行政院國家科學委員會專題研究計畫　期中進度報告

白色念珠菌分泌性 aspartyl proteinase 與臨床預後之相關性研究(1/3)

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-165-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院內科

計畫主持人：陳宜君
共同主持人：李芳仁，張上淳

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 6 月 10 日

A comparison of secreted aspartyl proteinase from different clinical isolates of *Candida albicans*

Abstract

The yeasts of the genus *Candida* are opportunistic pathogens associated with the rising incidence of life-threatening infections in immunocompromised individuals. Secretion of aspartic proteinases has been determined to be one of the virulence factors of the pathogenic *Candida* species. Besides, yeast-hypha morphogenesis is coregulated with *SAP4-6* gene products. Thus, Sap4-6 proteins are the potential targets for intervention. Our previous study demonstrated variation of fungistatic activity of anti-Sap5 antiserum and control serum against different bloodstream isolates of *C. albicans*. In this study, in order to analyze the extracellular proteolytic activities of a large number of *Candida* clinical isolates, we developed a screening system based on a solid medium containing bovine serum albumin as the sole nitrogen source. Using this system, we assessed 78 clinical isolates of *Candida* collected from patients treated at National Taiwan University Hospital, for the presence of secreted aspartic proteases (Saps). Implications of such differences on pathogenicity will be determined by evaluation of relationship between Sap levels and patient outcome.

Introduction

The incidence of nosocomial candidemia increased rapidly in the past two decades and the impact is substantial, with an attributable mortality rate of 43% in this hospital (Chen et al., 1997 & 2003). *Candida* spp. were the leading pathogens at National Taiwan University Hospital since 1993 (Chen et al., 1997 & 2003). Because the modes of management of such patients are limited, with the increased incidence of *C. albicans* infections **the investigation of virulence factors and the search for a new target for development of anti-fungal agents and immunotherapy has become increasingly important.** The most common species that causes candidiasis is *Candida albicans* (Hung et al., 1996; Chen et al., 1997 & 2003; Edmond et al., 1999). Furthermore, the species-specific case-fatality rate was highest (49.2%) for *C. albicans* than other *Candida* spp. (Hung et al., 1996). *C. albicans* are

commensal of the intestinal tract and can be found frequently in other mucocutaneous sites. Invasive candidiasis is considered to be of endogenous origin (Chen et al., 2001a) and occurs in compromised hosts or critically ill patients (Hung et al., 1996; Chen et al., 1997 & 2001b). There is no clear evidence, whether invasion of the yeast is solely related to a failure in the defense systems of the host or whether specific properties of certain yeast strains favor invasion. However, our study has confirmed the independent role of nosocomial *Candida* infection in ICU mortality (Chen et al., 2001b). These opportunistic infections can be a catastrophe for patients with potentially curable diseases, and are the major obstacles to adequate control of underlying diseases with good prognosis. However, nosocomial infection has a high potential for modification or control compared to other potential risk factors for ICU mortality. Knowledge regarding the epidemiology and pathogenesis of candidiasis is important, and may facilitate the control of these problems and thereby improve patient outcomes.

Data from infection models and null mutants generated by targeted mutagenesis suggest that the contribution of virulence factors by these organisms to disease development is significant. Ability of the fungus to form germ tubes and growth in mycelial phase has been discussed as factors of pathogenicity (Lo et al., 1997). A relationship between secretion of proteinase and pathogenicity has been suggested as well. Besides, growing evidences suggest that yeast-hypha morphogenesis is coregulated with other virulence factors, such as *SAP4-6* gene products (Hube et al., 1994; Chen et al., 2002) and Sap4-6 proteins are the downstream effector of the signaling pathways of morphogenesis (Schweizer *et al.,* 2000; Felk *et al.,* 2002; Chen et al., 2002). Thus, *Candida* spp., and especially *C. albicans*, may be considered a highly evolved group of pathogens that utilize their resources to persist in the normal host and invade when host immune deficiencies occur. Furthermore, our previous studies suggest that **Sap4-6 proteins are the potential targets for intervention.**

C. albicans possesses a gene family encoding secreted aspartyl proteinases (Saps) with unusually broad substrate specificity (Cutler, 1991; Hube, 1996; Hube & Naglik, 2001). These enzymes have been linked to the virulence of the fungus since their discovery (Staib, 1965; Kwon-Chung *et al.,* 1985; Cassone *et al.,* 1987; de Bernardis *et al.,* 1999). The proposed functions of these proteinases during infection include the digestion of host proteins for nutrient supply,

evasion of host defenses by degrading immunoglobulins and complement proteins, adherence, and degradation of host barriers during invasion (Hube, 1996).

Thus far, at least 10 closely related *SAP* genes were identified (White *et al.,* 1993; Hube *et al.,* 1994; Monod *et al.,* 1994 & 1998). Differences in expression of various *SAP* mRNAs have been investigated *in vitro* and in experimental animal models (Morrow *et al.,* 1992; Hube *et al.,* 1994; White *et al.,* 1995; Borg-von Zepelin *et al.,* 1998; Monod et al, 1998). Experimental infections with various mutants (Δ*sap1* to Δ*sap6*) generated by targeted mutagenesis suggested the importance of different *SAP*s in the virulence of *C. albicans* (Hube *et al.,* 1997; Sanglard *et al.,* 1997; de Bernardis *et al.,* 1999; Kretschmar *et al.,* 1999). These data suggest that the temporal and specific regulation of *SAP* expression might be important for the survival of *C. albicans* in its natural environment and thus in the pathogenesis of this fungus. Among the various *SAP* gene products, deduced amino acid sequences for Sap4-6, which have 75%-89% similarity to each other, form a group distinct from Sap1-3 (Hube *et al.,* 1994; Miyasaki *et al.,* 1994). This subfamily, Sap4-6, was a potential target for intervention because *SAP4-6* mRNAs were first identified during hyphae formation at neutral pH (Hube *et al.,* 1994). Blood and salivary pH are approximately neutral, and hyphae formation is important during tissue invasion (Edwards, 2000); therefore, Sap4-6 proteins were presumed to be important in disseminated candidiasis. Recently, it has been demonstrated that a Δ*sap4-6* triple mutant resulted in attenuated mortality after systemic infection in guinea pigs and mice (Sanglard *et al.,* 1997), and that less tissue damage occurred in a murine peritonitis model (Kretschmar *et al.,* 1999). These results suggest that the *SAP4-6* genes contribute to the development of disseminated infections.

Our previous study demonstrated variation of fungistatic activity of anti-Sap5 antiserum and control serum against different bloodstream isolates of *C. albicans*. In this study, in order to analyze the extracellular proteolytic activities of a large number of *Candida* clinical isolates, we developed a screening system based on a solid medium containing bovine serum albumin as the sole nitrogen source. Using this system, we assessed 78 clinical isolates of *Candida* collected from patients treated at National Taiwan University Hospital, for the presence of secreted aspartic proteases (Saps). Implications of such differences on pathogenicity will be determined by evaluation of

relationship between Sap levels and patient outcome.

Materials and Methods.

Yeast strains. The *C. albicans* wild-type strain SC5314 (Gillum *et al.,* 1984) and the *sap* null mutant strains *Δsap1, Δsap2*, *Δsap3*, *Δsap4, Δsap5, Δsap6, Δsap4-6* (Hube *et al.,* 1997; Sanglard *et al.,* 1997)*,* and *Δefg1/cph1* (Lo *et al.*, 1997) will be used in the development of in vitro assay system. *C. albicans* SC5314, a blood culture isolate from a patient with disseminated candidiasis, was used as the DNA template for cloning individual *SAP* genes and expression of His-tagged fusion proteins in *E. coli* (Chen et al., 2002). The clinical isolates of *C. albicans* includes those colonizing and/or infecting critically patients which were collected during previous surveillance study in ICU (Chen et al., 2001b) and those bloodstream isolates collected for evaluation of in vitro antifungal susceptibility testing (Chen et al., 2003).The study included 245 *Candida* strains collected from patients treated at National Taiwan University Hospital during 1997 to 2004 (see Table). Multiple isolates from one patient were included only when the specimens were obtained from separate body sites. Blood samples were cultured by inoculation into BACTEC fungal medium (Becton-Dickinson Microbiology Systems, Cockeysville, Md, USA) and tested daily for microbial growth by BACTEC 9240 system (BD Biosciences, Sparks, Md., USA). Organisms were identified by germ tube analysis and morphology on cornmeal-Tween 90 agar [9] or, when necessary, by standard biochemical testing with the API 20C system (API BioMerieux Vitek, Inc., Hazelwood, Mo.). Clinical isolates of *Candida* species were collected randomly from Jan 1, 2002 through Dec 31, 2002. All the isolates were kept at –70°C and were subcultured at least twice on Sabouraud dextrose agar at 35°C prior to being tested. If a patient with the same species in the same culture site was found during a 7-day period, only first isolate was tested.

Growth conditions. The following liquid media will be used: YPD (1% yeast extract, 2% peptone, and 2% glucose) complex medium for general use; YNB-BSA medium for induction of Sap2 protein (0.17% [wt/vol] yeast nitrogen base [Difco], 2% glucose, 0.2% bovine serum albumin [BSA] [Sigma] [pH 5.0]); and modified Lee's medium (pH 4.5 and pH 6.5) for induction of Sap4-6 proteins (Lee *et al.,* 1975; Morrow *et al.,* 1992). BSA (Sigma) was added as indicated. *C. albicans* was grown in an orbital

incubator at the indicated temperatures.

Induction of hyphae formation. For hyphae growth, *C. albicans* will be incubated in modified Lee's medium (pH 4.5) (Lee et al., 1975; Morrow et al., 1992) at 25°C for 48 h (stationary phase), transferred to pre-warmed modified Lee's medium (pH 6.5) containing 0.2% BSA (final cell density approximately $5x10^6$ cells/ml), then incubated at 37°C for 48-72 h. Growth was measured at OD_{600}, and the pH values of the culture media were determined at the indicated times. Hyphae formation was visualized by use of phase-contrast microscopy, and percentages of hyphae formation were calculated by counting of ~ 200 cells. For kinetic study, proteins prepared from culture media obtained from wild-type and mutant strains before and after hyphae induction were evaluated. To determine the expression of the intracellular Sap4-6, we prepared *Candida* total lysates by use of liquid nitrogen, followed by addition of lysis buffer as previously described (Lee et al., 1997).

Preparation of Yeast RNA . Total RNA is isolated according to the procedures in Current Protocols in Molecular Biology ( ). Briefly, grow the yeast cell in of medium to mid-exponential phase (OD_{600}=1.0) and centrifuge cells 3 min at 1500xg. Resuspend pellet in 1 ml ice-cold water and microcentrifuge 10 sec at 4°C. Remove supernatant and freeze pellet in -70°C. Resuspend cell pellet in TES solution. Add acid phenol and vortex vigourously 10 sec. Incubate 30 to 60min at 65°C. Place on ice for 5 min. Microcentrifuge 5 min at top speed, 4°C. Transfer aqueous phase to a 1.5-ml microcentrifuge tube and add acid phenol, and vortex. Transfer aqueous phase to a new tube and add chloroform. Vortex and microcentrifuge 5 min at top speed, 4°C. Transfer aqueous phase to a new tube, add 3M sodium acetate, pH 5.3, and ice-cold 100% ethanol and precipitate. Microcentrifuge 5 min at top speed, 4°C. Wash RNA pellet by vortexing briefly in ice-cold 70%°C. Resuspend pellet in DEPD H_2O. RNA concentration are measured by absorbance at 260 nm.

RT-PCR. Synthesis of cDNA was performed with 1μg of total RNA using StrataScript Reverse transcriptase (Stratagene, California, USA) following the manufacturer's instructions. The cDNA samples were subjected to 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50 to 54°C depending on the primer pairs used, and extension for 1 min at 72°C. Primers which are highly specific for all 10 sap genes were chosen (Felk et al, 2002). EFB1 is expressed in living C. albicans cellsto a similar extent under all conditions investigated and is therefore a internal control.

RT-PCT without the addition of reverse transcriptase to prove the absence of contaminating DNA and reactions with genomic DNA templates to prove the efficiency of the PCR.

Western blot analysis. For immunoblot analysis, anti-Sap antibodies will be diluted in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 and 5% dried skim milk. Sample proteins were separated by SDS-PAGE, transferred onto Immobilon-P membrane (Millipore Corp.), and incubated with the primary specific antibodies at room temperature for 60 min, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:5000). Bound antibodies were detected with the ECL system (Amersham) according to the manufacturer's instructions.

Preparation of Candida proteins from culture medium, total cell lysate, and cell wall proteins. Blastospores and mycelium will be obtained as previously described. Cells will be collected from the culture media by centrifugation at 3000 g for 10 min, washed five times with phosphate-buffered saline (PBS) at 4°C for 10 min (to remove BSA) and frozen in liquid nitrogen. Glass beads (5 mg beads per mg cells) and lysis buffer (1% Triton X-100, 100 mM Tris-Cl [pH6.8], 8M urea, 2% SDS) will be added. The suspension will be disrupted by vigorous vortex for 3 to 5 min. The total cell lysate will be sedimented by centrifugation at 12,000 g for 20 min and the concentration of proteins in the supernatant will be measured by BCA Protein Assay reagent (Pierce). To prepare cell wall proteins, glass beads and PBS will be added to frozen cells and vortexed for 3 to 5 min. Cell wall fraction will be collected by centrifugation at 1,200g for 15 min, resuspended in lysis buffer (1% Triton X-100, 100 mM Tris-HCl [pH6.8], 2% SDS, 8M urea), and will be boiled for 5 min. Then, cell wall proteins in the supernatant will be collected by centrifugation at 12,000 g for 20 min.

Proteinase assay in bovine serum albumin agar. The method adopted is according to Ruchel et al (Ruchel et al., 1982) with minor modification (Chakrabari et al., 1991). Secretion of proteinase by *C. albicans* strains will be tested by cultivation on Staib's BSA-agar, on yeast extract (BBL) -BSA agar and on yeast carbon base (YCB, Difco)-BSA-Protovita (Roche) agar. Single colony from the pure culture is inoculated into 5 ml of yeast extract peptone dextrose (YEPD) broth composed of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose. After incubating overnight, secretion of proteinase is assayed on a solid medium containing 2.34% YCB, 0.2% yeast extract,

2.5% (v/v) and 0.4% BSA and adjusted to pH 5.0. Filter sterilized medium is then mixed with equal amount of 4% autoclaved agar and 20 ml medium is poured in each sterile 90 mm Petri dish. Plates are stored inverted at 4℃ in a moist atmosphere up to 1 month before use.

A sterile filter paper disc of 8 mm diameter (Toyo Kaisha, Ltd) is soaked with the YEPD broth containing overnight growth of different isolates. A disc dipped in uninoculated YEPD broth placed in the center of the Petri dish serves as negative control. The plates after incubated for 5 days at 37 ℃, or for 24 h at 37℃ and for 5 days at 26℃, are stained with 1% amido black solution (Merck, 1g/L in 3.5M acetic acid) for 1 minute and are gently washed 5-10 times in tap water.

BSA proteolysis by *Candida* species is observed against ordinary light as a clear zone surrounding and under the colony. Proteinase activity is scored as grade – or + when no visible or very limited clarification of the agar under the disc is present, Grade 1+; when visible proteolysis is limited to 1-2 mm around the disc and Grade 2+; when the zone of proteolysis is more than 2 mm from the margin of the disc.

Results

SAP expression profile in vitro. The expression of *SAP1-10* varied slightly in different sap mutant under hypha-inducing conditions in vitro compared to wild-type cells. *SAP7* and *SAP8* RNA were not detectable. On the other hand, *SAP9* and *SAP10* were detected in wild type and mutants before and after induction.

Development and optimization of medium. The test medium used by Odds and Abbott (1980) contains BSA as the only source of nitrogen. The cleavage of BSA by Saps results in zones of clearance. In liquid protein-supplemented cultures of *C. albicans*, the characteristic drop in pH associated with Sap production has been observed previously (Chen et al., 2003). The plates containing the BSA were stained black/blue, but clear zones surrounding the disc of proteolytic *Candida* strains (n = 181) appeared. No color change was caused by nonproteolytic strains (n = 64). A *C. albicans* mutant strain lacking the *SAP1*, *SAP2*, and *SAP3* genes displayed limited growth and did not form any proteolytic zones.

Screening of *Candida* isolates for Sap production. We analyzed 245 clinical isolates of *Candida* species (Table 2). We examined the growth of these isolates and their ability to produce extracellular proteinase on the hemoglobin-supplemented medium. We focused on the species that are known to produce extracellular aspartic proteinases, i.e., *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. As *C. lusitaniae* was found to produce proteinase in liquid protein-supplemented media, we included also seven strains of this species. Isolates of *C. krusei*, *C. glabrata*, and *C. guilliermondii* were included, as they are allegedly nonproteolytic.

Discussion

Complexity of differential expression of *SAP* Gene family in *C. albicans.* To study the expression of the *C. albicans SAP* gene family during hyphae-inducing conditions, we performed RT-PCR of wild type strain (SC 5314), △sap4, △sap5, △sap6, and △sap4,5,6 triple mutant strains. Cells were induced by induction in 0.2% serum, pH- and temperature-regulated transition (from pH4.5under 25℃ to pH6.5under 37℃). The results showed that expression of *SAP3*, *SAP7* and *SAP8* were not detectable in the wild type cells. The expression patterns of *△sap4,* *△sap5,* *△sap6,* and *△sap4,5,6* triple mutant strains were similar to that of the wild type except that SAP1 were detected in △sap4 and △sap5 strains. The study of Sanglard et al.(Microbiology, 1998,144:2731-2737) showed that *SAP8* mRNA was expressed preferentially in yeasts at 25℃ after 6 and 9h growth in BSA-containing medium. Transcripts of *SAP9* were observed preferentially in later growth phases when *SAP8* expression had decreased.

Our previous study (Chen et al. MM 2003) revealed that (1) expression of Sap4/6 was reduced in *Δsap2* mutant; (2) There was only trace, if any, amount of Sap2 in the culture supernatants of *Δsap4,5,6* triple mutant, (3) Simultaneously expression of *SAP1-3* and *SAP4-6* mRNAs were shown in a model of oral candidiasis based on reconstituted human epithelium, and in the oral cavity of patients with *Candida* colonization or oral candidiasis (Schaller et al., 1999; Naglik et al., 1999). Thus, Sanglard et al. (1997) speculated that *SAP4-6* expression might be required in the process of Sap2 induction. On the other hand, much lower quantities of Sap4-6 proteins were detected in hyphal cells as compared to Sap1-3 proteins in yeast and hyphal cells walls (Schaller et

al., 1999). The tissue damage caused by the *Δsap4,5,6* triple mutant was at least as severe as those caused by SC5314 (Schaller et al., 1999). Thus, the interaction between *SAP1-3* gene subfamily and *SAP4-6* subfamily is more complex than previously thought. The mechanism of differential expression of different *SAP* gene family warranted further explored.

The pattern of *SAP* gene expression differed in some aspects from the results obtained by cell culture studies, in experimental vaginitis and in experimental human oral candidiasis (Schaller et al., 1998 & 1999). The programme of *SAP* gene expression in in vitro model is presumably related to progressive changes in environmental conditions, such as the availability of Sap-inducing substrates, the pH in the microniches and the development of hyphae of *C. albicans* during the infection process. The proteolytic activity of ***Δsap4-6*** triple mutant noted in previously may attribute to the.expression of SAP9 and SAP10.

Screening of *Candida* isolates for Sap production. In the proteolysis test, size of the clear zone around the disc was affected by the growth condition of colony. Condition was even serious when hyphae formed. Besides, the process of destain may wash the colony off and affected the size of the clear zone. Reading from the reverse side of the □etri dish did not help much. SC5314, *cph1* mutant, Δ*SAP1*, Δ*SAP2*, Δ*SAP3*, Δ*SAP4*, Δ*SAP5*, Δ*SAP6,* Δ*SAP4,5,6* showed hyphae formation and had a slight sag (Fig)on the Modified Lee's agar plates. On the modified Lee's agar plate, mutants lacking the *efg1* and *efg1/cph1* did not produce hyphal cells, while the mutants lacking *cph1* produced hyphal cells and showed a slight sag on the plates. In the study of Felk et al., intraperitoneal infection of a *cph1* mutant still produced long hyphae cells and showed invasive abilities .However, *efg1* mutants had a strongly reduced ability to produce hyphae cells in vivo and did not invade any parenchymal organ.

References.

Felk, A., Kretschmer, M.,Albrecht, A., Schaller, M., Beinhauer, S., Nichterlein, T., Sanglard, D., Kortin, H. C., Schafer, W., and Hube B. 2002. Candida albicans Hyphal formation and the expression of the Efg1-Regulated proteinases Sap4 to Sap6 are required for the invasion of parchymal organs. Infection and Immunology 70:3689-3700.

Fusek, M., E. A. Smith, M. Monod, B. Dunn, and S. I. Foundling. 1994. Extracellular aspartic proteinases from *Candida albicans*, *Candida tropicalis* and *Candida parapsilosis* yeast differ substantially in their specificities. Biochemistry **33:**9791-9799.

Haynes, K. 2001. Virulence in *Candida* species. Trends Microbiol. **9:**591-596.

Hube, B., and J. Naglik. 2001. *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiology **147:**1997-2005.

Koelsch, G., J. Tang, J. A. Loy, M. Monod, K. Jackson, S. I. Foundling, and X. Lin. 2000. Enzymic characteristic of secreted aspartic proteases of *Candida albicans*. Biochim. Biophys. Acta **1480:**117-131.

Monod, M., G. Togni, B. Hube, and D. Sanglard. 1994. Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. Mol. Microbiol. **13:**357-368.

Odds, F. C., and A. B. Abbott. 1980. A simple system for the presumptive identification of *Candida albicans* and differentiation of strains within the species. Sabouraudia **18:**301-317.

Pichová, I., L. Pavlíčková, J. Dostál, E. Dolejší, O. Hrušková-Heidingsfeldová, J. Weber, T. Ruml, and M. Souček. 2001. Secreted aspartic proteinases of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida lusitaniae*: inhibition with peptidomimetic inhibitors. Eur. J. Biochem. **268:**2669-2677.

Ray, T. L., and C. D. Payne. 1990. Comparative production and rapid purification of *Candida* acid proteinase from protein-supplemented cultures. Infect. Immun. **58:**508-514.

Ruchel, R., F. De Bernardis, T. L. Ray, P. A. Sullivan, and T. G. Cole. 1992. *Candida* acid proteinases. J. Med. Vet. Mycol. **30:**123-132.

Ruchel, R. 1981. Properties of a purified proteinase from the yeast *Candida albicans*. Biochim. Biophys. Acta **659:**99-113.

Smolenski, G., P. A. Sullivan, S. M. Cutfield, and J. F. Cutfield. 1997. Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. Microbiology **143:**349-356.

Stewart, K., and C. Abad-Zapatero. 2001. *Candida* proteases and their inhibition: prospects for antifungal therapy. Curr. Med. Chem. **8:**941-948.

Wu, T., L. P. Samaranayake, W. K. Leung, and P. A. Sullivan. 1999. Inhibition of growth and secreted aspartyl proteinase production in *Candida albicans* by lysozyme. J. Med. Microbiol. **48:**721-730.

Zaugg, C., M. Borg-von Zepelin, U. Reichard, D. Sanglard, and M. Monod. 2001. Secreted aspartic proteinase family of *Candida tropicalis*. Infect. Immun. **69:**405-412.

Chakrabarti A., N. Nayak & P. Talwar. (Title) 1991. Mycopathologia 114: 163-168.

Jií Dostál, Petr Hamal, Libuše Pavlíčková, Milan Souček, Tomáš Ruml, Iva Pichová, and Olga Hrušková-Heidingsfeldová. 2003. Simple Method for Screening *Candida* Species Isolates for the Presence of Secreted Proteinases: a Tool for the Prediction of Successful Inhibitory Treatment. J Clin Microbiol 41:712-716.