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Effect of Buffers on Testing of *Candida* Species Susceptibility to Flucytosine

STEPHEN D. MACKERROW,† JOANNE M. MERRY, AND PAUL D. HOEPRICH*

Section of Medical Mycology, Division of Infectious and Immunologic Diseases, Department of Internal Medicine, University of California Davis Medical Center, Sacramento, California 95817

Received 22 December 1986/Accepted 10 February 1987

Synthetic amino acid medium for fungi (SAAMF) is a totally defined, nutritionally adequate, macromolecule-free culture medium for fungi that is buffered with an organic weak acid-weak base pair: 2-(*N*-morpholino)-propanesulfonic acid (MOPS) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris). In 1984, it was reported that MOPS-Tris in SAAMF antagonized the activity of flucytosine against *Candida albicans* (D. L. Calhoun and J. N. Galgiani, Antimicrob. Agents Chemother. 26:364–367, 1984). Accordingly, we evaluated the buffering capacity of seven synthetic organic buffers and monobasic potassium phosphate, both singly and in pairs, over the pH range 7.4 to 6.0. Of these buffers, MOPS, BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminomethanesulfonic acid], a BES-MOPS combination, and KH_2PO_4 provided the best buffering. Growth of *C. albicans* in unbuffered SAAMF was equivalent overall to that in SAAMF containing buffers, singly or in pairs. Twelve strains of *C. albicans* and five strains of *Candida lusitaniae* were tested for susceptibility to flucytosine in SAAMF, with and without buffers. In the presence of Tris, the geometric mean MICs were 6.5- and 3.6-fold higher, respectively, for *C. albicans* and *C. lusitaniae*. We recommend replacing Tris with the nonantagonistic MOPS.

SAAMF (synthetic amino acid medium for fungi) is a totally defined, completely synthetic, macromolecule-free medium for fungi that was introduced 17 years ago for susceptibility testing in vitro (5). With experience, modifications of the medium were introduced, and the present formula, hereafter referred to as "conventional," was published in 1977 (8).

In 1984, Calhoun and Galgiani reported work implicating the buffers in SAAMF as causes for the high MICs of flucytosine (5FC) against 12 strains of *Candida albicans* (2). While MOPS-Tris [2-(*N*-morpholino)-propanesulfonic acid plus 2-amino-2-(hydroxymethyl)-1,3-propanediol] appeared to antagonize the anticandidal activity of 5FC, the individual buffers were not examined, and only strains of *C. albicans* were studied.

With the goal of furthering the standardization of fungal susceptibility testing, we evaluated the following in SAAMF: (i) the buffering capacity of seven synthetic organic buffers (including MOPS and Tris) and monobasic potassium phosphate (KH_2PO_4), both individually and in pairs, by titration; (ii) the growth of four strains of *C. albicans* with buffers maximally effective over the pH range 7.4 to 6.0; and (iii) the susceptibility to 5FC of 12 strains of *C. albicans* and 5 strains of *Candida lusitaniae* with the buffers used in (ii).

MATERIALS AND METHODS

Media and buffers. A 3× concentration of unbuffered SAAMF was prepared as previously described (8), except that cystine was replaced with an equimolar amount of cysteine. This stock solution of SAAMF was filter sterilized (average pore size of membrane, 0.45-μm; Millipore Corp., Bedford, Mass.) and stored in a refrigerator. In addition to MOPS and Tris (Sigma Chemical Co., St. Louis, Mo.), five other synthetic organic buffers (United States Biochemicals Co., Cleveland, Ohio), introduced by Good et al. (4), were tested. They were selected for their possible utility in providing buffering between pHs 8 and 6, either alone or in combination. All seven organic buffers are water soluble, are enzymatically and hydrolytically stable, form soluble complexes with cations and, with the exception of Tris, do not pass through biological membranes (4). Monobasic potassium phosphate (reagent grade; Matheson, Coleman & Bell, East Rutherford, N.J.) was also evaluated, as it is commonly used in culture media and is inexpensive. Some properties of the eight buffers evaluated are given in Table 1.

Fungi. John N. Galgiani (Medical Service, Veterans Administration Medical Center, Tucson, Ariz.) kindly supplied the 12 strains of *C. albicans* used in the work previously reported from his laboratory (2). All 12 strains of *C. albicans* were isolated from patients who had not received 5FC before cultures were obtained. Five strains of *C. lusitaniae*, also isolated from patients who had not been treated with 5FC, were kindly supplied by Michael G. Rinaldi, University of Texas Health Science Center, San Antonio. The fungi were stored in a refrigerator on slants of Sabouraud glucose agar.

Buffering capacity. For testing single buffers, SAAMF was made up with each of the buffers at a final concentration of 0.165 M. For testing buffer pairs, a concentration of 0.0825 M of each component was used, as in conventional SAAMF (8). Before titration, the initial pH of SAAMF with buffer(s) was adjusted to pH 7.4 with 15 N hydrochloric acid or 15 N sodium hydroxide. Titration was carried out with 0.1 N hydrochloric acid over the pH range 7.4 to 6.0, with constant stirring at 22°C and with a pH meter (model 125; Corning Glass Works, Corning, N.Y.) Unbuffered SAAMF and SAAMF buffered with seven organic buffers, potassium phosphate, and four organic buffer pairs (including MOPS-Tris) were titrated (Table 1).

* Corresponding author.
† Present address: Department of Medicine, School of Medicine, University of New Mexico, Albuquerque, NM 87131.

TABLE 1. Some properties of eight buffers (at 0.165 M) and two-buffer combinations (each component at 0.0825 M) evaluated in SAAMF, listed in order of increasing effectiveness in controlling the pH

Buffer[a]	pK_a (at 20°C)	Mol wt	mmol of HCl needed to lower pH 7.4 to pH 6.0
None (unbuffered SAAMF)			0.10
CAPS	10.40	221.3	0.10
BICINE	8.35	163.2	0.60
Tris	8.30	121.1	0.63
HEPES	7.55	238.2	1.39
MES	6.15	195.2	1.50
MOPS	7.20	209.3	1.96
BES	7.15	213.2	2.05
KH_2PO_4	7.21[b]	136.1	2.35
BICINE-BES			1.31
BICINE-MOPS			1.33
BES-Tris			1.40
MOPS-Tris			1.41
BES-HEPES			1.70
BES-MOPS			2.04

[a] CAPS, Cyclohexylaminopropanesulfonic acid; BICINE, *N,N*-bis(2-hydroxyethyl)-glycine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid.
[b] Determined at 25°C.

Growth. Four strains of *C. albicans* were incubated overnight in conventional SAAMF. On the basis of direct counts in a hemacytometer (Gram iodine diluent), dilutions were made in sterile distilled water to obtain 2×10^4 cells per ml. Aliquots (1 ml) of the candidal suspensions and 1-ml aliquots of 2× concentrations (without and with buffers) of SAAMF were combined in snap-top polycarbonate tubes (11 by 75 mm; Becton Dickinson Labware, Oxnard, Calif.). Incubation was carried out at 35°C without agitation. Growth was assessed by counting cells in a hemacytometer 24 h after inoculation.

Susceptibility. The 12 strains of *C. albicans* used by Calhoun and Galgiani (2) and 5 strains of *C. lusitaniae* were tested for susceptibility to 5FC (obtained from Hoffmann-La Roche Inc., Nutley, N.J.) at final concentrations ranging from 128 to 0.0078 μg/ml. The inocula were prepared as for the growth experiments described above, except that the final dilution was made in 2× SAAMF with the buffer(s) under evaluation. 5FC was prepared in sterile distilled water at twice the desired final test concentrations; the addition of 1 ml of 5FC solution to 1 ml of inoculum yielded the desired final test conditions. Control tubes contained no 5FC (1 ml of sterile distilled water added to the inoculum). The validity of the inocula was verified by quantitative culturing of 0.01-ml aliquots from control tubes immediately after mixing (surface inoculation of Sabouraud glucose agar). The tubes were incubated at 35°C without agitation after initial mixing; after 18 to 20 h, the lowest concentration allowing no growth as detected by visual examination was taken as the MIC.

RESULTS

Buffering capacity. Of the organic buffers, BES [*N,N*-bis(2-hydroxyethyl)-2-aminomethanesulfonic acid] was the most effective compound, with MOPS being only slightly less effective. The buffering capacity of MOPS exceeded that of Tris by more than threefold. The MOPS-Tris combination used in conventional SAAMF was not as effective as MOPS alone. Overall, buffer pairs secured no significant increase in buffering capacity as compared with MOPS or BES used alone. As compared with unbuffered SAAMF, SAAMF buffered with KH_2PO_4 was 23.5 times more effective over the pH range 7.4 to 6.0 (Table 1).

Growth. The growth of *C. albicans* was excellent in all of the formulations tested, with no apparent adverse effects from the buffers (Table 2).

Susceptibility. The MICs for all strains of both *C. albicans* and *C. lusitaniae* were higher when Tris was present in the medium, either alone or in combination with MOPS (Table 3). When compared with unbuffered SAAMF, the addition of MOPS-Tris or Tris alone resulted in 6.4- and 6.5-fold increases, respectively, in the geometric mean MICs for *C. albicans*. For *C. lusitaniae*, the increases were 3.6-fold. Buffering with either MOPS (two concentrations) or BES had little effect on the MICs, as compared with the results obtained with unbuffered SAAMF, whereas KH_2PO_4 resulted in slightly higher MICs.

DISCUSSION

From the work reported by Calhoun and Galgiani (2), it appeared that the use of MOPS-Tris in conventional SAAMF (8) resulted in higher MICs of 5FC for *C. albicans*. By examining MOPS and Tris separately with the same 12 strains of *C. albicans* examined by Calhoun and Galgiani (2), we showed that it was Tris that resulted in the higher MICs of 5FC. Further, we found that the effect was not species specific, as it was also demonstrable with five strains of *C. lusitaniae*. It is, however, reasonable to ask if the differences in MICs were great enough to have clinical significance. From the data listed in Table 3, the MICs in the Tris-containing media were consistently higher than those in the media without Tris, varying from four- to eightfold higher. With *C. albicans*, the falsely higher values were in the range of resistant for strain 10 and bordering on resistant for strain 12. More strains of *C. albicans* would need to be tested, especially strains thought to be resistant to 5FC, for valid

TABLE 2. Growth of four strains of *C. albicans* (sessile cultures at 35°C) in unbuffered and buffered SAAMF as assessed by direct counts in a hemacytometer[a]

Strain	No. of cells (10^7)/ml in SAAMF with:						
	No buffer	MOPS (0.08 M)	Tris (0.08 M)	MOPS (0.08 M)–Tris (0.08 M)[b]	MOPS (0.16 M)	BES (0.16 M)	KH_2PO_4 (0.16 M)
1	5.6	10.6	9.8	7.1	6.6	6.5	7.6
6	3.5	4.3	5.3	7.0	4.0	7.7	6.5
9	6.0	4.5	4.8	6.2	8.8	5.6	7.1
10	4.7	5.0	5.6	6.2	5.1	5.0	6.7
Geometric mean	4.9	5.7	6.1	6.6	5.9	6.1	7.0

[a] Starting inocula, 10^4 cells per ml; cells were counted 24 h after inoculation.
[b] As in conventional SAAMF (8).

TABLE 3. MICs of 5FC for *C. albicans* and *C. lusitaniae* in unbuffered and buffered SAAMF

Strain	MIC (μg/ml) in SAAMF with:						
	No buffer	MOPS (0.08 M)	Tris (0.08 M)	MOPS (0.08 M)–Tris (0.08 M)[a]	MOPS (0.16 M)	BES (0.16 M)	KH_2PO_4 (0.16 M)
C. albicans							
1	0.125	0.125	1.000	1.000	0.125	0.125	0.250
2	0.250	0.0625	1.000	1.000	0.25	0.250	0.500
3	0.125	0.125	1.000	1.000	0.125	0.125	0.250
4	0.250	0.250	4.000	2.000	0.250	0.250	0.500
5	0.125	0.125	1.000	1.000	0.125	0.125	0.250
6	0.250	0.250	2.000	1.000	0.250	0.250	0.250
7	0.125	0.125	1.000	1.000	0.250	0.250	0.250
8	0.500	0.500	2.000	2.000	0.500	0.500	0.500
9	0.500	0.500	4.000	4.000	0.500	0.500	1.000
10	4.000	8.000	16.000	32.000	8.000	4.000	8.000
11	0.500	0.500	4.000	4.000	0.500	0.500	0.500
12	4.000	4.000	16.000	16.000	4.000	4.000	8.000
Geometric mean	0.374	0.353	2.519	2.378	0.420	0.396	0.629
C. lusitaniae							
1	0.031	0.031	0.125	0.125	0.031	0.031	0.063
2	0.031	0.031	0.063	0.063	0.031	0.031	0.031
3	0.016	0.016	0.031	0.063	0.008	0.016	0.031
4	0.031	0.031	0.125	0.125	0.031	0.031	0.016
5	0.016	0.016	0.125	0.063	0.016	0.016	0.016
Geometric mean	0.023	0.023	0.082	0.082	0.020	0.023	0.027

[a] As in conventional SAAMF (8).

judgement as to the clinical significance of antagonism of the activity of 5FC by Tris.

With regard to non-*C. albicans Candida* spp., assessment of the clinical significance of the phenomenon also requires further work. Although we found falsely high MICs for all five of the *C. lusitaneae* strains that we examined, none were in the range of apparent resistance. Moreover, other non-*C. albicans Candida* spp. should be examined.

We did not try to find out if the activity of 5FC is affected adversely by Tris in the presence of other antifungal antimicrobial agents. It is a point of some interest, because 5FC is virtually never used as the sole agent in therapy. However, the agents with which it might be combined, e.g., the polyenes and the azoles, are active by entirely different mechanisms; their presence would probably not influence the antagonism of 5FC by Tris.

The mechanism of the antagonism of 5FC by Tris is obscure. We found no adverse effect of Tris or MOPS-Tris on the growth of *C. albicans*, i.e., some nonvital mechanism is involved. As the antifungal activity of 5FC depends on its oxidative deamination inside the fungal cell to yield 5-fluorouracil (6, 7, 11), it appears that Tris in some way blocks or diminishes this critical reaction. Two possibilities come to mind. The first relates to the fact that exogenous pyrimidines are actively transported into fungal cells (9); Tris might compete for transport, a construction that fits with the finding of Calhoun and Galgiani that the degree of antagonism of 5FC by MOPS-Tris was a function of the concentrations of the buffers (2). The second possibility relates to the fact that Tris, unlike the other organic buffers that were studied, is known to enter mammalian cells (4); if Tris also enters fungal cells, it may in some way interfere with the conversion of 5FC to 5-fluorouracil.

Elimination of Tris from SAAMF eliminated the antagonism of 5FC in the testing in vitro of two *Candida* spp. Fortunately, neither MOPS nor BES, two organic compounds with excellent buffering capacity in the pH range under examination, displayed antagonism of the anticandidal activity of 5FC. However, we showed that four strains of *C. albicans* grew as well in unbuffered as in buffered SAAMF; hence, it is reasonable to question the need for buffering. In our view, several factors favor the use of a buffered medium for susceptibility testing. A system that maintains a pH in the range of 7.4 to 6.0 may provide pH conditions akin to those prevailing at sites of infection. Some antifungal agents, e.g., amphotericin B (1), appear to be less active in an acidic environment. Also, *Candida* spp. do not represent the entire mycologic world. Indeed, some pathogenic fungi, such as *Coccidioides immitis* and *Cryptococcus neoformans*, normally inhabit alkaline environments in nature—the arid soils of the western United States (10) and pigeon feces (3), respectively; both *C. immitis* and *C. neoformans* grow well in SAAMF.

We support the continued use of SAAMF because it is a totally defined, completely synthetic medium that is precisely replicable, nutritionally adequate, exceptionally well buffered, isosmolal, and free of macromolecules. However, the conventional formula (8) should be modified by the reduction of the concentration of L-cystine to 0.24 g/liter (higher concentrations of this relatively insoluble amino acid may precipitate, especially when >1× formulations of the medium are stored in a refrigerator), the addition of L-cysteine in the amount of 1.00 g/liter, the elimination of Tris, and the equimolar replacement of Tris with MOPS to give a final concentration of 0.165 M MOPS (34.54 g/liter). The resultant medium is very well buffered and is isosmolal. While BES was marginally superior to MOPS over the pH range that was tested, BES costs about three times more than MOPS. The buffering capacity of KH_2PO_4 was superior but resulted in MICs that were higher than those obtained in

unbuffered SAAMF and in SAAMF with non-Tris organic buffers. Moreover, phosphate may form insoluble complexes with cations such as Ca^{2+}, unlike the organic buffers.

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