Journal of Clinical Microbiology

Killer system: a simple method for differentiating Candida albicans strains.

L Polonelli, C Archibusacci, M Sestito and G Morace

***J. Clin. Microbiol.* 1983, 17(5):774.**

JOURNAL OF CLINICAL MICROBIOLOGY, May 1983, p. 774–780
0095-1137/83/050774-07$02.00/0

Vol. 17, No. 5

Killer System: a Simple Method for Differentiating *Candida albicans* Strains

L. POLONELLI,* C. ARCHIBUSACCI, M. SESTITO, AND G. MORACE

Istituto di Microbiologia, Facoltà di Medicina e Chirurgia Agostino Gemelli, Università Cattolica del Sacro Cuore, 644-00168 Rome, Italy

Received 8 March 1982/Accepted 14 September 1982

The killer effect of 37 species of *Candida, Cryptococcus, Hansenula, Pichia, Rhodotorula, Saccharomyces*, and *Trichosporon* on 100 *Candida albicans* isolates of human and animal origin was studied. All of the *C. albicans* cultures were sensitive to one or more killer yeasts. The factors affecting the killer phenomenon on *C. albicans* were investigated for realizing a simple system for the differentiation of the 100 *C. albicans* isolates. By using this system, it was possible to differentiate up to 512 isolates of *C. albicans* according to their susceptibility to the killer effect of nine selected killer yeasts. The use of this method as an epidemiological marker in the case of presumptive nosocomial infections due to *C. albicans* is also reported.

In 1963 Bevan and Makower (2) reported, for the first time in yeasts, that a few isolates of *Saccharomyces cerevisiae* produced a substance lethal to other strains of the same species. This effect was named the killer phenomenon, and the substance was called the killer toxin. Since that time numerous studies have been carried out to determine the distribution of the killer phenomenon among yeasts, the modalities of its action, and the physiological and chemical properties of the toxin (1, 3, 6, 7, 12, 13, 16, 17, 19, 22, 25, 26).

On the basis of these reports we initiated a study of the killer phenomenon in *Candida albicans*, a well-known etiological agent of topical and systemic infections.

The finding of a convenient, sensitive, and reproducible method that could be used as an epidemiological marker for differentiating strains of *C. albicans* would be of value. Currently, the only method generally acceptable is based on the division of *C. albicans* isolates into two serological groups (A and B) (11). However, the majority of *C. albicans* isolates belong to group A, thus limiting the value of this method in epidemiological studies. For this reason, studies have been carried out on the differentiation of *C. albicans* isolates from one another (5, 10, 14, 15, 20, 21, 23, 24).

In this report we present a relatively simple procedure for differentiating *C. albicans* isolates based on the susceptibility of *C. albicans* strains to the toxic effects of nine different killer yeasts. This killer system has the potential for differentiation of up to 512 strain types of *C. albicans*.

MATERIALS AND METHODS

Cultures. One hundred *C. albicans* isolates were obtained during a 3-month period among yeasts isolated from clinical specimens and pigeon droppings (18) (Table 1). The *C. albicans* isolates were identified by their biochemical and morphological characteristics. They were maintained in our collection at room temperature in sterile distilled water.

During this time 9 killer yeasts were selected from 54 other yeasts graciously furnished by public and private collections (Table 2). Their selection was made according to their characteristics of reproducibility and heterogeneous behavior against the 100 sensitive isolates of *C. albicans*.

Media. A standard medium containing 2% dextrose, 2% peptone, 1% yeast extract, and 2% agar, buffered at pH 4.5 with 0.1 M citric acid and 0.2 M potassium phosphate dibasic anhydrous (YEPD), was used. Later, we replaced the YEPD agar with buffered Sabouraud glucose agar, modified (Difco Laboratories).

Test performance. The *C. albicans* isolates were initially incubated for 18 h at 25°C with shaking (120 rpm) in 10 ml of YEPD broth (pH 4.5). One milliliter from these broth cultures was diluted with 10 ml of fresh YEPD broth (pH 4.5). One milliliter of this suspension was then mixed with 20 ml of YEPD agar (pH 4.5) that contained 0.003% methylene blue and poured into a petri dish to obtain an agar-*C. albicans* suspension. The dye differentially stained dead yeast cells blue. For comparative purposes, 1 ml of a distilled water suspension (optical density of 25% at 530 nm) of 48-h-old cultures of *C. albicans* grown on modified Sabouraud glucose agar was mixed with 20 ml of modified Sabouraud glucose agar containing 0.003% methylene blue and buffered at pH 4.5. The killer yeasts were cultured for 48 h in YEPD agar and, later, in modified Sabouraud glucose agar and streaked onto the pour plates of *C. albicans*. The agar-*C.*

TABLE 1. Origin of the 100 *C. albicans* isolates studied

Material	No. of isolates	Collection no.
Vaginal swabs	61	1, 2, 4, 6, 7, 12, 14, 15, 18, 19, 21, 29, 31–33, 35–38, 40, 41, 43, 45–56, 60, 61, 63, 66–68, 70, 72, 73, 75–78, 80, 83, 86, 88, 90–92, 94–100
Sputum	19	3, 5, 9–11, 20, 30, 34, 57–59, 64, 71, 74, 79, 84, 85, 87, 93
Pigeon droppings	7	22–28
Pharyngeal swabs	4	16, 17, 39, 44
Tonsillar swabs	3	65, 69, 81
Pus	2	13, 42
Stools	2	62, 89
Bile	1	82
Urine	1	8

albicans dishes were successively incubated at 25°C for 72 h before the results were read.

Factors affecting the killer phenomenon on *C. albicans*. YEPD medium was used to determine the influence of pH, temperature, and medium concentration of dextrose, peptone, and yeast extract on the toxic effect of different isolates of killer yeasts against representative isolates of *C. albicans*.

Reading and interpretation of results. The killer effect was considered positive when either a clear zone of inhibition or a region of blueish-colored cells, or both, surrounded the streaked killer yeasts. A negative result was recorded if neither of these results was observed (Fig. 1 and 2). A code adopted in our institute for differentiating *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Proteus rettgeri* was used to record the combined effect of the nine killer yeasts adopted and arranged in triplets. Each triplet was examined for its pattern of activity against each isolate of *C. albicans* (4, 8, 9) (Table 3).

RESULTS

Of all yeasts studied, only the species of *Pichia* and *Hansenula* had different toxic effects on the 100 *C. albicans* isolates under standard conditions (Table 2). Each isolate of *C. albicans* was found to be sensitive to at least one killer yeast.

Temperature and pH were the most important factors affecting the killer phenomenon against

TABLE 2. Percentage of activity of yeast species tested as potential killers of isolates of *C. albicans*

Strain	Collection[a]	No.	%	Strain	Collection[a]	No.	%
Candida glabrata	UCSC	0		*H. dimennae*	AHEARN	WC 44	71
C. guilliermondii	UCSC	0		*H. fabianii*	CBS	5640	
C. krusei	UCSC	0		*H. fabianii*	AHEARN	WC 45	53
C. lipolytica	UCSC	0		*H. holstii*	CBS	4140	
C. maltosa	UCSC	0		*H. mrakii*	AHEARN	WC 51	89
C. parapsilosis	UCSC	0		*H. nonfermentans*	UM		
C. pseudotropicalis	UCSC	0		*H. petersonii*	AHEARN	WC 53	45
C. stellatoidea	UCSC	0		*H. subpelliculosa*	CBS	5767	
C. tropicalis	UCSC	0		*Pichia* sp.	STUMM	1035	98
Cryptococcus diffluens	UCSC	0		*Pichia carsonii*	CBS	810	
C. neoformans	CDC	B551		*P. farinosa*	CBS	185	26
C. neoformans	CDC	B977		*P. guilliermondii*	UT		
C. neoformans	UCSC	0		*P. guilliermondii*	CBS	2031	
C. neoformans	UCSC	1		*P. kluyveri*	STUMM	1002	23
C. neoformans	UCSC	2		*P. membranaefaciens*	UT		32
C. neoformans	UCSC	3		*P. membranaefaciens*	UM		4
C. neoformans	UCSC	4		*P. membranaefaciens*	CBS	107	5
C. neoformans	UCSC	5		*P. ohmeri*	CBS	5367	
Hansenula sp.	STUMM	1034	97	*P. spartiniae*	UCSC	0	
Hansenula anomala	UT		94	*Rhodotorula glutinis*	UCSC	0	
H. anomala	UM		7	*R. rubra*	UCSC	0	
H. anomala	CBS	5759	92	*Saccharomyces cerevisiae*	CDC	B2210	
H. anomala	AHEARN	UN 866	98	*Trichosporon beigelii*	UCSC	0	
H. bimundalis	CBS	5642		*T. beigelii*	UCSC	1	
H. bimundalis	AHEARN	WC 38	25	*T. capitatum*	UCSC	0	
H. californica	AHEARN	WC 40	73	*T. capitatum*	UCSC	1	
H. canadensis	AHEARN	WC 41	100	*T. penicillatum*	UCSC	0	

[a] UCSC, Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy; CDC, Centers for Disease Control, Atlanta, Ga.; STUMM, C. Stumm, University of Njimegen, Njimegen, The Netherlands; UT, Istituto di Igiene, Università di Torino, Torino, Italy; UM, Istituto di Igiene, Università di Milano, Milan, Italy; CBS, Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands; AHEARN, D. G. Ahearn, Georgia State University, Atlanta.

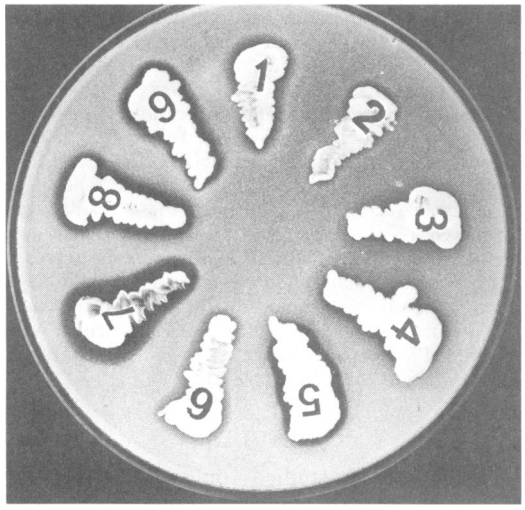

FIG. 1. Activity of the nine killer yeasts on *C. albicans* isolate no. 10, strain type 1 1 1.

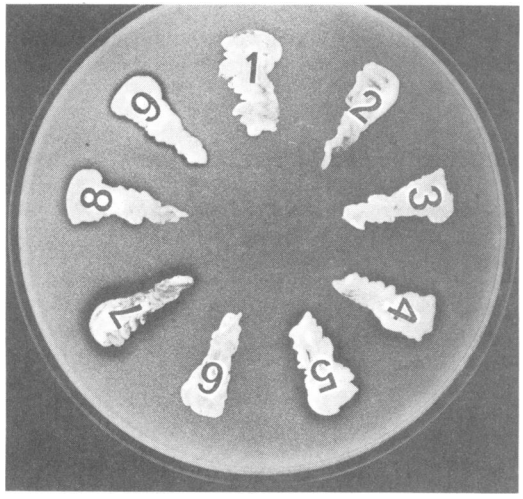

FIG. 2. Activity of the nine killer yeasts on *C. albicans* isolate no. 44, strain type 7 2 1.

C. albicans. The majority of the killer yeasts did not produce a killer factor at pH values higher than 5.6. Their optimal activity was in the range of pH 4 to 4.6, although the *C. glabrata* isolate showed activity at pH 6 (Fig. 3).

Selected strains of *C. albicans* generally were most sensitive to the killer yeasts when all cultures were incubated at 25°C. *C. albicans* isolate 38 appeared resistant to the killer factors at below 30°C (Fig. 4). Smaller differences were found when the concentrations of dextrose, peptone, and yeast extract in the medium were varied (Fig. 5, 6, and 7).

The substitution of buffered modified Sabouraud glucose agar for YEPD agar did not appreciably alter the results. Analogously, an inoculum of sensitive cells of *C. albicans* in a log phase was replaced by a water suspension of the same *C. albicans* isolate without loss of sensitivity of the test.

We were able to identify 25 different strain types among the 100 *C. albicans* isolates. These

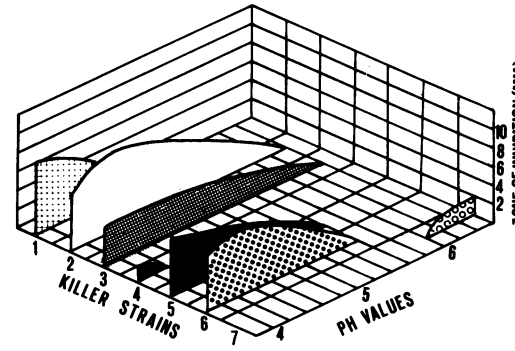

FIG. 3. Influence of pH of the growth medium on susceptibility of *C. albicans* to the killer toxins of various yeasts: 1, *P. kluyveri*; 2, *Hansenula* sp.; 3, *P. membranafaciens*; 4, *P. guilliermondii*; 5, *H. anomala*; 6, *Pichia* sp.; 7, *C. glabrata*.

TABLE 3. Triplet grouping and activity codes for killer strains

Activity of first triplet				Activity of second triplet				Activity of third triplet			
Yeast				Yeast				Yeast			
Hansenula sp. Stumm 1034	*Pichia* sp. Stumm 1035	*H. anomala* UM	Code	*H. anomala* CBS 5759	*H. anomala* Ahearn UN 866	*H. californica* Ahearn WC 40	Code	*H. canadensis* Ahearn WC 41	*H. dimennae* Ahearn WC 44	*H. mrakii* Ahearn WC 51	Code
+	+	+	1	+	+	+	1	+	+	+	1
+	+	−	2	+	+	−	2	+	+	−	2
+	−	+	3	+	−	+	3	+	−	+	3
−	+	+	4	−	+	+	4	−	+	+	4
+	−	−	5	+	−	−	5	+	−	−	5
−	+	−	6	−	+	−	6	−	+	−	6
−	−	+	7	−	−	+	7	−	−	+	7
−	−	−	8	−	−	−	8	−	−	−	8

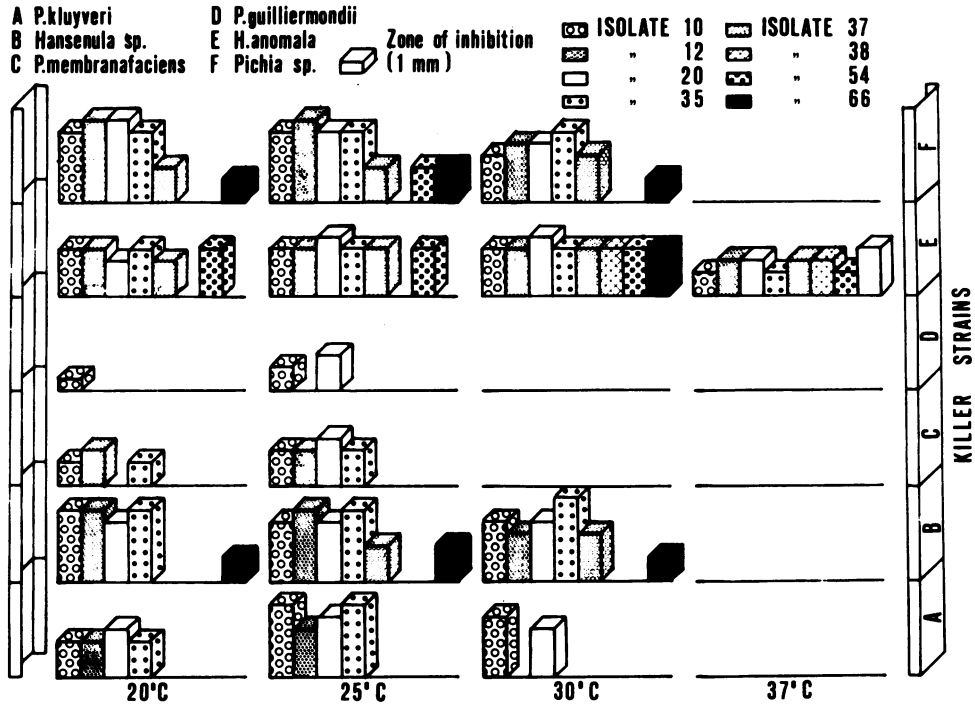

FIG. 4. Influence of growth temperature on susceptibility of *C. albicans* to killer toxins of various yeasts.

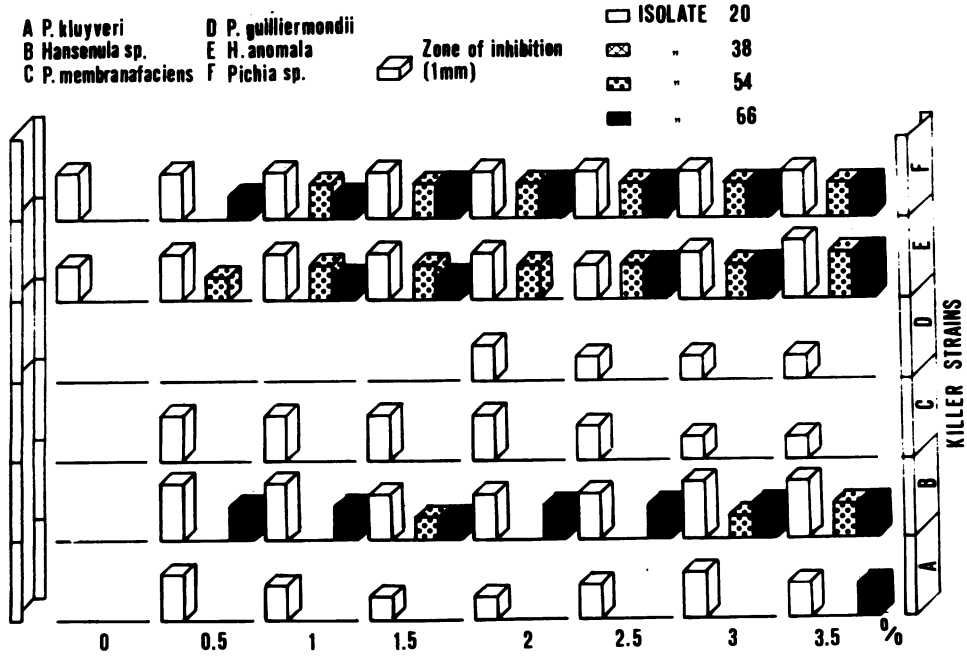

FIG. 5. Effect of glucose concentration of the growth medium on susceptibility of *C. albicans* to the killer toxin of various yeasts.

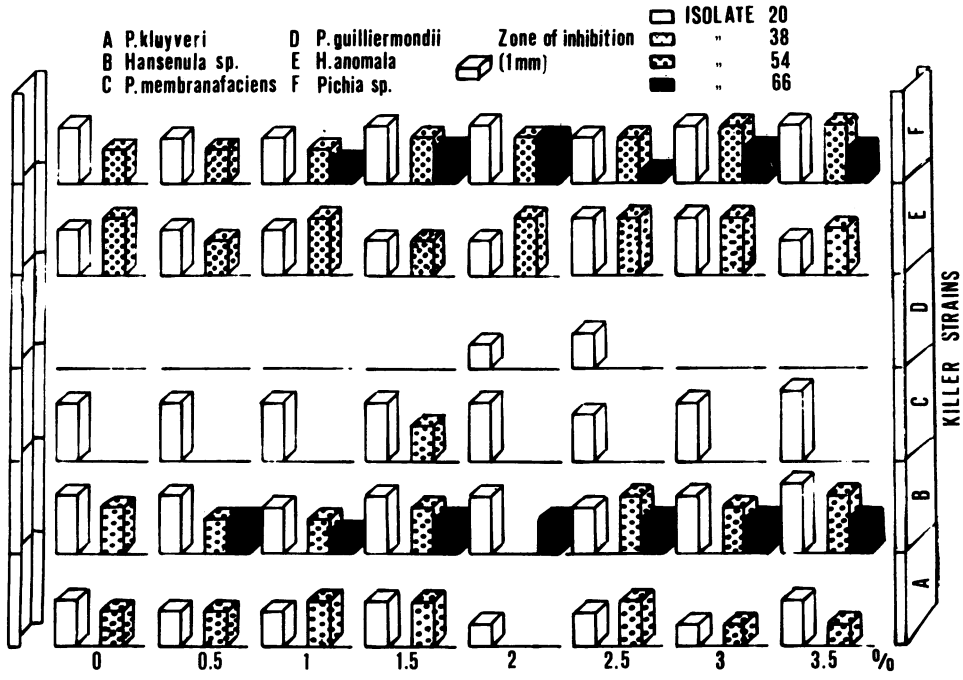

FIG. 6. Effect of peptone concentration of the growth medium on susceptibility of *C. albicans* to the killer toxins of various yeasts.

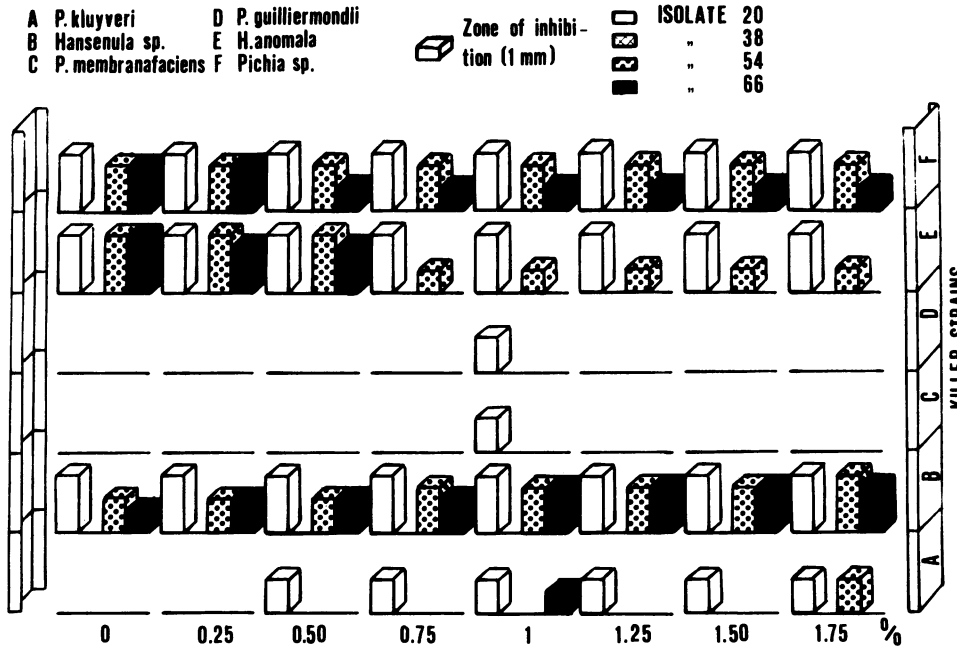

FIG. 7. Effect of yeast extract concentration of the growth medium on susceptibility of *C. albicans* to the killer toxins of various yeasts.

TABLE 4. Strain types of the 100 isolates of *C. albicans*

Strain type	No. of isolates	Collection no.
1 1 1	52	1–6, 8, 10, 11, 13–15, 18–20, 23, 25–27, 29, 34–36, 39, 41–43, 46, 47, 49, 52–56, 59, 60, 62, 65, 67, 70, 73, 75, 76, 80, 83, 84, 86, 95, 98–100
1 1 3	4	24, 30, 31, 33
1 2 3	4	40, 81, 92, 97
5 2 5	4	78, 79, 82, 91
7 2 5	4	37, 50, 58, 85
8 1 1	4	17, 28, 51, 66
8 2 3	3	22, 32, 69
8 2 5	3	63, 77, 89
2 1 1	2	12, 21
3 1 1	2	9, 57
3 2 1	2	45, 72
5 1 1	2	7, 16
7 1 1	2	61, 90
1 1 5	1	87
2 2 3	1	93
3 2 3	1	96
3 2 5	1	74
5 1 3	1	68
6 6 5	1	71
7 2 1	1	44
7 2 3	1	38
8 1 5	1	64
8 6 3	1	48
8 6 5	1	94
8 6 8	1	88

were correlated with neither their human or animal origin nor the clinical materials from which they had been isolated (Table 4). The strain type most frequently encountered was 1 1 1 (52%). It was sensitive to all nine killer yeasts.

By this method it was possible to study a few cases of presumptive nosocomial infections due to *C. albicans* encountered in the university's Agostino Gemelli polyclinic in Rome during the period of 13 March to 13 April 1981. For example, strain type 8 6 3 was isolated from a tonsillar swab and strain type 8 2 5 was isolated from a urine specimen from the same patient.

DISCUSSION

All of the *C. albicans* isolates were shown to be sensitive to at least one of the killer yeasts. This is in disagreement with a previous study (12), which did not report a sensitive isolate among 120 cultures of *C. albicans* investigated. This difference can be attributed to the different killer strains used.

The choice of our simplified conditions (modified Sabouraud glucose agar at pH 4.5 as medium and a water suspension of *C. albicans* isolates as inoculum) permitted the simple detection of the killer factors on *C. albicans*. The test conditions, however, may not be suitable for detecting the activity of other killer or sensitive yeasts. Our procedure, nevertheless, represents an effective screening method that could be easily applied to epidemiological investigations of infections due to *C. albicans*. The method is practical, it does not require tedious standardizations of test media or inocula, and the results are easy to read.

The system could be improved by utilizing a larger number of suitable killers or their purified toxins, a study which is now in progress in our institute. The use of a killer system as an epidemiological marker proved to be useful in the presumptive cases of nosocomial infections we studied.

ACKNOWLEDGMENTS

We thank the following individuals for providing the killer strains: D. G. Ahearn, Georgia State University, Atlanta; E. J. Middelbeek, University of Njimegen, Njimegen, The Netherlands; and R. A. Samson, Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands. We also thank L. Ajello, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga., for his invaluable interest in our work.

LITERATURE CITED

1. **Bevan, E. A., A. J. Herring, and D. J. Mitchell.** 1973. Preliminary characterization of two species of ds RNA in yeast and their relationship to the killer character. Nature (London) **245**:81–86.
2. **Bevan, E. A., and M. Makower.** 1963. The physiological basis of the killer character in yeast. *In* S. J. Geerts (ed.), Genetics today. XIth International Congress on Genetics **1**:202–203.
3. **Bevan, E. A., and J. M. Somers.** 1969. Somatic segregation of the killer (k) and neutral (n) cytoplasmatic genetic determinants in yeast. Genet. Res. **14**:71–77.
4. **Branca, G., S. Manzara, L. Scanu, and C. Chezzi.** 1979. La produzione di batteriocine come marker epidemiologico di "*Serratia marcescens*." Ig. Mod. **72**:467–473.
5. **Brown-Thomsen, J.** 1968. Variability of *Candida albicans* (Robin) Berkhout. I. Studies on morphology and biochemical activity. Hereditas **60**:355–398.
6. **Bussey, H.** 1972. Effects of yeast killer factor on sensitive cells. Nature (London) New Biol. **235**:73–75.
7. **Bussey, H., and N. Skipper.** 1975. Membrane mediated killing of *Saccharomyces cerevisiae* by glycoproteins from *Torulopsis glabrata*. J. Bacteriol. **124**:476–483.
8. **Chezzi, C., F. Ardito, and F. Leone.** 1979. Sulla tipizzazione di *Pseudomonas aeruginosa* in base alla produzione di piocina. Ig. Mod. **72**:490–501.
9. **Chezzi, C., G. Branca, S. Manzara, L. Scanu, and C. Tarquini.** 1980. Sulla tipizzazione batteriocinica di *Proteus rettgeri*. Ig. Mod. **73**:3–11.
10. **Drouhet, E., L. Mercier Soucy, and S. Montplaisir.** 1975. Sensibilité et resistance des levures pathogenes aus 5-fluoropyrimidines. I. Relation entre les phenotypes de resistance a la 5-fluorocytosine, le serotype de *Candida albicans* et l'ecologie de different especès de *Candida* d'origine humaine. Ann. Microbiol. **126B**:25–39.
11. **Hasenclever, H. F., and W. O. Mitchell.** 1961. Antigenic studies of *Candida*. I. Observation of two antigenic groups in *Candida albicans*. J. Bacteriol. **82**:570–573.
12. **Kandel, J. S., and T. A. Stern.** 1979. Killer phenomenon in pathogenic yeasts. Antimicrob. Agents Chemother. **15**:568–571.
13. **Middelbeek, E. J., J. M. H. Hermans, C. Stumm, and H.**

Muytjens. 1980. High incidence of sensitivity to yeast killer toxin among *Candida* and *Torulopsis* isolates of human origin. Antimicrob. Agents Chemother. **17**:350–354.

14. **Moreaud, S., and L. Friedman.** 1961. Pathogenicity of *Candida*. J. Bacteriol. **81**:550–556.
15. **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.
16. **Philliskirk, G., and T. W. Young.** 1975. The occurrence of killer character in yeasts of various genera. Antonie van Leeuwenhoek J. Microbiol. Serol. **41**:147–151.
17. **Pietras, D. F., and J. A. Bruenn.** 1976. The molecular biology of yeast killer factor. Int. J. Biochem. **7**:173–179.
18. **Polonelli, L., G. Morace, B. M. Barcaioli, and A. L. Cossu.** 1981. Survey of *Cryptococcus neoformans* diffusion in Italy. Ig. Mod. **76**:733–741.
19. **Somers, J. M., and E. A. Bevan.** 1969. The inheritance of the killer character in yeasts. Genet. Res. **13**:71–83.
20. **Staib, F.** 1963. Serum-proteins as nitrogen source for yeast-like fungi. Sabouraudia **4**:187–193.
21. **Steer, P. L., M. I. Marks, P. D. Klide, and T. C. Bickoff.** 1972. 5-Fluorocytosine: an oral antifungal compound. Ann. Intern. Med. **76**:15–22.
22. **Stumm, C., J. H. M. Hermans, E. J. Middelbeek, A. F. Croes, and G. J. M. L. De Vries.** 1977. Killer-sensitive relationships in yeasts from natural habitats. Antonie van Leeuwenhoek J. Microbiol. Serol. **43**:125–128.
23. **Van Uden, N., and H. Buckley.** 1970. *Candida* Berkhout, 893–1087. *In* J. Lodder (ed.), The yeasts. North-Holland Publishing Co., Amsterdam.
24. **Warnock, D. W., D. C. E. Speller, J. E. Day, and A. J. Farrell.** 1979. Resistogram method for differentiation of strains of *Candida albicans*. J. Appl. Bacteriol. **46**:571–578.
25. **Woods, D. R., and E. A. Bevan.** 1968. Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. J. Gen. Microbiol. **51**:115–126.
26. **Young, T. W., and M. Yagiu.** 1978. A comparison of the killer character in different yeasts and its classification. Antonie van Leeuwenhoek J. Microbiol. Serol. **44**:58–77.