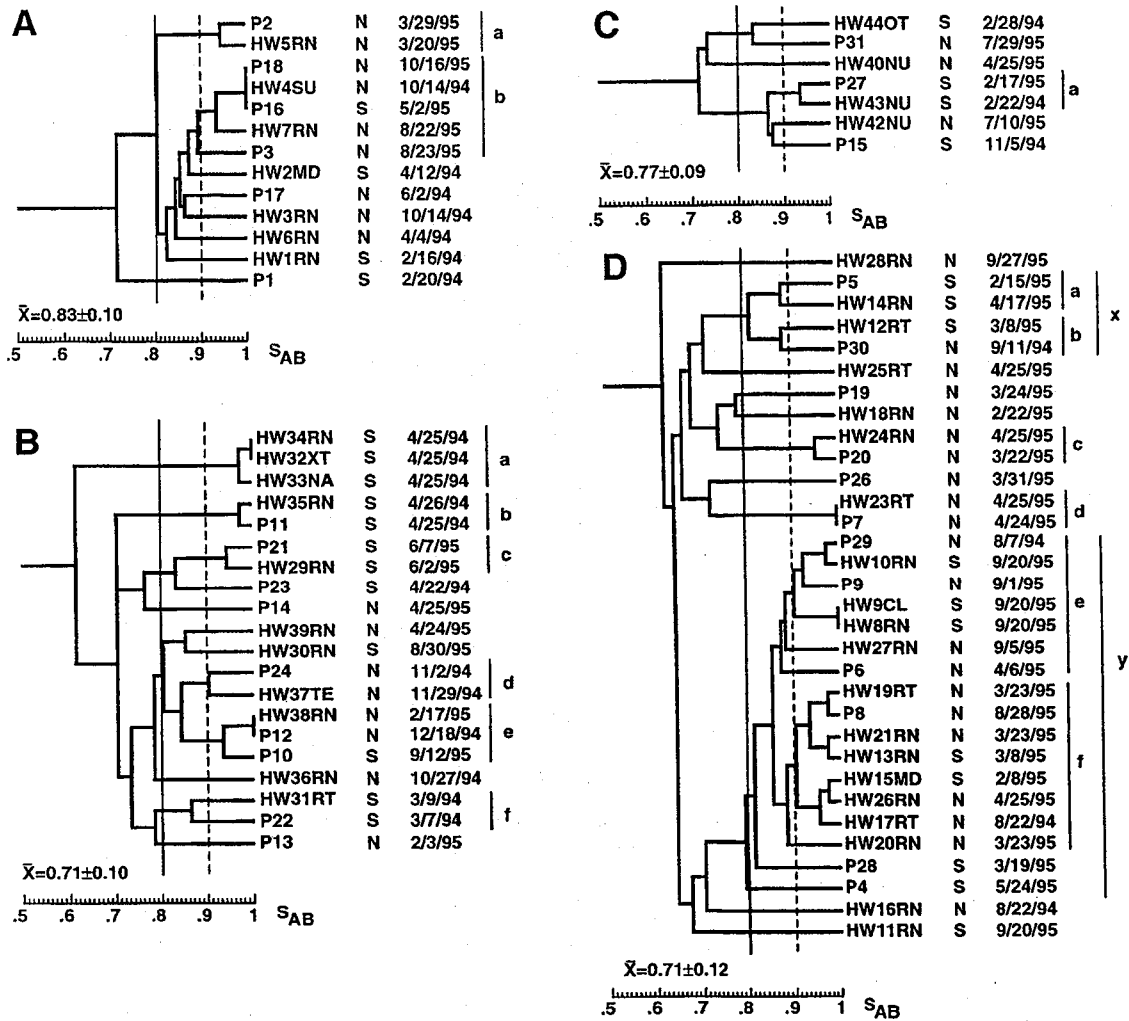


FIG. 6. Dendrograms for combined BSI isolates and HCW isolates from hospitals A (A), B (B), C (C), and D (D). Only one BSI isolate from each patient was used. Isolate labels are explained in Materials and Methods. Arbitrary S_{AB} thresholds are drawn at 0.80 (straight line) and 0.90 (dashed line). Clusters determined by a threshold of 0.90 are delineated to the right of each dendrogram.

infection with commensal organisms with BSI isolates from the same individuals. Stool and urine isolates collected prior to and after collection of the first BSI isolate were similar or identical to the BSI isolates in approximately 90% of the patients. However, because all isolates were obtained from each patient after the patient entered the SICU, we cannot be certain that in all patients the infecting strain originated from the commensal strain carried into the hospital by the patient. Indeed, a variety of strains rather than a single *C. albicans* strain may have become endemic in a particular hospital setting, leading to a variety of nosocomial isolate genotypes similar in diversity to the variety of genotypes of isolates carried as commensal organisms in healthy individuals. Therefore, similar levels of diversity (e.g., equal S_{AB} s) do not exclude the possibility that a variety of endemic hospital strains are responsible. A second study is therefore planned. In that study high-risk patients will be sampled prior to and after entering the hospital, and isolates from a control group of healthy individuals from the same geographical locale will be used for comparison. In the case of infants who acquire nosocomial infections in NICUs, *Candida* colonization must originate from either the mother or the hospital setting.

The proportion of patient isolate collections that included isolates with highly related but nonidentical patterns and that were therefore undergoing microevolution was 33%. This value is below the values of 66 and 55% previously observed for collections of commensal isolates and isolates that caused vaginitis, respectively (22). The difference may be due to the time frame of the study. Carriage of the same commensal strain usually continues for very long periods of time, and the same established strain is responsible for recurrent vaginal infections over periods of up to several years (22). Therefore, in both patients who carry commensal organisms and patients with recurrent yeast vaginitis, the colonizing strain has ample chance to diversify through microevolution. The lower figure for hospitalized patients suggests that the strains that colonize patients in the respective ICUs have not had ample time to diversify, supporting the idea either that they recently colonized their present hosts or that one commensal substrain recently dominated the colonizing population.

Possibility of endemic BSI strains in ICUs. The average S_{AI} for BSI isolates in each of the four test hospitals was similar to that for the unrelated control collection and could therefore be interpreted to support the conclusion that the isolates from

TABLE 3. Highly related BSI and HCW isolates collected from the same hospitals within 2 months of each other

Hospital	BSI patient	HCW	Type of ICU		S_{AB}	Time (no. of days before or after) isolation from HCW
			BSI patient	HCW		
A	P2	HW5RN	N	N	0.94	-9
	P3	HW7RN	N	N	0.89	-1
B	P11	HW35RN	S	S	0.97	+1
	P21	HW29RN	S	S	0.94	+5
	P24	HW37TE	N	N	0.90	+27
	P12	HW38RN	N	N	1.00	+62
	P22	HW31RT	S	S	0.87	+2
D	P5	HW14RN	S	S	0.88	+62
	P20	HW24RN	N	N	0.96	+33
	P7	HW23RT	N	N	1.00	+1
	P9	HW27RN	N	N	0.88	+4
		HW8RN	N	S	0.91	+19
		HW10RN	N	S	0.93	+19
		HW9CL	N	S	0.91	+19

" Time before (-) isolation of the BSI isolate or time after (+) isolation of the BSI isolate.

patients with candidemia in each hospital collection were unrelated and therefore did not emanate from the hospital environment. However, there were more isolates in clusters in three of the four hospital collections than in the control collection of BSI isolates. Therefore, while no single strain was responsible for the majority of nosocomial BSIs in any of the ICUs of the four hospitals in this study, BSI isolates showed more group relationships, on average. The latter point is reinforced by two additional observations. First, the collections of BSI isolates from hospitals A and D each contained a pair of identical isolates from different patients, while no identical isolates emerged in the collection of 29 unrelated control BSI isolates. Second, hospital D contained a cluster of 4 BSI isolates defined at an S_{AB} threshold of 0.90 that represented 33% of isolates from that hospital, while the largest cluster in the control BSI collection defined at that threshold contained 3 of 29 isolates, which represented only 10% of the collection. Schmid et al. (49) obtained similar results in an analysis of surveillance isolates from 32 patients in different wards of a hospital in New Zealand. Using Ca3 fingerprinting to analyze relatedness, they found that isolates in each ward were, on average, more highly related than isolates in general.

Strong relationships exist between BSI isolates and isolates obtained from the hands of HCWs. The isolates from the hands of HCWs in each of the four hospitals exhibited cluster characteristics similar to those of BSI isolates from the respective hospitals. The same two hospitals, hospitals A and C, exhibited the highest average S_{AB} s for both BSI and HCW isolates, and hospital A had the highest proportion of both BSI and HCW isolates in clusters at an S_{AB} threshold of 0.86, 67 and 71%, respectively, compared to 52% for the control collection of unrelated BSI isolates. There were several additional cluster characteristics that suggested that the BSI isolates and HCW isolates were related. In the HCW isolate collection from hospital A, six of the seven isolates grouped in one cluster at an S_{AB} threshold of 0.85, suggesting that an endemic strain had cross-contaminated the hands of hospital coworkers over a period of approximately 1 year and that this strain had undergone significant microevolution. In a combined dendrogram of patient and HCW isolates from hospital A, the four BSI isolates that formed a major cluster in the dendrogram for the BSI isolates mixed with the isolates in the HCW cluster. Iso-

lates in the mixed cluster were distributed between the SICU and NICU of hospital A, suggesting a general endemic strain. Isolates in this mixed cluster represented 69% of isolates in the combined collection of isolates from hospital A. An additional cluster of isolates from one patient and one HCW isolate with a node at an S_{AB} of 0.95 also emerged in the dendrogram for the mixed collection of isolates, raising the proportion of isolates from hospital A in mixed clusters defined at an S_{AB} threshold of 0.85 to 85%. This value was significantly higher than the value of 59% obtained for the control collection of BSI isolates at the same S_{AB} threshold. Similar results were obtained in the dendrograms for the mixture of patient and HCW isolates from hospitals B and D. Mixed clusters defined at an S_{AB} threshold of 0.85 dominated each mixed dendrogram. The proportions of isolates in clusters defined at an S_{AB} threshold of 0.85 for collections of isolates from hospitals B and D were 80 and 74%, respectively; again, both values were significantly higher than the value of 59% obtained for the control collection.

For 11 BSI patients from the four hospitals, isolates that were collected from HCWs within a 2-month period were related to the BSI isolates at an S_{AB} threshold of 0.86. In all but one of these cases, the BSI and HCW isolates were obtained in the same ICU. For example, isolates from HW38RN and patient P12 had an S_{AB} of 1.00 and were collected 2 months apart in the same NICU, and isolates from HW4SU and P18 had an S_{AB} of 1.00 and were collected 1 year apart in the same NICU. In the case of patient P9 in the NICU of hospital D, related isolates with S_{AB} s of ≥ 0.90 were obtained 19 days later from HCWs in the SICU of the same hospitals. Fifteen days earlier, a related isolate had been collected from an HCW in the NICU, suggesting cross-contamination between HCWs in the alternative ICUs. The times of isolation of identical isolates from patients and HCWs in the same ICUs were sometimes separated by several months. For example, an isolate from HW4SU in the NICU and an identical isolate from patient P16 in the SICU of hospital A were collected 7 months apart. Clustering in the mixed dendrograms for isolates from each hospital demonstrated cross-contamination between the hands of HCWs and patients in the same hospitals. In 12 of the 14 cases in which related BSI and HCW isolates were collected within 2 months in the same hospital, the HCW

isolate was collected after collection of the BSI isolate. These results suggest that in the majority of cases, transmission is from a BSI patient to an HCW, but in a minority of documented cases, HCW isolates were collected up to several months prior to collection of highly related BSI isolates, suggesting transmission from an HCW to a patient.

Conclusion. By computing average S_{AB} s and comparing the clustering characteristics of BSI isolates in the test collections and a control collection of unrelated BSI isolates, we can conclude that in none of the four test hospitals was a single endemic strain responsible for the majority of BSIs in an ICU. However, the characteristics of clustering in dendrograms suggest that endemic strains may account for some infections, and comparisons between BSI and HCW isolates demonstrate a high degree of relatedness in many cases that can only be interpreted as cross-contamination. Our evidence suggests that cross-contamination occurs between patients and HCWs, between HCWs in the same ICU, and between HCWs in different ICUs of the same hospital. The temporal sequence of isolation in most cases supports the conclusion that HCWs are contaminated by isolates from infected or colonized patients, but in a minority of cases transmission appears to be from HCW to patient. Our results demonstrate that DNA fingerprinting with the complex probe Ca3 provides the resolution necessary for such studies and provides the framework necessary for development of a surveillance study that will address, first, the origins of nosocomial infections in NICUs, second, the relationship between commensal organisms carried by healthy individuals prior to hospitalization and subsequent infecting strains in SICUs, third, the impact of transfer from HCW to patient, and, fourth, the microevolution of endemic strains in hospital settings.

ACKNOWLEDGMENTS

This research was supported by a grant from Pfizer Inc., and by Public Health Service grants AI39735 and DE10758 from the National Institutes of Health (awarded to D.R.S.). S.R.L. was supported by training grant AG00214 from the National Institutes of Health. Francesc Marco was partially supported by a grant from Fondo de Investigaciones Sanitarias (FIS 97/5144) and a Permiso de Ampliación de Estudios from Hospital Clínic, Barcelona, Spain.

REFERENCES

- Akova, M., H. E. Akalin, Ö Uzun, M. Hayran, G. Tekuzman, E. Kansu, S. Aslan, and H. Telatar. 1994. Efficacy of fluconazole in the treatment of upper gastrointestinal candidiasis in neutropenic patients with cancer: factors influencing the outcome. *Clin. Infect. Dis.* 18:298-304.
- Anderson, J., T. Srikantha, B. Morrow, S. H. Miyasaki, T. C. White, N. Agabian, J. Schmid, and D. R. Soll. 1993. Characterization and partial sequence of the fingerprinting probe Ca3 of *Candida albicans*. *J. Clin. Microbiol.* 31:1472-1480.
- Arif, S., T. Barkham, E. G. Power, and S. A. Howell. 1996. Techniques for investigation of an apparent outbreak of infections with *Candida glabrata*. *J. Clin. Microbiol.* 34:2205-2209.
- Arnavielle, A., A. Blanchard, M. Mallie, M. Quilci, and J. M. Bastide. 1997. Multilocus enzyme electrophoresis analysis of *Candida albicans* isolates from three intensive care units. An epidemiological study. *Mycoses* 40:159-167.
- Backeljau, T., L. Debruyne, H. DeWolf, K. Jordaens, S. Van Dongen, and B. Winnepenninckx. 1996. Multiple UPGMA and neighbor-joining trees and the performance of some computer packages. *Mol. Biol. Evol.* 13:309-313.
- Bart-Delabesse, E., H. van Deventer, W. Goessens, J. L. Poirot, N. Lioret, A. van Belkum, and F. Dromer. 1995. Contribution of molecular typing methods and antifungal susceptibility testing to the study of a candidemia cluster in a burn care unit. *J. Clin. Microbiol.* 33:3278-3283.
- Beck-Sague, C. M., and W. R. Jarvis. 1993. National Nosocomial Infections Surveillance System. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. *J. Infect. Dis.* 167:1247-1251.
- Branchini, M. L., D. C. Geiger, O. Fischman, and A. C. Pignatari. 1995. Molecular typing of *Candida albicans* strains isolated from nosocomial candidemia. *Rev. Inst. Med. Trop. Sao Paulo* 37:483-487.
- Bross, J., G. H. Talbot, G. Maislin, S. Hurwitz, and B. L. Strom. 1989. Risk factors for nosocomial candidemia: a case-control study in adults without leukemia. *Am. J. Med.* 87:614-620.
- Buffington, J., R. Reporter, B. A. Lasker, M. M. McNeil, J. M. Lanson, L. A. Ross, L. Mascola, and W. R. Jarvis. 1994. Investigation of an epidemic of invasive aspergillosis: utility of molecular typing with the use of random amplified polymorphic DNA probes. *Pediatr. Infect. Dis. J.* 13:386-393.
- Burnie, J. P. 1986. *Candida* and hands. *J. Hosp. Infect.* 8:1-4.
- Burnie, J. P., R. Matthews, W. Lee, J. Philpott-Howard, R. Brown, Damani, N., J. Breuer, K. Honeywell, and Z. Jordans. 1987. Four outbreaks of nosocomial systemic candidiasis. *Epidemiol. Infect.* 99:201-211.
- D'Antonio, D., B. Violante, A. Mazzoni, T. Bonfini, M. A. Capuani, F. D'Aloia, A. Iacone, F. Schioppa, and F. Romano. 1998. A nosocomial cluster of *Candida inconspicua* infections in patients with hematological malignancies. *J. Clin. Microbiol.* 36:792-795.
- Diekema, D. J., S. A. Messer, R. J. Hollis, R. P. Wenzel, and M. A. Pfaller. 1997. An outbreak of *Candida parapsilosis* prosthetic valve endocarditis. *Diagn. Microbiol. Infect. Dis.* 29:147-153.
- Doebbling, B. N., R. J. Hollis, H. D. Isenberg, R. P. Wenzel, and M. A. Pfaller. 1991. Restriction fragment analysis of a *Candida tropicalis* outbreak of sternal wound infections. *J. Clin. Microbiol.* 29:1268-1270.
- Doi, M., M. Homma, S. Iwaguchi, K. Horiba, and K. Tanaka. 1994. Strain relatedness of *Candida albicans* strains isolated from children with leukemia and their bedside parents. *J. Clin. Microbiol.* 32:2253-2259.
- Isenberg, H. D., V. Tucci, F. Cintron, C. Singer, G. S. Weinstein, and D. H. Tyras. 1989. Single-source outbreak of *Candida tropicalis* complicating coronary bypass surgery. *J. Clin. Microbiol.* 27:2426-2428.
- Karabinis, A., C. Hill, B. Leclercq, C. Tancrede, D. Baume, and A. Andre-mont. 1988. Risk factors for candidemia in cancer patients: a case-control study. *J. Clin. Microbiol.* 26:429-432.
- Khatib, R., M. C. Thirumoorthi, K. M. Riederer, L. Sturm, L. A. Oney, and J. Baran, Jr. 1998. Clustering of *Candida* infections in the neonatal intensive care unit: concurrent emergence of multiple strains simulating intermittent outbreaks. *Pediatr. Infect. Dis. J.* 17:130-134.
- Leenders, A., A. van Belkum, S. Janssen, S. de Marie, J. Kluytmans, J. Wielenga, B. Lowenberg, and H. Verbrugh. 1996. Molecular epidemiology of apparent outbreak of invasive aspergillosis in a hematology ward. *J. Clin. Microbiol.* 34:345-351.
- Lin, D., and P. F. Lehmann. 1995. Random amplified polymorphic DNA for strain delineation within *Candida tropicalis*. *J. Med. Vet. Mycol.* 33:241-246.
- Lockhart, S., J. J. Fritch, A. S. Meier, K. Schröppel, T. Srikantha, R. Galask, and D. R. Soll. 1995. Colonizing populations of *Candida albicans* are clonal in origin but undergo microevolution through C1 fragment reorganization as demonstrated by DNA fingerprinting and C1 sequencing. *J. Clin. Microbiol.* 33:1501-1509.
- Lockhart, S., B. Reed, C. Pierson, and D. R. Soll. 1996. Most frequent scenario for recurrent *Candida* vaginitis is strain maintenance with "sub-strain shuffling": demonstration by sequential DNA fingerprinting with probes Ca3, C1, and CARE2. *J. Clin. Microbiol.* 34:767-777.
- Lockhart, S., R., and D. R. Soll. Generation of species-specific DNA probes and their applications for analysis of fungal populations. In D. Coleman and K. Haynes (ed.), *Methods in molecular medicine series: medical mycology protocols*, in press. Humana Press, Totowa, N.J.
- Loudon, K. W., A. P. Coke, and J. P. Burnie. 1995. "Pseudoclusters" and typing by random amplification of polymorphic DNA of *Aspergillus fumigatus*. *J. Clin. Pathol.* 48:183-184.
- Moro, M. L., C. Maffei, E. Manso, G. Morace, L. Polonelli, and F. Biavasco. 1990. Nosocomial outbreak of systemic candidiasis associated with parenteral nutrition. *Infect. Control Hosp. Epidemiol.* 11:27-35.
- O'Connell, B., D. C. Coleman, D. Bennett, D. Sullivan, S. R. McCann, and C. T. Keane. 1995. An epidemiological study of *Candida* species infection in cancer patients using genetic fingerprinting and morphotyping. *J. Hosp. Infect.* 31:211-217.
- Pfaller, M., L. Cabezudo, F. Koontz, M. Bale, and R. Gingrich. 1987. Predictive value of surveillance cultures for systemic infection due to *Candida* species. *Eur. J. Clin. Microbiol.* 6:628-633.
- Pfaller, M. A. 1995. Epidemiology of fungal infections: current perspectives and future directions. *Clin. Infect. Dis.* 20:1525.
- Pfaller, M. A. 1995. Epidemiology of fungal infections: the promise of molecular typing. *Clin. Infect. Dis.* 20:1535-1539.
- Pfaller, M. A. 1996. Nosocomial candidiasis: emerging species, reservoirs and modes of transmission. *Clin. Infect. Dis.* 22:S89-S94.
- Pfaller, M. A., R. N. Jones, G. V. Doern, H. S. Sader, R. J. Hollis, and S. A. Messer. 1998. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and antifungal susceptibility of isolates collected in 1997 in the United States, Canada and South America for the SENTRY Program. *J. Clin. Microbiol.* 36:1886-1889.
- Pfaller, M. A., R. N. Jones, S. A. Messer, M. B. Edmund, R. P. Wenzel, and the SCOPE Participant Group. 1998. National surveillance of nosocomial blood stream infections due to species of *Candida* other than *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn. Microbiol. Infect. Dis.* 31:327-332.
- Pfaller, M. A., S. R. Lockhart, C. A. Pujol, J. A. Swails-Wenger, S. A. Messer, M. B. Edmund, R. N. Jones, R. P. Wenzel, and D. R. Soll. 1998. Hospital specificity, regional specificity and fluconazole resistance of *Candida albicans*.

- bloodstream isolates. *J. Clin. Microbiol.* 36:1518-1529.
35. Pfaller, M. A., S. A. Messer, A. Houston, M. S. Rangel-Frausto, T. Wilson, H. M. Blumberg, J. E. Edwards, W. Jarvis, M. A. Martin, H. C. Neu, L. Saiman, J. E. Patterson, J. C. Dibb, C. M. Roldan, M. G. Rinaldi, and R. P. Wenzel. 1998. National Epidemiology of Mycoses Survey: a multicenter study of strain variation and antifungal susceptibility among isolates of *Candida* species. *Diagn. Microbiol. Infect. Dis.* 31:289-296.
 36. Pujol, C., S. Joly, S. Lockhart, S. Noel, M. Tibayrenc, and D. R. Soll. 1997. Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive probe Ca3 for fingerprinting *Candida albicans*. *J. Clin. Microbiol.* 35:2348-2358.
 37. Pujol, C., S. Joly, B. Nolan, T. Srikantha, S. Lockhart, and D. R. Soll. Unpublished observations.
 38. Reagan, D. R., M. A. Pfaller, R. J. Hollis, and R. P. Wenzel. 1990. Characterization of the sequence of colonization and nosocomial candidemia using DNA fingerprinting and a DNA probe. *J. Clin. Microbiol.* 28:2733-2738.
 39. Reagan, D. R., M. A. Pfaller, R. J. Hollis, and R. P. Wenzel. 1995. Evidence of nosocomial spread of *Candida albicans* causing blood stream infection in a neonatal intensive care unit. *Diagn. Microbiol. Dis.* 21:191-194.
 40. Reef, S. A., B. A. Lasker, D. S. Butcher, M. M. McNeil, R. Pruitt, H. Keyserling, and W. R. Jarvis. 1998. Nonperinatal nosocomial transmission of *Candida albicans* in a neonatal intensive care unit: prospective study. *J. Clin. Microbiol.* 36:1255-1259.
 41. Richet, H. M., A. Andremon, C. Tancrede, J. L. Pico, and W. R. Jarvis. 1991. Risk factors for candidemia in patients with acute lymphocytic leukemia. *Rev. Infect. Dis.* 13:211-215.
 42. Robert, F., F. Lebreton, M. E. Bougnoux, A. Paugam, D. Wassermann, M. Schlotterer, C. Tourte-Schaefer, and J. Dupouy-Camet. 1995. Use of random amplified polymorphic DNA as a typing method for *Candida albicans* in epidemiological surveillance of a burn unit. *J. Clin. Microbiol.* 33:2366-2371.
 43. Rosner, B. 1995. *Fundamentals of biostatistics*. Duxbury Press, Belmont, Calif.
 44. Ruiz-Diez, B., V. Martinez, M. Alvarez, J. L. Rodriguez-Tudela, and J. V. Martinez-Suarez. 1997. Molecular tracking of *Candida albicans* in a neonatal intensive care unit: long-term colonizations versus catheter-related infections. *J. Clin. Microbiol.* 35:3032-3036.
 45. Sadhu, C., M. J. McEachern, E. P. Rustchenko-Bulgac, J. Schmid, D. R. Soll, and J. Hicks. 1991. Telomeric and dispersed repeat sequences in *Candida* yeasts and their use in strain identification. *J. Bacteriol.* 173:842-850.
 46. Sanchez, V., J. A. Vazquez, D. Barth-Jones, L. Dembry, J. D. Sobel, and M. J. Zervos. 1992. Epidemiology of nosocomial acquisition of *Candida lusitanae*. *J. Clin. Microbiol.* 30:3005-3008.
 47. Sanchez, V., J. A. Vazquez, D. Barth-Jones, L. Dembry, J. D. Sobel, and M. J. Zervos. 1993. Nosocomial acquisition of *Candida parapsilosis*: an epidemiologic study. *Am. J. Med.* 94:577-582.
 48. Schmid, J., E. Voss, and D. R. Soll. 1990. Computer-assisted methods for assessing *Candida albicans* strain relatedness by Southern blot hybridization with repetitive sequence Ca3. *J. Clin. Microbiol.* 28:1236-1243.
 49. Schmid, J., Y. P. Tay, L. Wan, M. Carr, D. Parr, and W. McKinney. 1995. Evidence for nosocomial transmission of *Candida albicans* obtained by Ca3 fingerprinting. *J. Clin. Microbiol.* 33:1223-1230.
 50. Schroepel, K., M. Rotman, R. Galask, K. Mac, and D. R. Soll. 1994. The evolution and replacement of *C. albicans* strains during recurrent vaginitis demonstrated by DNA fingerprinting. *J. Clin. Microbiol.* 32:2646-2654.
 51. Sherer, R. J., K. S. Gledhill, K. D. Hampton, M. A. Pfaller, L. B. Givner, J. S. Abramson, and R. G. Dillard. 1992. Outbreak of *Candida* blood stream infections associated with retrograde medication administration in a neonatal intensive care unit. *J. Pediatr.* 120:455-461.
 52. Sneath, P., and R. Sokal. 1973. *Numerical Taxonomy. The principles and practice of numeric classification*. W. H. Freeman & Co., San Francisco, Calif.
 53. Soll, D. R. The "ins and outs" of DNA fingerprinting the infectious fungi. Unpublished data.
 54. Soll, D. R., R. Galask, S. Isley, T. V. G. Rao, D. Stone, J. Hicks, J. Schmid, K. Mac, and C. Hanna. 1989. "Switching" of *Candida albicans* during successive episodes of recurrent vaginitis. *J. Clin. Microbiol.* 27:681-690.
 55. Soll, D. R., R. Galask, J. Schmid, C. Hanna, K. Mac, and B. Morrow. 1991. Genetic dissimilarity of commensal strains carried in different anatomical locations of the same healthy women. *J. Clin. Microbiol.* 29:1702-1710.
 56. Soll, D. R., M. Staebell, C. J. Langtimm, M. A. Pfaller, J. Hicks, and T. V. G. Rao. 1988. Multiple *Candida* strains in the course of a single systemic infection. *J. Clin. Microbiol.* 26:1448-1459.
 57. Strausbaugh, L. J., D. L. Sewell, R. C. Tjoelker, T. Heitzman, T. Webster, T. T. Ward, and M. A. Pfaller. 1996. Comparison of three methods for recovery of yeasts from hands of health care workers. *J. Clin. Microbiol.* 34:471-473.
 58. Tibayrenc, M., K. Neubauer, C. Barnabé, F. Guerrini, D. Skaresky, and F. J. Ayala. 1993. Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proc. Natl. Acad. Sci. USA* 90:1335-1339.
 59. Vazquez, J. A., D. Boikov, S. G. Boikov, and A. S. Dajani. 1997. Use of electrophoretic karyotyping in the evaluation of *Candida* infections in a neonatal intensive-care unit. *Infect. Control Hosp. Epidemiol.* 18:32-37.
 60. Voss, A., R. J. Hollis, M. A. Pfaller, R. P. Wenzel, and B. N. Doebbling. 1994. Investigation of the sequence of colonization and candidemia in non-neutropenic patients. *J. Clin. Microbiol.* 32:975-980.
 61. Walsh, T. J., and W. G. Merz. 1986. Pathologic features in the human alimentary tract associated with invasiveness of *Candida tropicalis*. *Am. J. Clin. Pathol.* 85:498-502.
 62. Warren, N. G., and K. C. Hazen. 1995. *Candida*, *Cryptococcus*, and other yeasts of medical importance, p. 723-737. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.), *Manual of clinical microbiology*, 6th ed. American Society for Microbiology, Washington D.C.
 63. Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel. 1989. Risk factors for hospital-acquired candidemia: a matched case-control study. *Arch. Intern. Med.* 149:2349-2353.

V.- RESULTATS i DISCUSSIÓ

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OBJECTIU 1. Avaluar l'activitat in vitro d'un nou antifúngic azòlic (voriconazol) i dues equinocandines (casposfungina, anidulafungina) enfront de diferents espècies de *Candida* aïllades en hemocultius

Articles 1,2 i 3.

In vitro activities of voriconazole (UK-109,496) and four other antifungal agents against 394 clinical isolates of *Candida* spp. Antimicrob Agents Chemother 1998; 42:161-163.

Activity of MK-0991 (L-743,872), a new echinocandin, compared with those of LY303366 and four other antifungal agents tested against blood stream isolates of *Candida* spp. Diagn Microbiol Infect Dis. 1998;31:33-37

Trends in frequency and in vitro susceptibilities to antifungal agents, including voriconazole and anidulafungin, of *Candida* bloodstream isolates. Results from a six years study (1996-2001). Diagn Microbiol Infect Dis. (enviat a publicar)

En els darrers anys hem assistit a un increment en la incidència de les infeccions fúngiques invasives, no tant sols en quan al nombre, sino també en el tipus d'agents implicats. Una clara conseqüència d'aquesta situació ha estat la necessitat de disposar de nous antifúngics per tractar aquestes infeccions i evitar els inconvenients dels antifúngics actuals, com la toxicitat (amfotericina B), espectre d'activitat limitat (fluconazol), problemes de biodisponibilitat (itraconazol) o el desenvolupament de resistències amb certa facilitat si s'administren sols (5-fluorocitosina).

En el nostre treball recerca hem tingut l'oportunitat d'estudiar l'activitat in vitro de tres nous antifúngics. El primer d'ells, el voriconazol, un nou derivat

triazòlic del fluconazol que properament es comercialitzarà en el nostre país. La capacitat d'inhibir la 14 α -demetilasa de lisats de *C. albicans* i *A. fumigatus* és de 1.6 a 160 cops superior a la de fluconazol (Hitchcock et al, 1995). Es caracteritza per un espectre d'activitat ampli que abarca *Candida* spp, *Aspergillus* spp, *C. neoformans*, fongs dimòrfics, i fongs emergents com algunes espècies de *Fusarium* i *Penicillium marneffeii*. Els altres dos compostos estudiats són dues equinocandines, la caspofungina (coneguda anteriorment com L-743,872 i MK-0991) i l'anidulafungina (coneguda previament com LY303366 i V echinocandin). Totes dues actüen inhibint de forma no competitiva l'enzim (1,3)- β -D-glucà sintasa i manifesten una potent activitat enfront de *Candida* spp, *Aspergillus* spp, *Histoplasma capsulatum* i *Pneumocystis carinii*. No tenen activat sobre *C. neoformans*.

L'activitat in vitro del voriconazol, anidulafungina i caspofungina es va avaluar enfront d'una col·lecció de 394 (voriconazol) i 400 (anidulafungina i caspofungina) aïllats de *Candida* spp procedents d'hemocultius de 31 institucions hospitalàries. Els dos primers antifúngics, voriconazol i anidulafungina, també els hem estudiat amb els 218 aïllats de *Candida* spp recuperats en els hemocultius practicats a l'Hospital Clínic en el període de 1996 a 2001.

Per determinar la CMI es va fer servir en els tres estudis el mètode descrit en el document M27A del NCCLS. En la nostra experiència creiem que és un mètode amb una elevada reproductibilitat i que va permetre el creixement de tots els aïllats estudiats. És cert però, que en determinats aïllats el creixement a les 24 h és escàs. Per determinar la CMI del voriconazol es va seguir les recomanacions que el document dóna per els altres azols (fluconazol i itraconazol). Es va considerar que la CMI de les dues equinocandines corresponia a aquella concentració en la que es produïa una inhibició total del creixement.

En una anàlisi global dels resultats de les CMI's obtingudes, el voriconazol va demostrar ser molt actiu enfront de totes les espècies de *Candida* estudiades, amb una CMI₉₀ de 0.5 µg/ml (article 1) i 0.25 µg/ml (aïllats de l'Hospital Clínic). L'activitat enfront de *C. albicans* és remarcable, amb una CMI₉₀ de 0.03-0.06 µg/ml. Només hem trobat una soca (< 1%) al nostre hospital, entre les 91 estudiades, amb una CMI de 8 µg/ml que caldria considerar-la com a resistent. En canvi, entre les 206 del primer article, 7 aïllats (3.4%) tenien una CMI > 16 µg/ml. L'activitat enfront de *C. parapsilosis* va ser clarament superior a la de fluconazol (32 cops més actiu) i itraconazol (2-8 cops més actiu) i no hem detectat cap soca amb una CMI > 1 µg/ml. Enfront de *C. tropicalis* es comporta com a 4 cops més actiu que l'itraconazol i de 16 a 32 cops més actiu que el fluconazol. Una de les soques de *C. tropicalis* aïllades al nostre centre va manifestar una CMI de 8 µg/ml i tres dels aïllats del primer treball una CMI ≥ 4 µg/ml.

L'activitat del voriconazol enfront de les espècies amb sensibilitat disminuïda al fluconazol (*C. glabrata*) o intrínsecament resistents al fluconazol (*C. krusei*) és elevada. En *C. glabrata*, la CMI₉₀ va ser de 0.5 a 1 µg/ml, de dos a 4 cops més actiu que l'itraconazol i 64 cops més actiu que el fluconazol. En el nostre centre no hem trobat aïllats amb una CMI > 1 µg/ml, en canvi en el primer article, en vàrem detectar 8. Tots els aïllats de *C. krusei* es van inhibir amb una CMI ≤ 1 µg/ml.

Estem davant d'un nou compost triazòlic amb una potent activitat in vitro, clarament més actiu que el fluconazol i l'itraconazol, però que amb la nostra experiència, quan la soca manifesta resistència al fluconazol (≥ 64 µg/ml), amb l'excepció de *C. krusei*, les CMI's també són elevades indicant que probablement es veu afectat pels mateixos mecanismes de resistència que el fluconazol. Hi ha però

autors (Barry et al, 1996; Ruhnke et al, 1997) que han trobat que el voriconazol pot ser actiu en soques de *C. albicans* resistents al fluconazol ($\geq 64 \mu\text{g/ml}$).

Les dues equinocandines estudiades (caspofungina i anidulafungina) han demostrat tenir una elevada activitat in vitro enfront de les diferents espècies de *Candida* estudiades, amb alguna excepció que comentarem. La CMI90 de la caspofungina enfront de *C. albicans*, *C. glabrata* i *C. tropicalis* se situa en un interval de 0.12 a 0.25 $\mu\text{g/ml}$. La corresponent CMI90 de l'anidulafungina per als mateixos aïllats d'aquestes espècies va ser lleugerament superior amb un interval de 0.25 a 0.5 $\mu\text{g/ml}$. Els resultats que hem obtingut amb l'estudi de l'activitat de l'anidulafungina amb els aïllaments d'aquestes tres espècies a l'Hospital Clínic són molt similars, amb un interval de CMIs90 de 0.12 a 0.25 $\mu\text{g/ml}$. Les CMIs de les dues equinocandines en *C. krusei* són lleugerament més elevades amb un interval de 0.12 a 1 $\mu\text{g/ml}$ per als dos compostos (article 2) i 0.12 a 0.5 $\mu\text{g/ml}$ per l'anidulafungina amb els aïllats del nostre centre. Les CMIs de *C. parapsilosis* són més elevades si ho comparem amb l'activitat que tenen sobre les altres espècies. Aquesta es una característica també observada amb una altra equinocandina, la micafungina (Tawara et al, 2000), però que és més manifesta amb l'anidulafungina (CMI90 $>2 \mu\text{g/ml}$). Això podria representar un inconvenient en els tractaments amb aquest derivat ja que les dades farmacocinètiques d'aquest fàrmac indiquen que els nivells assolits a sang són propers als valors de les CMIs obtingudes en *C. parapsilosis* (Brown et al, 2000).

Com era d'esperar pel seu mecanisme d'acció, les dues equinocandines van demostrar tenir la mateixa activitat amb independència de si els aïllats eren sensibles o resistents als compostos azòlics (fluconazol, itraconazol i voriconazol). En resum, tant la caspofungina com l'anidulafungina, són dos antifúngics amb un futur prometedor en el tractament de les candidèmies.

OBJECTIU 2. Conèixer la freqüència de les diferents espècies de *Candida* responsables de candidèmia en el nostre medi (1996-2001) i la seva sensibilitat als antifúngics, incloent-hi els nous compostos voriconazol i anidulafungina.

Article 3.

Trends in frequency and in vitro susceptibilities to antifungal agents, including voriconazole and anidulafungin, of *Candida* bloodstream isolates. Results from a six years study (1996-2001). *Diagn Microbiol Infect Dis.* (enviat a publicar)

Al llarg d'un període de sis anys, des de 1996 fins el 2001, hem tingut l'oportunitat d'estudiar les diferents espècies de *Candida* aïllades en els hemocultius practicats als malalts ingressats a l'Hospital Clínic. El nombre total de soques aïllades ha estat de 218. Globalment, l'espècie més freqüent va ser *Candida albicans* amb un 41.7% dels casos, seguida de *C. parapsilosis* (22%), *C. tropicalis* (16.1%), *C. glabrata* (11.9%), *C. krusei* (6%) i altres espècies (2.3%). A la taula 1 es resumeix el nombre de soques aïllades per any i els percentatges corresponents.

Taula 1.

Espècies	Nombre d'aïllats (%) per any						
	1996	1997	1998	1999	2000	2001	Total
<i>C. albicans</i>	16 (59.3)	12 (42.9)	12 (29.3)	18 (46.1)	15 (42.9)	18 (37.5)	91 (41.7)
<i>C. parapsilosis</i>	6 (22.2)	3 (10.7)	11 (26.8)	9 (23.1)	8 (22.9)	11 (22.9)	48 (22)
<i>C. tropicalis</i>	2 (7.4)	8 (28.6)	5 (12.2)	7 (18)	6 (17.1)	7 (14.6)	35 (16.1)
<i>C. glabrata</i>	2 (7.4)	3 (10.8)	9 (21.9)	3 (7.7)	4 (11.4)	5 (10.4)	26 (11.9)
<i>C. krusei</i>	1 (3.7)	1 (3.6)	4 (9.8)	2 (5.1)	2 (5.7)	3 (6.3)	13 (6)
<i>Candida spp</i>	-	1 ^a (3.6)	-	-	-	4 ^b (8.3)	5 (2.3)
Totes	27	28	41	39	35	48	218

a) *C. guilliermondii*; b) *C. guilliermondii* and *C. famata* (un aïllat cada una), *Candida spp* (dos aïllats).

Hi ha diversos aspectes a destacar sobre els resultats obtinguts. En primer lloc, cal fer esment que des de l'any 1997, els percentatges de candidèmia atribuïbles a *C. albicans* en el nostre centre se situen en valors inferiors al 50%. Un altre fet a comentar és que *C. parapsilosis* s'ha situat en segon lloc, després de *C. albicans*, com agent etiològic responsable de candidèmia i que *C. glabrata* i *C. krusei* se situen en quart i cinquè lloc després de *C. tropicalis*. Algunes d'aquestes observacions també han estat comunicades per altres autors. Nguyen i cols descriuen en un estudi multicèntric prospectiu realitzat entre els anys 1990 i 1994, que en la segona part del període estudiat, la incidència de candidèmia per espècies de *Candida* no-*albicans* va ser superior a la de *C. albicans* (Nguyen et al, 1996). Les dades corresponents a l'any 1996 d'un hospital de Madrid indiquen que només el 26.8% de les fungèmies eren degudes a *C. albicans* (Muñoz P, 1997). En un altre estudi multicèntric realitzat a Espanya entre els mesos d'abril i juny de l'any 1997, es van analitzar els agents etiològics de 153 episodis de fungèmia per llevats. Les dades aportades pels diferents hospitals participants indiquen que *C. albicans* i *C. parapsilosis* van ser responsables del 38.6% i 34.6% dels casos, respectivament (Rodríguez-Tudela et al, 1999). Més recentment, en un ampli estudi que forma part del programa SENTRY realitzat durant els anys 1997 i 1999, la candidèmia per *C. albicans* va ser la més freqüent als Estats Units, Canadà, Europa i l'Amèrica Llatina, tot i que, en aquesta darrera zona geogràfica, va ser superada per la suma de les espècies no-*albicans* (Pfaller et al, 2001). De fet, les dades corresponents a aquesta darrera zona geogràfica són bastant similars a les del nostre centre (Pfaller, et al, 2001). En relació a les dades globals corresponents als hospitals europeus participants s'objectiva una major proporció de candidèmia atribuïble a *C. albicans* que no pas en el nostre centre: 58% vs 39.4%.

Les diferències observades entre centres, països o àries geogràfiques són difícils d'explicar perquè poden estar implicades diverses raons. La utilització de compostos azòlics, principalment fluconazol, en pautes de profilaxi s'ha relacionat amb un augment d'infeccions per *C. glabrata* i *C. krusei* (Wingard et al, 1991;

Wingard et al, 1993; Abi-Said et al, 1997). En el nostre centre, la utilització de fluconazol o itraconazol com a profilaxi de les infeccions fúngiques en malalts amb immunodepressió està ben establerta des de fa molts anys. Els percentatges d'aïllaments de *C. glabrata* i *C. krusei* d'aquests sis anys és d'un 17.9% si englobem les dues espècies. La major part dels malalts en què es va aïllar *C. glabrata* i en tots els casos de *C. krusei* rebien o havien rebut pautes profilàctiques amb azols. De totes formes, els valors finals estan influenciats pel baix nombre d'aïllats recuperats cada any i pels valors elevats de l'any 1998. No obstant, és una situació que caldrà vigilar-ne la seva evolució en els propers anys. En l'augment del nombre d'infeccions per *C. parapsilosis* s'han implicat la utilització d'alimentació parenteral, problemes amb el manteniment dels catèters o l'incumpliment de les normes habituals de prevenció de les infeccions nosocomials (Levin et al, 1998). És molt probable que aquests dos últims factors siguin els més importants en el nostre centre i que altres casos, com els atribuïbles a contaminacions de l'alimentació parenteral siguin fets puntuals o anecdòtics, donat que els controls microbiològics periòdics que es realitzen d'aquestes sol.lucions sempre han estat negatius per aquest microorganisme. Un altre aspecte a comentar, sobre tot en el cas d'estudis multicèntrics i què pot influir en la interpretació dels resultats, és què el nombre d'aïllaments obtinguts o comunicats pels diversos centres participants sol ser molt variable. Crida l'atenció, al menys pel que fa referència als hospitals espanyols integrats al projecte SENTRY amb unes característiques similars a les del nostre centre, que el nombre d'aïllaments de *Candida* spp obtinguts en un període de tres anys, des de l'any 1997 al 1999 és realment baix. En el primer hospital es comuniquen 21 aïllaments essent un 67% d'ells *C. albicans*; en el segon, el nombre total de soques és de 14 i un 29% de *C. albicans* i el tercer hospital no arriba als 10 aïllaments en tres anys (Pfaller, et al, 2001). En la nostra opinió creiem que cal ser molt prudent a l'hora de valorar aquestes dades donat què és molt improbable que s'hagin comunicat tots els aïllaments obtinguts.

Tot i què ja hem comentat l'activitat del voriconazol i l'anidulafungina al fer esment dels resultats dels treballs inclosos a l'objectiu I, cal remarcar diversos fets referents a l'activitat dels antifúngics amb els aïllats de candidèmia del nostre centre.

En el nostre hospital, durant el període de sis anys estudiat (1996-2001), la resistència al fluconazol dels aïllats de *C. albicans* procedents d'hemocultius és inferior a l'1%. La resistència a aquest antifúngic en *C. tropicalis* va ser del 3%. No es va detectar cap aïllat resistent en *C. parapsilosis*. Tot i què no es va trobar cap aïllat de *C. glabrata* resistent al fluconazol (CMI \geq 64 $\mu\text{g/ml}$), un 23% dels aïllaments van manifestar una CMI de 16-32 $\mu\text{g/ml}$. Voriconazol ha demostrat ser molt actiu enfront de totes les soques estudiades inhibint a una concentració igual o inferior a 1 $\mu\text{g/ml}$, el 100% dels aïllaments de *C. parapsilosis*, *C. glabrata* i *C. krusei* i el 99% i 97% dels aïllats de *C. albicans* i *C. tropicalis*, respectivament. Si la soca manifestava una CMI \geq 64 $\mu\text{g/ml}$ al fluconazol, les CMIs de voriconazol eren elevades (8 $\mu\text{g/ml}$). Anidulafungina va inhibir tots els aïllats estudiats a una CMI \leq 0.5 $\mu\text{g/ml}$, amb l'excepció de *C. parapsilosis* (CMI₉₀: 4 $\mu\text{g/ml}$ i *C. guilliermondii*, CMI: \geq 32 $\mu\text{g/ml}$)

OBJECTIU 3. Avaluar l'activitat in vitro de dos nous antifúngics azòlics (voriconazol, posaconazol) i dues equinocandines (caspofungina, anidulafungina) enfront diferents espècies de fongs filamentosos aïllades en mostres clíniques.

Articles 4, 5 i 6.

Antifungal activity of a new triazole, voriconazole (UK-109,496), compared with three other antifungal agents tested against clinical isolates of filamentous fungi. *Med Mycology*. 1998;36:433-436.

In vitro activity of a new triazole antifungal agent, Sch 56592, against clinical isolates of filamentous fungi. *Mycopathologia*. 1998;141:73-77.

In vitro activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus*, *Fusarium*, *Rhizopus*, and other filamentous fungi. *Diag Microbiol Infect Dis.* 1998;30:251-255

En aquests tres articles hem valorar l'activitat in vitro de dos triazols, voriconazol i posaconazol (conegut previament com Sch 56592) i dues equinocandines, caspofungina i anidulafungina. El posaconazol és un anàleg de l'itraconazol, però com a mínim, és 10 cops més potent inhibint l'enzim 14 α -demetilasa d'*A. fumigatus* i *A. niger* (Munayyer et al, 1996). El seu espectre d'activitat és molt similar al de voriconazol.

L'activitat in vitro d'aquests quatre antifúngics es va determinar enfront de diversos fongs filamentosos procedents de diferents aïllaments clínics. En total es van estudiar 51 aïllats distribuïts de la següent forma: *A. fumigatus* (12), *A. flavus* (10), *Fusarium oxysporum* (5), *Fusarium solani* (5), *Fusarium* spp (3), *Pseudallescheria boydii* (5), *Rhizopus* spp (6), *A. niger* (1), *A. terreus* (1), *Acremonium* spp (1), *Paecilomyces* spp (1) i *Trichoderma* spp (1).

Quan es va fer l'estudi, el NCCLS encara no havia publicat el document M28P, però per determinar les CMI's es van fer servir les recomanacions del document M27A (NCCLS M27A, 1997) i les del treball multicèntric publicat per Espinel-Ingroff i cols que va servir per la posterior el.laboració del document M28P (Espinel-Ingroff et al, 1997). La lectura de les CMI's es va realitzar a les 48 i 72 h. Tots el aïllats van tenir un creixement adequat a les 48 h, excepte l'únic aïllat de *Trichoderma* spp, que va necessitar 72 h. Per als triazols i equinocandines es va considerar que la CMI corresponia a aquella concentració en la que es produïa una reducció del creixement d'un 75% comparat amb el creixement del control.

Si analitzem els resultats globalment, els dos nous triazols va ser més potents in vitro que l'itraconazol (2 a 8 cops més actius). Les CMI's de posaconazol obtingudes en el conjunt d'aïllats d'*Aspergillus* spp (24) van ser inferiors a les de voriconazol en una o dues dilucions. Així, si analitzem l'interval de CMI's obtinguts en *A. flavus*, el de posaconazol va ser de 0.06 a 0.25 µg/ml i el de voriconazol de 0.12 a 0.5 µg/ml. En *A. fumigatus*, aquests valors van ser 0.12 a 0.25 µg/ml i 0.25 a 0.5 µg/ml, respectivament. Tots dos antifúngics van tenir una activitat escassa enfront de *Fusarium* spp (CMI₅₀: 2 µg/ml). Els resultats obtinguts amb el cinc aïllats de *P. boydii* ens indiquen que voriconazol va ser més actiu que posaconazol (CMI₅₀: 0.12 vs 1 µg/ml), en canvi, en el sis aïllats de *Rhizopus* spp va ser el posaconazol el més potent (CMI₅₀: 1 vs 8 µg/ml). Cal destacar que, en aquests darrers aïllats, l'activitat in vitro de l'itraconazol va ser igual a la de posaconazol i per tant, superior a la de voriconazol.

Els resultats d'aquestes CMI's corresponen a una incubació de 72 h i el NCCLS, en el seu document M28P, recomana fer la lectura amb una incubació de 48 h. En el nostre cas, si la valoració de les CMI's s'hagués realitzat a les 48 h, els resultats haurien estat els mateixos o bé, una dilució menys. De totes formes les dades que hem obtingut són similars a les comunicades per altres investigadors (Espinel-Ingroff et al, 1997; Espinel-Ingroff et al, 1998; McGinnis et al, 1997, Oakley et al, 1997; Radford et al, 1997).

Les dues equinocandines van demostrar tenir una bona activitat enfront d'*Aspergillus* spp amb una major potencia intrínseca de l'anidulafungina. En *A. fumigatus* i *A. flavus*, la CMI₉₀ d'aquest últim compost va ser de 0.06 i 0.03 µg/ml, respectivament i la de caspofungina, 0.12 µg/ml en les dues espècies. Cap dels dos fàrmacs va demostrar tenir activitat enfront de *Rhizopus* spp i *Fusarium* spp (CMI's >2 µg/ml). L'activitat sobre *P. boydii* va ser discreta amb una CMI₅₀ de 0.5 µg/ml

(casporfungina) i 1 µg/ml (anidulafungina). Amb aquests antifúngics, la lectura de les CMI a les 72 h va ser practicament la mateixa de la realitzada a les 48 h en quasi tots els aïllats. De totes formes, la determinació de la CMI amb les equinocandines, en especial amb els fongs filamentosos, no està completament resolta. Els criteris utilitzats per establir la CMI ha variat des d'una inhibició prominent del creixement ($\leq 50\%$ comparat amb el control) fins a un 80% d'inhibició. Es probable que aquests criteris infravalorin l'activitat de les equinocandines ja que, a concentracions inferiors a la de la CMI, s'observen alteracions en les hifes (hifes escurçades, més ramificades i amb extrems deformats) que podrien reflexar millor l'activitat in vitro dels compostos (Douglas et al, 2000; Kurtz et al, 1994). Per això, alguns autors proposen la utilització del concepte concentració mínima efectiva (CME), que correspondria a aquella concentració en la es veurien les modificacions de les hifes (Arikan et al, 2001).

OBJECTIU IV. Aval.luar l'aplicació de la sonda semirepetitiva *Ca3* en l'estudi del grau de similitud dels aïllats de *Candida albicans* en pacients amb candidèmia i ingressats en Unitats de Cures Intensives.

Article 7.

Elucidating the origins of nosocomial infections with *Candida albicans* by DNA fingerprinting with the complex probe Ca3. J Clin Microbiol 1999;37:2817-2828.

En aquest article es va aplicar un mètode genotípic, l'anàlisi de l'ADN després de la hibridació amb una sonda semirepetitiva i complexa, com és la Ca3, per avaluar una col.lecció d'aïllaments de *C. albicans* obtinguts durant un període de 2 anys en dues UCIs (neonatal i quirúrgica) de 4 hospitals. Es van estudiar 35 aïllats corresponents a 30 malalts que van presentar una candidèmia i 75 aïllats recuperats en 28 d'aquests pacients a partir de mostres d'orina, femta, secrecions respiratòries o suc gàstric abans, durant o després de l'episodi de candidèmia. A

més a més, es van incloure 42 aïllats obtinguts de les mans de personal sanitari de les mateixes UCIs. La sonda Ca3 ha demostrat ser molt útil per analitzar el grau de relació genètica i la possible microevolució dels aïllats de *C. albicans* (Pujol et al, 1997). Les imatges obtingudes després de la hibridació es van analitzar amb el programa informàtic DENDRON (Soll DR, 2000) i es va calcular el coeficient de similitud (S_{AB}) entre els aïllats. Un S_{AB} de 1.00 indica que les soques formen part del mateix clon; S_{AB} entre 0.90 i 0.99 indica un grau de similitud molt elevat i generalment indica microevolució d'una sola soca quan els aïllats són obtinguts en un mateix pacient; S_{AB} entre 0.80 i 0.89 indica una menor relació entre els aïllats, i un $S_{AB} < 0.75$ indicaria que els aïllats no tenen cap relació entre ells. Els valors obtinguts permeten generar de forma automàtica un dendrograma i averiguar el grau de similitud. Amb 29 *C. albicans* recuperades en hemocultius d'altres hospitals i que no tenien cap relació entre elles es va generar un dendrograma control amb un S_{AB} de 0.72 ± 0.10 . La finalitat d'aquest grup control va ser la de tenir un grup comparatiu que ens permetés estimar el grau o ausència de relació entre els aïllats de candidèmia de l'estudi.

Comparació entre l'aïllat de l'hemocultiu i aïllats comensals.

Al comparar els aïllats de l'hemocultiu i els recuperats en altres mostres es van produir diverses situacions. En les figura 1 de la pàgina següent en tenim alguns exemples. En alguns malalts, l'aïllat recuperat a l'hemocultiu i a altres mostres (femta, orina) era idèntic (exemple A); en altres (exemple B), alguns dels aïllats presentaven una petita variabilitat que només reflexa l'existència de microevolució i finalment, en els exemples C i D podem veure com poden coexistir dos tipus d'aïllats o "clusters", suggerint colonització per dues soques no relacionades. L'anàlisi combinat de tots els resultats indiquen que, en aproximadament el 90% dels malalts, els aïllats recuperats a la femta i orina abans o després de la candidèmia, eren similars o idèntics al recuperat a l'hemocultiu (S_{AB} 0.91 – 1.00). En un 33% dels malalts es van recuperar aïllats que no eren idèntics al

de l'hemocultiu però tenien un grau de similitud molt elevat (S_{AB} 0.91 – 0.99), la qual cosa suggereix la presència de microevolució.

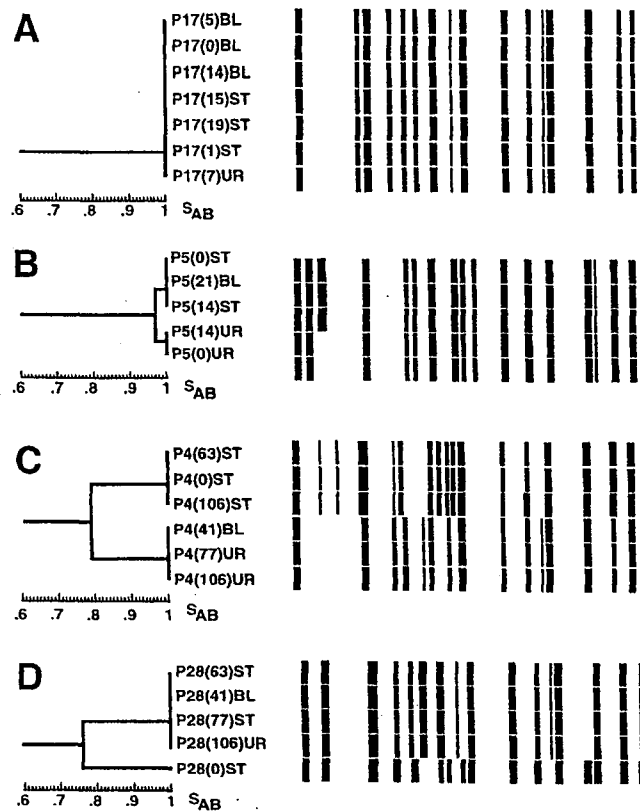


Figura 1. Exemples de dendrogrames dels aïllats obtinguts en un determinat pacient.

Relació genètica entre els aïllats responsables de candidèmia obtinguts en un mateix hospital.

Quan es van comparar els coeficients de similitud dels aïllats dels 4 hospitals amb el S_{AB} de les soques control es va veure que era similar, la qual cosa suportava la idea de que els aïllats dels malalts amb candidèmia de cada hospital no estaven relacionats i probablement no provenien del medi ambient hospitalari. No obstant, es va apreciar una major tendència a agrupar els aïllats que no pas amb la col.lecció control. En la figura 2 es mostren el dendrogrames obtinguts en els quatre hospitals (A, B, C i D).

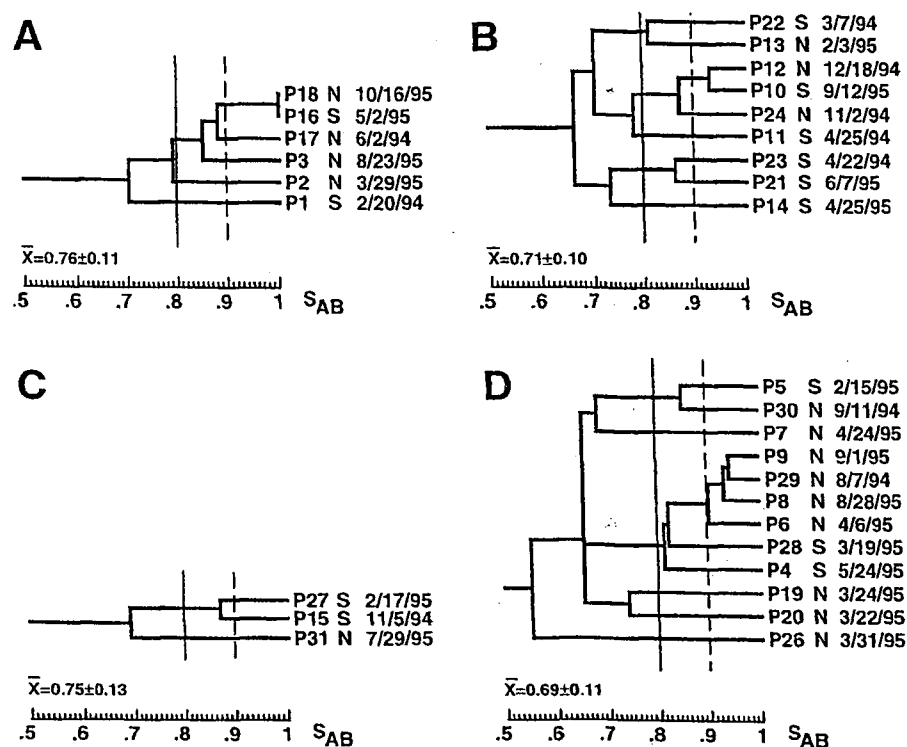


Figura 2. Dendrogrames dels aïllats recuperats als hemocultius en els quatre hospitals: A, B, C i D.

Relació genètica entre els aïllats recuperats en personal sanitari.

En la figura 3 es mostren els dendrogrames generats amb els aïllats procedents de les mans del personal sanitari. En dos hospitals (A i C) el S_{AB} generat amb els aïllats obtinguts va ser superior al del grup control, però només en l'hospital A va ser estadísticament significatiu ($p = 0.004$). Els dendrogrames generats amb els aïllats recuperats en el personal sanitari dels quatre hospitals tenien unes característiques similars als obtinguts amb els aïllats responsables de candidèmia. En aquest cas, però, es produeix una associació temporal en la recuperació d'aïllats en el personal sanitari. Així, en alguns casos, es va aïllar el mateix dia la mateixa soca o una altament relacionada, com va passar a l'hospital A, B i D. Aquests resultats demostren que es produeix transferència de soques entre personal sanitari o a partir del pacient al personal sanitari en una mateixa UCI.

Relació genètica entre els aïllats responsables de candidèmia i els recuperats en personal sanitari.

Per analitzar el grau de relació entre els aïllats de candidèmia i els del personal sanitari d'un mateix hospital es van generar dendrogrames mixtes incloent-hi un sol aïllat per pacient i treballador (Figura 4).

El coeficient de similitud obtingut a l'hospital A va ser $S_{AB} : 0.83 \pm 0.10$, significativament més elevat que el del grup control ($p = 0.002$). En aquest hospital es formen dos "clusters" utilitzant un valor de S_{AB} de 0.89. El cluster a, inclou dues soques (malalt i treballador sanitari) separades per nous dies de diferència. El "cluster" b conté 5 aïllats, dels quals tres són idèntics. És interessant comentar que aquests tres aïllats es van recuperar al llarg d'un període de temps d'un any, la qual cosa reforça la idea del establiment d'una soca endèmica.

A l'hospital B es van detectar 5 "clusters" amb un valor de S_{AB} de 0.90. El primer "cluster" (a) el van formar tres aïllats procedents de les mans del personal

sanitari i recuperats el mateix dia. Els tres “clusters” següents (b,c,d) estaven integrats per aïllaments d’un malalt i un treballador sanitari. El cinquè “cluster”, e, l’integraven dos aïllaments de dos malalts i un d’un treballador sanitari, separats per més d’un més de diferència entre ells. Un sisè “cluster” amb un de S_{AB} de 0.86 estava integrat pels aïllaments d’un malalt i un treballador sanitari recuperats amb dos dies de diferència.

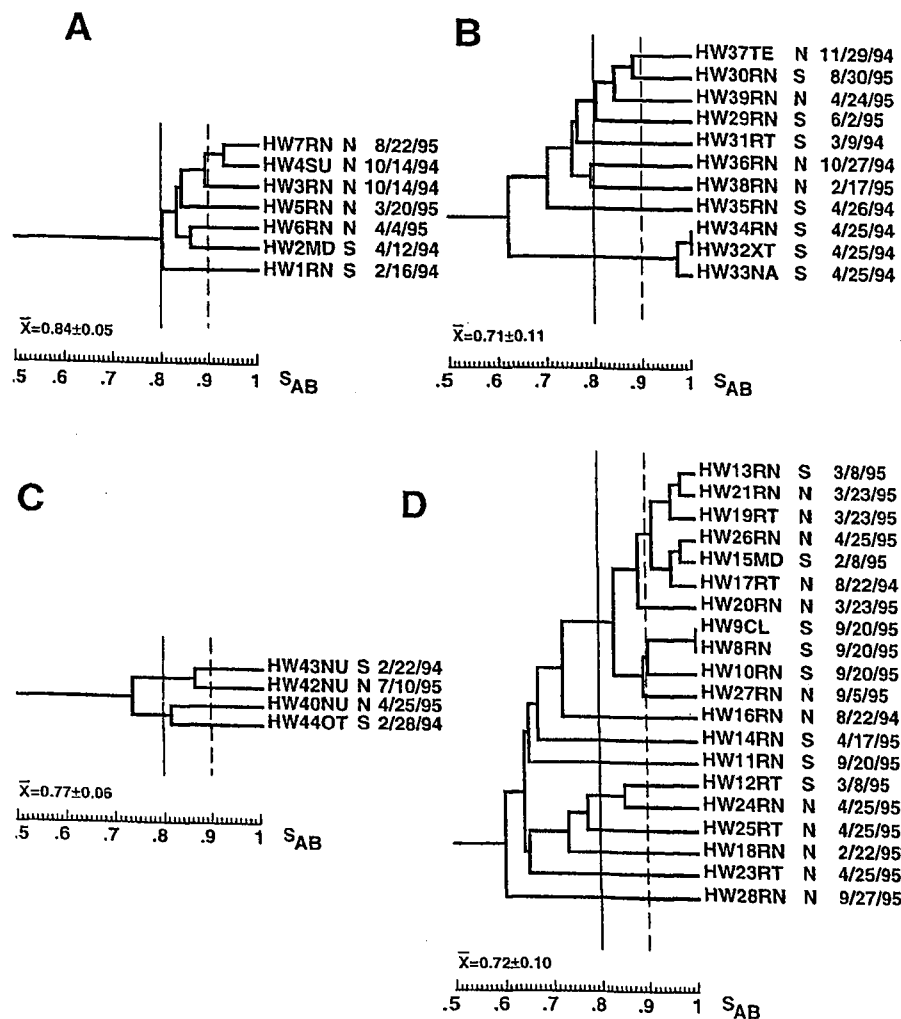


Figura 3. Dendrogrames dels aïllats recuperats en personal sanitari.

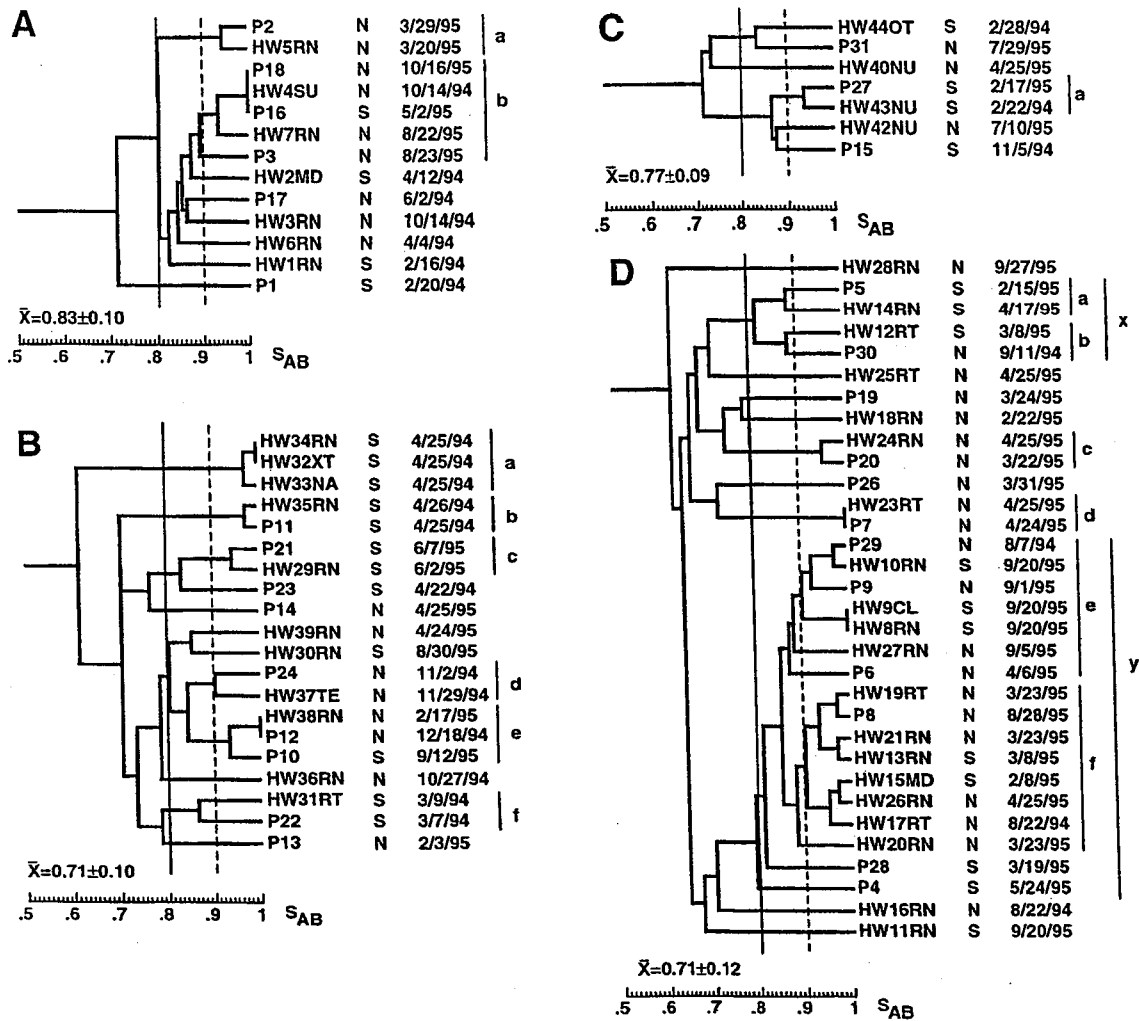


Figura 4. Dendrogrames combinats d'aïllats de candidèmia i del personal sanitari dels quatre hospitals (A, B, C i D)

A l'hospital C el dendrograma va demostrar l'existència d'un cluster amb dos aïllats. El procedent del treballador sanitari es va aïllar un any abans que el del pacient. A l'hospital D es van poder definir 6 "clusters" amb un S_{AB} de 0.88. Els quatre primers (a,b,c i d) estaven integrats cada un per un aïllat procedent d'un malalt i d'un treballador sanitari. El "cluster" e contenia aïllats de tres malalts i de quatre treballadors sanitaris. El "cluster" f el formaven 8 aïllats, 7 de treballadors sanitaris i 1 d'un malalt. L'anàlisi global d'aquests resultats suggereix que en la majoria dels casos, la transmissió es produeix del malalt cap al personal sanitari, pero en una minoria de casos les dades suggereixen que la transmissió és al contrari, del personal sanitari cap al malalt.

VI.- CONCLUSIONS

VI. CONCLUSIONS.

Objectiu 1.

1. Voriconazol és un nou antifúngic triazòlic amb una elevada activitat in vitro enfront de totes les espècies del gènere *Candida*, incloent-hi *C. glabrata* i *C. krusei* (CMI90 de $\leq 0.5 \mu\text{g/ml}$). Les nostres dades suggereixen que els mecanismes que confereixen resistència al fluconazol també afecten al voriconazol, ja que CMIs $\geq 64 \mu\text{g/ml}$ de fluconazol es corresponen amb CMIs $\geq 2 \mu\text{g/ml}$ de voriconazol.

2. Anidulafungina i caspofungina són dues equinocandines igualment actives enfront de *Candida* spp. La seva activitat és més reduïda enfront dels aïllats de *C. parapsilosis*, sobre tot en anidulafungina (CMI90: $> 2 \mu\text{g/ml}$).

Objectiu 2.

3. En els darrers sis anys (1996-2001), l'espècie aïllada amb més freqüència en els episodis de candidèmia diagnosticats en el nostre centre ha estat *C. albicans*. En segon lloc s'ha situat *C. parapsilosis* i a continuació, per ordre de freqüència, *C. tropicalis*, *C. glabrata* i *C. krusei*. En els darrers cinc anys (1997-2001), el percentatge de candidèmia atribuïda a espècies no-*albicans* ($> 50\%$) va superar al de *C. albicans*.

4. En el nostre medi, la sensibilitat al fluconazol dels aïllats de *C. albicans*, *C. parapsilosis* i *C. tropicalis* procedents d'hemocultius (80% del total) és del 94 al 100% (1996-2001). A una concentració $\leq 1 \mu\text{g/ml}$, la sensibilitat al voriconazol va ser del 97 al 100%.

Objectiu 3.

5. Tant voriconazol com posaconazol van demostrar tenir una bona activitat in vitro enfront d'*Aspergillus* spp (CMI₉₀: ≤ 0.5 i ≤ 0.25 $\mu\text{g/ml}$, respectivament). Voriconazol va ser més actiu que posaconazol enfront de *P. boydii* (CMI₅₀: 0.12 vs 1 $\mu\text{g/ml}$), en canvi, posaconazol i itraconazol van demostrar ser més potents que voriconazol enfront de *Rhizopus* spp (CMI₅₀: 1 vs 8 $\mu\text{g/ml}$).

6. Les dues equinocandines van demostrar la seva efectivitat inhibint tots els aïllats d'*Aspergillus* spp, amb una major potencia intrínseca de l'anidulafungina (CMI₉₀: ≤ 0.06 $\mu\text{g/ml}$) respecte a la caspofungina (CMI₉₀: 0.12 $\mu\text{g/ml}$). Cap de les dues va demostrar activitat enfront de *Fusarium* spp i *Rhizopus* spp.

Objectiu 4.

7. En cap dels quatre hospitals una única soca va ser la responsable de les candidèmies per *C. albicans* en les UCIs estudiades.

8. Les característiques d'agrupació en els dendrogrames suggereixen que determinades soques endèmiques poden ser les responsables d'algunes infeccions. La comparació entre els aïllats dels hemocultius i els del personal sanitari demostra en molts casos un elevat grau de similitud que només pot interpretar-se com una contaminació creuada.

9. El nostre estudi suggereix que la contaminació creuada té lloc entre els malalts i el personal sanitari, entre el personal sanitari d'una mateixa UCI i fins i tot, entre el personal sanitari de diferents UCIs del mateix hospital.

10. La seqüència temporal d'aïllament suporta la conclusió que el personal sanitari es contamina amb els aïllats dels malalts infectats o colonitzats, però en una minoria de casos la transmissió sembla que es produeix del personal sanitari al pacient.

11. La utilització de la sonda Ca3 com a mètode genotípic de tipificació i posterior anàlisi de les imatges amb un programa informàtic és d'enorme utilitat per analitzar problemes epidemiològics complexos com és l'origen de les infeccions nosocomials.

VII.- BIBLIOGRAFIA

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Alexopoulos CJ, Mims CW, Blackwell M. Introductory Mycology, 4th ed. John Wiley & Sons, Inc. New York, NY. 1996

Aller AI, Martin-Mazuelos E, Lozano F, Gómez-Mateos J, Steele-Moore L, Holloway WJ, Gutiérrez MJ, Recio FJ, Espinel-Ingroff A. Correlation of fluconazole MICs with clinical outcome in cryptococcal infection. *Antimicrob Agents Chemother.* 2000;44:1544-1548.

Anaissie E, Bodey GP. Nosocomial fungal infections: old problems and new challenges. *Infect Dis Clin North Am.* 1989;3:867-882.

Anaissie E. Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. *Clin Infect Dis.* 1992;14(Suppl 1):43-53.

Anaissie EJ, Paetzniek VL, Ensign LG, Espinel-Ingroff A, Galgiani JN, Hitchcock CA, LaRocco M, Patterson T, Pfaller MA, Rex JH, Rinaldi MG. Microbroth antifungal susceptibility testing of *Candida albicans* and *Cryptococcus neoformans* with and without agitation: An eight-center collaborative study. *Antimicrob Agents Chemother.* 1996;40:2387-2391.

Anderson JM, Mihalik R, Soll DR. Ultrastructure and antigenicity of the unique cell wall "pimple" of the *Candida* opaque phenotype. *J Bacteriol.* 1990;172:224-235.

Anderson MJ, Gull K, Denning DW. Molecular typing by random amplification of polymorphic DNA and M13 southern hybridization of related paired isolates of *Aspergillus fumigatus*. *J Clin Microbiol.* 1996;34:87-93.

Andriole VT. Current and future antifungal therapy: new targets for antifungal agents. *J Antimicrob Chemother.* 1999;44:151-162.

Arikan S, Gur D, Akova M. Comparison of Etest, microdilution and colorimetric dilution with reference broth macrodilution method for antifungal susceptibility testing of clinically significant *Candida* species isolated from immunocompromised patients. *Mycoses.* 1997;40:291-296.

Arikan S, Lozano-Chiu M, Paetzniek V, Rex JH. In vitro susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. *Antimicrob Agents Chemother.* 2001;45:327-330.

Arnavielhe S, Blancard A, Mallie M, Quilici M, Bastide JM. Multilocus enzyme electrophoresis analysis of *Candida albicans* isolates from three intensive care units. *Mycoses.* 1997;40:159-167.

Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol.* 1999;37:3332-3337.

Arthington-Skaggs BA, Warnock DW, Morrison CJ. Quantitation of *Candida albicans* ergosterol content improves the correlation between in vitro antifungal susceptibility test results and in vivo outcome after fluconazole treatment in a murine model of invasive candidiasis. *Antimicrob Agents Chemother.* 2000;44:2081-2085.

Asakura K, Iwaguchi S, Homma M, Sukai T, Higashide K, Tanaka K. Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *C. glabrata*. *J Gen Microbiol.* 1991;137:2531-2538.

- Aufauvre-Brown A, Cohen J, Holden DW.** Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. *J Clin Microbiol.* 1992;30:2991-2993.
- Barchesi F, Hollis RJ, Del Poeta M, McGough DA, Scalise G, Rinaldi MG, Pfaller MA.** Transmission of fluconazole-resistant *Candida albicans* between patients with AIDS and oropharyngeal candidiasis documented by pulsed-field gel electrophoresis. *Clin Infect Dis.* 1995;21:561-564.
- Barchesi F, Hollis RJ, Messer SA, Scalise G, Rinaldi MG, Pfaller MA.** Electrophoretic karyotype and in vitro antifungal susceptibility of *Cryptococcus neoformans* isolates from AIDS patients. *Diag Microbiol Infect Dis.* 1995;23:99-103.
- Barry AL, Brown SD.** Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. *J Clin Microbiol.* 1996;34:2154-2157.
- Barry AL, Pfaller MA, Brown SD, Espinel-Ingroff A, Ghannoum MS, Knapp C, Rennie RP, Rex JH, Rinaldi MG.** Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. *J Clin Microbiol.* 2000;38:3457-3549.
- Beck-Sague CM, Jarvis WR, and the National Nosocomial Infections Surveillance System.** Secular trends in the epidemiology of nosocomial fungal infections in the United States. 1980-1990. *J Infect Dis.* 1993;167:1247-1251.
- Blumberg HM, Jarvis WR, Soucie JM, Edwards JE, Patterson JE, Pfaller MA, Rangel-Frausto MS, Rinaldi MG, Saiman L, Wiblin RT, Wenzel RP, and the NEMIS Study group.** Risk factors for *Candidal* bloodstream infections in surgical intensive care unit patients: The NEMIS prospective multicenter study. *Clin Infect Dis.* 2001;33:177-186.
- Bodey GP.** The emergence of fungi as major hospital pathogens. *J Hosp Infect.* 1988;11(Suppl A):411-426.
- Boekhout T, Van Belkum A, Leenders AC, Verbrugh HA, Mukamurangwa P, Swinne D, Scheffers WA.** Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. *Int J Syst Bacteriol.* 1997;47:432-442.
- Boerlin P, Boerlin-Petzold F, Goudet J, Durussel C, Pagani JL, Chave JP, Bille J.** Typing *Candida albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. *J Clin Microbiol.* 1996;34:1235-1248.
- Bostock A, Khattak MN, Matthews R, Burnie J.** Comparison of PCR fingerprinting by random amplification of polymorphic DNA, with other molecular typing methods for *Candida albicans*. *J Gen Microbiol.* 1993;139:2179-2184.
- Branchini ML, Pfaller MA, Rhine-Chalberg J, Frempong T, Isenberg HD.** Genotypic variation and slime production among blood and catheter isolates of *Candida parapsilosis*. *J Clin Microbiol.* 1994;32:452-456.
- Brandt ME, Bragg SL, Pinner RW.** Multilocus enzyme typing of *Cryptococcus neoformans*. *J Clin Microbiol.* 1993;31:2819-2823.

- Brandt ME, Hutwagner LC, Kuykendall, Pinner RW.** Comparison of multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis for molecular subtyping of *Cryptococcus neoformans*. Cryptococcal Disease Active Surveillance Group. **J Clin Microbiol.** 1995;33:1890-1895.
- Brandt ME, Hutwagner LC, Klug LA, Baughman WS, Rimland D, Graviss EA, Hamill RJ, Thomas C, Pappas PG, Reingold AL, Pinner RW.** Molecular subtype distribution of *Cryptococcus neoformans* in four areas of the United States. Cryptococcal Disease Active Surveillance Group. **J Clin Microbiol.** 1996;34:912-917.
- Brawner DL.** Comparison between methods for serotyping of *Candida albicans* produces discrepancies in results. **J Clin Microbiol.** 1991;29:1020-1025.
- Buesching WJ, Kurek K, Roberts GD.** Evaluation of the modified API 20C system for identification of clinically important yeasts. **J Clin Microbiol.** 1979;9:565-569.
- Buffington J, Reporter R, Lasker BA, McNeil MM, Lanson JM, Ross IA, Mascola L, Jarvis WR.** Investigation of an epidemic of invasive aspergillosis: utility of molecular typing with the use of random amplified polymorphic DNA probes. **Pediatr Infect Dis J.** 1994;13:386-393.
- Burnie JP, Coke A, Matthews RC.** Restriction endonuclease analysis of *Aspergillus fumigatus* DNA. **J Clin Pathol.** 1992;45:324-327.
- Cantón E, Peman J, Carrillo-Muñoz A, Orero A, Ubeda P, Viudes A, Gobernado M.** Fluconazole susceptibilities of bloodstream *Candida* spp isolates as determined by National Committee for Clinical Laboratory Standards method M27-A and two other methods. **J Clin Microbiol.** 1999;37:2197-2200.
- Casal M, Linares MJ.** Preliminary investigations of *Candida albicans* biovars. **J Clin Microbiol.** 1983;18:430-431.
- Chen SC, O'Donnell ML, Gordon S, Gilbert GL.** Antifungal susceptibility testing using the E test: comparison with the broth macrodilution technique. **J Antimicrob Chemother.** 1996;37:265-273.
- Chindamporn A, Nakagawa Y, Mizuguchi I, Chibana H, Dol M, Tanaka K.** Repetitive sequences (RPSs) in the chromosomes of *Candida albicans* are sandwiched between two novel stretches, HOK, and RB2, common to each chromosome. **Microbiology.** 1998;144:849-857.
- Clancy CJ, Nguyen MH.** Comparison of a photometric method with standardized methods of antifungal susceptibility testing of yeasts. **J Clin Microbiol.** 1997;35:2878-2882.
- Clancy CJ, Nguyen MH.** Correlation between in vitro susceptibility determined by E-test and response to therapy with amphotericin B: results from a multicenter prospective study of candidemia. **Antimicrob Agents Chemother.** 1999;43:1289-1290.
- ClemonsKV, Shankland GS, Richardson MD, Stevens DA.** Epidemiologic study by DNA typing of a *Candida albicans* outbreak in heroin addicts. **J Clin Microbiol.** 1991;29:205-207.
- ClemonsKV, Feroze F, Holmberg K, Stevens DA.** Comparative analysis of genetic variability among *Candida albicans* isolates from different geographic locales by three genotypic methods. **J Clin Microbiol.** 1997;35:1332-1336.

Coleman D, Sullivan D, Harrington B, Haynes K, Henman M, Shanley D, Bennet D, Moran G, McCreary C, O'Neill L. Molecular and phenotypic analysis of *Candida dublinensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients. *Oral Dis*. 1997;3:96-101.

Coleman DC, Rinaldi MG, Haynes KA, et al. Importance of *Candida* species other than *Candida albicans* as opportunistic pathogens. *Med Mycol* 1998;1:156-165.

Cormican MG, Hollis RJ, Pfaller MA. DNA macrorestriction profiles and antifungal susceptibility of *Candida (Torulopsis) glabrata*. *Diag Microbiol Infect Dis*. 1996;25:83-87.

Cuenca-Estrella M, Díaz-Guerra TM, Mellado E, Monzón A, Rodríguez-Tudela JL. Comparative in vitro activity of voriconazole and itraconazole against fluconazole-susceptible and fluconazole-resistant clinical isolates of *Candida* species from Spain. *Eur J Clin Microbiol Infect Dis*. 1999;18:432-435.

Cuenca-Estrella M, Díaz-Guerra TM, Mellado E, Rodríguez-Tudela JL. Influence of glucose supplementation and inoculum size on growth kinetics and antifungal susceptibility testing of *Candida* spp. *J Clin Microbiol*. 2001;39:525-532.

Cuenca-Estrella M, Rodríguez-Tudela JL. Present status of the detection of antiungal resistance: the perspective from both sides of the ocean. *Clin Microbiol Infect*. 2001;7(Supl 2):46-53.

Currie BP, Freundlich LF, Casadevall A. Restriction fragment length polymorphism analysis of *Cryptococcus neoformans* isolates from environmental (pigeon excreta) and clinical sources in New York City. *J Clin Microbiol*. 1994;32:1188-1192.

Davey KG, Holmes AD, Johnson EM, Szekely A, Warnock DW. Comparative evaluation of FUNGITEST and broth microdilution methods for antifungal drug susceptibility testing of *Candida* species and *Cryptococcus neoformans*. *J Clin Microbiol*. 1998;36:926-930.

Davey KG, Johnson EM, Holmes AD, Szekely A, Warnock DW. In-vitro susceptibility of *Cryptococcus neoformans* isolates to fluconazole and itraconazole. *J Antimicrob Chemother*. 1998;42:217-220.

De Beule K, Gestel JV. Pharmacology of itraconazole. *Drugs*. 2001;61(Suppl 1):27-37.

Debono M, Gordee RS. Antibiotics that inhibit fungal cell wall development. *Ann Rev Microbiol*. 1994;48:471-497.

Denning DW, Hanson LH, Perlman AM, Stevens DA. In vitro susceptibility and synergy studies of *Aspergillus* species to conventional and new agents. *Diag Microbiol Infect Dis*. 1992;15:21-34.

Denning DW. Therapeutic outcome in invasive aspergillosis. *Clin Infect Dis*. 1996;23:608-615.

Denning DW, Radford SA, Oakley KL, Hall L, Johnson MJ, Warnock DW. Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection. *J Antimicrob Chemother*. 1997;40:401-414.

Diasio R, Bennet J, Myers C. Mode of action of 5-fluorocytosine. *Biochem Pharmacol*. 1978;27:703-707.

Dick JD, Merz WG, Saral R. Incidence of polyene-resistant yeasts recovered from clinical specimens. *Antimicrob Agents Chemother*. 1980;18:158-163.

- Dikema DJ, Messer SA, Hollis RJ, Wenzel RP, Pfaller MA. An outbreak of *Candida parapsilosis* prosthetic valve endocarditis. *Diag Microbiol Infect Dis.* 1997;29:147-153.
- Doebbeling BN, Hollis RJ, Isenberg HD, Wenzel RP, Pfaller MA. Restriction fragment analysis of a *Candida tropicalis* outbreak of sternal wound infections. *J Clin Microbiol.* 1991;29:1268-1270.
- Doebbeling BN, Lehmann PF, Hollis RJ, Wu LC, Widmer AF, Pfaller MA. Comparison of pulsed-field gel electrophoresis with isoenzyme profiles as a typing system for *Candida tropicalis*. *Clin Infect Dis.* 1993;16:377-383.
- Dominguez JM, Kelly VA, Kinsman OS, Marriott MS, Gómez de las Heras F, Martin JJ. Sordarins: A new class of antifungals with selective inhibition of the protein synthesis elongation cycle in yeasts. *Antimicrob Agents Chemother.* 1998;42:2274-2278.
- Dominguez JM, Martin JJ. Identification of elongation factor 2 as the essential protein targeted by sordarins in *Candida albicans*. *Antimicrob Agents Chemother.* 1998;42:2279-2283.
- Douglas CM, Bowman JC, Abruzzo G, Flattery AM, Gill CJ, Kong L, Leighton C, Smith JG, Pikounis VB, Bartizal K, Kurtz MB, Rosen H. The glucan synthesis inhibitor caspofungin acetate (CANCIDASTM, MK-0991) kills *Aspergillus fumigatus* hyphal tips in vitro and is efficacious against disseminated aspergillosis in cyclophosphamide-induced chronically leukopenic mice. (Abstr 1683). *Programs and Abstracts of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, 2000.* American Society for Microbiology: Washington DC: American Society for Microbiology.
- Druetta A, Freydiere A, Guinet R, Gille Y. Evaluation of five commercial antifungal susceptibility testing systems. *Eur J Clin Microbiol Infect Dis.* 1993;12:336-342.
- Ellsworth DL, Rittenhouse KD, Honeycutt RL. Artfactual variation in randomly amplified polymorphic DNA banding patterns. *Bio-Techniques.* 1993;14:214-217.
- Emori TG, Gaynes RP. An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin Microbiol Rev.* 1993;6:428-442.
- Espinel-Ingroff A, Dawson K, Pfaller MA, Anaissie E, Breslin B, Dixon D, Fothergill A, Paetznick V, Peter J, Rinaldi M, Walsh T. Comparative and collaborative evaluation of standardization of antifungal susceptibility testing for filamentous fungi. *Antimicrob Agents Chemother.* 1995;39:314-319.
- Espinel-Ingroff A, Rodríguez-Tudela JL, Martínez-Suárez JV. Comparison of two alternative microdilution procedures with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for in vitro testing of fluconazole-resistant and -susceptible isolates of *Candida albicans*. *J Clin Microbiol.* 1995;33:3154-3158.
- Espinel-Ingroff A, Pfaller MA, Erwin ME, Jones RN. Interlaboratory evaluation of Etest method for testing antifungal susceptibilities of pathogenic yeasts to five antifungal agents by using casitone agar and solidified RPMI 1640 medium with 2% glucose. *J Clin Microbiol.* 1996;34:848-852.
- Espinel-Ingroff A, Barlett M, Bowden R, Chin NX, Cooper Jr C, Fothergill A, McGinnis MR, Menezes P, Messer SA, Nelson PW, Odds Jr FC, Pasarell L, Peter J, Pfaller MA, Rex JH, Rinaldi MG, Walsh TJ, Weitzman I. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing for filamentous fungi. *J Clin Microbiol.* 1997;37:139-143.

Espinel-Ingroff A. Comparison of in vitro activities of the new triazole Posaconazole and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. *J Clin Microbiol.* 1998;36:2950-2956.

Espinel-Ingroff A, Pfaller MA, Messer SA, Knapp CC, Killian S, Norris HA, Ghannoum MA. Multicenter comparison of the Sensititre YeastOne Colorimetric Antifungal Panel with the National Committee for Clinical Laboratory Standards M27A reference method for testing clinical isolates of common and emerging *Candida* spp, *Cryptococcus* spp and other yeasts and yeast-like organisms. *J Clin Microbiol.* 1999;37:591-595.

Espinel-Ingroff A, Bartlett M, Chaturvedi V, Gannaoum M, Hazen KC, Pfaller MA, Rinaldi M, Walsh TJ. Optimal susceptibility testing conditions for detection of azole resistance in *Aspergillus* spp.: NCCLS collaborative evaluation. *Antimicrob Agents Chemother.* 2001;45:1828-1835.

Essayag SM, Baily GG, Denning DW, Burnie JP. Karyotyping of fluconazole-resistant yeasts with phenotype reported as *Candida krusei* or *Candida inconspicua*. *Int J Syst Bacteriol.* 1996;46:35-40.

Favel A, Peyron F, De Meo M, Michel-Nguyen A, Carriere J, Chastin C, Regli P. Amphotericin B susceptibility testing of *Candida lusitanae* isolates by flow cytofluorometry: comparison with the Etest and the NCCLS broth macrodilution method. *J Antimicrob Chemother.* 1999;43:227-232.

Franzot SP, Hamdam JS, Currie BP, Casadevall A. Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for focal genetic differences and a global clonal population structure. *J Clin Microbiol.* 1997;35:2243-2251.

Fridkin SK, Jarvis WR. Epidemiology of nosocomial fungal infections. *Clin Microbiol Rev.* 1996;9:499-511.

Fries BC, Chen F, Currie BP, Casadevall A. Karyotype instability in *Cryptococcus neoformans* infection. *J Clin Microbiol.* 1996;34:1531-1534.

Fung-Tome JC, Minassian B, Huczko e, et al. In vitro antifungal and fungicidal spectra of a new pradimicin derivative, BMS-181184. *Antimicrob Agents Chemother.* 1995;39:295-300.

Galgiani JN, Reiser J, Brass C, Espinel-Ingroff A, Gordon MA, Kerkering TM. Comparison of relative susceptibilities of *Candida* species to three antifungal agents as determined by unstandardized methods. *Antimicrob Agents Chemother.* 1987;31:1343-1347.

Gamis AS, Gudnason T, Gieink GS. Disseminated infection with *Fusarium* in recipients of bone marrow transplants. *Rev Infect Dis.* 1991;13:1077-1088.

Gehrt A, Peter J, Pizzo PA, Walsh TJ. Effect of increasing inoculum sizes of pathogenic filamentous fungi on MICs of antifungal agents by broth microdilution method. *J Clin Microbiol.* 1995;33:1302-1307.

Georgopapadakou NH, Walsh TJ. Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother.* 1996;40:279-291.

Georgopapadakou NH. Update on antifungals targeted to the cell wall: focus on β -1,3-glucan synthase inhibitors. *Expert Opin Investig Drugs.* 2001;10:269-280.

- Ghannoum MS, Ibrahim AS, Fu Y, Shafiq MC, Edwards Jem Criddle RS. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. *J Clin Microbiol.* 1992;30:2881-2886.
- Ghannoum M, Spellberg BJ, Ibrahim a, Ritchie JA, Currie B, Spitzer E, Edwards Jr JE, Casadevall A. Sterol composition of *Cryptococcus neoformans* in the presence an absence of luconazole. . *Antimicrob Agents Chemother.* 1994;38:2029-2003.
- Ghannoum MS, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev.* 1999;12:501-517.
- Girardin H, Latge JP, Srikantha T, Morrow B, Soll DR. Devepment of DNA probes for fingerprinting *Aspergillus fumigatus*. *J Clin Microbiol.* 1993;31:1547-1554.
- Girardin H, Sarfati J, Traore F, Camet JD, Derouin F, Latge JP. Molecular epidemiology of nosocomial invasive aspergillosis. *J Clin Microbiol.* 1994;32:684-690.
- Gooday GW. The potential of novel antifungl drugs for the treatment of disease in the immunocompromised host. *Expert Opin Investig Drugs.* 1995;4:679-691.
- Goodwin DS, Cleary JD, Walawander CA, Taylor JW, Grasela TH. Pretreatment regimens for adverse events related to infusion of amphotericin B. *Clin Infect Dis.* 1995;20:755-761.
- Green L, Petersen B, Steimel L, Haeber P, Current W. Rapid determination of antifungal activity by flow cytometry. *J Clin Microbiol.* 1994;32:1008-1091.
- Guarro J, Llop C, Aguilar C, Pujol I. Comparison of in vitro antifungal susceptibilities of conidia and hyphae of filamentous fungi. *Antimicrob Agents Chemother.* 1997;41:2760-2762.
- Guinet R, Nerson D, Declosets F, Dupouy-Camet J, Kures L, Marjollet M, Poirot JL, Ros A, Texier-Maugein J, Volle PJ. Collaborative evaluation in seven laboratories of a standardized micromethod for yeast susceptibility testing. *J Clin Microbiol.* 1988;26:2307-2312.
- Hamilton-Miller, JMT. Chemistry and biology of the polyene macrolide antibiotics. *Bacteriol Rev.* 1973;37:166-196.
- Harvey RL, Myers JP. Nosocomial fungemia in a large community teaching hospital. *Arch Intern Med.* 1987;147:2117-2120.
- Hasenclever HF, Mitchell WO. Antigenic studies of *Candida*. I. Observations of two antigenic groups in *Candida albicans*. *J Bacteriol.* 1961;82:570-573.
- Hasenclever HF, Mitchell WO. Antigenic studies of *Candida*. II. Comparative pathogenicity of *Candida albicans* group A, group B, and *Candida stellatoidea*. *J Bacteriol.* 1961;82:578-581.
- Hawser SP, Norris H, Jessup CJ, Ghannoum MA. Comparison of a 2,3-bis(2-metoxi-4-nitro-5-dulfofenil)-5-[(fenilamino) carbonil]-2H-hidroxiid de tetrazoli (XTT) colorimetric method with the Standardized National Committee for Clinical Laboratory Standards method of testing clinical yeasts isolates for susceptibility to antifungal agents. *J Clin Microbiol.* 1998;36:1450-1452.
- Hector RF, Schaller K. Positive interaction of nikkomycins and azoles against *Candida albicans* in vitro and in vivo. *Antimicrob Agents Chemother.* 1992;36:1284-1289.

Hellstein J, Vawter-Hugart H, Fotos P, Schmid J, Soll DR. Genetic similarity and phenotypic diversity of commensal and pathogenic strains of *Candida albicans* isolated from the oral cavity. *J Clin Microbiol.* 1993;31:3190-3199.

Herrerros E, Martínez CM, Almela MJ, Marriott MS, Gómez de las Heras F, Gargallo-Viola D. Sordarins: in vitro activities of new antifungal derivatives against pathogenic yeasts, *Pneumocystis carinii*, and filamentous fungi. *Antimicrob Agents Chemother.* 1998;42:2863-2869.

Herrerros E, Almela MJ, Lozano S, Gómez de las Heras F, Gargallo-Viola D. Antifungal activities and cytotoxicity studies of six new Azasordarins. *Antimicrob Agents Chemother.* 2001;45:3132-3139.

Hitchcock C, Dickinson K, Brown SB, Evans EG, Adams DJ. Interaction of azole antifungal antibiotics with cytochrome P450-dependent 14 α -sterol demethylase purified from *Candida albicans*. *J Biochem.* 1990;266:475-480.

Holz RW. The effects of the polyene antibiotics nystatin and amphotericin B on thin lipid membranes. *Ann N Y Acad Sci.* 1974;235:469-479.

Hunter PR. A critical review of typing methods for *Candida albicans* and their applications. *Crit Rev Microbiol.* 1991;17:417-434.

Ingram CW, Haywood HB, Morris VM, Allen RL, Perfect JR. Cryptococcal ventricular-peritoneal shunt infection: clinical and epidemiological evaluation of two closely associated cases. *Infect Control Hosp Epidemiol.* 1993;14:719-722.

Jessup CJ, Pfaller MA, Messer SA, Zhang J, Tumberland M, Mbidde EK, Ghannoum MA. Fluconazole susceptibility testing of *Cryptococcus neoformans*: comparison of two broth microdilution methods and clinical correlates among isolates from Ugandan AIDS patients. *J Clin Microbiol.* 1998;36:2874-2876.

Johnson EM, Ojwang JO, Szekely A, Wallace TL, Warnock DW. Comparison on in vitro antifungal activities of free and liposome-encapsulated nystatin with those of four amphotericin B formulations. *Antimicrob Agents Chemother.* 1998;42:1412-1416.

Joly S, Pujol C, Schröppel K, Soll DR. Development and verification of two species fingerprinting probes for *Candida tropicalis* amenable to computer analysis. *J Clin Microbiol.* 1996;34:3063-3071.

Joly S, Pujol C, Rysz M, Vargas K, Soll DR. Development and characterization of complex DNA fingerprinting probes for the infectious agent *Candida dublinensis*. *J Clin Microbiol.* 1999;37:1035-1044.

Kao AS, Brandt ME, Pruitt WR, et al. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin Infect Dis.* 1999;29:1164-1170.

Karyotakis NC, Anaissie EJ, Hachem R, Dignani MC, Samonis G. Comparison of the efficacy of polyenes and triazoles against hematogeneous *Candida krusei* infection in neutropenic mice. *J Infect Dis.* 1993;168:1311-1313.

Kelly SL, Lamb DC, Kelly DE, Loeffler J, Einsele H. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. *Lancet.* 1996;348:1523-1524.

- Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, Schumacher U, Einsele H. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol Delta(5,6) desaturation. *FEBS Lett.* 1997;400:80-82.
- Kerridge D. Antifungal drugs. *Drugs of Today.* 1988;24:705-715.
- Kersulyte D, Woods JP, Keath EJ, Goldman WE, Berg DE. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. *J Bacteriol.* 1992;174:7075-7079.
- Kertschmar M, Nichterlein T, Kuntz P, Hof H. Rapid detection of susceptibility to fluconazole in *Candida* species by a bioluminescence assay of intracellular ATP. *Diag Microbiol Infect Dis.* 1996;25:117-121.
- King D, Rhine-Chalberg J, Pfaller MA, Moser SA, Merz WG. Comparison of four DNA-based methods for strain delineation of *Candida lusitanae*. *J Clin Microbiol.* 1995;33:1467-1470.
- Kleinegger C, Lockhart ST, Vargas K, Soll DR. Frequency, intensity, species and strains of oral yeast vary as a function of host age. *J Clin Microbiol.* 1996;34:2246-2254.
- Kontoyiannis DP, Lewis RE. Antifungal drug resistance of pathogenic fungi. *Lancet.* 2002;359:1135-1144.
- Kuehnert MJ, Clark E, Lockhart ST, Soll DR, Chia J, Jarvis WR. *Candida albicans* endocarditis traced to a contaminated aortic valve allograft: implications for regulation of allograft processing. *Clin Infect Dis.* 1998;27:688-691.
- KurtzMB, Abruzzo G, Flattery A, Bartizal K, Marrinan JA, Li W, Milligan J, Nollstadt K, Douglas CM. Characterization of echinocandin-resistant mutants of *Candida albicans*: genetic, biochemical, and virulence studies. *Infect Immun.* 1996;64:3244-3251.
- KurtzMB, Health IB, Marrinan JA, Dreikorn S, Onishi J, Douglas CM. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)- β -D-glucan synthase. *Antimicrob Agents Chemother.* 1994;38:1480-1489.
- Lass-Florl C, Nagl CM, Speth C, Ulmer H, Dierich MP, Wurzner R. Studies of in vitro activities of voriconazole and itraconazole against *Aspergillus* hyphae using viability staining. *Antimicrob Agents Chemother.* 2001;45:124-128.
- Law D, Moore CB, Denning DW. Amphotericin B resistance testing of *Candida* spp: a comparison of methods. *J Antimicrob Chemother.* 1997;40:109-112.
- Le Guennec R, Reynes J, Mallie M, Pujol C, Janbon F, Bastide JM. Fluconazole- and Itraconazole-resistant *Candida albicans* strains from AIDS patients: multilocus enzyme electrophoresis analysis and antifungal susceptibilities. *J Clin Microbiol.* 1995;33:2732-2737.
- Lehmann PF, Lin D, Lasker BA. Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. *J Clin Microbiol.* 1992;30:3249-3254.

Li RK, Rinaldi MG. Antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. *Antimicrob Agents Chemother.* 1999;43:1401-1405.

Liao RS, Rennie RP, Talbot JA. Assessment of the effect of amphotericin B on the vitality of *Candida albicans*. *Antimicrob Agents Chemother.* 1999;43:1034-1041.

Lin D, Lehmann PF, Hamory BH, Padhye AA, Durry E, Pinner RW, Lasker BA. Comparison of three typing methods for clinical and environmental isolates of *Aspergillus fumigatus*. *J Clin Microbiol.* 1995;33:1596-1601.

Lin D, Lehmann PF. Random amplified polymorphic DNA for strain delineation within *Candida tropicalis*. *J Med Vet Mycol.* 1995;33:241-246.

Lin D, Wu LC, Rinaldi MG, Lehmann PF. Three distinct genotypes within *Candida parapsilosis* from clinical sources. *J Clin Microbiol.* 1995;33:1815-1821.

Lin SJ, Schranz J, Teutsch SM. Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis.* 2001;32:358-366.

Llop C, Pujol I, Aguilar C, Sala J, Riba D, Guarro J. Comparison of three methods of determining MICs for filamentous fungi using different end point criteria and incubation periods. *Antimicrob Agents Chemother.* 2000;44:239-242.

Lockhart SR, Fritch JJ, Meier AS, Schöppel K, Srikantha T, Galask R, Soll DR. Colonizing populations of *Candida albicans* are clonal in origin but undergo microevolution through C1 fragment reorganization as demonstrated by DNA fingerprinting and C1 sequencing. *J Clin Microbiol.* 1995;33:1501-1509.

Lockhart SR, Reed BD, Pierson DL, Soll DR. Most frequent scenario for recurrent *Candida* vaginitis is strain maintenance with "substrain shuffling": demonstration by sequential DNA fingerprinting with probes Ca3, C1, and CARE2. *J Clin Microbiol.* 1996;34:767-777.

Lockhart SR, Joly S, Pujol C, Sobel JD, Pfaller MA, Soll DR. Development and verification of fingerprinting probes for *Candida glabrata*. *Microbiology.* 1997;143:3733-3746.

Lott TJ, Keykendall RJ, Welbel SF, Pramanik A, Lasker BA. Genomic heterogeneity in the yeast *Candida parapsilosis*. *Curr Genet.* 1993;23:463-467.

Loudon KW, Coke AP, Burnie JP. "Pseudoclusters" and typing by random amplification of polymorphic DNA of *Aspergillus fumigatus*. *J Clin Pathol.* 1995;48:183-184.

Lozano-Chiu M, Nelson PW, Lancaster MV, Pfaller MA, Rex JH. Lot-to-lot variability of antibiotic medium 3 when used for susceptibility testing of *Candida* isolates to amphotericin B. *J Clin Microbiol.* 1997;35:270-272.

Lozano-Chiu M, Lancaster MV, Rex JH. Evaluation of a colorimetric method for detecting amphotericin B-resistant *Candida* isolates. *Diag Microbiol Infect Dis.* 1998;31:417-424.

- Lozano-Chiu M, Paetznick VL, Ghannoum MA, Rex JH.** Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: performance of three different media assessed by using E-test and National Committee for Clinical Laboratory Standards M27-A methodologies. *J Clin Microbiol.* 1998;36:2817-2822.
- Lozano-Chiu M, Arikan S, Paetznick VL, Anaissie EJ, Rex JH.** Optimizing voriconazole susceptibility testing of *Candida*: effects of incubation time, endpoint rule, species of *Candida*, and level of fluconazole susceptibility. *J Clin Microbiol.* 1999;37:2755-2759.
- Marichal P, Vanden-Bossche H, Odds FC, Nobels G, Warnock DW, Timmerman V, Van Broeckhoven C, Fay S, Mose Larsen P.** Molecular-biological characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrob Agents Chemother.* 1997;41:2229-2237.
- Marr KA, Rustad TR, Rex JH, White TC.** The trailing endpoint phenotype in antifungal susceptibility testing is pH-dependent. *Antimicrob Agents Chemother.* 1999;43:1383-1386.
- Martinez A, Ferrer S, Santos I, Jiménez E, Sparrowe J, Regadera J, Gómez de las Heras F, Gargallo-Viola D.** Antifungal activities of two new azasordarins, GW471552 and GW471558, in experimental models of oral and vulvovaginal candidiasis in immunosuppressed rats. *Antimicrob Agents Chemother.* 2001;45:3304-3309.
- McAlpin CE, Mannarelli B.** Construction and characterization of a DNA probe for distinguishing strains of *Aspergillus flavus*. *Appl Environ Microbiol.* 1995;61:1068-1072.
- McCreight MC, Warnock DW, Martin MV.** Resistogram typing of *Candida albicans* isolates from oral and cutaneous sites in irradiated patients. *Sabouradia.* 1985;23:403-406.
- McNeil M, Nash SL, Hajjeh R, et al.** Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. *Clin Infect Dis.* 2001;33:641-647.
- Meis J, Petrou M, Billie J, Ellis D, Gibbs D.** A global evaluation of the susceptibility of *Candida* species to fluconazole by disk diffusion. *Diag Microbiol Infect Dis.* 2000;36:215-223.
- Meletiadiis J, Meis J, Mouton JW, Donnelly JP, Verweij PE.** Comparison of NCCLS and 3-(4,5-dimethyl-2-tiazolil)-2-5-difenil-2H-bromur de tetrazoli (MTT) methods of in vitro susceptibility testing of filamentous fungi and development of a new simplified method. *J Clin Microbiol.* 2000;38:2949-2954.
- Merz WG, Khazan U, Jabra-Rizk MA, Wu LC, Osterhout GJ, Lehmann PF.** Strain delineation and epidemiology of *Candida (Clavispora) lusitaniae*. *J Clin Microbiol.* 1992;30:449-454.
- Messer SA, Pfaller MA.** Clinical evaluation of a dried commercially prepared microdilution panel for antifungal susceptibility testing. *Diag Microbiol Infect Dis.* 1996;25:77-81.
- Milewski S, Mignini F, Borowski E.** Synergistic action of nikkomycin X/Z with azole antifungals on *Candida albicans*. *J Gen Microbiol.* 1991;137:2155-2161.
- Moore CB, Walls CM, Denning DW.** In vitro activity of the new triazole BMS-207147 against *Aspergillus* species in comparison with itraconazole and amphotericin B. *Antimicrob Agents Chemother.* 2000;44:441-443.
- Muñoz P.** Candidemia: el reto que no cesa. *Rev Clin Esp.* 1997;197:795-797.

National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Proposed standard M27-P. **National Committee for Clinical Laboratory Standards.** Villanova, Pa. 1992.

National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts; tentative standard. NCCLS document M27-T. **National Committee for Clinical Laboratory Standards.** Wayne, Pa. 1995.

National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. NCCLS document M27-A. **National Committee for Clinical Laboratory Standards.** Wayne, Pa. 1997.

National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Proposed standard. NCCLS document M28-P. **National Committee for Clinical Laboratory Standards.** Wayne, Pa. 1998.

Nguyen MH, Clancy CJ, Yu YC, Yu VL, Morris AJ, Snyderman DR, Sutton DA, Rinaldi MG. Do in vitro susceptibility data predict the microbiologic response to amphotericin B? Results of a prospective study of patients with *Candida* fungemia. **J Infect Dis.** 1998;177:425-430.

Nguyen MH, Yu YC. Influence of incubation time, inoculum size, and glucose concentration on spectrophotometric endpoint determinations for amphotericin B, fluconazole, and itraconazole. **J Clin Microbiol.** 1999;37:2755-2759.

Odds FC, Abbott AB. A simple system for the presumptive identification of *Candida albicans* and differentiation of strains within the species. **Sabouradia.** 1980;18:301-317.

Odds FC, Abbott AB. Modification and extension of tests for differentiation *Candida* species and strains. **Sabouradia.** 1983;21:79-81.

Odds FC, Auger P, Krogh P, Neely AN, Segal E. Byotyping of *Candida albicans*: results of an international collaborative survey. **J Clin Microbiol.** 1989;27:1506-1509.

Odds FC, Messer SA, Woestenborghs F. Antifungal susceptibility testing of yeasts: evaluation of technical variables for test automation. **Antimicrob Agents Chemother.** 1995;39:2051-2060.

Olivo PD, McManus EJ, Riggsby WS, Jones JM. Mitochondrial DNA polymorphism in *Candida albicans*. **J Infect Dis.** 1987;156:214-215.

Osaka K, Ritov VB, Bernardo JF, Branch RA, Kagan VE. Amphotericin B protects cis-parinaric acid against peroxy radical-induced oxidation: amphotericin B as an antioxidant. **Antimicrob Agents Chemother.** 1997;41:743-747.

Pan S, Cole GT. Electrophoretic karyotypes of clinical isolates of *Coccidioides immitis*. **Infect Immun.** 1992;60:4872-4880.

Parkinson T, Falconer DJ, Hitchcock C. Fluconazole resistance due to energy-dependent drug efflux in *Candida glabrata*. **Antimicrob Agents Chemother.** 1995;39:1696-1699.

Patterson TF, Kirkpatrick WR, Revankar SG, McAtee RK, Fothergill AW, McCarthy DI, Rinaldi MG. Comparative evaluation of microdilution and chromogenic agar screening for determining fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol.* 1996;34:3237-3239.

Patterson TF, Revankar SG, Kirkpatrick WR, Dib O, Fothergill AW, Redding SW, Sutton DA, Rinaldi MG. A simple method for detecting fluconazole resistant yeasts with chromogenic agar. *J Clin Microbiol.* 1996;34:1794-1797.

Perfect JR, Schell WA. The new fungal opportunists are coming. *Clin Infect Dis.* 1996;22(Suppl 2):112-118.

Perfect JR, Cox GM. Drug resistance in *Cryptococcus neoformans*. *Drug Resistance Updates.* 1999;2:259-269.

Pfaller MA, Cabezudo I, Hollis RJ, Huston B, Wenzel RP. The use of biotyping and DNA fingerprinting in typing *Candida albicans* from hospitalized patients. *Diag Microbiol Infect Dis.* 1990;13:481-489.

Pfaller MA, Messer SA, Hollis RJ. Strain delineation and antifungal susceptibilities of epidemiologically related and unrelated isolates of *Candida lusitanae*. *Diag Microbiol Infect Dis.* 1994;20:127-133.

Pfaller MA, Messer SA, Hollis RJ. Variations in DNA subtype, antifungal susceptibility, and slime production among clinical isolates of *Candida parapsilosis*. *Diag Microbiol Infect Dis.* 1995;21:9-14.

Pfaller MA, Messer SA, Coffman S. Comparison of visual and spectrophotometric methods of MIC endpoints determinations by using broth microdilution methods to test five antifungal agents, including the new triazole D0870. *J Clin Microbiol.* 1995;33:1094-1097.

Pfaller MA. Nosocomial candidiasis: emerging species. Reservoirs, and modes of transmission. *Clin Infect Dis.* 1996;22(Suppl 2):89-94.

Pfaller MA, Messer SA, Bolmström A, Odds FC, Rex JH. Multisite reproducibility of the Etest MIC method for antifungal susceptibility testing of yeast isolates. *J Clin Microbiol.* 1996;34:1691-1693.

Pfaller MA, Arikan S, Lozano-Chiu M, Chen YS, Coffman S, Messer SA, Rennie R, Sand C, Heffner T, Rex JH, Wang J, Yamane N. Clinical evaluation of the ASTY colorimetric microdilution panel for antifungal susceptibility testing. *J Clin Microbiol.* 1998;36:2609-2612.

Pfaller MA, Messer SA, Bolmström A. Evaluation of E-test for determining in vitro susceptibility of yeast isolates to amphotericin B. *Diag Microbiol Infect Dis.* 1998;32:223-227.

Pfaller MA, Messer SA, Hollis RJ, Espinel-Ingroff A, Ghannoum MA, Plavan H, Killian S, Knapp CC. Multisite reproductibility of MIC results by the Sensititre (R) YeastOne colorimetric antifungal susceptibility panel. *Diag Microbiol Infect Dis.* 1998;31:543-547.

Pfaller MA, Messer SA, Mills K, Bolmström A. In vitro susceptibility testing of filamentous fungi: comparison of Etest and reference microdilution methods for determining itraconazole MICs. *J Clin Microbiol.* 2000;38:3359-3361.

Phongpaichit S, Mackenzie DW, Fraser C. Strain differentiation of *Candida albicans* by morphotyping. *Epidemiol Infect.* 1987;99:421-428.

- Polak A, Hartman PG. Antifungal chemotherapy-are we winning?. *Prog Drug Res.* 1991;37:181-269.
- Polak A, Scholer H. Mode of action of 5-fluorocytosine and mechanisms of resistance. *Chemotherapia.* 1975. 21:113-130.
- Polonelli L, Archibusacci C, Sestito M, Morace G. Killer system: a simple method for differentiating *Candida albicans* strains. *J Clin Microbiol.* 1983;17:774-780.
- Pore RS. Antibiotic susceptibility testing of *Candida albicans* by flow cytometry. *Curr Microbiol.* 1990;20:323-328.
- Pore RS. Antibiotic susceptibility testing by flow cytometry. *J Antimicrob Chemother.* 1994;34:613-627.
- Posteraro B, Romano L, Sanguinetti M, Masucci L, Morace G, Fadda G. Commercial systems for fluconazole susceptibility testing of yeasts: comparison with the broth microdilution method. *Diag Microbiol Infect Dis.* 2000;38:29-36.
- Poulain D, Hopwood V, Vernes A. Emerging variability of *Candida albicans*. *Crit Rev Microbiol.* 1985;12:223-270.
- Pujol C, Joly S, Lockhart SR, Noel S, Tibayrenc M, Soll DR. Parity of MLEE, RAPD, and Ca3 hybridization as fingerprinting methods for *Candida albicans*. *J Clin Microbiol.* 1997;35:2348-2358.
- Pujol C, Joly S, Nolan B, Srikantha T, Lockhart SR, Soll DR. Microevolutionary changes in *Candida albicans* identified by the complex Ca3 probe involve insertions and deletions of RPS units at specific genomic sites. *Microbiology.* 1999;145:2635-646.
- Quindós G, Lipperheide V, Barturen B, Alonso R, Bikandi J, San Millán S, Tellaetxe M, Ribacoba L, Pontón J. A new method of antibiotyping yeasts for subspecies discrimination and distribution in human clinical specimens. *Eur J Epidemiol.* 1996;12:55-62.
- Quindós G, Salesa R, Carrillo-Muñoz AJ, Lipperheide V, Jaudenes L, San Millán R, Torres-Rodríguez JM, Pontón J. Multicenter evaluation of ATB fungus: a standardized micromethod for yeast susceptibility testing. *Chemotherapy.* 1994;40:245-251.
- Ramani R, Ramani A, Wong SJ. Rapid flow cytometric susceptibility testing of *Candida albicans*. *J Clin Microbiol.* 1997;35:2320-2324.
- Ramani R, Chaturvedi V. Flow cytometry antifungal susceptibility testing of pathogenic yeasts other than *Candida albicans* and comparison with the NCCLS broth microdilution test. *Antimicrob Agents Chemother.* 2000;44:2752-2758.
- Ramsey H, Morrow B, Soll DR. An increase in switching frequency correlates with an increase in recombination of the ribosomal chromosomes of *Candida albicans* strain 3153A. *Microbiology.* 140:1525-1531.
- Rees J, Pinner R, Hajjeh R, Brandt M, Reingold A. The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992-1993; results of the population-based laboratory active surveillance. *Clin Infect Dis.* 1999;27:1138-1147.

Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG, Patterson TF. Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. *J Clin Microbiol.* 1998;36:153-156.

Rex JH, Cooper CR Jr, Merz WG, Galgiani JN, Anaissie EJ. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. *Antimicrob Agents Chemother.* 1995;39:906-909.

Rex JH, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum MA, Lancaster M, Odds FC, Rinaldi MG, Walsh TJ, Barry AL and Subcommittee on antifungal susceptibility testing of the National Committee for Clinical Laboratory Standards. Development of interpretative breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. *Clin Infect Dis.* 1997;24:235-247.

Rex JH, Nelson PW, Paetznick VL, Lozano-Chiu M, Espinel-Ingroff A, Anaissie EJ. Optimizing the correlation between results of testing in vitro and therapeutic outcome in vivo for fluconazole by testing critical isolates in a murine model of invasive candidiasis. *Antimicrob Agents Chemother.* 1998;42:129-134.

Rex JH, Pfaller MA, Walsh TJ, Chaturvedi V, Espinel-Ingroff A, Ghannoum MA, Gosey LL, Odds FC, Rinaldi MG, Sheehan DJ, Warnock DW. Antifungal susceptibility testing: practical aspects and current challenges. *Clin Infect Dis.* 2001;14:643-658.

Riesselman MH, Hazen KC, Cutler JE. Determination of antifungal MICs by a rapid susceptibility assay. *J Clin Microbiol.* 2000;38:333-340.

Robert F, Lebreton F, Bognoux ME, Paugam A, Wassermann D, Schlotterer M, Tourte-Schaefer C, Dupouy-Camet J. Use of random amplified polymorphic DNA as a typing method for *Candida albicans* in epidemiological surveillance of a burn unit. *J Clin Microbiol.* 1995;33:2366-2371.

Rodriguez E, De Meeus T, Mallie M, Renaud F, Symoens F, Mondon P, Piens MA, Lebeau B, Viviani MA, Grillot R, Nolard N, Chapuis F, Tortorano AM, Bastide JM. Multicentric epidemiological study of *Aspergillus fumigatus* isolated by multilocus enzyme electrophoresis. *J Clin Microbiol.* 1996;34:2559-2568.

Rodriguez-Tudela JL, Martínez-Suarez JV. Improved medium for fluconazole susceptibility testing of *Candida albicans*. *Antimicrob Agents Chemother.* 1994;38:45-48.

Rodriguez-Tudela JL, Martínez-Suarez JV. Defining conditions for microbroth antifungal susceptibility testing: influence of RPMI and RPMI-2% glucose on the selection of endpoint criteria. *J Antimicrob Chemother.* 1995;35:739-749.

Rodriguez-Tudela JL, Berenguer J, Martínez-Suarez JV, Sánchez R. Comparison of a spectrophotometric microdilution method with RPMI-2% glucose with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for in vitro susceptibility testing of amphotericin B, flucytosine, and fluconazole against *Candida albicans*. *Antimicrob Agents Chemother.* 1996;40:1998-2003.

Rodriguez-Tudela JL, Cuenca-Estrella M, Grupo de trabajo para el estudio de la fungemias. Estudio multicéntrico sobre fungemias por levaduras en España (abril-junio de 1997). *Rev Clin Esp.* 1999;6:356-361.

Rodríguez-Tudela JL, Martín-Díez F, Cuenca-Estrella M, Rodero L, Carpintero Y, Gorgojo B. Influence of shaking on antifungal susceptibility testing of *Cryptococcus neoformans*: a comparison of the NCCLS standard M27A medium, buffered yeast nitrogen base, and RPMI-2% glucose. *Antimicrob Agents Chemother.* 2000;44:400-404.

Ruiz-Díez B, Martínez V, Álvarez M, Rodríguez-Tudela JL, Martínez-Suárez JV. Molecular tracking of *Candida albicans* in a neonatal intensive care unit: long term colonizations versus catheter-related infections. *J Clin Microbiol.* 1997;35:3032-3036.

Ryder NS. Activity of terbinafine against serious fungal pathogens. *Mycoses.* 1999;42(Suppl 2):115-119.

Ryder N, Favre B. Antifungal activity and mechanism of action of terbinafine. *Rev Contemp Pharmacother.* 1997;8:275-287.

Sandven P. Detection of fluconazole-resistant *Candida* strains by a disc diffusion screening test. *J Clin Microbiol.* 1999;37:3856-3859.

Sanglard D, Kuchler K, Isher F, Pagani JL, Monod M, Bille J. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother.* 1995;39:2378-2386

Sanglard D, Ischer F, Monod M, Bille J. Susceptibility of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob Agents Chemother.* 1996;2300-2305.

Scherer S, Stevens DA. Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. *J Clin Microbiol.* 1987;25:675-679.

Scherer S, Stevens DA. A *Candida albicans* dispersed, repeated gene family and its epidemiological implications. *Proc Natl Acad Sci USA.* 1988;85:1452-1456.

Schmalreck AF, Kottmann I, Reiser A, Ruffer U, Scharr E, Vanca E. An evaluation of seven methods of in vitro susceptibility testing of clinical yeast isolates against fluconazole. *Mycoses.* 1995;38(Suppl 1):55-63.

Schmid J, Voss E, Soll DR. Computer-assisted methods for assessing strain relatedness in *Candida albicans* by fingerprinting with the moderately repetitive sequence Ca3. *J Clin Microbiol.* 1990;28:1236-1243.

Schmid J, Odds FC, Wiselka MJ, Nicholson KG, Soll DR. Genetic similarity and maintenance of *Candida albicans* strains in a group of AIDS patients demonstrated by DNA fingerprinting. *J Clin Microbiol.* 1992;30:935-941.

Schwab U, Chernomas F, Larcon L, Weems J. Molecular typing and fluconazole susceptibility of urinary *Candida glabrata* isolates from hospitalized patients. *Diag Microbiol Infect Dis.* 1997;29:11-17.

Schwartz DC, Cantor CR. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell.* 1984;37:67-75.

- Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. *Clin Microbiol Rev.* 1999;12:40-79.
- Shimokawa O, Nakayama H, Acetate-mediated growth inhibition in sterol 14 alpha-demethylation-deficient cells of *Candida albicans*. *Antimicrob Agents Chemother.* 1999;43:100-105.
- Shimokawa O, Nakayama H, Estimation of minimum sterol 14 alpha-demethylation-inhibitory concentration of azoles in *Candida* yeasts using acetate-mediated growth inhibition: potential utility in susceptibility testing. *J Clin Microbiol.* 2000;38:2893-2896.
- Soll DR, Langtimm CJ, McDowell J, Hicks J, Galask R. High-frequency switching in *Candida* strains isolated from vaginitis patients. *J Clin Microbiol.* 1987;25:1611-1622.
- Soll DR. Dimorphism and high frequency switching in *Candida albicans*. In Kirsch DR, Kelly R, Kurtz MB (ed). *The genetics of Candida*. Crc Press Inc. Boca Ratón, Fla. 1990;147-176.
- Soll DR, Anderson J, Bergen M. The developmental biology of the white-opaque transition in *Candida albicans*. In Prasad R (ed). *The molecular biology of Candida albicans*. Springer-VerlagKG, Berlin Germany. 1991;20-45.
- Soll DR. High-frequency switching in *Candida albicans*. *Clin Microbiol Rev.* 1992;5:183-203.
- Soll DR. The ins and outs of DNA fingerprinting the infectious fungi. *Clin Microbiol Rev.* 2000;13:332-370.
- Spitzer ED, Spitzer SG. Use of dispersed repetitive DNA element to distinguish clinical isolates of *Cryptococcus neoformans*. *J Clin Microbiol.* 1992;30:1094-1097.
- Steele PE, Carie GF, Kobayashi GS, Medoff G. Electrophoretic analysis of *Histoplasma capsulatum* chromosomal DNA. *Mol Cell Biol.* 1989;9:983-987.
- Stein GE, Sheridan VL, Magee BB, Magee PT. Use of rDNA restriction fragment length polymorphisms to differentiate strains of *Candida albicans* in women with vulvovaginal candidiasis. *Diag Microbiol Infect Dis.* 1991;14:459-464.
- Sullivan D, Bennett D, Henman M, Harwood P, Flint S, Mulcahy F, Shanley D, Coleman D. Oligonucleotide fingerprinting of isolates of *Candida* species other than *C. albicans* and of atypical *Candida* species from human immunodeficiency virus-positive and AIDS patients. *J Clin Microbiol.* 1993;31:2124-2133.
- Suzuki T, Kobayashi I, Kanbe T, Tanaka K. High frequency variation of colony morphology and chromosome reorganization in the pathogenic yeast *Candida albicans*. *J Gen Microbiol.* 1989;135:425-430.
- Swinne D, Raes-Wuytack C, Van Looveren K, Desmet P. Comparative evaluation of Fungitest (R), Neosensitabs (R) and M27T-NCCLS broth microdilution methods for antifungal drug susceptibility testing of *Candida* species and *Cryptococcus neoformans*. *Mycoses.* 1999;42:231-237.
- Szekely A, Jhonson EM, Warnock DW. Comparison of E-test and broth microdilution methods for antifungal drug susceptibility testing of molds. *J Clin Microbiol.* 1999;37:1480-1483.

- Van den Saffele JK, Boelaert JR. Zygomycosis in HIV-positive patients: review of the literature. *Mycoses*. 1996;39:77-84.
- Vanden Bossche H, Willemsens G, Marichal P. Anti-*Candida* drugs-the biochemical basis for their activity. *Crit Rev Microbiol*. 1987;15:57-72.
- Vanden Bossche H, Marichal P, Le Jeune L, Coene M, Gorrens J, Cools W. Effects of itraconazole on cytochrome P-450-dependent 14 α -demethylation and reduction of 3-ketosteroids in *Cryptococcus neoformans*. *Antimicrob Agents Chemother*. 1993;37:2101-2105.
- Vanden Bossche H, Marichal P, Odds FC. Molecular mechanisms of drug resistance in fungi. *Trends Microbiol*. 1994;2:393-400.
- Vanden Bossche H, Dromer F, Improvisi I, Lozano-Chiu M, Rex JH, Sanglard D. Antifungal drug resistance in pathogenic fungi. *Med Mycol* 1998;36(Suppl 1):119-128.
- Vazquez JA, Beckley A, Donabedian S, Sobel JD, Zervos MJ. Comparison of restriction enzyme analysis and pulsed-field gradient gel electrophoresis as a typing system for *Torulopsis glabrata* and *Candida* species other than *C. albicans*. *J Clin Microbiol*. 1993;31:2021-2030.
- Wald A, Leisenring W, van Burik JA, Bowden RA. Epidemiology of *Aspergillus* infections in a large cohort of patients undergoing bone marrow transplantation. *J Infect Dis*. 1997;175:1459-146....
- Wallace TL, Paetznick V, Cossum PA, Lopez-berenstein G, Rex JH, Anaissie E. Activity of liposomal nystatin against disseminated *Aspergillus fumigatus* infection in neutropenic mice. *Antimicrob Agents Chemother*. 1998;41:2238-2243.
- Walsh TJ, Melcher GP, Rinaldi MG, et al. Trichosporon beigelii, an emerging pathogen resistant to amphotericin B. *J Clin Microbiol*. 1990;28:1616-1622.
- Walsh TJ, Giri N. Pradimicins: a novel class of broad-spectrum antifungal compounds. *Eur J Clin Microbiol Infect Dis*. 1997;16:93-97.
- Wanger A, Mill K, Nelson PW, Rex JH. Comparison of E-test and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin R – resistant *Candida* isolates. *Antimicrob Agents Chemother*. 1995;39:2520-2522.
- Warnock DW. Fungal infections in neutropenia: current problems and chemotherapeutic control. *J Antimicrob Chemother*. 1998;41:95-105.
- Warnock DW, Jhonson EM, Rogers TRF. Multi-centre evaluation of the Etest method for antifungal drug susceptibility testing of *Candida* spp and *Cryptococcus neoformans*. *J Antimicrob Chemother*. 1998;42:321-331.
- Watanabe M, Hiratani T, Uchida K, et al. The in-vitro activity of an antifungal antibiotic benanomicin A in comparison with amphotericin B. *J Antimicrob Chemother*. 1996;38:1073-1077.
- Wenisch C, Linnau KF, Parschalk B, Zedtwitz-Liebenstein K, Georgopoulos A. Rapid susceptibility testing of fungi by flow cytometry using vital staining. *J Clin Microbiol*. 1996;35:5-10.
- Whelan WL, Kerridge D. Decreased activity of UMP pyrophosphorylase associated with resistance to 5-fluorocytosine in *Candida albicans*. *Antimicrob Agents Chemother*. 1984;26:570-574.

Whelan WL. The genetic basis of resistance to 5- fluorocytosine in *Candida* species and *Cryptococcus neoformans*. *Crit Rev Microbiol*. 1987;15:45-56.

White TC. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14-alpha-demethylase in *Candida albicans*. *Antimicrob Agents Chemother*. 1997;41:1482-1494.

White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev*. 1998;11:382-402.

Wilkinson BM, Morris L, Adams DJ, Evans EG, Lacey CJ, Walmsley RM. A new, sensitive polynucleotide probe for distinguishing *Candida albicans* strains and its use with computer assisted archiving and pattern comparison system. *J Med Vet Mycol*. 1992;30:123-131.

Williams JG, Kubelik AR, Livak KJ, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. 1990;18:6531-6535.

Willinger B, Apfalter P, Hirschl AM, Makristathis A, Rotter M, Seibold M. Susceptibility testing of *Candida* species: comparison of NCCLS microdilution method with Fungitest. *Diag Microbiol Infect Dis*. 2000;38:11-15.

Wills JW, Lasker BA, Sirotkin K, Riggsby WS. Repetitive DNA of *Candida albicans*: nuclear and mitochondrial components. *J Bacteriol*. 1984;157:918-924.

Wingard JR, Merz WG, Rinaldi MG, Johnson TR, Karp JE, Saral R. Increase in *Candida krusei* infection among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. *N Engl J Med*. 1991;325:1274-1277.

Wingard JR. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clin Infect Dis*. 1995;20:115-125.

Witt MD, Lewis RJ, Larsen RA, Milechik EN, Leal MAE, Haubrich RH, Richie JA, Edwards Jr JE, Ghannoum MS. Identification of patients with acute AIDS-associated cryptococcal meningitis who can be effectively treated with fluconazole: The role of antifungal susceptibility testing. *Clin Infect Dis*. 1996;22:322-328.

Witthuhn F, Toubas D, Beguinot I, Aubert D, Rouger C, Remy G, Pinon JM. Evaluation of the fungitest kit by using strains from human immunodeficiency virus-infected patients: study of azole drug susceptibility. *J Clin Microbiol*. 1999;37:864-866.

Wong-Beringer A, Jacobs RA, Guglielmo BJ. Lipid formulations of amphotericin B: clinical efficacy and toxicities. *Clin Infect Dis*. 1998;27:603-618.

Yang HC, Mikami Y, Yazawa K, Taguchi H, Nishimura K, Miyaji M, Branchini MLM, Aoki FH, Yamamoto K. Colorimetric MTT assessment of antifungal activity of D0870 against fluconazole-resistant *Candida albicans*. *Mycoses*. 1998

Taula 1. Principals grups taxonòmics i gèneres.

Classificació Taxonòmica	Gèneres representatius
Filum: <i>Zygomycota</i> Classe: <i>Zygomycetes</i> Ordre: <i>Mucorales</i> Ordre: <i>Entomophthorales</i>	<i>Mucor, Absidia, Rhizomucor, Rhizopus, Basidiobolus, Conidiobolus</i>
Filum: <i>Ascomycota</i> Classe: <i>Hemiascomycetes</i> Ordre: <i>Saccharomycetales</i> Classe: <i>Euascomycetes</i> Ordre: <i>Onygenales</i> Ordre: <i>Onygenaceae</i> Ordre: <i>Eurotiales</i> Ordre: <i>Microascales</i> Ordre: <i>Hypocreales</i> Ordre: <i>Pleosporales</i> Ordre: <i>Chatothyriales</i> Classe: <i>Archiascomycetes</i> Ordre: <i>Pneumocystidales</i>	<i>Sacharomyces, Pichia</i> (teleomorf* d'algunes <i>Candida</i> spp), <i>Geotricum</i> <i>Arthroderma</i> (teleomorfos de <i>Trichophyton</i> i <i>Microsporum</i> spp) <i>Ajellomyces</i> (teleomorfos de <i>Histoplasma</i> i <i>Blastomyces</i> spp), <i>Paracoccidioides, Coccidioides</i> , Teleomorfos d' <i>Aspergillus</i> i <i>Penicillium</i> . <i>Pseudallescheria boydii</i> (teleomorf de <i>S. apiospermium</i>). <i>Nectria, Gibberella</i> (teleomorf de molts <i>Fusarium</i> spp) <i>Neocomospora</i> (teleomorf de <i>Acremonium</i>) <i>Alternaria, Exserohilum, Bipolaris</i> <i>Phialophora, Exophiala</i> <i>Pneumocystis carinii</i>
Filum: <i>Basidiomycota</i> Classe: <i>Urediniomycetes</i> Ordre: <i>Sporodiales</i> Classe: <i>Hymenomycetes</i> Ordre: <i>Tremellales</i>	<i>Rhodosporidium</i> (teleomorf de <i>Rhodotorula</i>) <i>Filobasidiella</i> (teleomorf de <i>C. neoformans</i>), <i>Trichosporum</i>

*Teleomorf: forma sexual

Tot i ser organismes eucariotes, l'estructura i organització dels fongs és única. En el citoplasma podem trobar els nuclis, mitocondries, ribosomes, retícul endoplasmàtic i vacuoles que emmagatzemen diversos compostos i metabòlits. A destacar és el fet que el nucli dels fongs sol ser petit (2-3 µm de diàmetre), està embolcat per una membrana nuclear doble amb porus i conté un nucleòl amb gran quantitat d'ARN. Els fongs poden ser haploides (la majoria), diploides (alguns llevats com *C. albicans*) o alternar les dues fases. La membrana plasmàtica i la paret cel·lular dels fongs són dos components estructurals que mereixen especial consideració, si més no, perquè es tracta d'estructures que ofereixen diferents possibilitats teòriques d'intervenció farmacològica. A aquests dos components caldria sumar-hi, des d'un punt de vista terapèutic, una tercera via d'actuació: la inhibició de la síntesi proteica.

Membrana plasmàtica. La membrana plasmàtica fúngica és similar a la d'altres cèl·lules eucariotes, totes estan formades per una bicapa de fosfolípids juntament amb proteïnes i esterols. Hi ha però una diferència important. El principal esterol de la membrana fúngica és l'ergosterol, en canvi, en les cèl·lules humanes és el colesterol. Aquesta diferència és important a la pràctica, ja que com després es comentarà, la majoria dels antifúngics actuals actuen sobre l'ergosterol. El principal paper de la membrana plasmàtica radica en la regulació que exerceix sobre l'entrada i sortida de diversos substrats, nutrients o productes finals del metabolisme. La membrana té en la seva part externa diverses proteïnes (proteïnes integrals de membrana) que, com veurem posteriorment, intervenen en la síntesi de components de la paret cel·lular. Un tercer aspecte de la funció de la membrana plasmàtica és la d'actuar transmetent les senyals rebudes de l'ambient exterior cap a l'interior de la cèl·lula (transducció).

Paret cel·lular. Els fongs tenen una paret cel·lular que els determina la seva forma. La pèrdua d'aquesta estructura, per tractaments enzimàtics, condueix a la