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EVALUATION OF COHEMOLYTIC ACTIVITY IN STREPTOCOCCI, LISTERIA AND CORYNEBACTERIA BY USING SHEEP BLOOD AGAR PLATES AND A QUANTITATIVE MICROASSAY

Marcela de Freitas Lopes
Lúcia Martins Teixeira*

SUMMARY

Hemolytic synergism and antagonism among streptococci, listeria and corynebacteria were evaluated by a sheep blood agar assay, in the presence of *Staphylococcus aureus, Rhodococcus (Corynebacterium) equi* and *Corynebacterium haemolyticum* as indicator strains. Cooperative hemolysis with *R. (C.) equi* and hemolytic antagonism with *S. aureus* were observed with *C. Haemolyticum, Corynebacterium pseudotuberculosis, Corynebacterium ulcerans* and *Listeria ivanovii.* When these species were also tested as indicator strains, only *C. haemolyticum* yielded unequivocal results. *C. haemolyticum* was found to be useful in eliminating doubts caused by weakly positive or non-specific positive CAMP reactions. All strains of *Streptococcus agalactiae* and just one *Streptococcus uberis* were positive with *S. aureus;* however only *S. agalactiae* strains gave positive reactions with *C. haemolyticum. R. (C.) equi* and *Corynebacterium hoagii* (now also considered as *R. equi*) as well as *Corynebacterium renale* gave hemolytic synergism with *S. aureus.* When the latter was replaced by *C. haemolyticum* as indicator, *R. (C.) equi* and *C. hoagii,* but not *C. renale,* were positive. *C. haemolyticum* caused weakly positive results with *L. monocytogenes* and antagonism with *L. ivanovii,* whereas both *L. monocytogenes* and *L. seeligeri* produced weak reactions with *S. aureus. L. monocytogenes* strains were also positive or weakly positive with *C. equi.* An accurate microtiter plate assay was adapted for quantitative assessment of cohemolytic activity in culture supernatants. For that, sheep blood red cells sensitized with ß-lysin from S. aureus were allowed to react with the culture supernatant to be tested and the optical density of unlysed red cells was read after incubation at 37°C for 30 min. Cohemolytic activity titers correlated well with overall results obtained in solid medium. One important exception was the finding of high cohemolytic activity among pathogenic *Listeria,* which caused doubtful reactions in blood agar plates. Besides facilitating bacterial cocytolysins studies, quantitative cohemolytic determinations can be useful for the characterization of pathogenic *Listeria* species.

Key words: Cocytolysins, hemolytic synergism, cohemolytic activity, streptococci, listeria and corynebacteria.

INTRODUCTION

Studies on hemolytic synergism among bacterial cocytolysins, in particular those produced by streptococci, listeria and corynebacteria, have been applied in the identification of several spe-

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cies, including some important human and animal pathogens (6, 7).

Synergistic hemolytic interactions, originally described by Christie *et al.* (2) were proved to be useful for the presumptive identification of *Streptococcus agalactiae*, and are based on the ability of the CAMP factor to enhance sheep erythrocyte lysis induced by staphylococcal ß-lysin. However, some *Streptococcus uberis* strains can also give positive results in CAMP-like tests (11) and, under certain conditions, some group A, C and G streptococci cause weak false-positive reactions (4, 14). A test using *Corynebacterium pseudotuberculosis* as indicator strain has been proposed as an alternative to solve problems arisen from those non-specific results(11).

Cooperative hemolysis observed with both ß-lysin producing strains of *Staphylococcus aureus* and *C. pseudotuberculosis* have also been used as important instruments to identify *Rhodococcus* (*Corynebacterium*) *equi* (9). On the other hand, *C. pseudotuberculosis, C. haemolyticum* and *Corynebacterium ulcerans* produce substances with phospholipase D activity and cause inhibition of ß-lysin effects on sheep blood red cells (1, 13).

A synergistic hemolysis assay using two indicator strains: a ß-lysin producing *S. aureus* and a *R.* (*C.*) *equi* is considered to be a fundamental criterion for differentiating *Listeria* species (10, 12). Nevertheless, Vasquez-Boland *et al.* (15) have questioned the validity of such test, due to the lack of specificity and subjective readings. Based on synergistic lysis of human erythrocytes pre-treated with several bacterial exosubstances, a semiquantitative microtechnique has been proposed for the differentiation of *Listeria* species (3, 15).

In this report, selected strains of *S. aureus, R.* (*C.*) *equi* and *C. haemolyticum* were used, as indicators, in studies involving analysis of hemolytic synergism with several streptococci, listeria and corynebacteria strains. In addition, an accurate microtechnique was adapted to quantify cohemolytic activity in culture supernatants.

MATERIALS AND METHODS

Bacterial strains - A total of 66 bacterial strains (Table 1) were studied. Streptococci, listeria and corynebacteria strains obtained from the CDC (Centers for Disease Control, Atlanta, Georgia, EUA), ATCC (American Type Culture Collection, Rockville, Maryland, EUA), and UFG (University of Florida, Gainesville, Florida, EUA) were reference strains. *Listeria monocytogenes* strains provided by FIOCRUZ (Fundação Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil), streptococcal strains and *Staphylococcus aureus* R II (used as indicator strain) belonging to the culture collection of the IM-UFRJ (Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil) were isolated from clinically significant animal or human specimens, as well as from human carriers. The purity of the strains was verified through observation of colonies and cellular characteristics. For storage, 24 h growth was harvested in sterile sheep blood and kept at -20°C.

Screening of cohemolytic activity on solid medium - Synergism hemolytic assays were performed as previously described (10) on sheep blood (5% v/v) agar plates, by using Columbia Agar Base (Merck) and Casoy Agar (Merck) as base media. Plates were inoculated with the indicator strains (*S. aureus* RII, *Corynebacterium equi* KC1610, *Corynebacterium haemolyticum* KC1384) and the strains to be tested for cohemolytic activity as depicted in Figure 1. Tests were read after 24 and 48 h of incubation at 37°C.

Detection of cohemolytic activity in culture supernatants - Bacterial strains were initially grown on sheep blood agar plates for 24 h at 37°C and transferred to tubes containing saline. Optical density of each cell suspension was adjusted to 0.85 at 620 nm. Suspensions (0.1 ml) were inoculated into 2 tubes (capacity for 50 ml) containing 8 ml of Columbia broth (Difco) each, and incubated at 37°C, with constant shaking (150 rpm), for 24 and 48 h. After centrifugation, supernatants were collected and stored frozen until use.

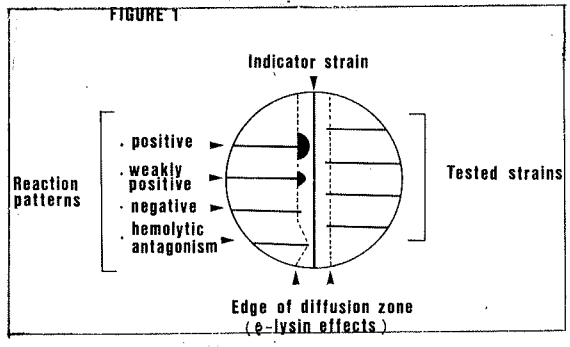

FIGURE 1 - Diagramatic representation of synergistic and antagonistic (inhibition) hemolytic reactions on sheep blood agar plates. *S. aureus, C. equi* and *C. haemolyticum* were used as indicator strains. The possible patterns of reaction (when B-lysin producing *S. aureus* is used as indicator strain) are shown, being complete hemolysis denoted as black areas.

Cohemolytic activity was determined in culture supernatants by using a microtechnique adapted from a method previously described (5). Briefly, a 1% sheep erythrocyte suspension was prepared in PBS (pH 7.2) containing ß-lysin from *S. aureus* R II (5 CHU/ml) and 1 mM $MgCl_2$, and incubated at 37°C for 30 min. Tests were performed in 96 well, flat bottom microtiter plates. One volume (0.1 ml) of ß-lysin treated erythrocyte suspension was added to each well containing one volume of a serial two-fold dilution of culture supernatant in PBS pH 7.2.

After incubation at 37°C for 30 min, hemolysis was evaluated by reading the optical density of unlysed erythrocytes at 620 nm in a vertical multichannel spectrophotometer (Titertek Multiskan). The titer, in cohemolytic units/ml (CHU/ml), was calculated as the inverse of LD50, i.e., dilution giving 50% of lysis, by the two-point interpolation method proposed by Kanclerski & Möllby (5). When untreated sheep red cells were used, titers were referred as hemolytic units/ml (HU/ml).

Determination of ß-lysin activity was performed by the same method, except that red cells were first treated with supernatants of *R. (C.) equi* KC1610 instead of ß-lysin.

RESULTS AND DISCUSSION

Table 1 shows the results of the screening tests for cohemolytic activity on blood agar plates, after 24 h incubation. After 48 h incubation, reading was unprecise due to the lytic effect of ß-lysin from *S. aureus* on blood agar plates prepared with Columbia Agar Base. This problem was similar to that observed by Vasquez-Boland *et al.* (15), by using Brain Heart Infusion as base medium. However, since high background lysis with blood agar plates containing Casoy agar was not observed, its use can be convenient when reading after 48 h is necessary.

Table 1. Cohemolytic activity of streptococci, listeria and corynebacteria strains determined on sheep blood agar plates in the presence of *Corynebacterium equi* KC 1610, *Corynebacterium haemolyticum* KC 1384 and *Staphylococcus aureus* R II as indicator strains.

Species	Number of strains tested	Number of strains grouped[a]	Cohemolytic activity[b]		
			C. equi	*C. haemolyticum*	*S. aureus*
S. agalactiae	29	3	–	(+)	+
		26	–	+	+
S. uberis	3	1	–	±	+
		2	–	–	–
Other streptococci					
Group A	2	2	–	–	(+)
C	2	2	–	–	(+)
D	4	4	–	–	(+)
G	2	2	–	–	(+)
"S. milleri"	3	3	–	–	(+)
L. monocytogenes	8	4	+	(+)	(+)
		4	(+)	(+)	(+)
L. ivanovii	1	1	+	(–)	(–)
L. seeligeri	1	1	–	–	(+)
L. welshimeri	1	1	–	–	–
L. innocua	1	1	–	–	–
C. equi	3	3	–	+	+
C. hoagii	1	1	–	+	+
C. renale	2	2	–	–	+
C. haemolyticum	1	1	+	–	(–)
C. pseudotuberculosis	1	1	+	–	(–)
C. ulcerans	1	1	+	–	(–)

a, Strains presenting indicated reaction pattern after incubation at 37°C for 24 h on sheep blood agar plates prepared with Columbia Agar Base

b, +, positive; (+), weakly positive; –, negative; (–), negative with hemolytic antagonism

When *C. equi* was used as indicator strain (Table 1), *L. Monocytogenes* strains gave either positive or weakly positive reactions, whereas *L. ivanovii* were positive, in agreement with results obtained by other authors (15). *C. haemolyticum, C. pseudotuberculosis* and *C. ulcerans* gave positive results, thus conforming the validity of using *R. (C.) equi* (hemolytic synergism) in conjunction with *S. aureus* (hemolytic antagonism) as indicator strains for the detection of phospholipase D-producing strains (1). All other strains were negative with *R. (C.) equi.*

We have also tested *L. ivanovii, C. pseudotuberculosis, C. ulcerans* (data not shown) and *C. haemolyticum* as indicator strains for detecting hemolytic interactions. Formation of two hemolytic zones, one complete and another incomplete, was observed around *L. ivanovii* growth. According to Vasquez-Boland *et al.* (16) those zones of hemolysis are caused by 2 different *L. ivanovii* exosubstances, respectively: ivanolysin O and sphingomyelinase C. This effect impaired the use of *L. ivanovii* as an indicator strain and it was probably responsible for the high titers obtained with the *L. ivanovii* strain in the microassay (Table 3). Furthermore, among the other strains tested, besides *R. (C.) equi* and *S. aureus,* only *C. haemolyticum* gave reproducible and clear reactions as shown in Table 1.

S. agalactiae strains produced positive or weakly positive results with *C. haemolyticum,* but other streptococci were negative. These reactions have also been previously demonstraded by Skalka *et al.* (11), with a different phospholipase D-producing strain (*C. pseudotuberculosis*), except that, in their case, a doubtful reaction was obtained with *Streptcococcus pyogenes* strains. Therefore, we suggest that *C. haemolyticum* can be useful in the presumptive differentiation of *S. agalactiae* and *S. uberis* CAMP positive strains, as well as in eliminating doubts related to other streptococci that give weakly positive results in CAMP-like tests, since these weak reactions are conventionally considered as negative in tests with streptococci.

L. monocytogenes produced weakly positive reactions, whereas the exosubstance from *L. ivanovii* was inhibited by *C. haemolyticum* phospholipase D, allowing differentiation of these species, as previously reported (8). However *L. monocytogenes, L. seeligeri* and *L. ivanovii* produced doubtful reactions with *S. aureus* in accordance to observations already mentioned in the literature (15).

Among all other strains, only *R. (C.) equi* and *C. hoagii* - the latter being now considered as *R. equi* by some authors (12) - presented hemolytic synergism with *C. haemolyticum* and *S. aureus,* whereas *C. renale* strains were positive with *S. aureus.* We concluded that *C. haemolyticum* may be useful as an instrument for identifying *R. (C.) equi* in the same way described for S. aureus and *C. pseudotuberculosis* (9).

By using sheep erythrocytes sensitized with ß-lysin, results obtained in solid medium with *S. aureus* as indicator strain (Table 1) were compared to cohemolytic activity present in supernatants obtained after 24 and 48 h of growth (Tables 2 and 3).

Comparison of *S. agalactiae* isolated from carriers and from clinically significant specimens showed no meaningful differences regarding to cohemolytic activity in supernatants (Table 2).

Cohemolytic titers from strains considered positive on sheep blood agar plates ranged from 4.62 (Table 2) to 643.65 CHU/ml (Table 3) in the microtiter assay. Although group A *Streptococcus* strain SS-635 and most of the previously negative and weakly positive strains gave titers below 2.62 CHU/ml group A *Streptococcus,* strain S-235, and all *L. monocytogenes* strains tested, with characteristically weak pattterns of reaction in solid medium, had higher titers in their supernatants (Tables 2 and 3). In addition, only *L. ivanovii* and *L. monocytogenes* presented detectable hemolytic activity during the period of time used for performing the tests (data not shown).

Use of cohemolytic microtiter plate assay allowed pathogenic species *L. ivanovii* and *L. monocytogenes* to be readly differentiated from nonpathogenic *Listeria,* such as *L. seeligeri,* by virtue of their cohemolytic titer, as already observed by Vasquez-Boland *et al.* (15).

The finding of increased areas of synergistic hemolysis after 24 h of incubation, was observed especially when *R. (C.) equi* and *C. hoagii* were tested with *S. aureus,* and was confirmed in supernatants obtained from these strains (Table 3).

Regarding to other species, substantial variability in supernatants from different strains was observed when comparing activities after 24 and 48 h of incubation. However, the titers obtained from each individual strain were reproducible.

Some of these results were further confirmed by more detailed studies about cohemolytic activity determined during the growth cycles of *S. agalactiae, S. uberis, L. monocytogenes, R. (C.) equi, C. hoagii* and *C. renale* (manuscript in preparation).

In conclusion, we have adapted an accurate and reproducible method to determine cohemolytic

Table 2. Cohemolytic activity ofstreptococci strains determined in culture supernatants obtained after 24 and 48 h of growth in Columbia broth at 37°C.

Species	Strain Number	Source	Cohemolytic activity (CHU/ml)[a]	
			24 h	48 h
S. agalactiae	SS–615	CDC	10.35	6.28
	SS–617	CDC	9.93	6.94
	SS–618	CDC	16.69	12.04
	SS–619	CDC	32.47	28.10
	SS–620	CDC	27.76	26.23
	SS–700	CDC	12.64	12.05
	SS–1070	CDC	30.24	38.33
	SS–1240	CDC	56.73	52.78
	578–89	CDC	35.73	46.07
	579–89	CDC	18.75	19.55
	580–89	CDC	49.13	73.72
	80–119[b]	IM–UFRJ	18.62	9.17
	80–121	IM–UFRJ	13.43	11.39
	80–178	IM–UFRJ	18.57	6.39
	80–335	IM–UFRJ	13.64	12.16
	81–322	IM–UFRJ	19.54	27.84
	81–1294	IM–UFRJ	20.78	14.01
	CL–499[c]	IM–UFRJ	11.67	5.96
	90–35	IM–UFRJ	17.74	15.89
	90–36	IM–UFRJ	19.01	24.62
	90–44	IM–UFRJ	14.97	17.85
	90–60	IM–UFRJ	18.34	17.52
	90–88	IM–UFRJ	20.23	17.88
	90–90	IM–UFRJ	11.54	9.76
	90–92	IM–UFRJ	22.56	25.22
	90–93	IM–UFRJ	14.64	13.35
	90–94	IM–UFRJ	13.27	12.61
	90–104	IM–UFRJ	5.59	4.62
	90–115	IM–UFRJ	11.23	12.12
S. uberis	85851[d]	IM–UFRJ	7.19	5.81
	SS–847	CDC	1.55	1.57
	SS–1001	CDC	1.56	1.56
Other streptococci				
Group A	SS–235	CDC	46.77	38.24
	SS–635	CDC	2.62	2.54
Group C	SS–188	CDC	1.80	1.68
	26RP66	UFG	1.67	1.72
Group D	CL–312	IM–UFRJ	1.71	1.68
	CL–323	IM–UFRJ	1.78	1.68
	CL–395	IM–UFRJ	1.93	1.76
	CL–396	IM–UFRJ	1.85	1.77
Group G	SS–13	CDC	1.66	1.55
	SS–565	CDC	1.74	1.76
"S. milleri" Group F	SS–194	CDC	1.28	1.77
ß–non groupable	SS–549	CDC	1.56	0.00
	SS–763	CDC	1.52	1.44

a, Determined by a method adapted from that described by Kanclerski & Möllby (5) and expressed as cohemolytic units/ml (CHU/ml
b, Strains listed under 80–119 to 81–1294 were isolated from human carriers.
c, Strains listed under CL–499 to 90–115 were isolated from clinical significant human specimens.
d. Isolated from a case of bovine mastitis.

Table 3. Cohemolytic activity of listeria and corynebacteria strains determined in culture supernatants obtained after 24 and 48 h of growth in Columbia broth at 37°C.

Species	Strain Number	Source	Cohemolytic activity (CHU/ml)	
			24 h	48 h
L. monocytogenes	KC1703	CDC	47.94	72.74
	774812	FIOCRUZ	11.95	18.06
	1394	FIOCRUZ	55.89	36.64
	1872	FIOCRUZ	48.11	36.82
	2643	FIOCRUZ	6.01	4.50
	3910	FIOCRUZ	21.87	16.58
	3983	FIOCRUZ	30.48	36.07
	6871	FIOCRUZ	35.68	47.63
L. ivanovii	KC1786	CDC	1303.52	1450.96
L. seeligeri	KC1785	CDC	2.28	1.64
L. welshimeri	KC1825	CDC	1.82	1.55
L. innocua	KC1783	CDC	1.67	1.35
C. equi[a]	KC1289	CDC	12.58	22.84
	KC1610	CDC	300.79	643.65
	KC1611	CDC	57.89	82.85
C. hoagii[b]	KC1369	CDC	170.35	297.44
C. renale	ATCC19412	ATCC	15.93	11.59
	KC312	CDC	132.72	205.34
C. haemolyticum[c]	KC1384	CDC	1.61	1.72
C. pseudotuberculosis[d]	KC1365	CDC	0.00	1.79
C. ulcerans	F9910	CDC	8.81	10.29

a, Reassigned as *Rhodococcus equi* (12)
b, Considered as *Rhodococcus equi* (12)
c, Reassigned as *Arcanobacterium haemolyticum* (12)
d, Yet frequently referred as *Corynebacterium ovis* (12)

activity, which combines microtechnique advantages with precision of quantitative tests, thereby facilitating investigations of bacterial cocytolysins.

However, due to the high sensitivity of the test described (false-positive group A streptococci and *C. ulcerans* reactions), preliminary studies on sheep blood agar plates are necessary. For this purpose, *S. aureus, C. equi* and *C. haemolyticum* can be very useful as indicator strains of hemolytic interactions involving streptococci, listeria and corynebacteria.

RESUMO

Avaliação da atividade co-hemolítica de estreptococos, listerias e corinebactérias usando placas de agar sangue de carneiro e uma microtécnica quantitativa.

Sinergismo e antagonismo hemolíticos entre estreptococos, listérias e corinebactérias foram avaliados em placas de agar sangue de carneiro, na presença de *Staphylococcus aureus, Rhodococcus (Corynebacterium) equi* e *Corynebacterium haemolyticum* como amostras indicadoras. Hemólise sinérgica com *R. (C.) equi* e antagonismo hemolítico com *S. aureus* foram observados com *C. haemolyticum, Corynebacterium pseudotuberculosis, Corynebacterium ulcerans e Listeria ivanovii*. Quando estas últimas foram também testadas como amostras indicadoras, somente *C. haemolyticum* forneceu resultados inequívocos. *C. haemolyticum* pode ser útil para eliminar dúvidas causadas, no teste de CAMP, pelas reações fracas ou não específicas. Todas as amostras de *Streptococcus agalactiae* testadas e uma de *Streptococcus uberis* foram positivas com *S. aureus*, mas apenas *S. agalactiae* deu reações positivas com *C. haemolyticum.,R. (C.) equi, Corynebacterium hoagii*(esta agora, também, considerada como *R. equi*) e *Corynebacterium renale* apresentaram sinergismo hemolítico com *S. aureus*. Quando esta indicadora foi

substituída por *C. haemolyticum, R. (C.) equi* e *C. hoagii* foram positivas, mas não *C. renale. C. haemolyticum* apresentou resultados fracamente positivos com *Listeria monocytogenes* e antagonismo com *L. ivanovii*, enquanto tanto *L. monocytogenes*, quanto *Listeria seeligeri* forneceram reações fracas com *S. aureus*. Amostras de *L. monocytogenes* também foram positivas ou fracamente positivas com *R. (C.) equi*. Uma microtécnica precisa foi adaptada para a determinação quantitativa da atividade co-hemolítica em sobrenadantes de cultura. Para tal, hemácias de carneiro sensibilizadas com a ß-lisina de *S. aureus* foram empregadas e a densidade ótica das hemácias não lisadas foi determinada após incubação a 37°C por 30 min. Os títulos de atividade co-hemolítica correlacionaram bem com os resultados obtidos em meio sólido. Uma exceção importante ocorreu com a atividade co-hemolítica detectável entre listérias patogênicas, as quais apresentaram reações duvidosas em placas de agar sangue. Além de facilitar o estudo das co-citolisinas bacterianas, a determinação quantitativa da atividade co-hemolítica pode ser importante para diferenciar espécies patogênicas de *Listeria*.

Palavras-chave: Co-citolisinas, sinergismo hemolítico, atividade co-hemolítica, estreptococos, listérias e corinebactérias.

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EFFECT OF *STAPHYLOCOCCUS AUREUS* ON THE DEVELOPMENT OF A SARCOMA IN RATS

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SUMMARY

The antitumoral effect of *Staphylococcus aureus* against a transplantable rat sarcoma was demonstrated.

Adult rats of an inbred line were inoculated s.c. with sarcoma E –100 (S-E 100) and received 8 x 10^8 killed *S. aureus* s.c.

Different experimental groups were designed: 1) S-E 100 plus *S. aureus* 7 days before; 2) S-E 100 plus *S. aureus* simultaneously; 3) S-E 100 plus *S. aureus* 7 days later; 4) S-E 100 (controls). The S-E 100 incidence was not significantly different in the experimental groups.

Tumor size was measured periodically on days 7, 14, 21 and 28 after S-E 100 inoculum and it was observed that the tumor burden of rats pretreated with *S. aureus* was sifnificantly lower than in the control group. No differences were detected when compared tumor size of group 3 with those of the control group.

Animals from groups 1 and 2 had a survival period significantly longer than controls although in these groups important differences in mortality percentages were not observed.

Histological examination showed an increased necrotic area in tumors of rats treated before or at the same time of tumor challenge with *S. aureus* compared with those measured in the control group.

It is concluded that *S. aureus* given prior or simultaneously to S-E 100 challenge is able not only to inhibit S-E 100 growth but also to increase tumor necrotic process.

Key Words: adjuvant, antitumoral activity, *Staphylococcus aureus*.

INTRODUCTION

It is well known the antitumoral and immunoadjuvant activity of several microorganisms and their cell wall skeleton which has been particularly studied utilizing BCG and *Corynebacterium* (11, 13, 19, 20). These bacteria and the components of their cell wall have been widely used as immune therapeutic agent in cancer treatment.

It has been pointed out the adjuvant effect of *Lactobacillus* exerted both on humoral and cellular immune mechanisms. That stimulating activity of different species of *Lactobacillus*, particularly *Lactobacillus casei*, on several tumor lines has also been reported (20). We have recently demonstrated that *Proteus mirabilis* has an immunopotentiator activity that is manifested as an antitumoral effect, since it produces a markedly inhibition of the tu-

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mor's growth (17), and it could also produce a stimulation of the mononuclear phagocytic system, as well as the increase of hemagglutinin levels against sheep red blood cells (SRBC) in rats (5).

Recently it was studied that exists a renewed interest in biological response modifiers to elevate the host's natural defenses against tumors as a modality of cancer therapy, While "passive" administration of Interleukin-2, monoclonal antibodies and Interferon has produced some successes in cancer therapy (8), their toxicity and high cost have limited their general usefulness. Havas reported in 1990 the effect of mixed bacterial vaccine (MBV) prepared from *Streptococcus pyogenes* and *Serratia marcesens* on the immune system of mice and demonstrated that MBV caused regression of 20-100% of a transplantable mouse tumor, sarcoma 37 (8). Thus, the use of immunostimulant agents with either a preventive role or as antineoplastic therapy is proposed (15).

The immunostimulant role of *Staphylococcus aureus* has been already studied and it was also demonstrated by means of treatment with killed *S. aureus* as well as its capacity to induce, to a certain extent, the inhibition of growth and necrosis in a variety of human and animal tumors (9, 14, 18). The effect was also confirmed in murine leukemia experimental treatment (8).

In the present work the antitumoral effect of *S. aureus* on a rat sarcoma growth and on the survival time of animals was studied concomitantly with histological examination to measure the capacity of this bacteria to induce tumor necrosis that might reduce the S-E 100 size.

MATERIALS AND METHODS

Animals: Adult male rats, 9-12 weeks of age, with a high coefficient of inbreeding (line "m") have been used throughout the experiment. The latter is a local line obtained from outbred rats submitted later to inbreeding and maintained by Houssay and co-workers; they are registered as IIM in the VI Supplement of the International Survey in the Supply Quality and Use of Laboratory Animals, November 1964.

Tumor: A transplantable encapsulated fibrosarcoma (S-E 100) spontaneously originated in outbred rats maintained by serial subcutaneous transplantation in line "m" rats has been used. The tumor has an incidence around 98-100% between day 7 and 14 after tumor transplantation (average incidence: 97,5%) (6). All the animals received a counted inoculum of S-E 100 viable cells ($1,5x10^6$) subcutaneously.

Staphylococcus aureus: *S. aureus* Cowan I strain grown in a nutritious agar medium, submitted to tyndallization (70°C for 1h for 3 days) were used. The absence of viability was checked by cultures performed after the above-mentioned treatment. The microorganisms were susppended in Phosphate Buffer Solution (PBS) and counted in a hemocytometer. The animals were inoculated subcutaneously with $8x10^8$ *S. aureus*.

Experimental groups: The animals were submitted to the following set of experiments:

1) S-E 100 plus *S. aureus* 7 days prior to inoculation. The rats were inoculated subcutaneously (s.c.) with $1,5x10^6$ S-E 100 cells. Seven days prior to inoculation they received a 0,2 ml saline suspension of 8×10^8 killed microorganisms in the ipsilateral flank.

2) S-E 100 plus *S. aureus*: The animals received simultaneously the tumor inoculum and a 0,2 ml saline suspension of $8x10^8$ microorganisms s.c.

3) S-E 100 plus *S. aureus* 7 days later: The animals were given the same tumor inoculum and 7 days later a 0,2 ml saline suspension of *S. aureus* s.c.

4) S-E 100 control group: The rats received only the tumor inoculum as previously mentioned.

Evaluation of tumor's growth: The evaluation of tumor's growth in the animals was performed based on the following parameters:

a) *S-E 100 incidence*: The tumor take percentage was assessed 10 days post inoculation.

b) *Tumor size*: The surface of the S-E 100 was evaluated. The measurement of the tumors were performed on days 7, 14, 21 and 28 post inoculation by a caliper and the results were expressed in mm^2 (major diameter x minor diameter).

c) *Survival period and mortality*: The percentage of dead animals was registered when all the animals died. The survival period (average survival days) was calculated in all the experimental groups. In both determinations the data were statistically evaluated by means of a Student T test for no paired data.

d) *Histological examination of S-E 100*: In order to evaluate the effect of treatment with *S. aureus* on the S-E 100 morphology in the rat, that might alter tumor's growth, a histological examination was carried out in tumors of treated animals and their controls when the rats died. Peripheral and central samples of the tumor were taken. The size was approximately the same. Fixation in 10% formalin, dehydration and embedding in paraffin was carried out in a conventional manner. The sec-

tions were stained with H.E. and PAS.

The area of necrotic tissue was determined by counting the number of necrotic foci and measuring their area by planimetry that was assessed by projection with a photographic amplifier. Charts of the histological sections were drawn in milimetric paper in order to evaluate precisely the areas of viable and necrotic tissue respectively.

RESULTS

Tumor incidence: The treatment with *S. aureus* did not modify the incidence of the S-E 100 since no significant differences in the take percentage recorded on day 10 post inoculation were evident, i.e.: Group I: 85,4%, 47/55; Group II: 89,47%, 34/38; Group III: 91,44%; 32/35; Group IV: 100%, 33/33. This evaluation was made by X^2 test.

Tumor size: The protector effect of *S. aureus* was markedly manifested as an inhibition of the S-E 100 growth either in the animals inoculated simultaneously with the microorganisms or 7 days before the tumor challenge.

On Table 1 are presented the tumor average surface in the experimental groups at different days of measurement.

Table 1: Effect of *S. aureus* on the development of the *Tumor Size* (surface in mm^2) of sarcoma in rats.

Day		7	14	21	28
Groups 1 S – E 100 + *S. aureus* 7 days before n= 47	$\bar{x}$ ± s.e.	2.56 ± 0.34	91.15 ± 12.95	774.23 ± 61.96	1143.91 ± 86.03
2 S – E 100 + *S. aureus* simultaneously n= 38	$\bar{x}$ ± s.e.	2.71 ± 0.36	112.48 ± 24.84	813.26 ± 68.47	1222.44 ± 102.97
3 S – E 100 + *S. aureus* 7 days later	$\bar{x}$ ± s.e.	3.65 ± 0.81	157.65 ± 26.82	1429.24 ± 78.77	1884.38 ± 104.88
4 S – E 100 Control n= 33	$\bar{x}$ ± s.e.	5.69 ± 0.81	244.73 ± 34.43	1383.66 ± 82.21	2079.91 ± 119.66

$\bar{x}$: Tumor size average s.e.: standard error

The average tumor surface on Groups I and II was significantly lower than the control group on days 7, 14, 21 and 28 post inoculation.

In Figure 1 is plotted the tumor's growth rate on Groups I, II, III and control. It can be observed that the tumor's growth rate is higher in the animals of the control group, regarding those that received *S. aureus* either 7 days prior to or simulteneously with the S-E 100 inoculum.

The comparison of the difference on tumor size on day 28 and 14 post inoculation showed a significant discrepancy ($p \ll 0.001$) regarding the control group respectively. Whilst in the third experimental group the tumor's growth rate showed no discrepancy with the control group.

Histological examination: S-E 100 is basically a highly aggresive fibrosarcoma. The histological sections from non-treated animals revealed that the sarcoma consisted of fusiform cells with ovoidal or vesiculated nuclei and prominent nucleoli and exhibited a high mitotic index (1-2 mitosis per field, 500 x). The cells of uniform size, moderately acidofilic cytoplasm and ill-defined limits were infiltrating either as band-like or compact foci. The reticulin stain showed a loose network that surrounds completely the cellular infiltrates. The Masson trichromic point out the scarce production of thin connective tissue.

The tumor grows building up a pseudo capsule in the periphery. In 12 days sarcomas, the ischemic recrosis in the central area is paralelled to the tumor's growth. Isolated focal necrotic lesions in 10-20% of the tumor mass were observed.

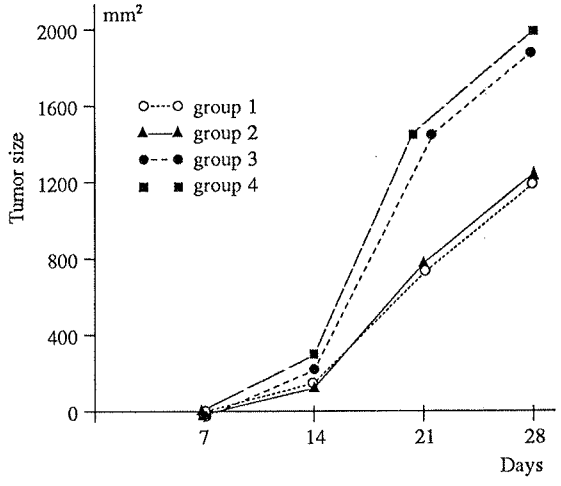

Figure 1 – Tumor growth rate of transplantable rat sarcoma. See text for de tails of groups.

Evaluation of the necrotic surface of the tumor.

This was evaluated through the determination of the respective area of viable and necrotic zones separately in tumors from treated rats and their controls. Data summarized in Table 2 points out the difference in necrosis percentage in groups I, II and IV, which was confirmed by statistical analysis (X^2 test, p« 0.001).

Table 2: Effect of *S. aureus* on the development of the SARCOMA E – 100 Viable and necrotic tissue surface

a) Tumor from *S. aureus* treated rats

		Marginal area		Central area	
	Group	Surface (mm^2)	%	Surface (mm^2)	%
Viable tissue	1	3600	80	525	27.3
	2	3200	79.2	630	26.3
Necrotic tissue	1	400	20	1400	72.7
	2	840	20.8	1770	73.7

Group 1: *S. aureus* 7 days before S–E 100 inoculus (n = 13)
Group 2: *S. aureus* simultaneously with S–E 100 inoculus (n =

b) Tumor from control group (n = 12)

	Surface (mm^2)	%	Surface (mm^2)	%
Viable tissue	2875	97.6	2800	83.6
Necrotic tissue	50	2.4	550	16.3

Survival and mortality in the animals

Treatment with *S. aureus* increased significantly survival time compared with control animals (p« 0.05). The survival period is expressed by x ± SE (standard error). Group I (n=47): 49 ±10,5(*); Group II (n=38): 48 ± 8 (*); Group III (n=39): 42 ± 10,2; Group IV (n=33): 41 ± 9,2. Asterisks indicate where value is significantly different (p« 0.01) from control rats.

DISCUSSION

The results obtained throughout these experiments demonstrate that *S. aureus* has an antitumoral effect when administered under certain conditions that imply either previous or simultaneous inoculation of killed microorganisms, i.e. 7 days prior to or on the same day of the S-E 100 transplantation. This suggests that the microorganism exerts its adjuvant activity when administered to the host as a vaccine or simultaneously with the tumor challenge. The *S. aureus* activity was manifested as a decrease in the tumor size associated with histological alterations and an increase in the necrotic tissue of the sarcoma under study, that could be one of the possible causes of size reduction.

The histological examination of the S-E 100 showed large areas of necrosis and abundant hemorrhagic foci. The assessment of the area of both necrotic and viable tissue has demonstrated a significant increase in necrotic tissue, particularly in the central region of the tumor.

The exacerbation of the tumoral necrosis allows us to postulate the hypothesis that the increase of such alteration could be attributable to the production of a certain factor with capacity to reduce the size of the sarcoma E-100, perhaps a sort of tumor necrosis factor (TNF). This factor has been described and characterized as a cytotoxic protein that causes an hemorrhagic necrosis (1, 16). The factor could be either cytolitic or cytotoxic against several tumor lines (5).

In the present experiment the TNF might have induced the necrosis of the transplantable sarcoma in the animals inoculated with *S. aureus* but this hypothesis requires experimental evidence in order to be confirmed. This event was demonstrated by other authors, since Bloksma and co-workers reported in 1981 that utilizing vaccines of different strains of *Corynebacterium* and *Propionibacterium* is possible to induce the macrophage function and the release of TNF (3).

Apparently, this inhibitory and necrotizing action could be attributed to the protein A present in important amounts in the cell wall, since non protein A strains are unable to generate such an effect. The mechanism of action of protein A producing inhibition of the tumor's growth has been widely discussed by other authors (14, 18) and it seems it could be attributed to biological effects not completely investigated.

Morphological, histological and immunohistological tumor changes described as well as the increase of survival time, led us attempt further experiments in order to use this agent in the future not only to prevent but also in treatment of tumors. The objective of these trials will be also directed

to elucidate the mechanisms induced by *S. aureus* that modulate host's immune response.

RESUMO

Efeito do *Staphylococcus aureus* no desenvolvimento de sarcoma em ratos.

Comprovado experimentalmente o efeito antitumoral do *Staphylococcus aureus* sobre um sarcoma transplantado em ratos (S-E 100).

Ratos adultos endocriados e inoculados s.c. com S-E 100 receberam via s.c. uma suspensão de 8×10^6 *S. aureus* mortos por tindalização.

Grupos experimentais: 1) S-E 100 + *S. aureus* 7 dias antes: 2) S-E 100 + *S. aureus* simultaneamente; 3) S-E 100 + *S. aureus* 7 dias depois; 4) S-E 100 (grupo de controle). A incidência do S-E 100 não modificou de forma significativa nos grupos experimentais.

As medidas efetuadas nos dias 7, 14, 21, 28 depois de inoculado o tumor evidenciaram que os animais desafiados, prévia e simultaneamente com *S. aureus* apresentavam tumores com um tamanho significativamente menor que o grupo de controle. Nos animais que pertenciam aos grupos 1 e 2, o período de sobrevivência foi significativamente superior ao grupo controle, mas a porcentagem de mortalidade não foi diferente entre os grupos.

O estudo histológico do tumor evidenciou uma maior superfície de tecido necrosado, sete (7) dias antes do desafio tumoral com o microorganismo, e nos ratos inoculados simultaneamente, em relação aos animais que pertenciam ao grupo de controle.

Conclusão: *S. aureus* quando é inoculado antes (vacina) ou em forma simultânea ao desafio com sarcoma E-100 exerce uma inibição do crescimento do tumor e também induz um aumento no processo de necrose do tumor além de aumentar o período de sobrevida dos animais.

Palavras-chave: atividade antitumoral, participação, *Staphylococcus aureus*, necrose do tumor.

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SERRATIA MARCESCENS: STUDIES ON NORMAL HUMAN SERUM RESISTANCE, SEROGROUPING AND PATHOGENICITY FOR MICE

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SUMMARY

Serratia marcescens isolated from nosocomial infections of different serogroups (01, 03, 04, 05, 010, 013, 016, 019 and 06,14) were analyzed for the resistance to the bactericidal activity of normal human serum (HS). Of the 60 strains studied, 55 (91,6%) were HS-resistant at a serum concentration of 50%, and only strains of serogroup 06,14 were HS-sensitive. All strains isolated from blood, eyes and bones were HS-resistant. Two strains of the serogroup 06,14 with different sensivities to HS were tested for the pathogenicity in mice and both strains were lethal at the same LD_{50} ($3,5 \times 10^7$). These results suggest that normal human serum resistance is not the principal determinant in the virulence of *S. marcescens*.

Key words: *Serratia marcescens*, serum resistance, nosocomial infections, O-antigen.

INTRODUCTION

Serratia marcescens is known as an important opportunistic pathogen frequently isolated from nosocomial infections (01, 17, 18), but the factors which contribute for the virulence of this specie are not yet clear.

A cell-bound hemolysin activity was observed among clinical isolates of *S. marcescens* (04, 15, 23), and in spite the presence of beta-hemolysin these strains were not associated with higher levels of virulence in mice (06).

The siderophore aerobactin production, also considerated as an important virulence factor in several pathogens, has not been found in nosocomial isolates of *S. marcescens* (06).

It has been suggested that normal human serum resistance is an important factor in the virulence of *S. marcescens* isolated from clinical specimens (24).

Additionally serum resistance has also been ascribed as a major virulence factor for most gram-negative bacteria (16). The serum resistance is a process developed by many bacterial pathogens to overcome host defenses (08), and one of the main components of the humoral host defense system is the complement (14). The lethal activity of HS results mostly from the action of the complement system on the bacterial surface on account of its activation either by classical or alternative pathways. Although the basis of serum resistance is not well understood, it has been suggested that a number of components can be involved, such as lipopolysaccharides (LPS) (10, 13, 19), outer membrane proteins (12, 25), capsular antigens (09, 21) and microbial proteases (14).

In this work, 60 strains of *S. marcescens* isolated from nosocomial infections were screened for serum resistance. In addition a possible relationship between different isolated serotypes and their respective sources was studied.

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MATERIALS AND METHODS

Bacterial strains - *S. marcescens* isolated from clinical cases were obtained from Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto-USP (HCFMRP). The bacterial strains were isolated from urinary and respiratory tract infections, osteomyelitis, keratitis and several other infections and serotyped at Instituto Adolfo Lutz, São Paulo. *E. coli* K12-711 HS-sensitive was used as negative control.

Serum resistance assays - Human blood was collected from 10 healthy volunteers, allowed to clot at room temperature for 30 min. and then placed at 4°C for 2 h. Followed the centrifugation at 4°C, sera were pooled and stored in aliquots of 0,5 ml at -20°C before the use. The determination of bacterial resistance to the lethal activity of HS was determined by a rapid turbidimetric assay in microtitration plates (20), with modifications. Overnight bacterial cultures grown in Luria Broth (LB) were diluted 1/100 in fresh prepared LB medium and incubated at 37°C for 90 min. with shaking. Cultures were cooled down, centrifuged (2,500 x g, 15 min.), and resuspended in cold phosphate-buffered saline pH 7.4 (10^7 bact./ml), and kept in ice bath until the use. The cell suspension was pipetted into the wells of a microtiter plate followed by the addition of HS at 36% and 50% final concentrations. Plates were briefly shaked and incubated at 37°C. Absorbance of the samples were read at 0, 30, 60, 90, 120, 150, and 180 min. in a spectrophotometer (Titertek Multiskan, model 340) at 620 nm in triplicate. The strains were considered either HS-sensitive or HS-resistant by measuring the bacterial lysis demonstrated during the HS exposure.

Lethality studies - The LD_{50} of *S. marcescens* strains were determined by injecting ten-fold dilutions of broth culture (10^4-10^8/0.5 ml) into the mouse peritoneal cavity. Lots of ten animals were used per dilution. The LD_{50} was calculated as previously described (22).

RESULTS

Assay for bactericidal activity of the serum - Of 60 strains of S. marcescens tested, numbered in crescent order from 1 to 60, 55 (91.6%), were resistant to the lytic action of HS. It was found that only five strains of the serogroup 06,14 were HS-sensitive and the others serogrouped as 01, 03, 04, 05, 010, 013, 016, 019 were HS-resistant (Table 1).

Table 1. Serum resistance and the correlation with diferent serogroups of *S. marcescens*

Serogroup	Nº tested	HS – Resistant (Nº)	(%)
01	4	4	(100)
03	2	2	(100)
04	2	2	(100)
05	3	3	(100)
010	1	1	(100)
013	1	1	(100)
016	1	1	(100)
019	3	3	(100)
06,14	44	39	(88,6)
Total	60	55	(91,6)

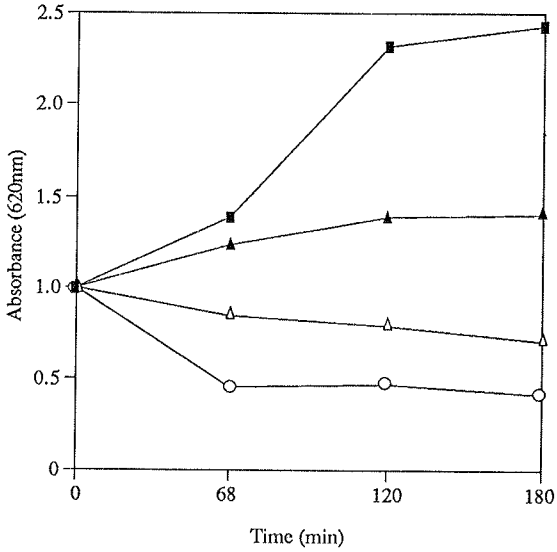

Figure 1. Bactericidal effects of 50% NHS on the representative strains of *S. marcescens*: Serratia 35 HS-resistant (■); Serratia 52 HS-sensitive with HS (Δ), or heat-inativate (▲). The sensitive strain *E. coli* K12 (○) was used as control. Indicated values correspond the mean of three determinations.

Figure 1 shows that isolated strain 52 of *S. marcescens* and *E. coli* K12-711 are sensitive to lysis by complement whereas strain 35 in comparison is resistant. These strains 52 and *E. coli* K12-711 grow in heat-inactivated HS. All HS-resistant strains behaved like strain 35. As a matter of fact strain 35 was resistant to higher serum concentrations, such as 36%, 50% and 80%, after 3h incubation, whereas the strain 52 and *E. coli* K12-711 were lysed at those serum concentrations (Fig. 2). The strains 53, 54, 55, 57 and 52 all HS-sensitive were isolated from urine,

Table 2. Serum resistance and source of *S. marcescens* isolates.

Site of isolate	Nº tested	HS – Resistant (Nº)	(%)
Blood	7	7	(100)
Urine	10	9	(90)
Bones	11	11	(100)
Sputum	4	3	(90)
Skin	11	9	(81,8)
Eye	4	4	(100)
Secretions	13	12	(92.3)

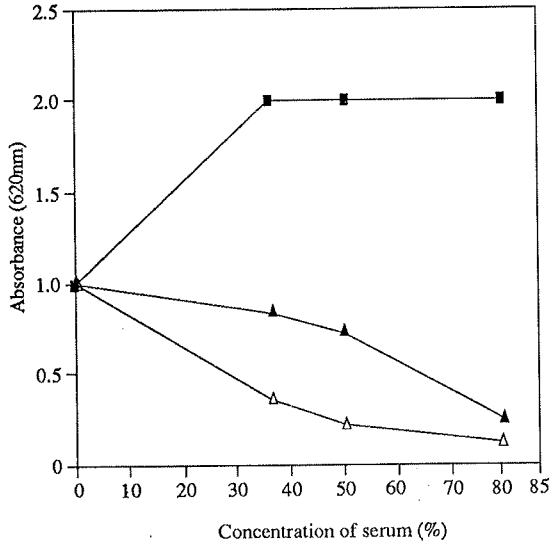

Figure 2. Effect of serum concentration on the Serratia HS-resistant 35 (■), Serratia HS-sensitive 52 (▲) and E. coli K12 (Δ). Indicated values correspond the mean of three determinations.

sputum, skin and secretions. None of the isolates from blood and bones were HS-sensitive.

Pathogenicity tests - Strains of the serogroups 01, 03, 04, 05, 010, 013, 016, 019 and 06,14 were virulent for mice at the same LD_{50} (3,5 x 10^7 bacteria).

Strains of *Serratia marcescens* classified as serogroup 06,14, two HS-sensitive and two HS-resistant also were lethal for mice LD_{50} (3,5 x 10^7 bact.).

DISCUSSION

The resistance to HS bactericidal activity is one factor that contributes to the virulence of many gram-negative pathogen (05). It was previously reported that of 54 clinical isolates of *S. marcescens* 77.8% were resistant to HS (24). Similarly, we found a high incidence (91.6%) of HS-resistant strains in contrast with the results reported elsewhere (03).

In this work, all strains isolated from blood, eyes and bone infections were HS-resistant similarly as observed in blood culture isolates (24). Conversely, both HS-resistant and HS-sensitive agents were found among sepsis-causing strains (11).

We have found that HS-sensitive *S. marcescens* are killed by heat-labile factors presumably complement. However, it has been difficult to ascertain true factors which determine the HS-resistance or sensitivity. It could be assumed that different components of the outer bacterial envelope avoid the penetration and binding of the serum bactericidal factors into the cell membrane of the HS-resistant strains (21).

Studies with susceptible strain of *S. marcescens* demonstrated that killing by serum is associated with early changes in the permeability of the microbial envelope (02). It has been demonstrated in *E. coli* that some serogroups such as 06 have smooth LPS which by themselves can render the agents resistant to serum whereas other serogroup such 012 have smooth LPS phenotypes that alone cannot confer serum resistance but require additional factors, for instance encapsulation (07).

In this work, we compared the effects of HS in various strains of *S. marcescens* belonging to different serogroups. We have only found five HS-sensitive strains out of 44 strains of the serogroup 06,14. These results suggested that sugar components of the serogroup 06,14 LPS, of Serratia were not sufficient to prevent complement-mediated bacteriolysis. It was also found HS-sensitive strains with smooth LPS indicating that the length of the LPS was not the only factor determining the HS-resistance of *S. marcescens* (11).

Strains carrying the complex serogroup - antigen 06,14 have previously been shown to be more virulent for mice than strains of other serogroup (26), however we verified that the virulence for mice not was associated with different serogroups tested.

Two strains HS-sensitive and two HS-resistant of the serogroup 06,14 were tested for virulence in mice and all strains showed the same LD_{50} (3,5 x 10^7 bact.). These results suggest that the serum resistance is not the principal determinant of virulence of *S. marcescens* at least regarding to LD_{50} assays carried out in mice. In addition strains HS-sensitive may become HS-resistant under nutrient-limiting conditions which occurs "in vivo".

RESUMO

Resistência de diferentes sorogrupos de *Serratia marcescens* ao soro humano normal e sua patogenicidade para camundongos.

Isolados nosocomiais de *Serratia marcescens* pertencentes a diferentes sorogrupos (01, 03, 04, 05, 010, 013, 016, 019 e 06,14) foram analisados quanto a resistência à ação bactericida do soro humano (HS). De 60 amostras estudadas, 55 (91,6%) foram HS-resistentes para uma concentração de 50% de soro e somente amostras do sorogrupo 06,14 foram HS-sensíveis. Todos os isolados de sangue, olhos e ossos foram resistentes ao HS. Amostras com diferentes sensibilidades ao HS foram testadas para a patogenicidade e todas foram letais para camundongos com a mesma DL_{50} (3,5 x 10^7). Esses resultados sugerem que a resistência ao soro não é o principal determinante na virulência de *S. marcescens*.

Palavras-chave: *Serratia marcescens*, soro-resistência, infecções hospitalares, antígeno 0.

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IDENTIFICATION OF *CORYNEBACTERIUM DIPHTERIAE* BY INDIRECT IMMUNOFLUORENCE TECHNIQUE[1]

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SUMMARY

A recent increase in number of outbreaks of diphtheria in Brazil stimulated the search for a realiable laboratory test for the differential diagnosis of tonsillitis.

Our investigation compared the indirect immunofluorescence method with the direct fluorescent-antibody assay, with commercial antiserum.

The evaluation studies showed that a specific immunofluorescence staining can be prepared and used to identify *C. diphtheriae* accurately and rapidly. The organisms were detected in pure culture and in smears made from young cultures of throat swabs of patients whose illness was clinically diagnosed as diphtheria. Fluorescent staining may prove to be helpfull also in examining family contacts.

Key words: *Corynebacterium*, diphtheria, immunofluorescence, diagnosis.

INTRODUCTION

Between 1980-89 diphtheria outbreaks were reported in such diverse geographic areas in Brazil (Piauí, Ceará, Rio Grande do Norte, Pernambuco, Amazonas and Santa Catarina). Morbidity was associated with poor vaccination status and preschool-age children. Clinical disease occurred in both nonvaccinated susceptible persons and persons who had a history of some previous vaccination. These cases led to consider diphtheria a national health priority (13).

Patients with skin lesions may be more infectious than those with faucial or nasal diphtheria (3,4).

A total of 1007 school children were examined with the purpose of verifying the prevalence of *C. diphtheriae* in cutaneous lesions in the city of Rio de Janeiro, Brazil. *C. diphtheriae* skin carriers, (13.9% with nontoxigenic and 1.19% with toxigenic bacilli) were found. Three regions with different incidence rates of respiratory diphtheria, were evaluated. The greatest prevalence was found in Santa Cruz, region that presented most cases of respiratory disease: 22.9% of nontoxigenic and 1.8% of toxigenic bacilli. Examining close contacts of bacteriologically confirmed diphtheria, 14.8% were *C. diphtheriae* carriers, with 8.3% carrying toxigenic bacilli (19, 20, 21).

The isolation of toxigenic and nontoxigenic *C. diphtheriae* from sperm and cutaneous ulcers due to *Leishmania* had been also reported recently (10,15).

The possibility is discussed that under certain conditions nontoxigenic *C. diphtheriae* may cause a diphtheria-like disease (9).

There has been much debate over the need for

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routine screening for diphtheria from patients with pharyngitis. A recent investigation suggests that routine culture for the organism should be done, specially for those who could be immunocompromissed (24).

The skin is a reservoir of Gram-positive pleomorphic organisms similar to *C. diphtheriae*, therefore, nontoxigenic bacilli may be considered a diphtheroid without adequate specification (7,9,11).

In the routine laboratory examination of cultures for diphtheria bacilli, it has been customary to discard as diphtheroids those organisms that ferment both dextrose and sucrose. Possible error due to this practice may occur. In contrast to most other countries, sucrose-fermenting strains are highly prevalent in Rio de Janeiro, Brazil (14).

Immunofluorescence could be usefully and economically applied to the examination of large number of clinical specimens in area where diphtheria is still endemic or epidemic, where the number of specimens tested in the laboratory may soon increase considerably. If applied to bacterial colonies obtained on primary isolation agree completely with definitive bacterial identification (10).

In this paper, an indirect immunofluorescence assay for identifying *C. diphtheriae* in pure cultures and in clinical materials was conducted and is described. A fluorescent absorbed antisera was prepared against somatic antigens of the bacilli. We evaluated the indirect IF method by its comparison with the IF technique with commercial fluorescent antiserum.

MATERIALS AND METHODS

Bacteriological methods - Fifty *C. diphtheriae*, and seven diphtheroids strains isolated in Rio de Janeiro and Bahia, Brazil, were examined besides *C. diphtheriae*, var. gravis (E6651), var. mitis (E8392) and var. intermedius (CD7920), *C. ulcerans* (KC279), *C. minutissimum* (F46), *C. renale* (A1214), *C. striatum* (F378), *C. equi* (KC1611), *C. hoagii* (KC1369), and *C. xerosis* (E8081) obtained from the Communicable Disease Center, Atlanta, Georgia, USA. Throat swabs obtained from 36 patients with clinical diagnosis of diphtheria were also examined. Heterologous organisms examined included three strains of Pseudomonas aeruginosa and a strain of Salmonella typhi all isolated in Rio de Janeiro.

Bacteriologic methods described previously (6,7,8,9,11) were followed in the laboratory for isolation and identification of *C. diphtheriae*. The strains were biotyped with the help of morphology, cultural characteristics and biochemical reactions. The microorganisms were cultured on Pai egg medium slant, sheep blood agar and chocolate tellurite agar plates. The organisms identification was based on U.V. fluorescence test (screening test), DAU medium (glucose, maltose and urea), nitrate reduction, toxigenicity test (radial immunodiffusion method), and PYZ - pyrazine carboxylamidase activity (11,22).

Immunological methods (1,2,12) - Antigens preparation for immunization and agglutination tests.

The antigenic suspensions were obtained from a *C. diphtheriae* var. gravis (E6651) and a sucrose fermenting strain (strain number 236) isolated in our laboratory. Microorganisms were cultivated in 250 ml of BHI medium (Brain Heart Infusion Broth (Baltimore Biological Laboratory, Inc.) supplemented with 0.2% yeast extract plus 0.02% tween 80. After incubation under aerobical conditions with gentle shaking for 72h at 37°C., growth was centrifuged 10000 x g for 15 min. at 4°C. Growth was then suspended also in 0,85% NaCl and heated for two hours at 100°C. Microorganisms were pelleted by centrifugations and cells were suspended in 0.85% NaCl. Antigenic suspensions added of 0.5% phenol were stored at 4°C.

Antigens for immunization - cells were suspended in esteril 0.85% NaCl, and the turbidity was ajusted to contain approximately 1.5 x 10 cells per ml.

Antigens for agglutination tests - the turbidity was adjusted to contain about 9 x 10 cells per ml.

Animals'immunizations - antisera were produced in rabbits by three consecutive days intravenous inoculations every week up to four weeks, by injecting 0.05, 0.1, 0.2 ml (first week); 0.3, 0.4, 0.5 ml (second week); 0.5, 0.5, 0.5 ml (third week); 1.0, 1.0, 1.0 ml (fourth week) and one 1.0 ml inoculation in the sixth and eight weeks. After that up to ten weeks, according to titers in test-bleeding of the animals those that showed a significant antibody titer were bled out. Serum was collected aseptically and preserved with sodium azide.

Agglutination tests - serum dilutions from 1/10 to 1/2560 in 0.5 ml quantities were prepared in physiological saline, and 0.5 ml of antigen was added to each. The tubes were shaken by hand and incubated in a water bath at 55°C for 4 h.. Readings were made at 4 h and after continued incubation overnight at 0 to 5°C. Antigens' control was included.

The final titer was taken to be intermediate

between the last clear agglutination pattern and the following questionable patern.

Absorption of antisera - Antisera used for absorption were diluted (1/5) in physiological saline. Antisera were absorbed with 2 ml concentrated freshly grown and washed cells of *C. equi* Kc1611 or C. hoaggi Kc1369. Suspensions were incubated at 55°C for 2 h., under manual agitation at 15 min. intervals, followed by overnight incubation at 4°C. The cells were removed by centrifugation. To prevent cross-reactivity with other corynebacteria sera absorption procedures of antisera were carried out for several times. Sera were divided into small portions and stored at -20°C.

Immunofluorescence methods - Microorganisms and clinical swabs were incubated in 2 ml BHI-medium (Brain Heart Infusion - Difco) prior to making smears for staining (17). The direct and indirect staining methods were performed withing 4 and 24 h. The direct method made use of commercial fluorescent antisera Bacto FA *C. diphtheriae*, Difco - code 320. For indirect staining *C. diphtheriae* serum absorbed with *C. equi* or *C. hoagii* was used as the primary reagent. A fluorescein isothiocyanate-labeled goat anti-rabbit globulin (Bhering) was used as the secondary reagent. The slides were examined for presence of fluorescent organisms with a Biological microscope Labophot-Nikon. Microscope was equipped with halogen lamp with an oil immersion objective giving a magnification of x 1000. The positive strains showed variations on intensity of fluorescence reactions graded by plus values from 1+ to 4+. Slides which were doubtful (less than 2+) were reported as negative.

RESULTS AND DISCUSSION

Corynebacterium strains - biochemical characteristics *C. diphtheriae*, and related species were correlated with literature dates. Twelve *C. Diphtheriae* strains isolated from throat, and eight from skin lesions were toxigenic. From thirty nontoxigenic strains isolated from skin, eight were isolated from leishmaniotic ulcer.

Absorption studies - agglutinin titers obtained with homologous and heterologous strains ranged from 40 to 2560. The *C. diphtheriae* monospecific serum was obtained by absorption with *C. equi* or *C. hoagii*. Absorbed antisera agglutinated with variable titers (20 to 640) all toxigenic and most nontoxigenic diphtheria bacilli strains. Titers from 80 to 160 were observed more frequently.

Immunofluorescence reactions - the prior incubation of pure cultures or swabs in broth for 4 and 24 h it was necessary. Smears for fluorescence staining can be satisfactory prepared from saline suspensions (9 x 10 cells per ml) of organisms grown on king's medium (UV-fluorescence test).

Direct and indirect immunofluorescence were positive for diphtheric bacilli in 76% strains. We observed correlation and specificity for both IF techniques. 14.2% diphtheritic bacilli strains were observed only by the indirect-IF technique versus 3.5% obtained with direct-IF technique.

BHI-medium and Pai medium are the media of choice for obtaining maximal fluorescence staining of the greatest number of *C. diphtheriae* strains (18). Our Data show that equivalent results can be obtained with king's medium.

Staining of organisms other than *C. diphtheriae* compounds the lack of specificity. Pharyngeal associated microbiota may contribute for lower intensity of fluorescence reaction (5, 18, 23).

Serum antibodies against *C. diphtheriae* are believed to react with strong affinity against other members of the genus. Measures can be taken to alleviate such problems by sorbing the reagent with troublesome heterologous bacteria (16).

C. diphtheriae, C. equii, C. hoagii are more close related to each other than to the remaining species. (1,2)

After performance of *C. equi/C. hoagii* absorpation procedure, all *C. diphtheriae* shoqed positive with variable titers. Satisfactory results were found after 1 to 5 absorptions.

No immunofluorescence was observed in any organism that could be confused with *C. diphtheriae*. This mono-specific antisera did not result in fluorescent staining with *C. xerosis* or other bacteria commonly found in the pharyngeal tract. However, *Corynebacterium ulcerans* and *Pseudomonas* strains showed positive reactions. The important point is that *C. ulcerans* is a *Corynebacterium* that differs from *C. diphtheriae* in morphological appearance, biochemical reactions, and the ability to produce an unrelated toxigenic factor, but, has in common the ability to produce diphtheria toxin.

The cross-reactivity between C. tenuis and *C. diphtheriae* was previously observed, but seems of minor significance (12).

In 18 cases (50%) *C. diphtheriae* strains were not detected by both bacterial culture and immunofluorescence preceded by incubation of the swabs. Eight cases (21%) were positive only by direct bacterioscopy, and diphtheroids were detected by culture in two opportunities.

We detected a few positive cases (50%) from clinical specimens. It may be a result of antibiotic therapy begun before hospitalization; misdiagnosis; or routine cultures of throat swabs for diphtheria from patients with pharyngitis.

There were 3 instances (8.3%) in which bacterial culture and immunofluorescence reaction were positive while negative in direct bacterioscopy. Positive immunofluorescence was observed in 3 other cases (8.3%) in which direct bacterioscopy and culture were both negatives. Unfortunately in two other cases with positive immunofluorescence reactions (5%) *C. diphtheriae* isolation was not attempted on the culture. Two cases with positive culture, were negative by both direct bacterioscopy and immunofluorescence methods (5.5%).

The results of IF and bacterial culture showed 68.8% agreement in 77 positive specimens examined during an epidemic of diphtheria in Texas, USA. (17). In Brazil, where diphtheria still endemic, 3% and 8% agreement was reported in Recife (16) and Rio de Janeiro, respectively. We reported three positive specimens (8.3%) by IF alone similarly to the results observed by Mc Cracken et al., 1971 (5.2%) during epidemic (17). Three *C. diphtheriae* strains showed negative fluorescence when applied both fluorescent antibody techniques.

The value of the IF test lies in the rapid presumptive diagnosis of diphtheria. One major advantage of FA over conventional procedures is that pure cultures are not required. The success or failure of FA techniques is determined largely by the quality of the labeled reagents. The FA test will not, and should not be expected to supplant conventional bacteriologic procedures. IF must be used with cultural procedures.

The eventual diagnosis of *C. diphtheriae* as the etiological agent may require 2-3 days of bacteriological work. Utilization of UV-fluorescence test (King's medium), and the fluorescence antibody technique can provide economical identification of this organism. UV fluorescent diphtheroids are rarely recovered from natural human reservoirs of diphtheria bacilli.

Indirect-IF assay with antisera prepared against somatic antigens are applicable to the detection of *C. diphtheriae* to persons with sore throats or a recent clinical diagnosis of diphtheria, or their close associates. This method would appear to be as reliable as and more rapid than other reactions for diagnosis of bacterial species. The indirect-IF method is suitable for large-scale screenings, especially during diphtheria outbreaks.

RESUMO

Identificação de *Corynebacterium diphtheriae* pela técnica de imunofluorescência indireta.

Os surtos de difteria, que recentemente ocorreram no território brasileiro, nos levaram a estudar diferentes testes laboratoriais, empregados no diagnóstico diferencial das amidalites.

Comparamos a técnica de imunofluorescência indireta, a partir de imune-soro absorvido preparado no nosso laboratório, com a técnica direta utilizando imune-soro comercial.

Demonstramos que a imunofluorescência pode ser satisfatoriamente utilizada na identificação rápida do *C. diphtheriae*. Os microorganismos podem ser observados em esfregaços feitos de culturas puras ou mesmo naqueles obtidos com swabs de pacientes, que foram cultivados por poucas horas em meio líqüido. A técnica pode também ser de grande utilidade no exame dos contactos familiares.

Palavras-chave: *Corynebacterium*, difteria, imunofluorescência, diagnóstico.

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ANTIMICROBIAL SUSCEPTIBILITY IN THREE GROWTH MEDIA OF ISOLATES OF THE *BACTEROIDES FRAGILIS* GROUP OBTAINED FROM HUMANS AND *CALLITHRIX PENICILLATA* MARMOSETS

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SUMMARY

The susceptibility of 104 strains of *Bacteroides fragilis* group recovered from humans (52) and *Callithrix penicillata* marmosets (52) to clindamycin, metronidazole, penicillin G, and mercuric chloride was determined in three different growth media using agar and broth dilution methods. Brain heart infusion agar supplemented with either hemin or blood was used for the former method and brain heart infusion broth for the latter. On the agar media, only 10% of the human isolates and none of the marmoset isolates was resistant to clindamycin. Of the four drugs tested, metronidazole was the most effective antimicrobial agent against all the microorganisms tested in the three media used. The marmoset isolates showed higher levels of resistance to penicillin G in every medium tested. Mercuric chloride presented the lowest activity on brain heart infusion blood agar medium, the MICs ranging from 2 to 128 ug/ml.

Key words: Anaerobic bacteria, *Bacteroides fragilis* group, Antimicrobial drugs.

INTRODUCTION

Anaerobic bacteria belonging to the *Bacteroides fragilis* group represent the numerically dominant organisms recovered from the intestinal tract of humans and non-human primates (20). Members of this indigenous microbiota have been implicated as the causative agents in several types of human and animal infections of endogenous origin.

In the past several years, worldwide increase of the resistance levels of anaerobes have been noted, particularly in strains of the *B. fragilis* group, to several antimicrobial drugs (7). Mercuric compounds may have an important role in the selection of resistant microorganisms (16). The mercurial resistance of *Bacteroides spp.* appears to be determined by plasmid carrying multiple antibiotic resistance genes (15). As a result of this increase in the resistance levels, it would be desirable that clinical laboratories consider instituting antimicrobial susceptibility testing of anaerobic bacteria in routine procedure (1).

Development of reliable tests for antimicrobial drug susceptibility implies that several parameters be taken into consideration, with adequate control (1,2,4). Published data show discrepancies

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between agar and broth dilution method for a variety of microorganism-drug combination (1,3). Although standardization of inoculum is considered to be critical in the antimicrobial drug susceptibility tests, it does not evaluate the growth peculiarities of the microbial species (1,2).

Recently, Finegold & The National Committee for Clinical Laboratory Standards (8) have reviewed the problems associated with the determination of the antimicrobial susceptibility of anaerobic bacteria and the clinical relevance of such tests. Inconsistencies in testing underscore the need for strict quality assurance programs for standardization of the methods (6).

In this report, we compared the susceptibility of strains of the *B. fragilis* group in three different media by agar dilution and broth dilution methods.

Considering the significance for Comparative Microbiology, human and *C. penicillata* marmoset strains were tested.

MATERIALS AND METHODS

Microorganisms. A total 104 intestinal strains of the *B. fragilis* group obtained from healthy adult humans and 2-to 3-year-old *C. penicillata* marmosets kept in captivity were tested: 52 human strains (32 *B. fragilis*, 6 *B. distasonis*, 5 *B. vulgatus*, 5 *B. thetaiotaomicron*, and 4 *B. ovatus*), and 52 marmoset strains (32 *B. fragilis*, 7 *B. vulgatus*, 5 *B. distasonis*, 4 *B. thetaiotaomicron*, and 4 *B. ovatus*). The species were identified recording to the biochemical standard criteria (11,13).

Antimicrobials. Standard laboratory drug powders were supplied as follows: penicillin G (Fontoura-Wyeth S.A., São Paulo, Brazil), clindamycin and metronidazole (Rhodia S.A., São Paulo, Brazil), and mercuric chloride (Inlab S.A., São Paulo, Brazil). The antimicrobial solutions were prepared daily.

Antimicrobial assays - 1. *Agar Dilution Method* - The agar dilution method described by Sutter et al. (22) was used as reference. Two-fold serial dilutions of the antibiotics were prepared in brain heart infusion agar (BHI - Biobrás Laboratories, Montes Claros, MG, Brazil) supplemented with 0.5% yeast extract and 0.5 ug/ml hemin as a modification of Sutter's method, and BHI agar enriched with 5% blood. Both media were prepared 2 to 4 h before the tests. Concentrations of the antibiotics ranged from 0.25 to 256 ug/ml. Agar plates without antibiotics were used as controls. The inocula were prepared by using BHI broth, either supplemented with 0.5% yeast extract and 0.5 ug/ml hemin, or without hemin: each strain was grown on blood agar and incubated at 37°C in an anaerobic atmosphere into 5 ml of BHI broth, supplemented or not with hemin. The broth cultures were incubated overnight at 37°C and used for standardization of the inocula (10^8 CFU). The inoculum size was verified by performing colony counts. Each plate was then inoculated using a Steers replicator (21) which delivered a final inoculum of approximately 105 CFU per spot. The plates were incubated under anaerobic conditions at 37°C for 48 h. The MIC was defined as the lowest concentration of antimicrobial agents capable of inhibiting growth or allowing just a barely visible haze caused by the development of the microorganisms.

2. *Broth Dilution Method (Macrodilution Method)* - The MIC endpoints were determined by a broth macrodilution method in test tubes containing 5 ml of BHI supplemented with 0.5% yeast extract and 0.5 ug/ml hemin. Two-fold serial dilutions of the antibiotics ranged from 0.25 to 256 ug/ml. Broth medium without antibiotic was used for sterility and organism growth controls. The inocula were prepared from the same BHI broth cultures, supplemented or not with hemin, used in the agar dilution method. Tubes were inoculated with a semiautomatic dispenser (100 ul) delivering a final inoculum of approximately 10^7 CFU per tube. The inoculum size was verified by performing colony counts. All tubes were incubated under anaerobic conditions for 48 h. The MIC was defined as the lowest concentration of antimicrobial agents capable of inhibiting growth or allowing just a barely visible haze caused by the development of the microorganisms.

3. *Quality Assurance* - Quality assurance of each susceptibility run was controlled by testing *B. fragilis* ATCC 23745 and *B. vulgatus ATCC* 8482.

RESULTS

1. *Standardization of Inoculum* - The standardization of the inoculum size showed that in broth without hemin the cellular number per ml (CFU) was approximately two-fold lower than that determined in broth medium with hemin.

2. *Comparison of Broth and Agar Sensitivity* - We observed that the resistance to all antimicrobials tested in broth was lower than that in the agar supplemented with hemin (reference method) or with blood.

3. *Broth and Agar MICs* - Table 1 shows the distributions of MICs required for inhibition of

growth of 50% and 90% of the human and animal strains (MIC_{50} and MIC_{90}) for the drugs tested.

All the human (52) and marmoset (52) strains showed MIC_{50} and MIC_{90} for clindamycin between 0.5 and 4 ug/ml on agar media, and between 0.25 and 1 ug/ml on the BHI broth medium.

Only five of the human isolates (10%) were resistant to this drug, and had higher MICs values than 4 ug/ml of clindamycin (breakpoint).

For metronidazole all of the 104 strains showed MICs ranging between 0.25 and 1 ug/ml on the broth medium. The strains were all sensitive at the breakpoint (16 ug/ml).

The percentages of human isolated strains resistant to penicillin G in all media tested was lower than that isolated from the marmoset. MICs for all human and *C. penicillata* marmoset strains ranged from 1 to 128 ug/ml on the agar media and between 1 and 32 ug/ml on the broth medium.

The MIC_{50} and MIC_{90} of mercuric chloride for human strains were respectively 16-fold and eight-fold lower in BHI agar plus hemin than in BHI blood agar; and likewise eight-fold and 16-fold lower for the marmoset isolates.

Table 1. Comparison of the activities of clindamycin, metronidazole, penicillin G, and mercuric chloride in various media, against 104 human and *C. penicillata* marmoset isolates of bacteria belonging to the *B. fragilis* group.

Antimic Agent[a]	Test medium	MIC (ug/ml) Human isolates (52) Range	50%	90%	% R[b]	Marmoset isolates (52) Range	50%	90%	% R[b]
Cl	BHIHA[1]	0.25–32	0.5	4	10	0.25– 4	2	4	0
	BHIBA[2]	0.25–32	0.5	4	10	0.25– 4	2	4	0
	BHIHB[3]	0.25– 1	0.25	0.5	0	0.25– 2	0.25	1	0
Mz	BHIBA	2 –16	2	4	0	1 –16	4	8	0
	BHIBA	2 –16	2	4	0	1 –16	4	8	0
	BHIHB	0.25– 1	0.5	0.5	0	0.25– 1	0.5	0.5	0
Pe	BHIHA	2 –128	16	32	37	1 –128	16	64	48
	BHIBA	2 –128	16	32	37	1 –128	16	64	44
	BHIHB	1 – 32	4	16	8	1 – 32	8	32	21
$HgCl_2$	BHIHA	0.5– 16	4	8	65	2 – 16	4	8	98
	BHIBA	2 – 64	64	64	92	8 –128	32	128	100
	BHIHB	0.5– 4	2	2	8	1 – 4	2	2	4

[a] Isolates were tested with each of the antimicrobial agents.
[b] Numbers are the percentages of resistant isolates at breakpoint as follows: clindamycin (Cl), 4ug/ml; metronidazole (Mz), 16 ug/ml; penicillin G (Pe), 16ug/ml; mercuric chloride ($HgCl_2$), 2ug/ml.
[1] BHI agar plus hemin (0.5 ug/ml).
[2] BHI agar plus blood (0.5%).
[3] BHI broth plus hemin (0.5 ug/ml).

DISCUSSION

The antimicrobial activities of clindamycin, metronidazole, penicillin G, and mercuric chloride against strains of the *B. fragilis* group isolated from humans and marmosets were determined by the macrodilution method. The macrodilution MIC values were then compared against the MIC values obtained by the agar dilution reference method.

From the methodological point of view the preparation of inocula is simpler for aerobic or facultative anaerobic than with strict anaerobic bacteria, specially because of the good and rapid growth of the former bacteria in Mueller-Hinton, or tryptic soy agar, with the adequate amount of time needed for the execution of the tests. Unfortunately, this is a problem for the majority of the slow growing strict anaerobic bacteria (23). Fortunately, this is not the case for the *B. fragilis* group bacteria. Because a better growth in broth medium supplemented with hemin was observed, this medium was chosen for the standardization of the inocula.

Some of the relative values of MIC obtained do not agree with the values for the *B. fragilis* group cited in the literature concerning human isolates (1); such discrepancies could be due to different methods used in the susceptibility tests for anaerobes, and to other variables that need further studies including growth media, inoculum size, incubation conditions, pH, temperature and supplements added (1).

Regarding the marmoset isolates, there is no data avaliable in the literature.

Comparison of the anaerobic susceptibility obtained by the broth dilution method with two different media by the agar dilution method, confirm the observations that different methods can give very distinct values that appear to be dependent on the culture media and antibiotics (14).

Obviously, nutritional requirements of the organisms tested should also be considered, such as peptides, hemin, vitamin K or succinate for some fastidious anaerobes (12,19). It is not known at which extent these growth factors could interfer with the susceptibility of the microbial populations.

The lower MICs observed in the broth dilution method could be due to a better diffusion of the drug in the medium allowing for a more intimate physical contact between the microorganisms and the antibiotic.

A correlation between the MICs in the BHI blood agar medium and BHI agar medium supplemented with hemin was observed for clindamycin.

In BHI broth, the MIC_{50} and MIC_{90} were lower that in the reference method. Considering the breakpoint for clindamycin (4 ug/ml), only 10% of the human isolates showed resistance on the two agar media tested. Fox & Phillips (9) reported that clindamycin resistance is uncommon: 1% of *B. fragilis* and 10% of the other *B. fragilis* isolates were resistant to 2 ug/ml clindamycin. De Almeida & De Uzeda (7) reported resistance to clindamycin in a number of human isolates of the *B. fragilis* group some of which reached higher MIC levels than 512 ug/ml.

Metronidazole was active against human and marmoset isolates in accordance with the literature (1,9).

Among the beta-lactams, moderate resistance of human isolates is common (9,14). The marmoset isolates showed a higher level of resistance to penicillin G than the human ones, in all tested media. MICs for all strains in both agar media were four-fold and two-fold (MIC_{50} and MIC_{90}, respectively) higher than that from the broth medium. The human isolates showed MIC_{90} two-fold lower than that seen with the isolates recovered from marmosets. The majority of the isolates were beta-lactamase positive (unpublished data) by the iodometric method (24).

B. fragilis was more susceptible to clindamycin, penicillin G, and metronidazole, on the different tested media, than the other members of the group, in accordance with the literature (5).

Mercury and mercuric compounds have been used in many industries, in medicines and antiseptics, and also as a component of dental amalgam. These compounds may have an important role in the selection of resistant microorganisms (16).

It was shown, by using an agar dilution technique, that *Bacteroides spp.* would appear far more resistant to heavy metals than their facultative counterparts, including mercuric ion (17, 18).

The majority of the 104 human and marmoset isolates were resistant to the mercuric chloride when determined by the reference method, but not by the broth dilution method, probably due to better drug-microorganism contact. Mercuric chloride showed a very poor activity when the agar medium was supplemented with blood, probably due to components that could interfer with the action of the mercuric ion.

The current reference agar dilution method (22) was shown to be reproducible and also established quality control guidelines. However, for most clinical laboratories this method is excessively laborious, time-consuming, and costly. On the other hand, broth media provide a better drug diffusion.

Some laboratories have instituted the use of technical variations for the broth dilution method in an effort to eliminate some of the disadvantages of the reference method (1).

In ecologial terms, certainly the iterative exposition of man and animals microflora to the antimicrobials should be considered critical in the selection of resistant microorganisms.

Regarding the relevance of the intra and interspecies transfer of drug resistance, considering the ecologial distribution the *B. fragilis* group, it should be important to perform additional comparative studies of strains isolated from human intestine and from wild and captive marmosets.

RESUMO

Susceptibilidade a antimicrobianos em três meios de cultivo, de cepas do grupo *Bacteroides fragilis* isoladas de humanos e de micos *Callithrix penicillata*.

A susceptibilidade de 104 cepas do grupo *B. fragilis* isolados de humanos (52) e de micos *C. penicillata* (52) para clindamicina, metronidazol, penicilina G e bicloreto de mercúrio, foi determinada em três diferentes meios de cultivo, pelos métodos de diluição em ágar e diluição em caldo. O ágar infuso cérebro coração, suplementado com hemina ou sangue, foi usado para o primeiro método e, o caldo infuso cérebro coração para o último. Nos meios sólidos, somente 10% dos isolados humanos e, nenhum dos isolados de micos foram resistentes para clindamicina. O metronidazol foi o antimicrobiano mais eficaz contra todos os microrganismos testados nos três meios usados. As cepas isoladas de micos apresentaram níveis elevados de resistência para penicilina G em todos os meios testados. O bicloreto de mercúrio apresentou menor atividade em ágar sangue, apresentando faixas de CIMs de 2 a 128 ug/ml.

Palavras-Chave: Bactérias anaeróbias; Grupo *Bacteroides fragilis*; Drogas antimicrobianas.

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ELECTROPHORETIC STUDY OF THE GENOME OF PORCINE ROTAVIRUSES FROM SÃO PAULO, BRAZIL.

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SUMMARY

An electrophoretic study of the genome of porcine rotavirus was made in São Paulo, Brazil. Faecal samples were collected from April to August, 1985, in 5 different municipal districts (group X), and in April, 1986, in Bragança Paulista (group Y). Rotaviruses were identified in 45 of 140 diarrhoeic samples analyzed by polyacrylamide gel electrophoresis and enzyme immunoassay. All strains identified belonged to the group A rotavirus. Group X included 113 samples from different municipal districts, collected in different dates, with 27 (23.9%) rotavirus positive samples and 3 different eletropherotypes. Group Y included 27 samples collected from two herds in the same municipal districts, in one day, resulting in 18 (66.7%) rotavirus positive samples, 14 of which belong to and 4 were suggestive of the same electropherotype. The results in group Y indicate the occurrence of an epizootic episode of diarrhea in this district, with the same virus strain; this is the first report of such occurrence in Brazil. The susceptibility of rotavirus infection increased with age, beginning at 15 days old, in both X and Y groups.

key words: rotavirus, swine, diarrhea, electrophoretic types.

INTRODUCTION

Rotaviruses are now well recognized as an important cause of diarrhoea in young pigs (10, 18, 21), as well as in the youngs of numerous other species, including humans (5, 6), in many parts of the world. The genome of rotaviruses consists of 11 segments of double-stranded RNA (dsRNA) which form a characteristic pattern on polyacrylamide gel electrophoresis (8). Differences in the electrophoretic mobility of the individual genome segments from various isolated strains of virus can be used to classified electrophoretic types of rotaviruses (11).

Early serological studies indicated that rotaviruses possessed a common group antigen regardless of their host species (22). Recently, however, viruses with rotavirus morphology but which lack this group antigen have been described and although these rotaviruses have 11 segments of ds RNA their characteristic electrophoretype migration patterns differ considerably from normal rotavirus genome (2, 12).

Comparative serological and nucleic acid studies were carried out on rotaviruses with and without the group antigen and led to the definition of distinct groups, A, B and C, and possibly D and E (13, 14).

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Several serological assays have been developed for the diagnosis of rotavirus infection and immunoelectronmicroscopy (IEM), radio and enzyme imunoassays (RIA and EIA) have demonstrated similar degrees of sensitivity, being the enzyme immunoassay the most commonly used in routine diagnosis (16). Polyacrylamide gel electrophoresis (PAGE) is another non-serological technique that presented degrees of sensitivity similar to enzyme immunoassay and immunoelectron microscopy (16) and furthermore has led to the diagnosis of all rotaviruses groups.

In the present study we have used polyacrylamide gel electrophoresis and enzyme immunoassay to detect and classify electrophoretically rotavirus RNA genomes in diarrhoeic faecal samples from piglets, in São Paulo State, Brazil.

MATERIALS AND METHODS

A total of 140 porcine diarrhoeic faecal samples were obtained from herds in 5 municipal districts (Bragança Paulista, Charqueada, Guarulhos, Pirassununga and Ribeirão Pires) from São Paulo State, Brazil. The collection period lasted from April to August, 1985 (group X) and during April, 1986, from Bragança Paulista, in two herds at the same day (group Y). Only one sample was obtained from each animal. Approximately 10% faecal suspension in 0.1 M Tris/HCl pH 7.3 buffer were clarified by centrifugation at 5000 g for 30 min. Supernatants were homogenized with and equal volume of Freon 113, centrifuged as above and deproteinized by extraction with phenol-chloroform and ethanol precipitation.

Poliacrylamide gel electrophoresis (PAGE) of dsRNA was carried out by Laemmli's technique with modifications described by Pereira et al. (17). The simian rotavirus strains SA11, obtained in MA104 cell cultures, was included in all electrophoretic runs as a standard. Gels were photographed with 32 ASA Pan-Atomic film (Kodak).

Faecal samples were also analyzed for the presence of rotavirus group A antigens, with the enzyme immunoassay (EIARA) described by Pereira et al.(15). The samples were considered positives if result by PAGE and/or EIARA was positive.

RESULTS

Of the 140 faecal samples examined by both techniques, 45 were positive for rotaviruses; 31 were positive by polyacrylamide gel electrophoresis (PAGE), 35 were positive by the enzyme imunnoassay and 21 were positive by both techniques. Samples were distributed in two groups X and Y, the first with 113 samples, collected in 5 different municipal districts and the other with 27 samples obtained in Bragança Paulista at the same day, in two herds. The results for the two groups are shown in Table 1. In group X, 27 out of 113 samples (23.9%) were positive for rotavirus. In group Y, a higher percentage of rotavirus positive samples (18/27, 66.7%) was found.

The age distribution of positive results (Figure 1) in groups X showed a peak in samples ob-

Table 1. Distribution of positive rotavirus samples by municipal distrits, São Paulo, SP, 1985-1986.

Group	Municipal districts	Samples analysed	Rotavirus positive (N)
X	Bragança Paulista	48	13
	Charqueada	9	2
	Guarulhos	30	8
	Pirassununga	11	1
	Ribeirão Pires	15	3
	Subtotal	113	27
Y	Bragança Paulista	27	18
Total			

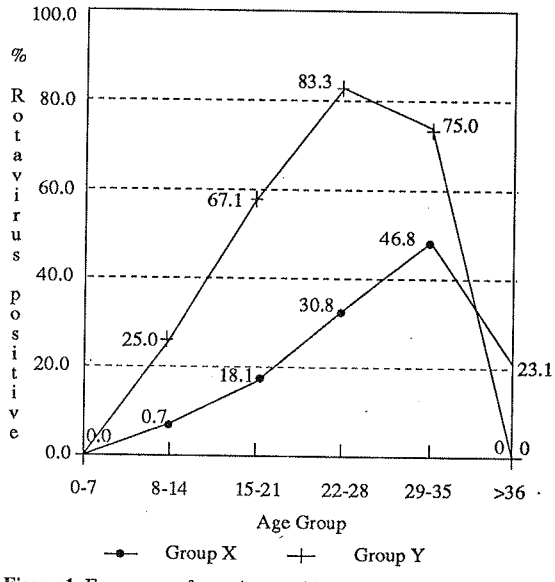

Figure 1. Frequency of rotavirus positive samples in relation to age of swine.

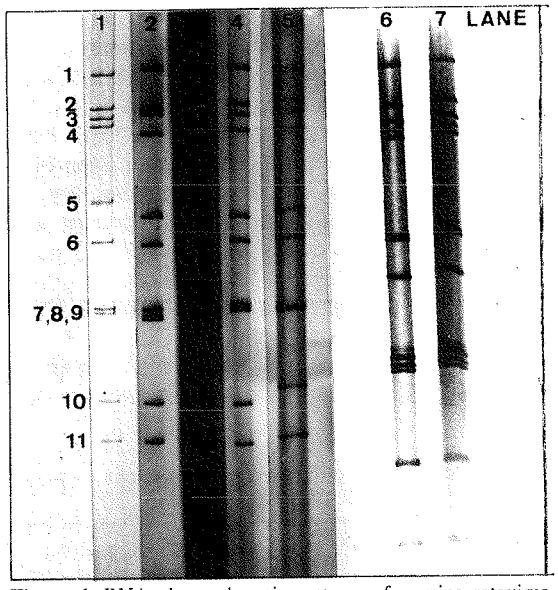

Figure 1. RNA electrophoretic patterns of porcine rotavirus, classified according to Lourenço et al. (11).
lane 1–SA11; Lane 2–IbIIaIIIcIVa (2 samples); lane 3–IaIIaIIIeIVa (1 sample); lane 5–IaIIaIIIgIVa (14 samples); lanes 6-7*–mixed rotavirus samples.
* lanes 6 and 7 from different electrophoretic runs

tained from animals between 29 - 35 days old. In group Y samples, the peak of positive rotavirus samples occurred in samples from animals between 22 - 28 days of age.

From samples positive by PAGE, 20 samples in which all 11 genome segments were demonstrable could be classified according to the system of Lourenço et al. (11). The remaining samples could not be classified due to low clearness in some visible bands. The electrophoretic pattern of genomic RNA from classified samples is shown in Figure 2 (lanes 2-5). All samples exhibited patterns characteristic of group A rotaviruses. Comparison of RNA migration patterns of the porcine rotaviruses indicate differences mainly in triplet 7, 8 and 9 and minor differences in segments 2 and 3.

All the samples with pattern Ia IIa IIIg IVa (Fig. 2, lane 4) were obtained from group Y.

Two of the samples examined showed more than the 11 characteristic bands of the rotavirus genomic RNA (Fig.2, lanes 6 and 7), representing a mixed rotavirus infection.

DISCUSSION

Results obtained from this study show the importance of rotaviruses as aetiological agents of porcine diarrhoea in different areas of the State of S. Paulo, Brazil. Rotaviruses were detected in 45 of the 140 stools specimens examined by PAGE and enzyme immunoassay. In group X, with samples from 5 municipal districts, the percentage of positive samples was 23.9%, similar to the percentage found by other authors in the State of São Paulo, Brazil (7, 19). Rotaviruses was identified in all the 5 municipal districts from which samples was obtained (Table 1).

The analysis of RNA by polyacrylamide gel electrophoresis of 20 samples showed that several different electropherotypes were circulating in the communities studied. The differences in migration of dsRNA segments were mainly in triplet 7, 8 and 9 minor differences in segments 2 and 3, similar to results observed by other authors (9, 20). This polymorphism of genomic RNA segments is already well known (1, 7), and reflects the co-circulation of different strains of rotavirus in the communities studied.

Rotavirus was not observed in the faeces of pigs less than one week old in both groups X and Y, and the results obtained in terms of age susceptibility to rotavirus infection showed an increase of rotavirus detection in pigs after 15 days of age, with a peak between 29 and 35 days of age for group X (Table 1), confirming the relation of rotavirus infection with the weaning process, described by other authors (3, 20).

In group Y samples, from two herds in Bragança Paulista, all collected in the same day, we found 18 out of 27 samples positive for rotavirus (66.7%), a higher percentage than in group X. From 18 positive samples, the electropherotype Ia IIa IIIg IVa (Figure 2, lane 5) was detected in 14 (77.7%), and the remaining 4 was suggestive of this same electropherotype, although it could not be established due to absence of some bands (data not show). The age distribution of positive samples in group Y was similar to that of group X, with much higher percentages in each group (Figure 1). The susceptibility increased after 15 days of age, but with a peak in the age of 22-28 days. The overall results in group Y indicate the occurrence of an epizootic episode of diarrhea in this district, caused by the same virus strain. This is the first report of such occurrence in Brazil. This results confirm the importance of using PAGE of rotavirus RNA as a tool in epidemiological studies, demonstrating the similarity in the genome of rotaviruses identified in the same place and day, indicating a probable common origin of the strain in the epizootic episode (1, 5).

Two of the samples examined showed more than the 11 characteristic bands of the rotavirus genomic RNA (Fig. 2, lanes 6 and 7), representing a mixed rotavirus infection, situation already found in pigs (7, 19) and humans (4).

RESUMO

Estudo eletroforético do genoma de rotavírus de suínos do Estado de São Paulo, Brasil.

Foi realizado um estudo sobre os eletroferótipos de RNA de rotavírus em suínos, no Estado de São Paulo, Brasil. As amostras de fezes foram coletadas de abril a agosto de 1985, em 5 municípios (grupo X) e em abril de 1986, em Bragança Paulista (grupo Y). Os rotavírus foram identificados em 45 amostras de um total de 140 amostras analisadas pela eletroforese em gel de poliacrilamida (PAGE) e ensaio imunoenzimático (EIARA). Todas as amostras identificadas pertenciam ao grupo A de rotavírus. O grupo X incluiu 113 amostras de 5 diferentes municípios, coletadas em datas diferentes, com 27 (23,9%) amostras positivas para rotavírus e 3 tipos eletroforéticos diferentes. O grupo Y incluiu 27 amostras, coletadas de 2 granjas no mesmo município, em um único dia, resultando em 18 (66,7%) amostras positivas para rotavírus, 14 das quais pertenciam ao mesmo tipo eletroforético e 4 foram sugestivos deste mesmo tipo. Os resultados do grupo Y indicam a ocorrência de um surto epizoótico neste município, causado pelo mesmo tipo de vírus: este é o primeiro relato de tal ocorrência no Brasil. Foi detectado um aumento da susceptibilidade dos animais à infecção por rotavírus a partir de 15 dias de idade, em ambos os grupos estudados.

Palavras-chave: rotavírus, suínos, diarréia, eletroferótipos

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INFLUENCE OF pH ON HEMOLYTIC ACTIVITY OF AN AVIAN SAMPLE OF INFLUENZA A VIRUS

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SUMMARY

The pH-dependent fusion activity between an avian sample of Influenza A virus and chicken erythrocytes was investigated using the hemolysis (HL) assay. Hemolysis was based on the quantification of the hemoglobin (Hb) released as a consequence of virus/erythrocyte fusion. Influenza virus hemolytic activity could be demonstrated at an acidic range of pH (5.0 - 5.8), which is related to the occurence of a conformational change in the hemagglutinin (HA) protein at low pH. Fusogenic activity was rapidly inactivated after a low-pH preincubation of virus at 37°C in the absence of erythrocytes. It suggests that the structural change of HA may be the cause of activation and/or inactivation of influenza virus fusion capacity. The evaluation of HL assay showed that increasing erythrocyte concentration while the virus concentration was kept constant a raise of hemoglobin (Hb) released could be observed which suggests that the virus had a better probability of finding more quantity of susceptible cells. Since the Hb colour changes at different pH values, a correction factor was introduced after the colorimetric assay.

Key Words: Influenza A virus, hemolytic activity, pH influence

INTRODUCTION

The fusogenic or hemolytic activity, mediated by the surface components of enveloped animal viruses, is a crucial event in which these viruses deliver their genomes into host cell for replication. The influenza virus (orthomyxovirus), enters cell by a receptor-mediated endocytosis process, routing the virus particle into the endosomal/lysosomal compartment of the cell (8,18). Subsequently, induced by the low pH in this compartment, the virus membrane fuses with the endosomal/lysosomal membrane, releasing the nucleocapsid into the cytosol. The initial adhesion of the virus to the membrane as well the fusion reaction are mediated by viral spike glycoproteins(16).

For this virus, both activities are confined to the hemagglutinin (HA) glycoprotein, which in its active form consists of two subunits, HA1 and HA2. The receptor-binding site is located on HA1, while fusion activity is thought to reside on the HA2 subunit. It has been demonstrated that low pH induces an irreversible conformational change in the HA molecule, exposing a hydrophobic sequence of amino acid residues on the HA2 subunit (12).

In order to gain more insight into the characteristics of this process, low-pH-induced fusion of an avian sample of Influenza A virus was investi-

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gated, using the hemolysis (HL) assay.

MATERIAL AND METHODS

Virus – The H3N8 sample of influenza virus used in this work was isolated from stools of ducks (*Dendrocygna viduata*) in 1980 at Rio de Janeiro Zoo's Garden. The virus was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs and incubated for 48h at 37°C. Harvested allantoic fluid was clarified by the centrifugation at 7,000 x g for 30 min (Sorvall, rotor model HS4). Clarified fluid was concentrated by ultra centrifugation at 80,000 x g for 60 min (Sorvall rotor model SW27), partially purified in potassium tartrate (40% - 0% in glycerol 5%) density gradient by centrifugation at 80,000 x g for 120 min, in a SW41 Beckman rotor. The virus band was collected, diluted 1:10 in 0.01M Tris, 0.001M EDTA, 0.0006M Cystein, Saline (TEC) pH 8.4 and centrifuged again for 60 min at 100,000 x g. The pellet was ressuspended with TEC and stored at -20°C until used(17).

Electron microscopic examination – The virus preparation was negatively stained with 4% phosphotungstic acid, pH 7.2 as described by Brener & Horne (2) and a Phillips 301 electron microscope was used for virus observation.

Erythrocytes – The erythrocytes used were from horse, guinea-pig, chicken, pigeon and human (group 0 Rh+). They were collected in Alsever solution and stored at 4°C. After washing three times, the number of erythrocytes were determined using a Coulter Counter (model SSr), and the preparations were adjusted to a concentration of 10% (v/v) in physiological saline (0.15M NaCl). At the time of use, the erythrocytes were adequately diluted in phosphate-buffered saline (PBS) free of Ca+2 Mg+2 for hemaglutination assay, or in 0.15M NaCl for hemolysis assay.

Hemagglutination assay – 25 µl of virus was diluted serially with 25 µl of physiological saline and to each dilution of virus was added 25 µl of a 1% mammal erythrocyte suspension or a 0.5% bird erythrocyte suspension. Titers have been reported as the reciprocal of the highest dilution of virus giving complete agglutination after the controls have been settled at 4°C(17).

Hemolysis assay – Hemolysis (HL) was determined in a total volume of 3.0 ml containing 1.0 ml of virus in the desired concentration, 1.0 ml of a 1% chicken erythrocyte suspension, both in physiological saline, and 1.0 ml of Phosphate Sorensen buffer (13) at different pH values. The pH values were measured using a potenciometer (Micronal B274). The tubes were capped, mixed by repeated inversion, and incubated for 20 min at 4°C followed by 60 min at 37°C with frequent mixing. After incubation, the tubes were centrifuged for 10 min at 1500 rpm and the amount of hemoglobin released in the supernatant was estimated by absorbance (Ab) measurement at 545 nm, using a spectrophotometer (spectronic 88 Bausch & Lomb). The percentual of cellular lysis was determined by comparison of the Ab values produced by virus action with that produced by lysis of 1.0 ml of erythrocyte suspension in 2.0 ml buffer solution containing Nonidet P-40 at 0.01% (maximum hemolysis control - MH). Another assay control consisting of 1.0 ml of the erythrocyte suspension in 2.0 ml buffer solution was done (residual hemolysis - RH) (7). These experiments were realized in duplicate.

RESULTS

Microscopic observation of the virus and study of hemagglutinating activity – Figure 1 shows the electron microscopy of the purified influenza virus preparation. A disrupted virus parti-

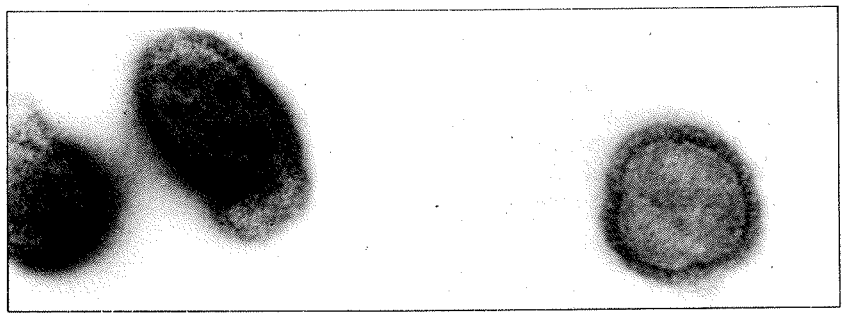


Figure: Influenza virus preparation after purification process. Negative stsain. X171,000.

cle can be seen behind an integral one.

This preparation was evaluated by the hemagglutination (HA) test with different types of erythrocytes. As shown in Table 1, the virus preparation differed up to 8 times from the hemagglutination titer (1280 and 10240) when it was tested with different chicken erythrocyte suspensions. Differences could also be observed in the HA titer when erythrocytes from other animals were used.

Hemolysis at different pH values – Fusion of influenza virus with chicken erythrocytes was first investigated as a function of pH. Figure 2 shows that at neutral pH fusion was negligible. A rise in the rate of fusion was noted below pH 5.8 reaching its maximum at pH 5.0-5.2. Upon further lowering of the pH (5.2) a slight decrease on the rate fusion was observed when 512 hemagglutinating units/ml of virus were used. It did no occur with lower concentrations of virus (64, 128 and 512 hemagglutination units/ml).

Changes on the colour of hemoglobin (Hb) which caused changes in Ab values measured could be noted when the same Hb concentration was tested at different pH values used for HL assay (Table 2). So, for the correct analysis of HL curve (Fig. 2) it was necessary to introduce a correction factor (CF) which was calculated in function of the maximum hemolysis (MH) control detected at pH 8.0.

$$\text{Correction Factor (CF)} = \frac{\text{MH pH 8.0}}{\text{MH pH of working}}$$

Then,
Virus Hemolysis (VH) corrected = VH X CF
Maximum Hemolysis (MH) corrected = MH X CF

TABLE 1 – Hemagglutination test of Influenza virus with different types of erythrocytes.

Erythrocytes	Hemagglutination titer[a]
dog	10240[b]
horse	640
guinea-pig	1280
chicken (1)	1280
chicken (2)	10240
human (1)	1280
human (2)	1280
pigeon	10240

a – Hemagglutination titer is expressed as the reciprocal of the highest dilution of the virus required to give complete agglutination.
b – Numbers in table expresses the mean of three experiments.

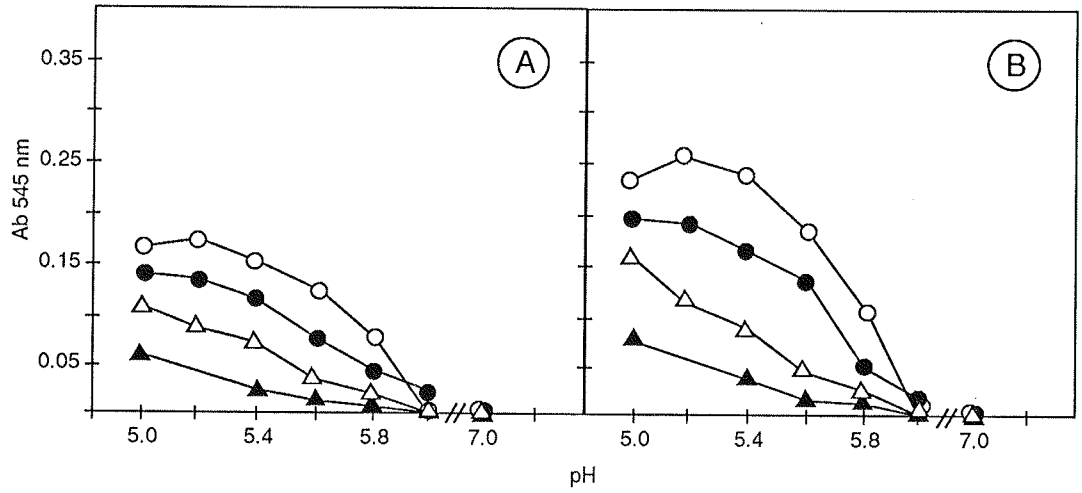


FIGURE 2 – Fusion of influenza virus with chicken erythrocytes at different pH values. Ab 545 represents virus hemolysis without (A) and with (B) correction factor. "Symbols denote virus concentrations: 64 (▲——▲), 128 (△——△), 256 (●——●) and 512 (○——○) hemagglutinating units/ml.

TABLE 2 – Absorbance of Hb released in the supernatant at different pH values.

pH	5.0	5.2	5.4	5.5	5.6	5.8	6.0	6.5	7.0	7.5	8.0
Ab 545	0.32	0.32	0.33	0.34	0.35	0.36	0.38	0.42	0.47	0.48	0.48

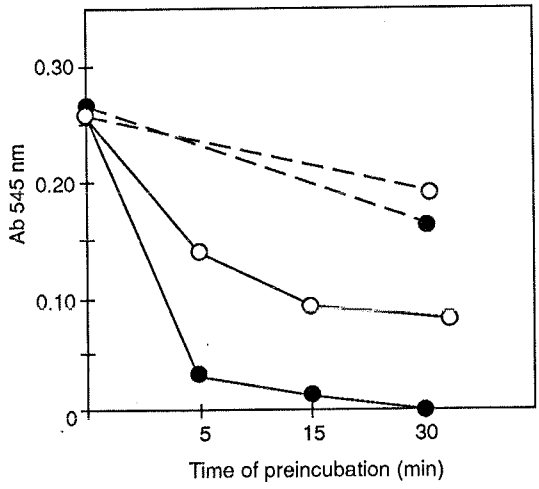
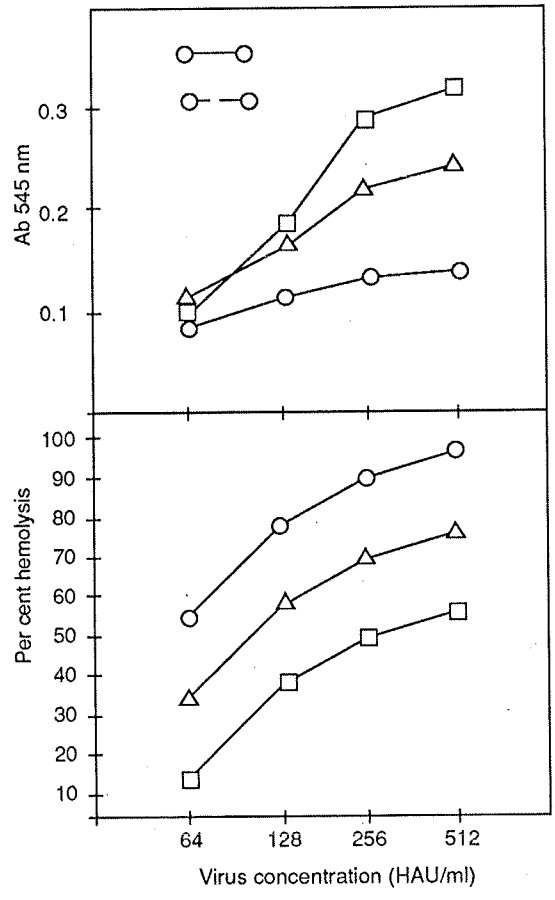


FIGURE 3 – Fusion activity of influenza virus towards chicken erythrocytes after preincubation of virus at low pH. Fusion was measured after preincubation of the virus at pH 5.2 at 37°C (●—●) and 4°C (○—○) in the absence of erythrocytes. Preincubation of the virus in physiological saline at 37°C (●– –●) and 4°C (○– –○) as assay control.



In order to examine the stability of viral fusion activity toward chicken erythrocytes at acidic pH, hemolysis was measured after preincubation of the virus at 37°C and 4°C in the absence of erythrocytes. Figure 3 shows that preincubation of virus at 37°C caused a rapid inactivation on viral fusion activity. In this system after 5 min of incubation the rate of Hb released dropped to 90% of its original value. On the other hand, after 30 min of virus preincubation at 4°C, only a minor inactivation of the fusion activity could be observed.

Hemolysis at different virus and erythrocyte concentrations – Figure 4 shows the relationship between virus concentration and the extent of hemolysis when different erythrocyte concentrations (5%, 10% and 20%)were used. It could be noted when virus concentration was increased gradually while erythrocyte concentration was kept constant, a raise of Hb released as also the percentage of cells lysed occurred. However, increasing erythrocyte concentration while virus concentration was kept constant, a raise in the quantity of Hb released was observed but it resulted in lysis of a lower percentage of erythrocytes.

DISCUSSION

It has been known that virus purification is a process necessary for any basic study of virus characteristics. The sedimentation technique was chosen because this proceeding causes leaks in the virus envelope structure (Fig. 1). Hemolysis can only be observed when virus particles which envelope are disrupted fuses with erythrocytes membranes (4).

The propagation of influenza virus in different types of cell: embryonated chicken eggs or mammalian cell culture, frequently leads to the isolation of viruses possessing antigenically and structurally distinct hemagglutinin (HA) molecules (6, 10), which may differ from each other only by one or two amino acids. Nevertheless, single amino acid substitution in the HA molecule not only may affect the virus antigenicity, but also, its receptor-binding specificity or affinity(11).

For these reasons, we decided to investigate the behavior of this influenza virus sample in binding to different cells receptors, so erythrocytes from several animals were used in the hemagglutination test. Table 1 shows that the virus preparation had different ability of binding to the eryth-

rocytes. It could be observed that using erythrocytes from different chickens the HA titers were also different. These results suggest that some erythrocytes have more quantity of receptors with affinity to the virus particles in the preparation. It was demonstrated that the influenza virus used in this work binds preferentially to cells containing N-acetylneuraminic acid into the α 2.3 galactose linkage (data not shown). Any alteration in the structure of the HA molecule which enhances the binding properties of a virus in a particular host cell, may result in the expansion of the virus subpopulation making its detection possible(6).

Enveloped animal viruses possess membrane fusion activity which they use to deliver their genomes into host cell for replication (18). The hemolysis assay was used to understand the fusion process of an avian influenza virus with chicken erythrocytes.

Embryonated chicken eggs were chosen for virus propagation because a pos-translational cleavage on the HA precursor, HAO, into the active form of the molecule, HA1 and HA2, is necessary for virus/cell fusion occurs. The protease recquired for this activation is available in the allantoic membrane of the chick(3).

Subsequently to proteolytic cleavage, a new N-terminus is generated on the HA2 subunit and this segment contains an unusually hydrophobic stretch of amino acid residues(9). It has been demonstrated that at low pH in which influenza virus hemolytic activity were detected (Fig. 2), a drastic, irreversible conformational change in the HA molecule occurs. It results in exposure of the hydrophobic sequence such that the apolar segment of HA would become acessible to interact with the target membrane. This condition is required for fusion to occur(1). Figure 2 also shows a slight relative decrease on the rate of Hb at pH 5.2 when higher concentrations of virus were used (512 hemagglutinating units/ml). It was probably due to inactivation of virus(5, 14).

The existence of a correlation between the pH dependence of viral fusion and inactivation of fusion capacity could be observed after preincubation of the virus alone at 37°C (Fig. 3). This correlation suggests that both fusion in the presence of target membranes and inactivation in their absence are initiated by the conformational change in viral HA. It has been suggested that low pH induces mutual interactions between hydrophobic segments of adjacent HA molecules, leading to a rapid clustering of the protein. Clustering would be energetically favourable as it reduces the area of hydrophobicity moiety exposed to the aqueous medium. As a result of clustering, viral fusion activity can no longer be detected(15).

It has also been observed that for optimal fusion activity it is essencial that the HA molecules are mobile in the plane of viral membrane(5). This mobility of HA is strongly temperature dependent as also the inactivation of the fusion capacity of virus (Fig. 3). So the hemolytic activity was not inhibited so rapidly after preincubation of the virus alone at 4°C, probably because the mobility of HA molecules is restricted at low temperatures (15).

The study of the hemolysis assay shows that the extent to which virus and erythrocyte concentrations affect the hemolysis reaction depends on the way in which the HL is measured. If measured in terms of the hemoglobin released, the extent of HL may be determined by the absolute concentrations of both virus and erythrocytes. It could be demonstrated that raising erythrocyte concentration while virus concentration was kept constant, there was an increase on the quantity of Hb released, however, it resulted in lysis of a lower percentage of erythrocytes (Fig. 4). This difference is probably the result of lysis from a large number, but smaller percentage of erythrocytes.It should be emphasize that the virus preparation used had been propagated in chicken embrionated eggs, and the efficiency of hemolysis depends on the relation between cleaved/uncleaved particles as also the previous interaction of the virus with cell surface receptors.

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RESUMO

Influência do pH na atividade hemolítica de uma amostra aviária de vírus influenza A.

A atividade de fusão, dependente de pH, de uma amostra aviária de Vírus Influenza A com eritrócitos de galinhas, foi investigada usando o

teste de hemólise (HL). Este teste é baseado na quantificação da hemoglobina(Hb) liberada como conseqüência da fusão vírus/eritrócito. A atividade hemolítica desta preparação viral pode ser demonstrada na faixa de pH ácido (5.0-5.8), o que está relacionado à ocorrência de uma mudança conformacional nas moléculas da hemaglutinina (HA) sob estas condições. A atividade fusogênica era rapidamente inativada após incubação do vírus, em pH ácido, a 37°C, na ausência de eritrócitos, sugerindo que a mudança conformacional da HA do vírus pode ser responsável pela ativação e/ou inativação da capacidade fusogênica. O estudo da reação de HL mostra que elevando-se a concentração de eritrócitos, enquanto a concentração de vírus permanece constante, pode-se observar um aumento na liberação de hemoglobina (Hb), o qual sugere que, provavelmente, os vírus tiveram a chance de encontrar um maior número de células susceptíveis. Uma vez que a Hb em solução muda de tonalidade em função do pH em que se encontra, um fator de correção foi utilizado após o ensaio colorimétrico.

Palavras-chave: vírus influência A, atividade hemolítica, infuencia do pH.

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BEHAVIOR AND CHARACTERISTICS OF A WILD STRAIN OF *METARHIZIUM ANISOPLIAE*.

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Elza Áurea de Luna Alves-Lima
Wilma Terezinha Gadelha de Assunção
Débora Maria Massa Lima

SUMMARY

The entomopatogenic *Metarhizium anisopliae* (Metsch.) Sorokin strain PL_{43} was grown on complete medium (CM) and potato dextrose agar (PDA) in order to study its vegetative growth and analyse the reproductive structures. Hyphae multinucleate were observed on CM and binucleate on PDA. Generally uninucleate conidia without significant differences in size and shape were observed on the media.

Key words: *Metarhizium anisopliae*, cytological characteristics, entomopatogenic fungus, cytological characteristics.

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin is currently being used to control spittlebug insect pests such as *Mahanarva posticata* in sugar-cane plantations in Northeastern and *Deois flavopicta* in pasture grasses in Central Brazil (4, 5, 15). The strain from this and other regions reveal a large genetic variability, that makes difficult the interpretation of the ecological response, when the target-insect. It is known that the microscopic and macroscopic characteristics fo fungal structures, precisely at the phase of reproduction have been a good parameter for identifying differences among genera and species. However the same is not valid for characterization at strain's level (3).

The strain PL_{43} is used to control sugar-cane leaf spittlebug in Northeastern Brazil. Biochemical research has been done on this strain to identify its characteristics (15, 16), but no research has been done from the cytological point of view.

The present work aims to study the cytological characteristics and the behavior of this strain in two different culture media.

MATERIALS AND METHODS

Fungal strain: ***Metarhizium anisopliae*** PL_{43} was obtained from the culture collection of the Sector of Entomology IAA, PLANALSUCAR at Carpina, Pernambuco, Brazil.

Culture media: Complete medium (CM) (2) and Potato dextrose agar (PDA) (6).

Dialysis membrane preparation (6, 13): suspensions of conidia ($1,6 \times 10^7$) were put on both media (CM and PDA) in a Petri dish and spread with the help of a Drigalsky handle, 5 pieces of sterilized di-

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alysis membrane were laid on the culture.

HCl-Giemsa staining for vegetative structures (7, 8): previously sterilized membrane dialysis were transferred aseptically to an albumine solution (5%) during 60 minutes. Then the membranes were put on the medium (CM and PDA) where 0.1 ml of conidia suspension was spread previously with the help of Drigalsky handle. The Giemsa staining was done after 72 hours of growth.

HCl-Giemsa staining for reproductive structures: albumine film (5%) was laid on the glass and was left to dry. The side containing albumine was pressed against the fungal culture. The Giemsa staining was prepared according to Paes de Barros (12).

Growth in culture media: the fungus was inoculated on the center of Petri dish containing CM and PDA and incubated at room temperature. Four repeated measurements of growth were taken with a milimeter scale during the period of 5-10, 10-15, 15-20, 20-25 days.

Photographs were taken using a Zeiss Photomicroscope and Kodak Kodacolor Gold 100-film.

RESULTS AND DISCUSSION

Under laboratory conditions and at room temperature better growth of *M. anisopliae* strain PL_{43} was obtained on CM media (Fig. 1).

The results of this study are similar to the ones reported by Alves (1), Luna (6), Matos (9) and Sosa Gómez (15). They worked in controlled temperature and fotoperiod, whereas the present work was done under laboratory conditions at room temperature. These results demonstrate the great capacity of adaptation of that strain and justify its preference in laboratory studies aiming the biological control of insects pests.

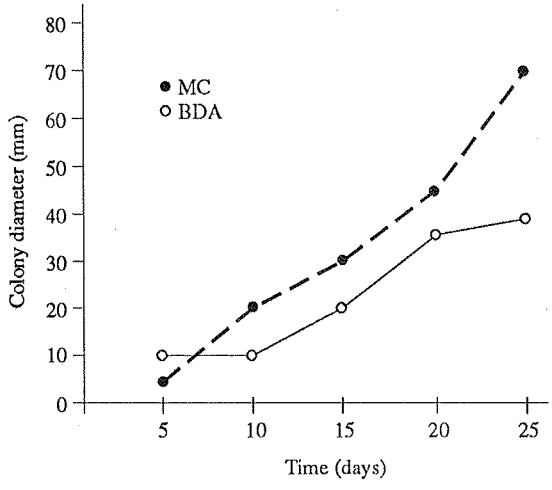

Figure 1 – Comparison of colony diameters of *metarhizium anisopliae* on potato dextrose agar (PDA) and complete medium (CM).

There were morphological differences among the colonies developed either on CM or PDA media. However it was observed differences on conidial dispersion. On PDA groups of conidia were easily released from the conidiophores, while on CM the mass of conidia were strongly fixed on the conidiophores making it difficult the liberation of the spores.

The hyphae showed variation in nuclear condition. On CM, uninucleate and binucleate segments predominated in relation to the trinucleate ones (Fig. 2). This characteristic is relevant because the hyphae of *M. anisopliae* were considered to be uninucleate (18).

Anastomoses (Fig. 3) were observed with little frequency in the both media. The same did not occur in other strains of *M. anisopliae*, in which the presence fo anastomoses was abundant (6). The establishment of anastomoses is an important factor for this fungus since it does not apparently produce structures of sexual reproduction. In Deuteromycotina the formation of anastomoses help directly the establishment of heterokaryon, proportioning genetic variability (11, 19, 20).

The conidiophores observed on CM and PDA did not show variation in shape and the phialides were always uninucleate confirming the observations of Luna (6) and Subramanian (17) in *M. anisopliae* var. *anisopliae*. The following measurements were observed: on CM 11,06 x 3,29 µm. On

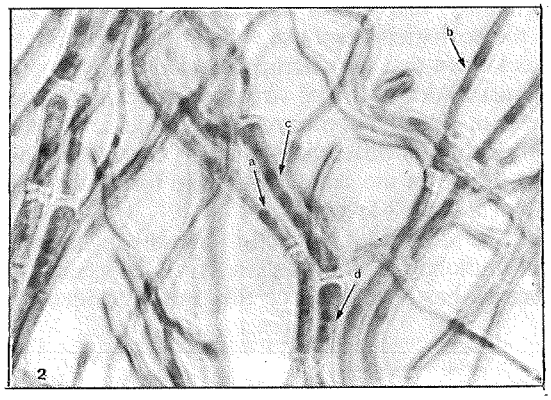

Figure 2 - Vegetative hyphae of segments uni-, bi - and trinucleate (a, b, c) 72h growth on CM. HCL-Giemsa staining. x840.

PDA 10,40 x 3,12μm. These data media this author determined for this strain the following measurements 7,41 x 2,36μm. However, both data are in the range determined by Tulloch (21) for *M. anisopliae* var. *anisopliae*. This divergence may be due to a physiological response of the revival of this strain.

The shape of the conidia remained unchanged in both media. Most of the conidia were short and cylindrical and uninucleated. However there were some long, curved and septate conidia (Fig. 4, 5, 6), such as the ones found by Luna (6) and Luna and Azevedo (8) in a strain isolated from *Mahanarva posticata* Stal of the Northeastern of Brazil. However this strain has presented a high percentage of multinucleate conidia. These data reinforce the necessity of cytological studies for the characterization of the taxon at the strain level.

Uninucleate condition has persisted as a characteristic of the strain PL_{43}, but it was verified on PDA the existence of 0.25% of bionucleate conidia. These data agree with those obtained by Messias and Azevedo (10) and Silveira (14).

Uninucleate condition of this strain is a desirable characteristic for genetic strains. The low rate of binucleate conidia helps to obtain mutants when mutagenic agents are applied to induce genetic variability.

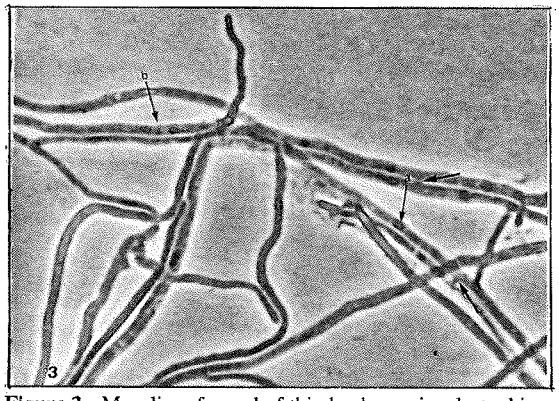

Figure 3 - Mycelium formed of thin hyphae uninucleate, binucleate (a, b) and anastomoses (arrow) 72h growth on PDA with dialysis membrana. x840.

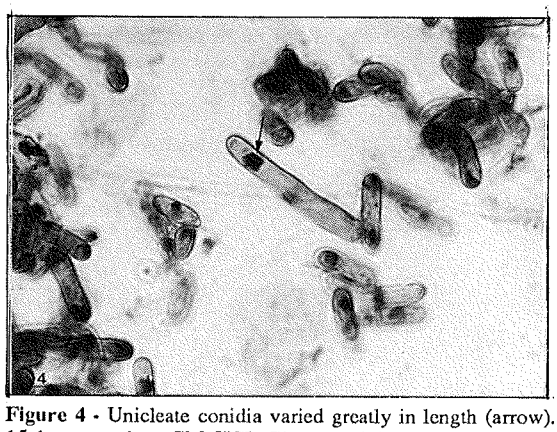

Figure 4 - Unicleate conidia varied greatly in length (arrow). 15 days growth on CM. HCL-Giemsa staining. x840.

RESUMO

Características de crescimento e morfologia de uma linhagem selvagem de *Metarhizium anisopliae*.

A linhagem PL_{43} do fungo entomotogênico *Metarhizium anisopliae* (Metsch.) Sorokin foi crescida em meio completo (MC) e batata-dextrose-ágar (BDA), para estudo do comportamento e da

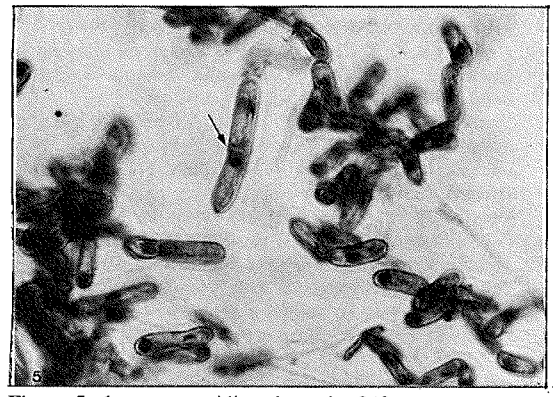

Figure 5 - 1-septate conidium (arrow). x840.

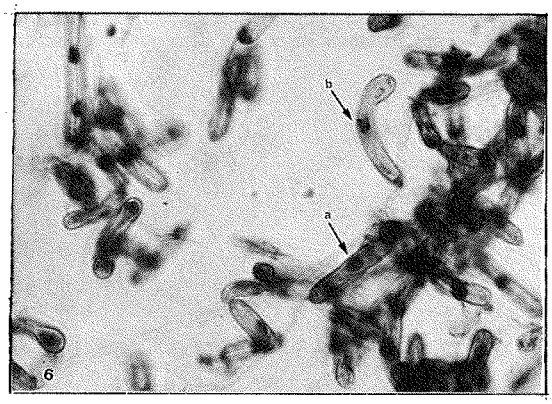

Figure 6 - Uninucleate conidia showing cylindrical (a) and long curved (b) forms. x840.

análise das estruturas vegetativas e reprodutivas. As hifas apresentaram-se multinucleadas em MC mas, freqüentemente binucleadas em BDA. Os conídios eram geralmente uninucleados. Não foram evidenciadas diferenças relevantes na forma e tamanho dos conídios nos dois meios estudados.

Palavras-chave: *Metarhizium anisopliae*, fungos entomopatogênicos, características citológicas.

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ISOLATION OF AUXOTROPHIC MUTANTS OF *Candida tsukubaensis*.

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SUMMARY

Auxotrophic mutants of *Candida tsukubaensis* were obtained from U. V. mutagenized cultures. Two methods were used to recover them, such us, nistatin enrichement and total isolation. The enrichement method was shown to be four times better than the total isolation method. With both methods used, the frequency of the mutants with requirement for adenine was much higher than that obtained for the other markers. The results indicate that the strain in study is an ade+/ade- heterozygote and, under the action of UV light, segregate after mitotic crossing-over, giving origin to auxotrophic and prototrophic colonies.

Key words: *Candida tsukubaensis*, auxotrophic mutants, heterozygous.

INTRODUCTION

One of the first steps in the elaboration of parasexual crosses is the isolation of auxotrophic and/or morphological mutants that may complement each other in the heterokaryon. In this respect, Sarachek (12) was the first to describe the isolation and characterization of simple and multiple auxotrophic mutants of *Candida albicans*. Since then, several auxotrophic mutants have been obtained for some yeast species belonging to the genus *Candida* using different mutagens such as ethyl methanesulfonate, nitrous acid, nitrosoguanidine and UV light (1, 14, 13, 9, 10, 3, 8, 2).

This strain has biotechnological potential because it produces an enzyme complex denoted Renital which is of interest for milk coagulation in the manufacturing of cheese (5).

The objective of the present investigation was to obtain auxotrophic mutants of *Candida tsukubaensis* to be latter used in parasexual crosses.

MATERIAL AND METHODS

Biological material - The strain used in the present study was isolated from soil by the Industrial Fermentation section of "Instituto de Tecnologia de Alimentos", Campinas, SP, and identified accord to Kreger-van Rij (4) by Micological Institute of "Universidade Federal de Pernambuco", Recife, PE.

Culture media - The complete medium (CM) used was YEPD described by Mortimer and Hawthorne (6) and minimal (MM) was that described by Reaume and Tatum (11), with some modifications. When necessary, MM was supplemented with vitamins (1-5mg/ml), amino acids (50-100mg/ml) and nucleic acids (50mg/ml).

UV light survival curve - A cell suspension containing approximately 10^6 cells/ml was prepared in saline solution (0.85% NaCl, w/v). A 1.0 ml aliquot of this solution was removed and used as control and the rest was exposed to UV light

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(Mineralight 115 volts, Ultra-Violet Prod. Inc., San Gabriel, CA, USA) for 30, 60, 120, 180 and 240 seconds. 1 ml aliquot was removed after each period of irradiation. These aliquots, as well as the control, were diluted in saline and 0.1 ml of each dilution was plated onto CM. After 48 hours of incubation at 30°C, colony counts were performed. Percent survival was estimated in relation to the number of colonies obtained for the control treatment, which was taken to represent 100% survival.

Ethyl methanesulfonate (EMS) survival curve - A cell suspension containing approximately 10^6 cells/ml was prepared in 3 ml saline and then divided into two portions of 2 and 1 ml, respectively. After the addition of 4 ml 2% EMS, the 2 ml portion was incubated at 30°C for 1, 3, 4 and 5 minutes and 1 ml aliquots were removed after each treatment and diluted in saline. The other (1 ml) cell suspension portion was used to prepare a control solution by adding 2 ml sterilized water instead of EMS. The treated suspension and the control suspension were then plate onto CM. Percent survival was estimated by the same methodology described above (see UV light survival curve).

Reversion test - A cell suspension containing 10^6 cell/ml saline was prepared for each auxotrophic mutant to be characterized. One ml of this solution was plated onto MM. After 5 days of incubation at 30°C, colonies were counted and the frequency of following equation: mean no. of colonies in MM/mean no. of colonies in CM.

Derivation and characterization of auxotrophic mutants;

a) *Total isolation* - A cell suspension containing approximately 10^6/ml saline was submitted to mutagenic treatment with UV light or EMS at a dose permitting approximately 5% survival. After the mutagenic treatments, 0.1 ml of appropriate dilutions were plated onto CM. Plates were incubated at 30°C for 48 hours and the colonies obtained were transferred to 26-point MM plates with the aid of sterilized toothpicks. Plates were incubated for 48 hours and the agar blocks of the points where growth was not observed were again transferred to CM plates. The colonies unable to grow in MM were latter characterized for auxotrophy.

b) *Enrichment by nistatin treatment* - A suspension of 10^7 cells/ml saline was prepared and irradiated with UV light for a period of time permitting 5% survival. One ml of the irradiated suspension was then inoculated into flasks containing 9 ml nitrogen-free liquid MM which were incubated at 30°C and centrifuged at 150 rpm. After 18 hours, 0.1 ml nistatin solution (20mg/10ml 70% alcohol) was added to the culture and incubation was continued for an additional 60 minutes. 0.1 ml of appropriate dilutions of these culture were plated onto CM. Plates were incubated at 30°C for 48 hours and replications were performed on MM to confirm the derivation of mutants. The auxotrophic mutants obtained were characterized by the auxonography technique (7).

RESULTS AND DISCUSSION

The UV light survival curve for *Candida tsukubaensis* cell is presented in Fig. 1. The time of UV light treatment permitting 5% survival corresponded to 210 seconds and was used to obtain mutants.

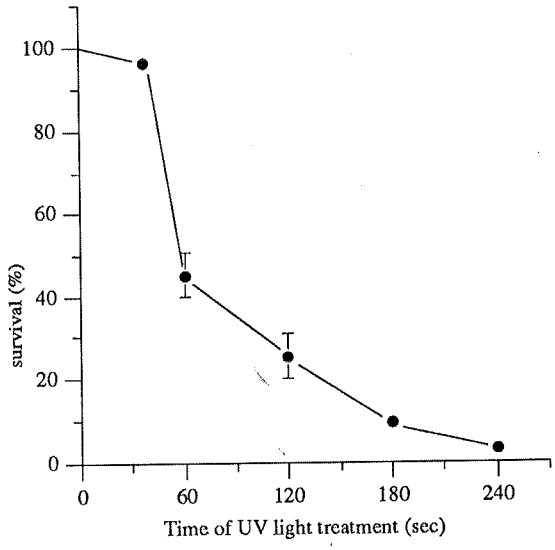

FIGURE 1 – UV light survival curve for *Candida tsukubaensis*.

The frequency of auxotrophic mutant derivation by total isolation and nistatin enrichment is presented in Table 1. The enrichment method yielded auxotrophic mutants at a four-fold higher frequency. It is interesting to note that, with both methods used, the frequncy of mutants with requeriment for adenine was much higher than that obtained for the other markers. Of the 26 auxotrophic mutants obtained, 14 presented requirement for adenine.

In an attempt to clarify the results referring to the high frequency of adenine-deficient mutants obtained with UV light, a chemical mutagen was used to induce auxotrophic mutations in *Candida tsukubaensis*. Thus a curve was constructed for survival of EMS treatment in order to determine the treatment that permits approximately 5% cell survival (Fig. 2).

Treatment of *Candida* cells with EMS for a period of 5 minutes permitted approximately 5% survival and therefore was utilized for the induction of auxotrophic mutants. A total of 1206 colonies treated with EMS were analyzed for auxotrophy and no auxotrophic mutant was detected.

TABLE 1 - Frequency of auxotrophic *Candida tsukubaensis* mutants obtained by total isolation and enrichment with nistatin

Method	Nº of colonies analyzed	% Auxotrophic mutants	Nutritional requirement	Nº of mutants/ nº of colonies analyzed (%)
Total Isolation	2506	0.68	arginine	0.12
			adenine	0.28
			uracil	0.04
			nicotinamide	0.04
			p-aminobenzoic acid	0.04
			methionine	0.08
			biotin	0.04
			pyridoxine	0.04
Nistatin enrichment	348	2.59	arginine	0.29
			adenine	2.01
			methionine	0.29

These results indicate that the high frequency of adenine-deficient mutants obtained was related to UV light treatment.

Similarly, some *Candida albicans* strains produced a large number of auxotrophic mutants (0.2 to 1.7%) after treatment with UV light, all of them of the same type (14, 15). This is due to the fact that these strains are diploid and heterozygous for a small number of recessive auxotrophic genes

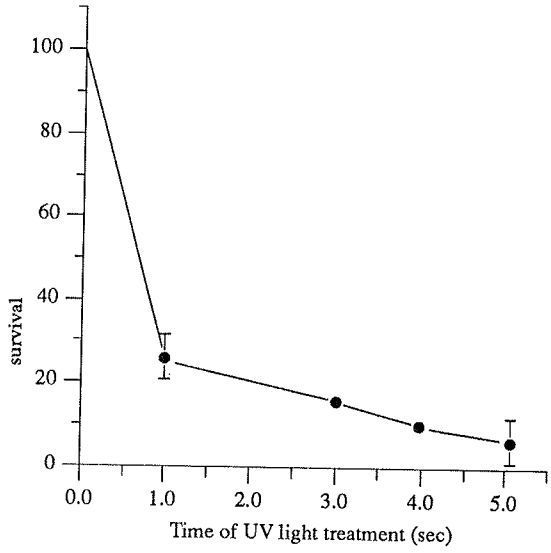

FIGURE 2 – EMS survival curve for *Candida tsukubaensis*.

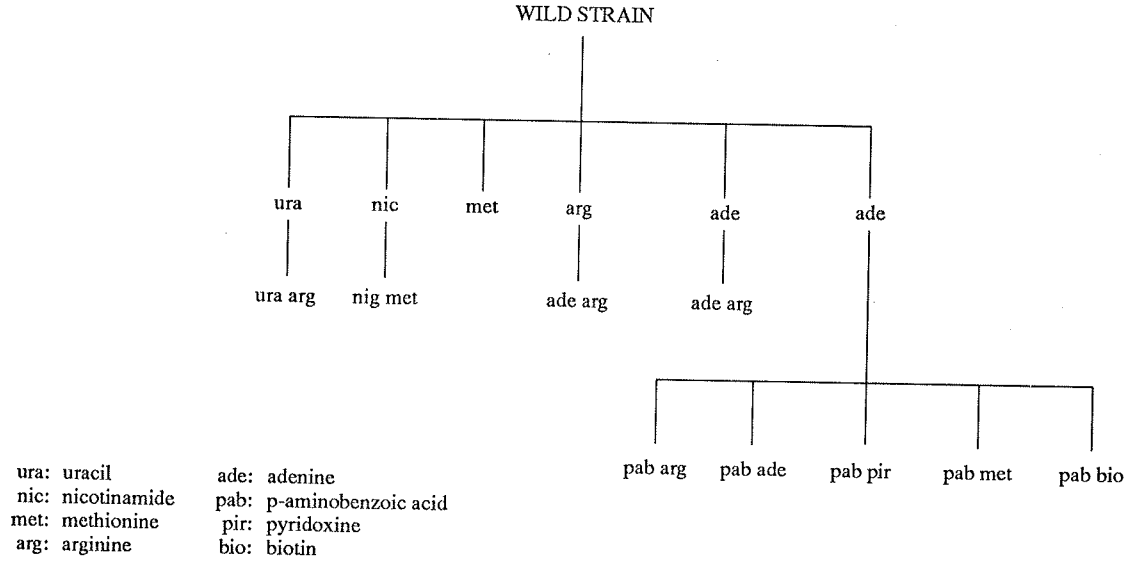

FIGURE 3 – Genealogy of the auxotrophic mutants obtained

which, under the action of UV light, an agent know to induce mitotic crossing-over, segregate and give origin to auxotrophic and prototrophic colonies.

Since the level of ploidy of the strains studied here is unknown, the same hypothesis as that advanced for *C. albicans* may explain the production of ade- mutants at high frequency. The *Candida tsukubaensis* strain may be ade$^+$/ade$^-$ heterozygote and, under the action of UV light, the mutant and wild alleles may segregate after mitotic crossing-over, giving origin to auxotrophic (ade$^-$/ade$^-$) and prototrophic (ade$^+$/ade$^+$) colonies. On the basis of this idea, the chromosome bearing the adenine gene may be disomic. However, considering that other auxotrophic markers were obtained at a higher frequency than expected for a homozygous disomic state, we may assume that this strain is monosomic for some chromosomes, and the yeast may therefore be an aneuploid, at least n + 1.

Since EMS does not induce mitotic permutation, it was not possible to obtain mutants auxotrophic for adenine with the use of this mutagen. The fact that no other types of auxotrophic mutants were obtained with EMS may have been due to the small number of colonies analyzed.

Some of the simple auxotrophic mutants were submitted to a new treatment with UV light in order to obtain colonies with double auxotrophy. Figure 3 shows the genealogy of the mutants obtained.

When the auxotrophic markers obtained were evaluated for frequency of reversion to the wild state, all of them (ura, nic, met, arg, pab, pir and bio) except ade, presented a reversion frequency of less than 1×10^{-6}. The auxotrophic mutation for the ade gene reverted at a frequency of 2.44×10^{-6}, thus indicating that the use of this marker in crosses should be avoided.

RESUMO

Isolamento de mutantes auxotróficos de *Candida tsukubaensis*.

Mutantes auxotróficos de *Candida tsukubaensis* foram obtidos após tratamento com luz ultravioleta. Dois métodos foram utilizados para a seleção destes mutantes: o de enriquecimento com nistatina e o de isolamento total. O primeiro método mostrou rendimento cerca de quatro vezes maior em relação ao de isolamento total. Os dois métodos seletivos deram uma freqüência de mutantes deficientes para adenina, bastante superior aquela obtida para outras marcas. os resultados sugeriram que a linhagem estudada é um heterozigoto ade$^+$/ade$^-$ que, sob a ação de luz ultravioleta, segrega após permuta mitótica, dando origem a colônias auxotróficas e prototróficas.

Palavras-chave: *Candida tsukubaensis*, mutantes auxotróficos, heterozigotos.

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EFFECT OF MEDIUM COMPOSITION ON THE ANTIFUNGAL SUSCEPTIBILITY TESTS TO LAPACHOL, β-LAPACHONE, PHTHIOCOL AND ITS SYNTHETIC ANALOGUES

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A.A. Silva[1] & L. Bieber[1]

SUMMARY

The effect of the culture medium composition on the antifungal activity is described. This was followed by using Casitone, Sabouraud, and Synthetic media. The results showed very high antifungal activity in β-Lapachone, Phthiocol and Synthetic analogue. The synthetic medium was selected as a simple, reproducible assay and more antifungal susceptible.

Key words: Lapachol, β-Lapachone, phthiocol, antifungal activity, effect of medium.

INTRODUCTION

Lapachone was obtained from heartwood of *Tabebuia avellanedae*, it is an antimicrobial substance chemically constitued by 2-hydroxy-3 (3-methyl-2-butenyl)-1, 4-naphthoquinone (7). A number of substances were isolated from the same plant, as β-Lapachone (3, 4-dehydro-2, 2 dimethyl-2H-naphtol/1, 2, 5/pyran 5, 10 dione), referred inhibiting microorganisms (7, 8, 9, 10). Phthiocol (2-hydroxy-3 methyl-naphthoquinone 1,4) and its analogues (2-hydroxy-3-alkyl-naphthoquinone-1,4: 2 hydroxy-3-n-butyl naphthoquinone-1,4,2-hydroxy 3-benzyl-naphthoquinone-1,4) were obtained by synthesis and used in the course of this investigation. The naphthoquinones and benzoquinones are described as increasing the production of superoxide anion hydrogen peroxide *in vitro* (2, 4, 12). The present report describes an assay using different culture media on the antifungal activities of Lapachol, β-Lapachone, Phthiocol and its analogues.

MATERIAL AND METHODS

Microorganisms: Table 1 shows the microorganisms used in this investigation belong to a Collection Mycology of Pharmacy Grénoble (CMPG) and Pasteur Institute (PI), resistant to Amphotericin B (polyen antibiotic).

Substances: Lapachol (I) and β-lapachone (II) were extracted from heartwood *Tabebuia avellanedae* according Gonçalves de Lima method (9). Phthiocol (2-hydroxy-3-methyl-naphthoquinone-1,4) (III) and its analogues [2-hydroxy-3-allyl-naphthoquinone-1,4 (IV); 2-hydroxy-3-n-butyl-naphthoquinone-1,4 (V); 2-hydroxy-3-benzyl naphthoquinone-1,4 (VI)], were synthetized by L. Bieber

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TABLE 1. Microorganisms used in this investigation

Other fungi	
Fusarium aqueductum	CMPG 319
Fusarium moniliforme	CMPG 253
Fusarium oxysporum	CMPG 250
Fusarium solani	CMPG 321
Geotricum candidum	CMPG 596
Yeast	
Candida albicans	CMPG 684[1]
Candida parapsilosis	CMPG 680
Candida tropicalis	R2 PI[2]
Cryptococcus neoformans	CMPG 682
Torulopsis glabrata	CMPG 681
Dermatophytes	
Trichophyton floccosum	CMPG 601
Microsporum gypseum	CMPG 448
Trichophyton rubrum	CMPG 597
Trichophyton interdigitatum	CMPG 599
Trichophyton mentagrophytes	CMPG 598

1 CMPG: Collection Mycology Pharmacy Grénoble.
2 PI: Pasteur Institute; R2: Anphotericin B - resistent.

(Personal communication) analogues of Phthiocol. Ketoconazole was used as antifungal control.

Culture media used: In this investigation 3 media were used, as follows: a) Synthetic Medium (5) - Asparagine 1.5g; L-Histidine 10 mg; DL-Methionine 20mg; DL-Tryptophane 20mg; Ammonium Sulphate 3.5g; D-Glucose 10g; Potassio Phosphate 1g; Magnesium Sulfate 0.5g; Sodium Chloride 0.1g; Biotine 10-8 mole/l; Thiamine 10-6 mole/l; Calcium Pantothenate 10-6 mole/l; Pyridoxine 10-6 mole/l; Nicotinic acid 10-6 mole/l; Inositol 10-5 mole/l: Oligoelements solution (Berthelot) 10 drops, Agar 20g and Distilled water 1000 ml. Final pH=5.6; b) Sabouraud/Dextrose Agar (Institute Pasteur Production) containing: Glucose 40g, Mycological peptone 10g and Agar 15g, to 1000ml; c) Casitone complexe medium (5) as: Casitone Difco 90g; Yeast extrat 5g, Trisodium citrate 10g; Disodium phosphate 1g; Monosodium phosphate 1g; D-Glucose 10g; Agar 10g and Distilled water 1000 ml pH=6.6.

Antifungal Activity: The antifungal activity was tested on solid media in Petri dishes (90mm of diameter) containing 25ml of medium according to the standard diffusion disc technique. The standard disc technique was performed by pouring 1ml of calibrated suspensions of the test strain spores (10^6/ml) in Agar (0.6%) on the solid media. Discs (6mm of diameter) impregnated different compounds (64 mg/disc) were prepared and placed on the inoculated plates. Incubation time at 24°C ranged from 48h to 4-5 days depending on the microorganism. Inhibition diameters were measured in mm and each test was repeated 3 times.

RESULTS AND DISCUSSION

Figure 1 shows the activities of Lapachol (I), β-lapachone (II), Phthiocol (III) and its analogues (IV, V, VI) on the growth in synthetic media. High activity was observed in β-lapachone (II), Phthiocol (III), analogues IV and VI. Higher activity will be observed in β-lapachone (II), Phthiocol (III) analogues (IV), specially against *Cryptococcus* neoformans, *Candida tropicalis* (polyen resistant). The results suggest that the synthetic medium is more indicated to antifungal susceptibility. The previous results obtained by Gonçalves de Lima et al. (7), Kurylowicz (10) indicated Lapachol as inactive against *Candida* and *Ctyptococcus genus*, but our results showed activities in *C. tropicalis*, resistent to polyene antibiothic and *C. neoformans* on Casitone, Sabouraud and Synthetic media. Galgiani (6) reports the lack of reproducilitity among different laboratories performing antifungal susceptibility testing the antifungal activities of compounds I to VI, against specifically dermatophytes growth are presented in Figure 2. All substances tested indicated very high activities against dermatophytes except the substances I and V. Similar results were obtained for compounds II, III and IV, specially against *I. interdigitatum*, *M. gypseum*, *T. mentagrophytes*, *T. rubrum*. Although the variability of the results of the 3 media, the use of synthetic medium showed entirely consistent of results. The test using other fungi against the compounds I to VI, indicated activities in II, III, IV, VI and inactive in I and II (Figure 3). All compounds did not inhibit the growth of *F. moniliforme* in Casitone medium. However, the use of Synthetic medium showed higher activity indicating inhibition of the growth in F. aquaeductum, *F. moniliforme*, *F. oxysporum*, *F. solani* and *G. candidum* by II, III and IV compounds. In general, the components of the medium may be antagonize drug activity (1,3,11,13,14,15). Galgiani (6) reports that fluorcytosine can be antagonized by purines and pyrimidines which may be found in Sabouraud or other complex media and certain buffers. The essential difference in the results between naphtoquinone compounds used in this investigation were found to be due the composition of the Sabouraud of Casitone media antagonise the activity was supported by Galgiani (6). The evaluation results

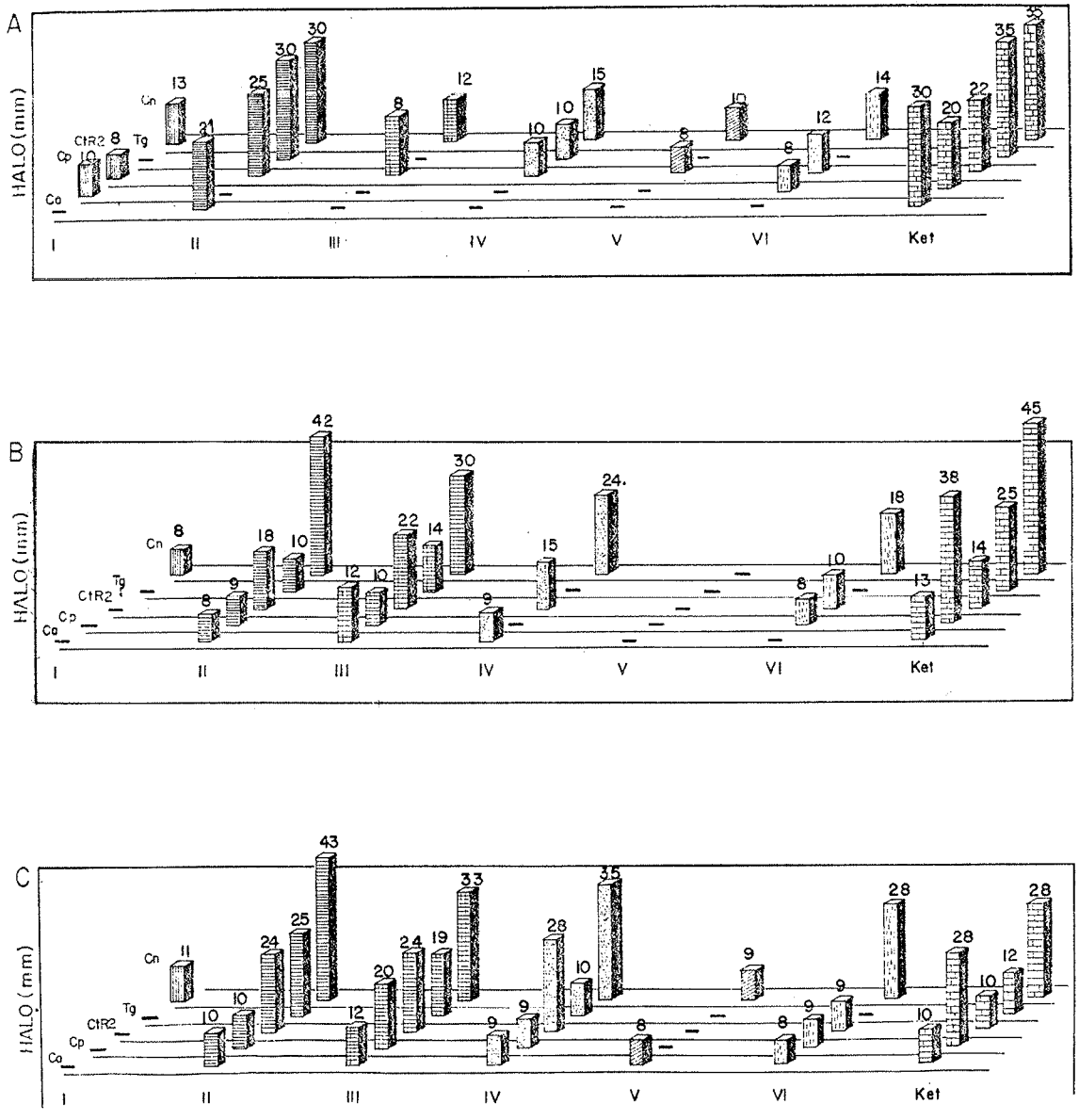

Figure 1 - Antifungal activities of compounds I-VI against yeasts.
Candida albicans (Ca); *C. parapsilosis* (Cp); *C. tropicalis* (Ct); *Torulopsis glabrata* (Tg); *Cryptococcus neoformans* (Cn). A - Casitone, B - Sabouraud, C - Synthetic medium.

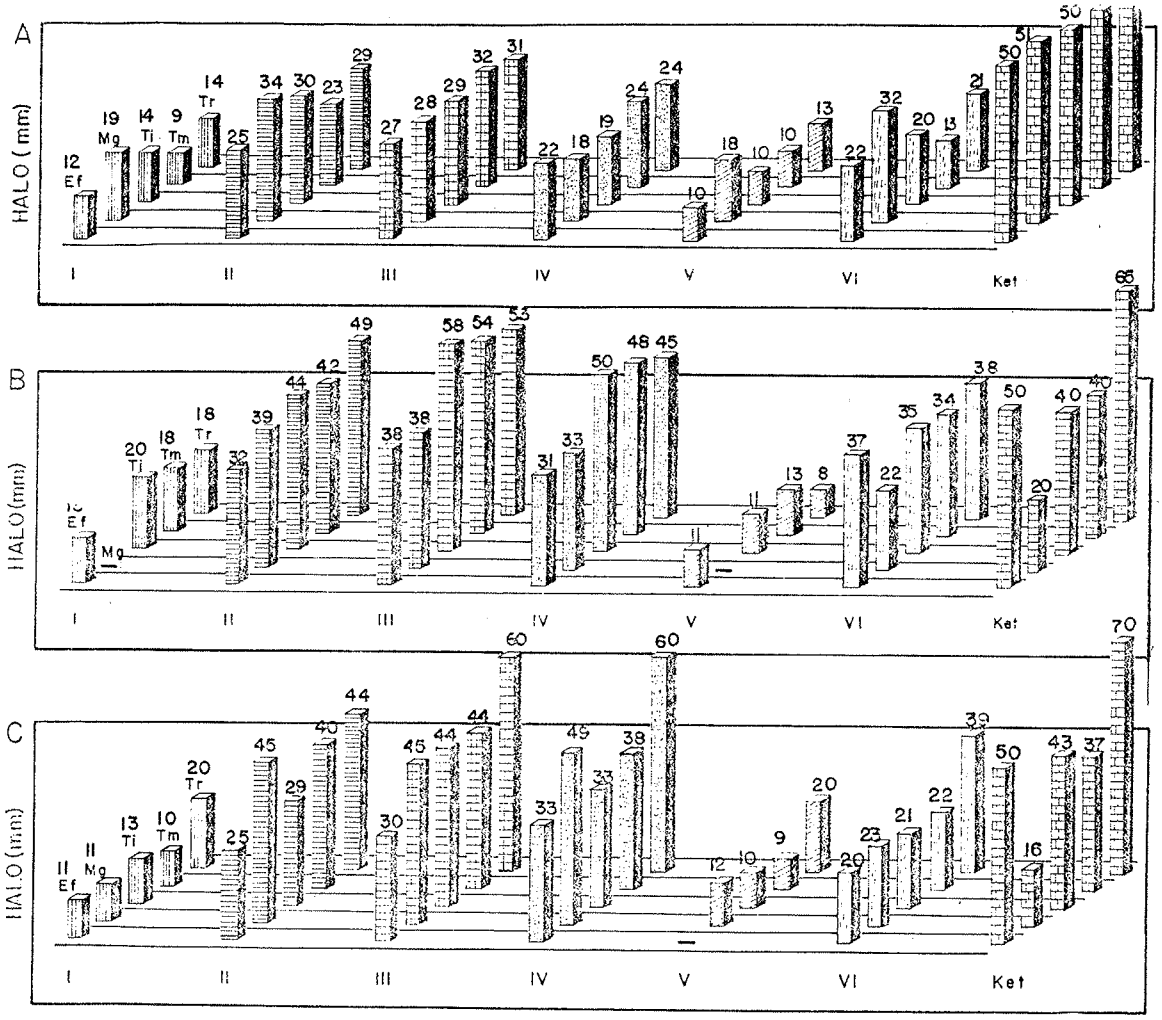

Figure 2 - Antifungal activities of compounds I - VI and Ketoconazole against Dermatophytes. *Epdermophyton floccosum* (Ef); *Microsporum gypseum* (Mg); *Trichophyton interdigitatum* (Ti); *T. mentagrophytes* (Tm); *T. rubrum* (Tr). A - Casitone, b - Sabouraud, C - Synthetic medium.

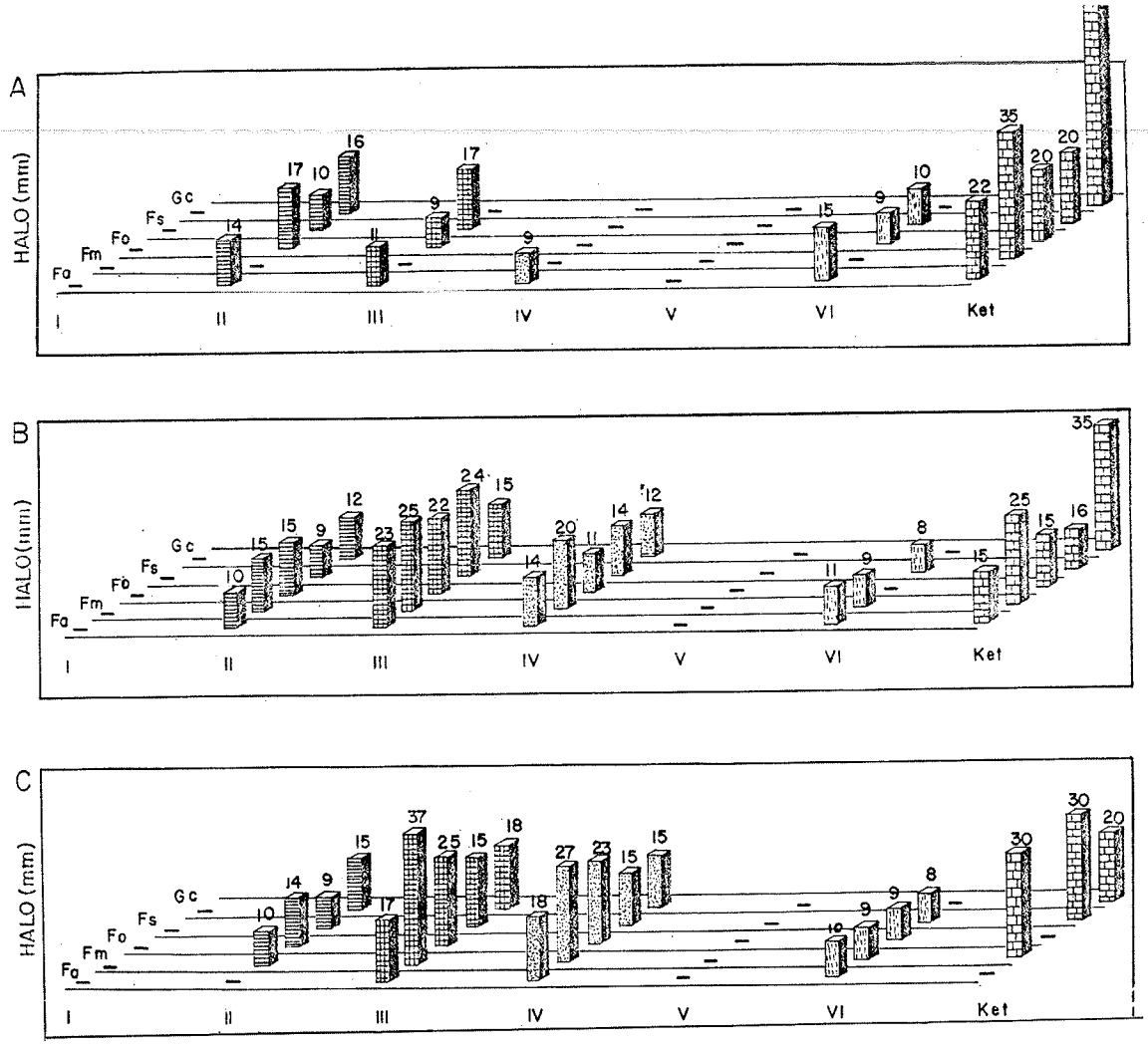

Figure 3 - Antifungal activities of compounds I - VI against Fusarium and Geotrichum. *Fusarium aquaeductum* (Fa); *F. moniliforme* (Fm); *F. oxysporum* (Fo); *F. solani* (Fs); *Geotrichum candidum* (Gc). A - Casitone medium, B - Sabouraud medium, C - Synthetic medium.

of the antifungal susceptibility indicated that Synthetic medium addequated to the tests, and the compounds II (β-lapachone, natural product from *Tabebuia avellanedae*), III and IV (Phthiocol and its analogue) showed the best activity. The lack of agreement with the previous investigation by Gonçalves de Lima (7,8,9) can be possible reflected different medium used, the inoculum sizes and several susceptibility endpoints.

RESUMO

Efeito da composição do meio nos testes de sensibilidade antifúngica para lapachol, β–lapachona, fitiocol e seus análogos sintéticos.

O efeito da composição do meio de cultura na atividade antifúngica é descrito. Este foi seguido usando os meios Casitone, Sabouraud e Sintético. Os resultados mostraram alta atividade antifúngica em β-Lapachona, fitiocol e análogos sintetizados. O meio sintético foi selecionado como simples ensaio, de fácil reprodução e maior sensibilidade para os antifúngicos.

Palavras-chave: Lapachol, β-Lapachona, Fitiocol, atividade antifúngica, efeito do meio.

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FUNGI FLORA OF FROG MEAT (*Rana Catesbeiana*, SHAW-1802) SLAUGHTERED FOR HUMAN CONSUME.

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Lenice Jung[4]

SUMMARY

Ninety-six samples of frog meat were collected from slaughterhouses of brazilian states. the mycological study of the samples reveled 61 mould and 240 yeast strains. The yeasts were identified in 10 different genera distributed into 28 species. The isolation of *Candida albicans* and *Candida tropicalis* should be emphasized because they are potential pathogens and because they are not common on meat and derivatives. The results showed that the fungi flora on frog meat was composed mainly by yeasts belonging to genera *Candida*. The most frequently specie isolated was *Kloechera apiculata*. No statistical difference was observed on the total number of yeasts isolated from samples obtained from different slaughterhouses.

Key words: *Rana catesbeiana*; meat fungi; yeasts.

INTRODUCTION

Rana Catesbeiana, known as Bull-frog is a specie selected for commercial production.

Originated from North America and introduced in Brazil in 1935, it adapted to our climatic conditions, showing fast growing and precocious reproduction. The climatic advantage associated with frog breading modern technology on capacitivity (3, 12) allowed frog raising in Brazil to occupy a space in the agriculture sector because it doesn't have the predatory hunt for sustenance.

The main product of frog business is meat which is easily deteriorated because its composition. Its microbiological quality depends on breeding conditions, the sanitary state of the houses and how it is handled (1).

Studies with the purpose to check the microbiological quality of frog meat have demonstrated the presence of bacteria belonging to genera *Salmonella* (14) and the lack of moulds and yeasts in frog meats raised in laboratory (1).

Frog and other amphibian species have natural susceptibility and are reservoir for pathogenic fungi (10). Fungi of the genus *Basidiobolus* (6) and yeasts (6, 12) have been isolated from the intestinal content of amphibians. Yeasts have often been cultured from several organs of these animals (6, 10). The dematiaceous are the most important group of fungi responsible for diseases in amphibians (11).

This justify the need to evaluate periodically the occurrence of fungi in frog meat used for human consume. The present work had the aim of isolating and identifying fungi in frog meat stored

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in freezing temperature and obtained from slaughterhouses under and without Sanitary Inspection.

MATERIAL AND METHODS

Sample Origin - Ninety-six samples were obtained from 9 slaughterhouses, 10 each (except slaughterhouse A, with 16) from different states of Brazil (Rio de Janeiro, Minas Gerais, Goiás, São Paulo, Rio Grande do Sul and Alagoas). Between the slaughterhouses studied, three were submitted to Federal Inspection and six were not. The samples were taken to the laboratory in the same original conditions, in plastic bags and frozen as they are commercialized.

Isolation and Identification of Fungi - After melting, one thigh of each sample was submitted to manual maceration in 100 ml of sterile saline solution.

From each macerated sample obtained a volume of 5 ml was pre-enriched in 5 ml of Sabouraud-broth with 0,5% of sodium pyruvate and prepared in double concentration.

On days 3th and 14th post-incubation at 28°C, an aliquot of 100 µl was smeared on agar malt extract mixed with 200 mg/l of cloramphenicol and 5g/l of yeast extract and on selective medium for *Candida albicans* (mCA) according to Buck & Bubucis (4). The plates were incubated at 28°C and 37°C, respectively, and they were analysed by the development of colony and by the appearance of brown and black colours in mCA, from days 3th and 14th post-incubation.

The characteristic colonies obtained were purified in Agar Sabouraud mixed with penicilin (40.000 U/l) and streptomycin (40 mg/l).

Each representative type was kept in glucose-yeasts extract-peptone agar (GYP) for further identification.

Conventional methods were used to characterize the yeasts (9).

Statistical Analyse - The statistical description of data was made through the percentage of yeast isolation in each sample and slaughterhouse studied. The statistical analysis consisted in the study of frequence dispersion in a contingence table through a chi-square test (15).

RESULTS AND DISCUSSION

Studies about the occurrence of fungi on meat and derivatives have been made trying to explain, mainly, the processes of deterioration caused by this group of microorganisms when techniques to reduce the bacterial number are used (2, 8).

However, the microorganisms become "damaged" when they submitted to sub-lethal treatment used to preserve the food, resulting in the lost of its multiplication ability (5).

The recognition of damaged microorganisms in important to evaluate and to interprete the microbiological quality of food because the microorganisms used to reveal the hygienic quality of food and the ones producing toxins may escape to detection.

Considering that the fungi on frog meat could be "damaged" by freezing, it was decided to use pre-enrichment of samples.

Two-hundred forty yeast and 61 mould strains distributed into 10 and 3 genera respectively were isolated (Table 1 and 2), using described methodology.

The composition of micoflora of frog meat is similar to that found in other kinds of meat. Thus, *Candida*, *Rhodotorula*, *Trichosporon*, *Cryptococcus*, *Torulopsis* and *Debaryomyces* have been isolated from meat products (2, 8). However, the

Table 1. Percentage distribution of 240 yeasts isolated from 96 samples of frog thigh (*Rana catesbeiana*) slaughtered in Brazil from 1988 to 1990.

Genus	Number	%
Candida	129	53,75
Rhodotorula	27	11,25
Kloechera	27	11,25
Trichosporon	26	10,83
Debaryomyces	15	6,25
Hansenula	5	2,08
Cryptococcus	4	1,66
Kluyveromyces	3	1,25
Geotrichum	2	0,83
Saccharomyces	2	0,83
Total	240	100,00

Table 2. Moulds distribution among 96 samples of frog thigh (*Rana catesbeiana*) slaughtered for human consume in Brazil from 1988 to 1990.

Moulds	Slaughterhouses									Total	
	A	B	C	D	E	F	G	H	I	Number	%
Fusarium	–	–	5	4	–	–	–	3	–	12	19,67
Mucor	11	10	–	7	1	1	–	–	4	34	55,73
Paecilomyces	–	–	–	8	–	–	–	2	5	15	24,59
TOTAL	11	10	5	19	1	1	–	5	9	61	100,00

Table 3. Distribution of yeasts species isolated from 96 samples of frog meat used for human consume in Brazil, from 1988 to 1990, according to the slaughterhouses.

	isolation number/slaughterhouses									Total	
	A	B	C	D	E	F	G	H	I	number	%
Kloechera apiculata	–	3	–	6	8	2	3	5	–	27	11,25
Trichosporon cutaneum	2	8	5	1	4	3	3	–	–	26	10,83
Rhodotorula rubra	1	8	–	4	2	–	8	–	1	24	10,00
Candida rugosa	12	–	–	–	–	–	–	1	10	23	9,50
Candida intermedia	–	–	–	–	4	6	1	10	1	22	9,16
Candida albicans	–	–	10	–	–	–	–	5	–	15	6,25
Debaryomyces hansenii	–	–	–	5	6	1	–	–	3	15	6,25
Candida parapsilosis	–	1	–	5	–	–	6	–	1	13	5,41
Candida guilliermondii	–	–	–	–	–	4	–	3	1	8	3,33
Candida fennica	1	–	1	–	–	4	–	–	2	8	3,33
Candida castelli	–	–	–	–	–	6	–	–	–	6	2,50
Candida glabrata	–	–	–	–	6	–	–	–	–	6	2,50
Candida krusei	1	3	–	–	1	–	–	–	1	6	2,50
Candida tropicalis	2	1	–	–	–	1	–	–	1	5	2,08
Hansenula anomala	–	–	–	2	–	–	–	–	3	5	2,08
Candida catenulata	4	–	–	–	–	–	–	–	–	4	1,66
Candida guilliermondii var. *membranefaciens*	–	–	–	1	–	2	1	–	–	4	1,66
Candida lipolytica	–	–	–	–	–	–	–	3	1	4	1,66
Cryptococcus albidus var. *albidus*	2	1	–	–	–	–	–	1	–	4	1,66
Rhodotorula glutinis	–	1	–	1	1	–	–	–	–	3	1,25
Candida famata	–	–	–	–	–	–	1	1	–	2	0,83
Candida lusitaniae	–	1	–	–	–	–	–	–	1	2	0,83
Geotrichum candidum	–	1	–	–	–	–	–	1	–	2	0,83
Kluyveromyces lodderi	–	–	2	–	–	–	–	–	–	2	0,83
Candida sorboxylosa	–	–	–	1	–	–	–	–	–	1	0,41
Saccharomyces exiguus	–	–	–	–	1	–	–	–	–	1	0,41
Saccharomyces cerevisiae	–	–	–	–	–	–	1	–	–	1	0,41
Kluyveromyces marxianus var. *marxianus*	–	–	–	–	–	–	–	–	1	1	0,41
Total	25	28	18	26	33	29	24	30	27	240	

genera *Koechera*, *Hansenula* and *Kluyveromyces* are not been found on these foodstuffs, representing 11.25%, 2.08%, and 1.25% in the present research, respectively. This suggests that these genera are peculiar to frog meat considering the methodology used.

Table 1 reveals that genus *Candida* represent 53.75% of the total yeasts isolated. Studying the distribution of species, it was seen (Table 3) that *Kloechera apiculata* was isolated more frequently. This result doesn't agree with those obtained by other authors when researching different kind of meat (2, 8). According to Smith (9), *Kloechera apiculata*, is usually isolated from soil, fruit juice and deteriorated strawberry.

Analysing Table 3, one can verify the presence of yeasts involved in human and animal pathologic alterations: *T. cutaneum*, *C. albicans*, *C. parapsilosis*, *C. guilliermondii*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *G. candidum*, *C. rugosa* and *S. cerevisiae* (13).

One emphasizes however, the importance of isolation of *C. albicans* and *C. tropicalis* because they can be of Public Health concern and because up to present time they weren't isolated from meat derivatives.

The results obtained in this work concerning to the isolation of *T. cutaneum*, *C. rugosa*, *C. tropicalis*, *C. krusei*, *C. catenulata*, *C. lusitaniae*, *S. cerevisiae*, *C. parapsilosis*, *C. glabrata*, *C. famata*, *G. candidum*, *R. glutinis* and *C. guilliermondii* agree with those showed by Mok & Morato de Carvalho (10) and Reis et. al. (12) when authors isolated those species in internal organs of amphibians and intestine of clinically health *Rana catesbeiana*. These findings suggest that the presence of yeasts in frog thigh meat may be explained by contamination from intestinal tract or from in-

Table 1. Percentage distribution of 240 yeasts isolated from 96 samples of frog thigh (*Rana catesbeiana*) slaughtered in Brazil from 1988 to 1990.

Genus	Number	%
Candida	129	53,75
Rhodotorula	27	11,25
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Cryptococcus	4	1,66
Kluyveromyces	3	1,25
Geotrichum	2	0,83
Saccharomyces	2	0,83
Total	240	100,00

NI: Not Inspected
I: Inspected

ternal organs during the slaughter operation.

Reis et al. (12) observed the importance of food handling on qualitative determination of yeastlike flora of intestinal tract of *Rana catesbeiana*. Between several foodstuffs used for this animals, each froggery adopted one more appropriated to their conditions and expectations. As the primary source of yeast on frog meat is probably the intestinal tract, a variation of the predominance species between the slaughterhouses, as shown on Table 4, in understandable.

The slaughterhouses C, despite showing the lowest number of isolations (Table 4) showed a high frequence of *C. albicans* (55,5%). As this slaughterhouse is considered a model of reference for frog slaughter, with technique and hygiene of workers strictly observed, the occurrence of *C. albicans* suggests contamination related to water.

C. albicans was isolated from fresh water, chlorinated water and sea water probably contamined with recent faecal material or with other human materials of non faecal origin (7).

The handler can not be discharged as source of contamination, once several yeasts in this work can be associated with colonization of men'skin and nails (13). Therefore, the routine examination of workers doesn't eliminate the possibility of assyntomatic carriers.

On conclusion, the results for this study suggest that the fungi flora of frog meat is composed mainly by yeasts, some showing pathogenic potential to man and animals. One could verify that the difference between the total number of isolated yeasts wasn't statistically significant in slaughterhouse under and without inspection. These results recommend the need for yeast quantitative studies as indicator of microbiological quality of frog meat.

RESUMO

Microbiota fúngica em carnes de rãs (*Rana Catesbeiana*, SHAW-1802) abatidas para o consumo humano.

Estudou-se 96 amostras de carnes de rãs procedentes de abatedouros sob Inspeção Sanitária e sem Inspeção Sanitária de diferentes estados do Brasil, com isolamento de 61 bolores e 240 leveduras. As leveduras foram identificadas em 10 gêneros diferentes, distribuídos em 28 espécies. Ressalta-se a ocorrência de *Candida albicans* e *Candida tropicalis* pelo potencial patogênico e por não serem de ocorrência comum em carnes e derivados. Os resultados indicam que a microbiota fúngica em carnes de rãs é predominantemente composta de leveduras do gênero *Candida* mas, a espécie mais freqüentemente isolada *Kloechera apiculata*. As diferenças entre o número total de leveduras isoladas de acordo com os tipos de abatedouros estudados não foram, estatisticamente, significativas.

Palavras-chave: *Rana Catesbeiana*, fungos em carnes, leveduras.

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FREEZE-DRYING OF INDUSTRIAL YEAST STRAINS: INFLUENCE OF GROWTH CONDITIONS, COOLING RATES AND SUSPENDING MEDIA ON THE VIABILITY OF RECOVERED CELLS

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SUMMARY

Ten yeast strains used for the production of ethanol in Brazil were studied for the purpose of achieving higher post-freeze-drying viability levels. The following three factors were examined: growth conditions, cooling rates, and suspending media. In order to obtain a better evaluation of the factors tested, freezing was dissociated from the drying parts of the freeze-drying process, which are frequently analyzed together. Cell viability was determined prior and after freezing and also after complete freeze-drying.

In the two cryoprotectants tested, agar-grown cells proved more resistant after both fast and slow cooling rates than those grown in shaken cultures. Post-freeze-drying results were better after slow cooling (1°C/min), although the rate of cooling *per se* did not seem to be exclusively responsible for cell death. However, when fast cooling was used for shaken culture cells, decreased viability was more evident only after freeze-drying. That is, freezing in itself did not seem to cause losses but the lowest post-freeze-drying viability values obtained were those corresponding to cells grown in shaken cultures and fast-frozen.

Key words: Freeze-drying, yeast, freezing, lyophilization.

INTRODUCTION

Although extensive study on the freezing and freeze-drying of yeasts has been carried out over the last decade (1, 2, 4), recent reports have emphasized the need to improve certain parameters so as to obtain higher viability levels after preservation of microorganisms by the above-mentioned methods (10, 12). Culture conditions and age, suspending medium, cooling rate, rehydration temperature, storage conditions, and residual moisture are among the parameters to be optimized.

Our prior experience with freeze-drying of yeast strains resulted in low cell survival, and literature on the subject is often controversial. Some authors recommend cultivation in YM broth without aeration (3), while others state that shaker-grown cells are more resistant to freezing and

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freeze-drying than those from static broth cultures. These authors also suggest that agar-grown cells may be even less resistant to damage stemming from freezing and freeze-drying (1, 11).

The aim of this investigation is to select the best procedures for obtaining higher post-freeze-drying cell viability levels by means of analysis of two different growth conditions, two cooling rates, and two suspending media.

MATERIALS AND METHODS

Microorganisms – Ten yeast strains were studied, the following five of which are commercial strains used in Brazilian distilleries for the production of ethanol from sugar cane juice and molasses:

- IZ-1904 AB 008 (*Saccharomyces uvarum* – Instituto Zimotécnico de Piracicaba);
- ZANITA AB 013 (Escola Superior de Agricultura Luiz de Queiroz ESALQ – USP);
- VALOSEP AB 014 (CRPAA – Cooperativa Regional dos Produtores de Açúcar de Alagoas);
- Fermento Fleischmann AB 015 (Produtos Alimentícios Fleischmann e Royal Ltda); and
- Fermento Itaiquara AB 016 (Usina Itaiquara de Açúcar e Álcool S. A.).

The other five strains listed below are from culture collections or laboratories:

- *Saccharomyces cerevisiae* AB 001 (CCY 21.4.36 Czechoslovak Collection of Yeasts);
- *Saccharomyces bayanus* AB 009 (INRA - Institut National de la Recherche Agronomique, France);
- *Saccharomyces sake* AB 010 (INRA – Institut National de la Recherche Agronomique, France);
- *Saccharomyces cerevisiae* AB 011 (UG-5 INSA – Institut National des Sciences Apliquées, France); and
- *Saccharomyces formosensis* AB 012 (NEDO – New Energy Development Organization, Japan).

Culture conditions – Cells were grown to stationary phase for 48h at 30°C under the following conditions: 1) in YM broth shaken at 150 rpm; and 2) on YM (DIFCO 0712-01-08) agar slants.

Suspending media – The suspending media tested were: 1) 10% skimmed milk plus 5% sodium glutamate; and 2) a 3.75% final concentration of glucose in horse serum.

Inoculation of ampoules – The cells grown on agar were carefully removed from the surface of the medium and suspended in each cryoprotectant.

The culture of cells grown in YM broth was mixed with an equal volume of double concentrated suspending medium. The suspensions thus obtained were transferred to sterile glass ampoules (0.1 ml per ampoule) and submitted to two different cooling rates: 1) slow cooling (1°C/min) in a Labconco 75250 shell-freezer; and 2) fast cooling by means of direct immersion in a -50°C cooling bath.

A Labconco 5-75050, 7510, 75180 apparatus was used for overnight freeze-drying. Ampoules were vacuum-sealed.

Viability assessment – Frozen cell suspensions were quickly thawed and diluted into sterile distilled water at room temperature (25°C) to viability determinations. Freeze-dried cells were ressuspended and diluted in sterile distilled water. Cell viability was determined by plating appropriate dilutions in YM agar (8) before and after freezing, after lyophilization and after one year of storage at refrigerator temperature. Colony forming units were enumerated (cfu/ml) after 48 h of incubation at 30°C.

RESULTS AND DISCUSSION

The data given in Figure 1 show viability of yeast cells suspended in skimmed milk plus sodium glutamate after testing under the various conditions. Each result is the average of nine observations. Figure 2 shows results achieved when the yeast cell suspending medium was glucose in horse serum.

Figure 1 shows that cells cultivated on agar produced higher after freezing and after freeze-drying viability levels than those from shaken cultures. The fact that responses to freezing and freeze-drying of agar-grown cells may differ from those obtained with freezing and freeze-drying of shaken cultures may be due to physiological changes during cultivation. It has been mentioned that aeration may be one of the determining factors of yeast cell resistance to freezing and freeze-drying (1). Some authors have also attributed this resistance to membrane composition and fluidity (5).

Comparison of cooling rates showed that slow-cooling was less detrimental to cell viability, and that slow-cooled cells maintained the same viability levels after 2 and 4 hours of freezing (data not shown). Cell damage, especially to cells grown in shaken culture was greater when quick-cooling was used, and was even more pronounced

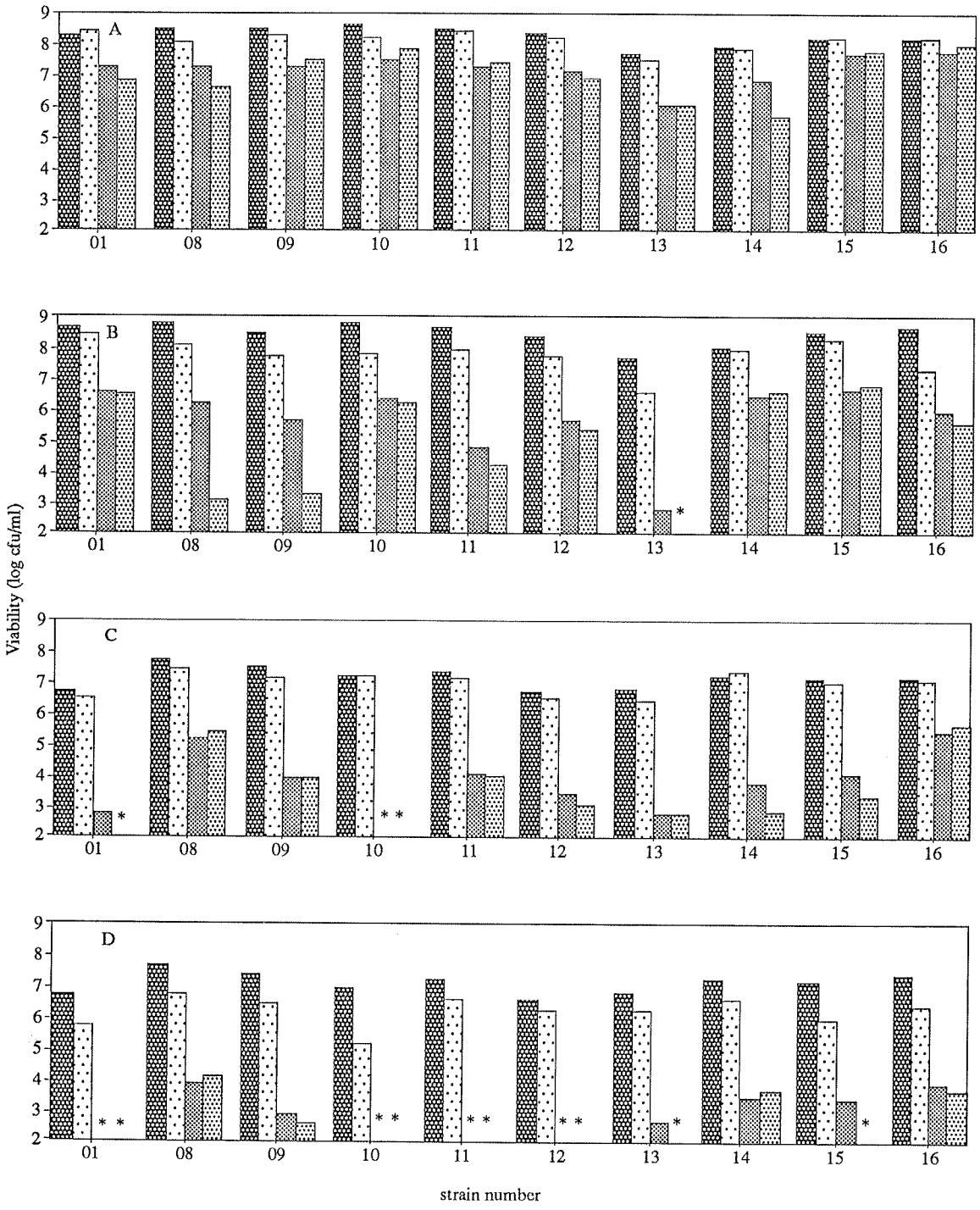

Figure 1. Viability of yeast cells (log cfu/ml) suspended in skimmed milk plus sodium glutamate prior (▦) and after freezing (□), after freeze-drying (▨) and 12 month storage (▤). Agar grown cells submitted to slow (A) and fast (B) cooling. Shaker cultured cells submitted to slow (C) and fast cooling (D).

* Cells not recovered.

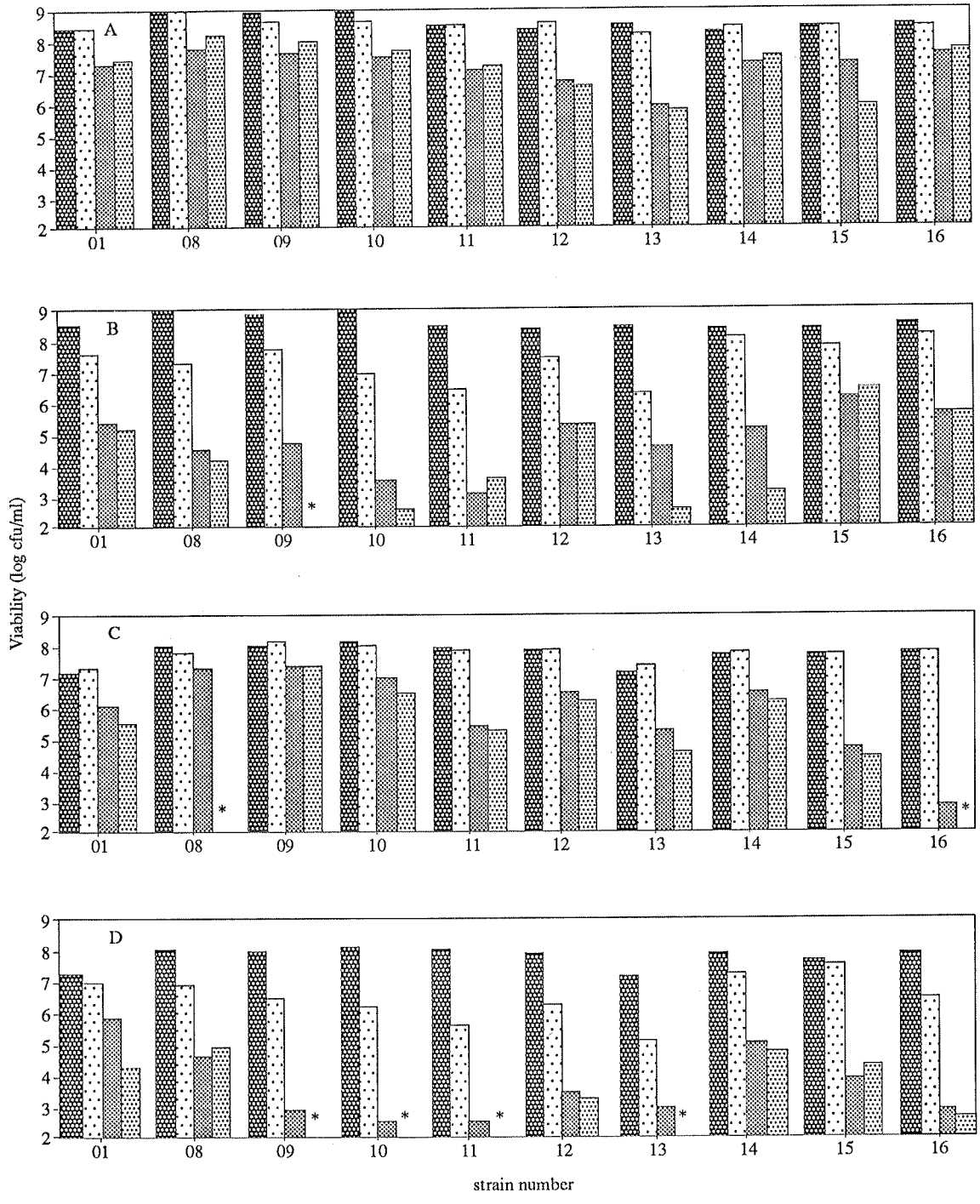

Figure 2. Viability of yeast cells (log cfu/ml) suspended in glucose in horse serum prior (▭) and after freezing (▭), after freeze-drying (▭) and 12 month storage (▭). Agar grown cells submitted to slow (A) and fast (B) cooling rates. Shaken cultures submitted to slow (C) and fast (D) cooling.

* Cells not recovered.

after freeze-drying. These results support the opinion that there is a need for further research on suspending media capable of protecting cells from damage due to freezing and freeze-drying (13).

In relation to cooling rates, results obtained are in agreement with those found in the literature on this subject: slow cooling (from 1 to 10°C/min) is less harmful to *S. cerevisiae* cells, and cell death at over 10°C/min can be attributed to nucleation of intracellular ice (6).

In many laboratories, the standard lyophilization procedure employed consists of first quick-freezing cell suspensions to a temperature of - 60°C and then dry them at -10°C, a procedure that may be harmful to yeast (7).

It has already been shown that quick-freezing produces a significant decrease in the respiratory activity of whole *S. cerevisiae* cells due to migration of cytochrome c + c_1 from mitochondria to cytosol (9).

The present experiment showed that when using skimmed milk plus sodium glutamate, the best results were obtained with slow-cooled agar-grown cells. Slow cooling made it possible to recover high viability levels in all strains tested. The second best results were obtained with quick-frozen agar-grown cells, followed by slow – and quick-cooled shaken –culture cells. These resulted in respective recoveries of 9, 8 and 4 strains after lyophilization and after one year's storage. The highest viability recovery obtained with slow-cooled agar-grown cells suggests this as the choice condition in freeze-drying yeast cells, although strain specificity must always be taken into account.

Figure 2 shows that when glucose in horse serum was used as cryoprotectant, slow cooling produced the best results under both culture conditions. When compared to the former cryoprotectant, there was an improvement in cell recovery for shaken-culture strains; however, agar-grown cells still proved to be the most resistant. In the case of glucose in horse serum, despite non-recovery of certain strains after storage, the second best results were achieved with slowly cooled broth cultures, followed by quick-cooled agar-grown cells, and rapidly cooled broth cultures.

Comparison showed that agar grown cells yielded similar results with both cryoprotectants which leads us to conclude that the choice of cryoprotectant could be based on factors such as cost and ease of handling. Results showed that for cells grown in shaken cultures, glucose in horse serum is a better cryoprotectant than skimmed milk plus sodium glutamate.

The findings of this study make it clear that, given an appropriate combination of cultivation condition, cryoprotectant, and cooling rate, even higher levels of post-freeze-drying cell recovery can be achieved. The dissociation of the freezing from the drying parts of freeze-drying process was shown to be a valuable procedure both to better evaluate the influence of the parameters tested as well to control freezing rate before drying cell suspensions.

RESUMO

Liofilização de leveduras de interesse industrial: influência das condições de cultivo, velocidade de congelamento e meio protetor, na viabilidade celular após a liofilização.

Dez linhagens de leveduras empregadas na produção de etanol no país foram estudadas, visando a obtenção de maiores níves de viabilidade celular após a liofilização.

Foi testada a influência de três parâmetros: condições de cultivo, velocidades de congelamento e meio protetor.

Nos dois protetores testados, células cultivadas em meio sólido mostraram-se mais resistentes aos congelamentos rápido e lento do que aquelas crescidas sob agitação.

A velocidade de congelamento em si parece não ser o único fator de morte celular. Entretanto, os melhores resultados de viabilidade após a liofilização foram obtidos quando o congelamento lento foi previamente empregado.

O emprego do congelamento rápido a células oriundas de culturas agitadas mostrou uma grande perda em viabilidade, evidenciada apenas após a liofilização. Assim sendo, embora esta forma de congelamento não pareça causar perdas, o mesmo, quando associado a células cultivadas em shaker foi responsável pelos mais baixos valores de viabilidade celular.

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ANALYSIS OF THE KINETIC PATTERN OF GLUCOAMYLASE PRODUCTION REGARDING THE ASPERGILLUS AWAMORI PRESERVATION TIME*

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Maria Cândida Reginato Facciotti
Willibaldo Schmidell

SUMMARY

In the industrial production of amyloglucosidase, it is frequent the employ of microorganisms belonging to the genus *Aspergillus*. This paper presents some batch process cultures conducted with *Aspergillus awamori* NRRL 3112, preserved in soil. It was verified, through batch cultures, a stability of the microorganism behavior concerning the growth and the enzyme production capacity after 3 years storage. For higher times of microorganism preservation, there was a little reduction of the final cellular concentration, namely, 10%, as well as the enzyme activity in the broth, corresponding to 36 to 43%.

Key-words: *Aspergillus awamori*, amyloglucosidase, preservation of microorganisms.

INTRODUCTION

The proper preservation of microorganisms of industrial interest has fundamental importance for a fermentation process. The proper preservation means maintenance of viability and reproduction capacity, as well as the product synthesis capacity of the microorganisms for a long time period. However, the various preservation techniques are frequently evaluated taking into account only the microorganisms growth capacity.

Even the researchers who use recent preservation techniques such as ultra low temperature storage of microorganisms, evaluate the viability on a solid medium (6, 9).

It is unusual to find recommendations as Chang & Elander (3) did, for the observation of microorganism viability and stability taking into account the yield for a metabolite of interest, by cultures in a pilot scale, or even, in the industrial reactor.

The works of Park & Santi (10), and Fortnay & Thoma (5) are good examples, although experiences are performed on a bench scale. The former authors observed a gradual reduction of amyloglucosidase synthesis capacity of *A. awamori*, when grown in a solid medium. Fortney & Thoma verified that the increasing of storage time and temperature of lyophilized *Streptomyces griseus*, result decreasing on the production of streptomycin.

In the past, the long time preservation of fungus spores was recommended to be made in soil (7). Backus & Staufer (2) for example, have not referred to any reduction of the penicillin production for different varieties of *P. chrysogenum*, after 10 years of spores storage in soil. However, according to the batch runs now presented, 5 years of *A. awamori* NRRL 3112 storage time in soil,

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leads to a severe reduction of amyloglucosidase synthesis capacity.

MATERIALS AND METHODS

Microorganism – In this work *A. awamori* NRRL 3112, which was preserved in tubes containing earth and sand, was employed.

Beginning with lyophilized microorganism in an ampoule, the preservation in soil was the following: suspension of the lyophilized microorganism in water; inoculation on a modified Czapek medium (11) in Petri dish, and incubation for 2 days at 30°C; after that, the same medium was inoculated in slanted tubes, and incubated for 4 to 5 days. This growth step in tubes was repeated once more. Finally the spores were suspended in water and introduced in tubes containing earth and sand. The microorganism prepared in such manner was stored in the refrigerator.

For each run in fermentation reactor, 100 ml of Czapek medium was inoculated with a little portion of microorganism in earth and sand, followed by an incubation of 6 days at 30°C. The spores were suspended in water and transfered to liquid medium in a flask and kept in a shaker for 24h, (250 r.p.m., 35°C) and finally transfered to the fermenter.

Culture medium – The culture medium for the shaken flasks and the fermenter contained polysaccharide with an initial concentration (S_o) between 20 to 80g TRS/1 (TRS= total reducing sugars, measured as glucose). The initial polysaccharide concentration was adjusted adding an adequate volume of a filtrated syrup prepared from cassava flour (1).

The culture medium has the following nutrients, in g/g of TRS: yeast extract, 0.005; $MgSO_4.7H_2O$, 0.025; $(NH_4)_2\ SO_4$, 0.25; $Na_2HPO_4.12H_2O$, 0.190; KH_2PO_4, 0.175.

Reactor cultures - In this work the results of 5 batch runs were presented. Each run was conducted in a Biolafitte fermenter of 15 l of capacity.

The operation conditions were as the following: reaction volume= 10 l; inoculum fraction (v/v)= 1%; agitation rate= 700 r.p.m.; air supply rate= 10 l/min; head pressure= 0.2 atm; pH, 4.0 or 5.0; temperature= 35°C.

Assay – The cellular concentration (X), was determined by measuring the mass of dry matter in the sample (14); glucose (G) was assayed by glucose-oxidase method adapted to a Technicon Auto-Analyser II (16); total reducing sugars (TRS), were determined by the glucose concentration after enzymatic hydrolysis of the polysaccharide in the sample (13); and amyloglucosidase activity (A), was also determined (15).

One glucoamylase activity (U) is defined as the quantity of enzyme that releases 1 g of glucose in 1 h, in a 4% (W/V) starch solution, at a temperature of 60°C and pH = 4.2.

RESULTS AND DISCUSSION

Table 1 shows the results of 5 batch runs including the S_o values, pH and microorganism preservation time in soil.

The maximum enzymatic activity (A_{max}), and final cellular concentration (X_f) were very close in the case of runs D1 and D2, such as 470 U/l and 8.1 g/l, and 500 U/l and 8.8 g/l respectively. The t_f and t'_f values which were given in Table 1, were also very close for the two runs, indicating that there was no change in the kinetic pattern. Thus, a storage time between 0.6 and 3.4 years doesn't affect the cell metabolism concerning growth and

Table 1: Results obtained in *A. awamori* NRRL 3112 batch cultures.

Runs	Ø (years)	S_o (g/l)	pH	t_f (h)	X_f (g/l)	t'_f (h)	A_{max} (U/l)
D1	0.6	21	4	17	8.1	16	470
D2	3.4	22	4	16	8.8	18	500
D3	5.0	20	4	22	7.6	24	310
D4	1.5	80	5	42	15.2	48	4800
D5	4.8	81	5	40	13.5	44	3650

Ø : storage time of microorganism in earth and sand
t_f : time to exhaust carbon source
X_f : cellular concentration in t_f
t'_f : time to attain maximum enzymatic activity
A_{max} : amyloglucosidase activity in t'_f

enzyme activity. This time interval was used for studies of the pH and S_o influences on the batch cultivations of this specie of *Aspergillus* (4, 12).

For the same culture conditions as runs D1 and D2, the run D3 was realized after 5 years of microorganism storage in soil, and gave 36% less enzyme activity (A_{max}) compared to the average calculated from the runs D1 and D2 (Table 1). The decrease of 10% for the final cellular growth (X_f) can be laid to the experimental fluctuation, meaning therefore no influence on the X_f value. However, the time interval needed to reach to X_f (t_f) was 33% higher than for the average for the runs D1 and D2. To reach A_{max} the time t'_f was 41% higher. These time increases indicate changes on the kinetic pattern of the process which can also be analyzed taking into account the values of specific rate of growth, and specific rate of amyloglucosidase production, defined respectively by:

$$\mu = \frac{1}{X}\frac{dX}{dt}\ (h^{-1}) \text{ and } \mu_A = \frac{1}{X}\frac{dA}{dt}\ (U/g.h)$$

The growth rate (dx/dt), and the amyloglucosidase production rate (U/l.h) were respectively calculated from the X vs t and A vs t plots (8).

Figure 1 shows the μ values of runs D2 and D3, and Figure 2 the μA values of the same runs. These graphs shows clearly the reduction in the growth capacity, as well as the enzyme production capacity of the microorganism stored during 5 years. The specific growth rate for run D2 given in Figure 1, shows a maximum rate phase about 8 h long, but this phase was not observed for the run D3. Figure 2 shows that the μ_A values for the microorganism stored for 5 years were strongly reduced since the beginning of the run, despite of the nutrients excess in the culture medium.

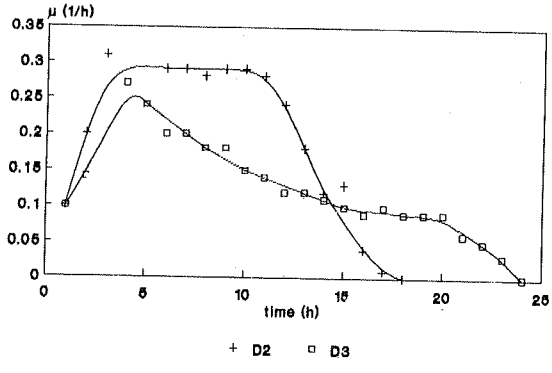

FIGURE 1: Specific growth rate, μ, for runs D2 and D3.

The runs D4 and D5 are analysed together due to the changes in conditions of S_o and pH.

The run D4 carried out with So value of 80 g/l, and microorganism storage time of only 1.5 year, as can be seen in Table 1. The A_{max} of 4,800 U/l for the run D4 was higher than the A_{max} values for runs with S_o value of 20 g/l. This increase is due to higher S_o, but also because of the change in the culture pH from 4 to 5. The results published in a previously work, showed the remarkable effect of pH on the amyloglucosidase synthesis by *A. awamori* NRRL 3112 (12).

The run D5 was realized with the same So and pH values of the run D4, (80 g/l and 5.0 respectively), but microorganism employed had 4.8 years of storage time. As in runs D1 to D3, the reduction on X_f value of only 10% can be imputed to the experimental fluctuation, but the A_{max} value reduction, 24%, was more significant. However, the time values, t_f and t'_f were about the same as before the microorganism attenuation.

For the μ values, Figure 3 shows that they were little affected, and it's possible to admit that the values were almost the same for both runs. Obviously,

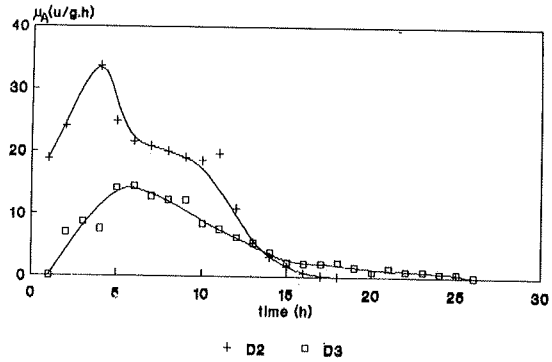

FIGURE 2: Specific amyloglucosidase production rate, μA, for runs D2 and D3.

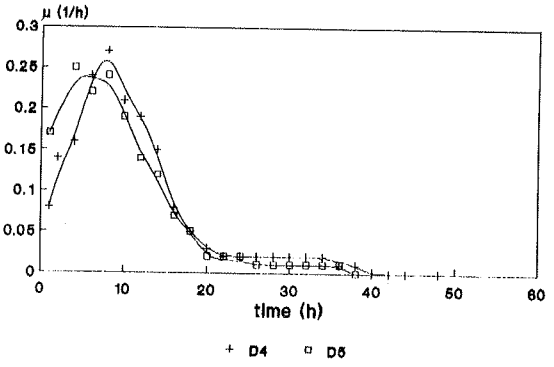

FIGURE 3: Specific growth rate, μ, for runs D4 and D5.

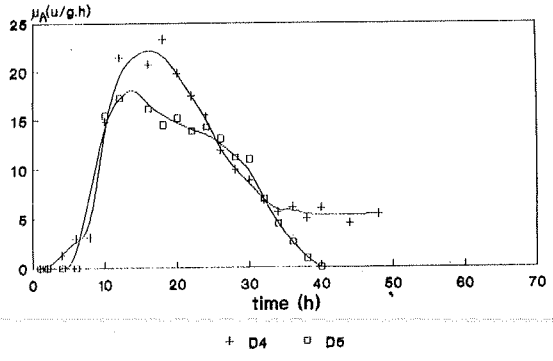

FIGURE 4: Specific amyloglucosidase production rate, μA, for runs D4 and D5.

this type of behavior is a result of no reduction in X_f (11%), and no modification of the time t_f.

The reduced microorganism attenuation effect on μ for S_o of 80 g/l when compared with S_o of 20 g/l, showed in Figures 1 and 3, may be an influence of the dissolved oxygen limitation that occurs with S_o values higher than 20 g/l (4). So, the microorganism growth capacity is strongly reduced in runs D4 and D5. This kind of limitation is often verified in shaker cultivation.

For the enzyme production however, the μ_A values of run D5 were lower than those observed for run D4, as Figure 4 shows. Besides that, the run D5 shows no enzyme production after 40 h of culture, while the microorganism of run D4 continues with positive values of μ_A until 48 h. These higher μ_A values at advanced time culture has strong influence on A_{max} values, taking into account that the X values were high in this culture stage.

It's important to note through these results that taking into account only the X_f values of Table 1, and even the μ values of runs D4 and D5, it's difficult to conclude that the microorganism is attenuated. Nevertheless, the enzyme synthesis capacity was strongly reduced, according to the results obtained.

As mentioned before, microorganism preservation techniques are frequently evaluated on the basis of the viability of the stored microorganism on Petri dishes. For the *A. awamori* NRRL 3112, the growth on the solid media, prior to the shaker incubation, did not show any change in the 4.8 years of storage time. And even the growth in fermenter shows any modification, as run D5 showed.

Than, the microorganism stability must be evaluate in the production conditions, mainly for stock cultures of industrial interest.

RESUMO

Análise da cinética de produção de amiloglicosidase em função do tempo de preservação de *Aspergillus awamori.*

Na produção industrial de amiloglicosidase é freqüente o emprego de microrganismos pertencentes ao gênero *Aspergillus*. Este trabalho apresenta alguns ensaios em processo descontínuo, com *A. awamori* NRRL 3112 preservado em terra e areia. Verificou-se através de cultivos descontínuos, uma estabilidade no comportamento do microrganismo relativa a capacidade de crescimento e produção da enzima após 3 anos de armazenamento da cêpa.

Para tempos superiores de conservação dos esporos, houve redução na concentração celular final entre 10 e 20%, bem como na atividade em amiloglicosidase acumulada no caldo, entre 36 a 43%.

Palavras-chave: *Aspergillus awamori*, amiloglicosidase, preservação de microrganismos.

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CYCLODEXTRIN GLYCOSYLTRANSFERASE PRODUCTION BY ALKALOPHILIC *BACILLUS LENTUS*.

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Yong Kun Park[2]

SUMMARY

A novel bacterial isolate, identified as *Bacillus lentus*, having the highest extracellular cyclodextrin glycosyltransferase (CGTase, E.C. 2. 4. 1. 19) activity, was selected after a screening of microorganismis obtained from soil of the Campinal area, using alkalophilic culture medium. A crude enzyme preparation was obtained by growing the microorganisms in liquid medium, pH 10.3, under agitation, at 37°C for 4 days. The optimum pH and temperature rangers for the formation of cyclodextrins by crude enzyme were 6.5 to 7.5 and 45 to 55°C, respectively. Analysis of the cyclodextrins by high Perfomance Liquid Chromatography showed the presence of α, β and γ forms in the ratio 1:67:1.6, respectively.

Key words: *Bacillus lentus*, cyclodextrin glycosyltransferase, CGTase.

INTRODUCTION

In the late years, the number of researches involving microorganisms or enzymes that can act in adverse conditions such as high temperatures and pH extremes, has increased. This may result in benefits like the control of microbial contaminations during fermentative processes or enzyme utilization. Thus, there are published papers about alkaline xylanase (4), thermostable lipase (3), alkaline amylase (5) and alkaline pectinase (6). Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) production by alkalophilic microorganisms was reported by Nakamura & Horikoshi (10) and Nomoto & et al. (12). CGTase hydrolyses starch and related carbohydrates, producing cyclic oligosaccharides known as cyclodextrins (2). Cyclodextrins (CD) are able to produce molecular encapsulation (inclusion), altering physical and/or chemical characteristics of the encapsulated molecules (15). The molecular inclusion by cyclodextrins has an enormous potential for use in food an pharmaceutical industries (15). However, the number of microorganisms that produce CGTase, alkalophilic or not, is still limited in the literature (1, 7, 8, 9, 10, 12, 14).

The objective of this work was to search for a new species of alkalophilic microorganism with high CGTase production.

MATERIAL AND METHODS

Isolation of the microorganism with high CGTase production – Using the culture medium of Park et al. (13), 114 soil samples of the city of Campinas (SP) were assayed, from which 57 cultures of alkalophilic bacteria CGTase productors were isolated. In order to evaluate the capacity of CGTase production, these microorganisms were

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grown under agitation for 4 days at 37°C, in 20 ml of alkaline medium, pH 10.3, with the following composition: 2% soluble starch (Reagen), 0.5% peptone (Difco), 0.5% yeast extract (Difco), 0.02% $MgSO_4.7H_2O$ (Reagen), 0.1% K_2HPO_4 (Merck), 1% Na_2CO_3 (Reagen) according to Nakamura & Horikoshi (11). After removal of the cells by centrifugation (11200 x g x 10 min at 5°C), the crude CGTase, present in the supernatant, was quantified by cyclodextrins formation, with the trichroloroethylene cyclodextrin (CD-TCE) method (12). In this method, the enzyme solution was successively diluited with 50 mM borax – HCl buffer pH 7.5 to obtain dilutions of $1:2^n$. One ml of each diluted enzyme solution was mixed with 5 ml of a 2% soluble starch solution prepared in the same buffer, and the mixture was incubated at 55°C for 24h. After incubation, 2.5ml of TCE was added with shaking and the suspension held at room temperature overnight. CD-forming activity of the enzyme solution is expressed by the dilution rate ($1:2^n$) that is capable of producing a CD-TCE complex precipitate. By this way, cultures were selected with CD-TCE activity $1:2^8$ (1:256), which produced the enzyme at higher level, according to the criteria proposed by Nomoto et al. (12).

Taxonomic Studies – In order to identify the species, the cultures that presented activity 1:256 (2^8) in the CD-TCE test (12), were submitted to morphological, physiological and biochemical studies, according to Sneath (17). Since the microorganisms were alkalophilic and there is no specific culture medium for taxonomic studies, the culture medium described by Sneath (17), was supplemented with 1% Na_2CO_3, pH 10.3.

After this study, a strain identified as alkalophilic *Bacillus lentus*, was submitted to the studies further described.

Temperature effect on the production of CGTase – Erlenmeyer flasks of 250ml, containing 70ml of the medium described in item 2.1, were inoculated with *B. lentus* and incubated at temperatures of 30, 37 and 40°C for 96h, on a reciprocal shaker. During the incubation, samples of 3ml were removed and centrifuged at 11200 x g x 10 min, at 5°C. The CGTase activity was determined in the supernatant by the CD-TCE method (12).

Crude CGTase obtention – The microorganism was aerobically grown for 96h at 37°C, in 1000ml of the medium described in item 2.1. After incubation, the cells were separated by centrifugation. Ammonium sulphate was added to the supernatant up to a 80% saturation. The precipitate obtained was separated by centrifugation, suspended with a borax buffer 50 mM, pH 8.5 and dialysed at 5°C for 48h in distilled water. The CGTase present in this crude preparation was quantified by the dextrinizing activity method, described below.

Dextrinizing activity – The enzymatic unit (U) was calculated by the dextrinizing activity of the CGTase on starch, according to Pongsawaski & Yagisawa (14). This procedure was carried out by adding 0.1ml of the enzyme conveniently diluted, to 0.3ml of 0.2% soluble starch, both prepared in acetate buffer 50mM, pH 5,8. After incubation at 55°C for 10 min, the reaction was stopped by adding 4 ml HCl 0,2M. Iodine reagent (0.2% KI and 0.02% I_2), was added 0.5ml. The mixture was diluted to 10ml in distilled water and the absorbance determined at 700nm. The enzyme was added to the control tube after HCl addition. The enzymatic unit was defined as the quantity of enzyme that reduces in 10% the blue color of the complex starch-iodine, after 10 min incubation at 55°C, in acetate buffer 50mM, pH 5.8.

Effect of pH and temperature on the crude CGTase activity – The pH effect on the cyclodextrins formation was determined with 0.5ml of CGTase solution (~163U), by the CD-TCE method (12), with incubation for 24h at 55°C. The following buffers were used at 50mM: acetate buffer (pH 3.5 - 5.8), phosphate buffer (pH 6 - 7), borax buffer (pH 7.5 - 9), borax-NaOH buffer (pH 9.5 - 10.2).

The effect of temperature on the cyclodextrins formation was studied with 0.5ml CGTase (~163U) in borax buffer 50mM, pH 7.5, by the CD-TCE method (12), incubating for 24h at temperature range of 35 to 70°C.

Determination of the types of cyclodextrins produced - The types of cyclodextrins produced by CGTase were determined by High Performance Liquid Cromatography (HPLC), according to Sato et al. (16). Six hundred unities of crude CGTase and $CaCl_2$ 10mM (11) were added to 100ml 1% soluble starch suspension in borax buffer 50mM, pH 7.5. The mixture was incubated at 55°C, and after 24 and 48h incubation, samples of 2ml were removed and treated with glucoamylase (NOVO), from *Aspergillus niger*, in order to hydrolyse the starch not converted into cyclodextrins. Then, 10µL of each sample were submitted to HPLC in the following equipment and conditions.

A Waters cromatograph composed by a 6000A model bomb, U6K injector, R-041 refraction index detector, Hp 3390(VDC) integrator, and ZORBAX-NH_2 column (4.6 x 250 mm), from Dupont, was used. The elution was performed with acetonitrille-water (65:35) at room temperature, in

1mL/min flow. The samples were compared to patterns 0.4% of α, ß and γ –cyclodextrins (Sigma), prepared in distilled water.

RESULTS AND DISCUSSION

Temperature effect on the CGTase production – Figure 1 shows the temperature effect on the CGTase production by *B. lentus*. The enzyme seems to be produced more rapidly at 30°C, but at the end of the incubation the CD-TCE activity was 1:a28, lawer than at 37 and 40°C, in which the CD–TCE activity reached 1:256. Initially, the CGTase production was slower at 40°C. A larger cellular growth with satisfactory CGTase production was observed at 37°C. The temperature effect on the CGTase production has not been reported by other researchers. Temperatures between 30 and 40°C have been reported to be used for CGTase production by other microorganisms (8, 9, 10, 12).

pH effect on the cyclodextrins formation – Figure 2 shows the pH effect on the crude CGTase activity at 55°C. It was noted that the cyclodextrins formation, measured by CD-TCE activity (12), was larger at pH 6.5 to 7.5. Therefore, *B. lentus* CGTase may be considered as a neutral enzyme. The optimum pH of the CGTase varies according to the microorganism species. The *Bacillus sp* CGTase (ATCC 21783), studied by Nakamura & Horikoshi (11), showed optimum pH in a medium of high acidity (pH 4.5 to 4.7). On the other hand the CGTase produced by *B. subtilis* (7), had optimum pH in alkaline medium (pH 8).

Temperature effect on the cyclodextrins formation – It can be seen from figure 3 that the enzyme presented the larger cyclodextrins formation activity at 45 to 55°C. This result is similar to that

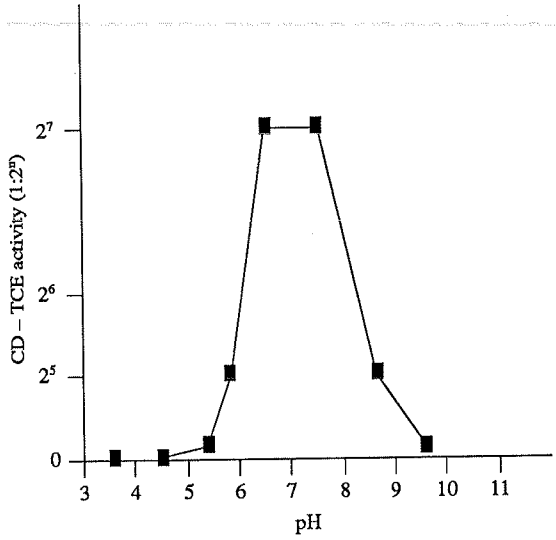

Figure 2. pH effect on the B*acillus lentus* CGTase cyclodextrins formation.

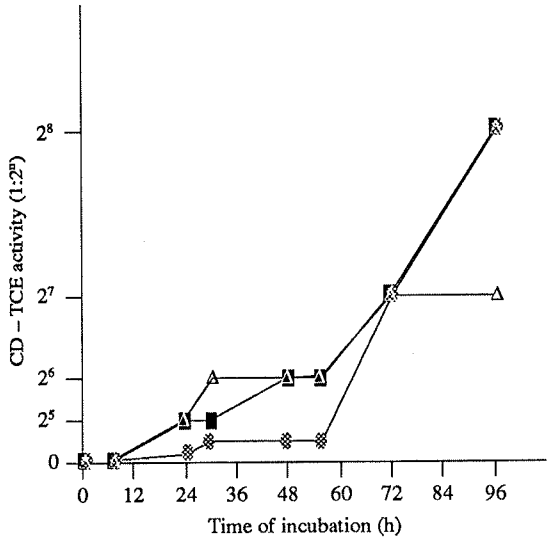

Figure 1. Temperature effect on the B*acillus lentus* CGTase production (CD–TCE activity at 30° C, Δ; 37° C, ■ ; 40°C, ❄)

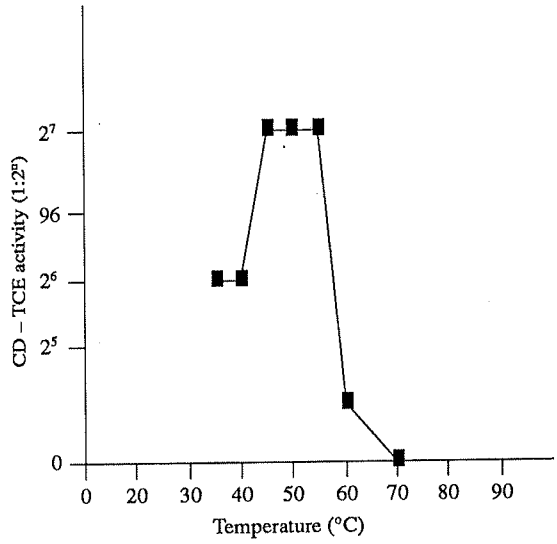

Figure 3 – Temperature effect on the B*acillus lentus* CGTase cyclodextrins formation.

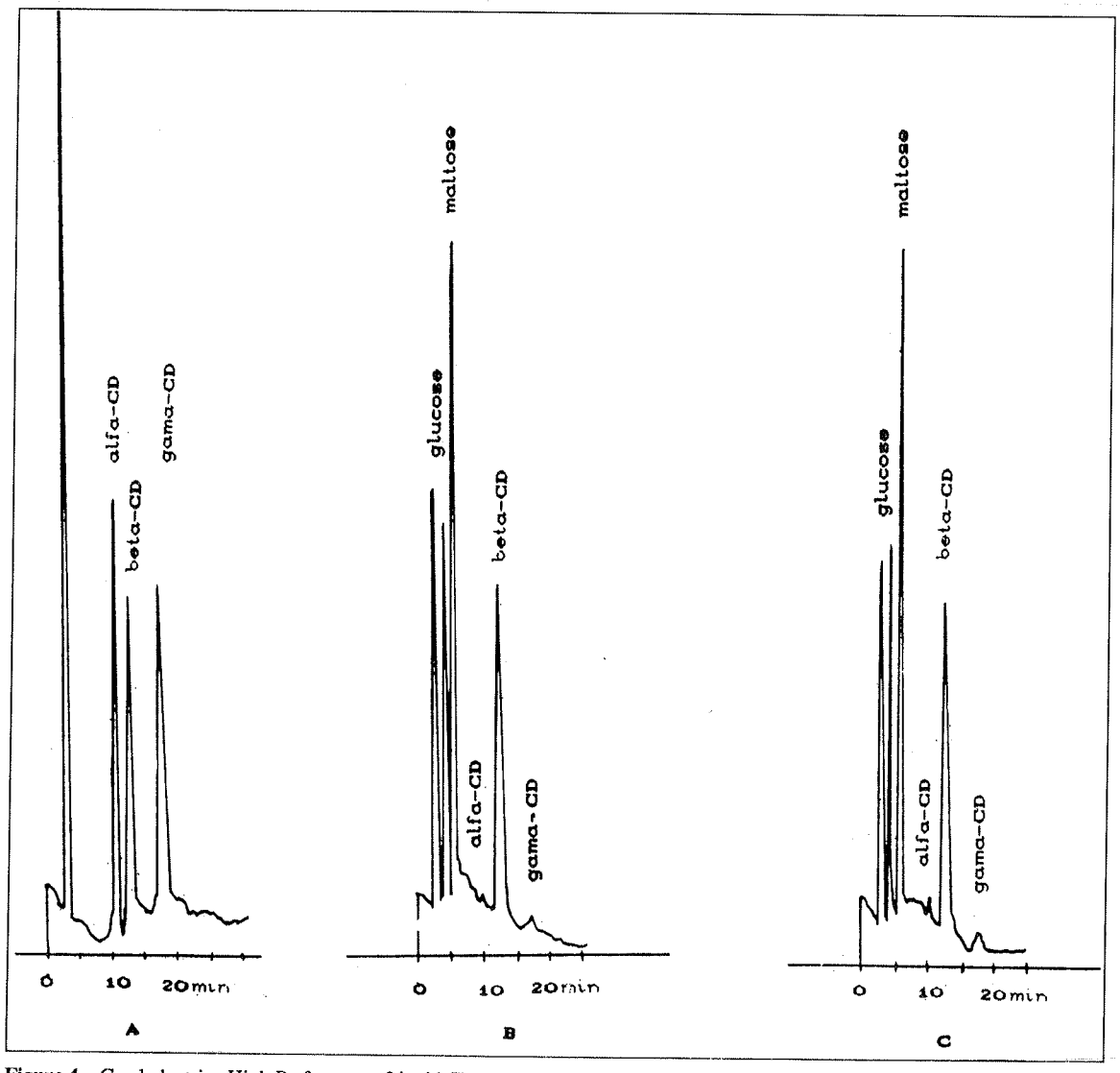

Figure 4 – Cy clodextrins High Performance Liquid Choromatography (HPLC). (A) – Elution of standard cyclodestrins (10 µl mixture containing 0,4% of each cyclodextrin). (B) and (C) – Elution of the hydrolysated (1% soluble starch), after 24 and 48h incubation with CGTase, respectively.

obtained by other researchers (7, 14). The CGTase produced by *B. macerans* (1) and *B. megaterium* (8), especially, presented optimum activity at 55°C.

Proportion of the types of cyclodextrins produced by CGTase – Figure 4 shows the cromatograms of the standard cyclodextrins and that obtained after hydrolysis of 1% soluble starch with *B. lentus* CGTase, for 24 and 48h at 55°C, respectively. In the cromatogram of figure 4A, the first peak corresponds to cyclodextrins solvent (water). In the cromatograms 4B and 4C, the first peak corresponds to water, the second and the third ones correspond to glucose and maltose, produced by glucoamylase from the starch not converted into cyclodextrins, according to Sato et al. (16). It can be noted that the ß -CD is almost exclusively produced, independent of the time of incubation. Based on the area percentage formed by each cyclodextrin, were calculated the proportions of 1:67:1.6 and 1.6:54:1 to α, β and γ -CD (figures 4B and 4C), after 24 and 48h incubation, respectively. It can be observed that *B. lentus* CGTase produced mainly the β -CD, in a proportion not

yet reported in the literature. This is an important characteristic, for permitting the commercial preparation of a more purified β -CD.

Kitahata & Okada (8) verified that the CGTases from *B. megaterium* and *B. macerans* produced the cyclodextrins in the proportions 1: 2.4 :1 and 2.7 : 1:1, respectively. For *B. circulans* CGTase, the proportion was 1:10.5:0 (14). Kato & Horikoshi (7), reported that *B. subtilis* CGTase produced only y -CD.

In conclusion, these results suggest that *B. lentus* CGTase is an enzyme that has potential to the specific production of ß -cyclodextrin. Studies to optimizate the production and characterize the purified enzyme are being performed, and will be presented later.

RESUMO

Produção de ciclodextrina glicosiltransferase por *Bacillus lentus* alcalofílico.

Uma linhagem de *Bacillus lentus* mesofílico e alcalofílico contendo alta atividade extracelular de ciclodetrina glicosiltransferase (CGTase, E. C. 2.4 1.19), foi obtida de uma coleção de microrganismos isolados de solos da cidade de Campinas (SP), utilizando-se um meio de cultivo alcalino. Uma preparação bruta da CGTase foi obtida cultivando-se o microrganismo por 4 dias a 37°C, sob aeração em meio líquido contendo 2% amido solúvel, 0,5% peptona, 0,5% extrato de levedura, 0,1% K_2HPO_4, 0,02% $MgSO_4.7H_2O$ e 1% Na_2CO_3 (pH 10,3). Os ótimos de pH e temperatura da enzima para a formação de ciclodextinas, situaram-se entre 6,5 a 7,5 e 45 a 55°C, respectivamente. As ciclodextrinas α, β e γ foram produzidas numa proporção de 1:67:1,6, respectivamente.

Palavras-chave: *Bacillus lentus*, ciclodextrina glicosiltransferase, CGTase.

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CANDIDA ALBICANS FROM AIDS PATIENTS: SUSCEPTIBILITY "*IN VITRO*" TO ANTIFUNGAL AGENTS

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SHORT COMMUNICATION

SUMMARY

Candida albicans strains susceptibilities from AIDS patients were determined by MIC (Minimal inhibitory concentration) and MFC (Minimal fungicidal concentration) to Amphotericin B, Nystatin, Ketoconazole and Miconazole. Semi solid agar dilution technique was the procedure employed. The authors did not find resistant strains of *Candida albicans* to antifungal agents tested. The basis of Candida resistance to polyene and imidazoles are discussed.

Key words: *Candida albicans*, susceptibility tests, antifungal agents, AIDS.

Candida albicans susceptibility to polyenes and imidazoles antifungal agents has been determined by several authors, with specimens from different diseases. Despite the lack of a universal pattern and a confusing results, some studies show the emergence of resistant *Candida* to these agents and emphasize the importance of these tests (Dick et al., Antimicrob. Agents Chemother., 18(1): 158-163, 1980). AIDS patients show oportunistic *Candida* infections with rates varying from 80% to 90%; though the antifungal sistemic therapeutic with polyenes and imidazoles, the morbidity and mortality by these infections persist (Meunier, F.; Reviews of Infected Diseases, 9: 408-416, 1987).

The goal this study was to verify the susceptibility of *Candida albicans* isolated from AIDS patients, to polyene and imidazoles antifungal agents and also relate the results to the previous use of these agents by those patients.

Ninety-three strains of *Candida albicans* were isolated from faeces of AIDS patients. The classic methods indicated on the Medical Mycology were employed for isolation and identification of the yeasts (McGinnis, M.R. –Laboratory handbook of Medical Mycology, Academic Press Inc., 1980). These strains were so divided: Group I –22 strains isolated from AIDS patients who used polyene antifungal agents; Group II –13 strains sourcing from AIDS patients who used imidazoles antifungal agents; Group III –58 strains proceeding from AIDS patients without antifungal chemotherapy.

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All strains were maintained on Sabouraud dextrose agar at 30°C.

The antifungal agents, Amphotericin B, Nystatin, Ketocanazole, Miconazole, were dissolved in Dimethylsulfoxide, and imediately diluted in Yeast Nitrogen Base (DIFCO) in order to obtain concentrations ranging from 0.25 ug/ml to 128 ug.ml of each antifungal agent in assay medium (Shadomy, S. et al., in Lennette, E et al., Manual of Clinical Microbiology, Am. Soc. Microbiol., 1985).

Semi solid agar dilution technique using Yeast Nitrogen Base with glucose buffered at pH 7,0 was the procedure employed (Cury et al., Rev. Microbiol., São Paulo, 20: 102-107, 1989). The Petri dishes with assay medium were inoculated with 1 x 10^5 cfu/ml from the strains studied, and incubated at 30°C during 24h. MIC was defined as the lowest concentration in which there was no visible growth of yeasts. The reported MICs were obtained in two or three repeated tests. The inoculated places that did not show any growth were subcultivated to determine th Minimal fungicidal concentration (MFC). MFC was defined as the lowest concentration at which was no growth of yeasts after the subculture on Sabouraud dextrose agar free from antifungal agents. In order to characterize the susceptibilities or resistance, the geometric means and Qui-square tests were applied.

The MIC and MFC ranges of Candida albicans strains which were studied are showed in the Table. The comparisons of MICs and MFCs among groups I, II and III concerning the antifungal agents studied did not show significant differences when Qui-square test was employed. The geometric mean did not show any tendency that could be understood as resistance either.

The polyene resistance can be a consequence of the previous use of imidazoles agents that block the ergosterol synthesis way; therefore the membranes become deficient in this type of sterol which is the primary site of polyenes action (Pappagianis, D. et al., Antimicrob. Agents Chemother., 16: 123-126, 1979). Other studies (Sokol-Anderson et al., Antimicrob. Agents Chemother., 29: 701-702, 1986) show that the frequent use of fractionated doses of polyenes can determine the increase of intracelular catalase. Consequently it will turn the yeasts resistant to the oxidative damage caused by these antifungal agents. On the other hand, the yeast resistance to imidazoles has not been well known yet but studies with Citochrome P450 indicate that this enzyme can be involved in these mechanisms (Smith, K.J. et al., J. Med. and Vet. Mycol., 24: 133-144, 1986). This knowledge suggests that in AIDS patients the development of *Candida* resistant to antifungal agents can occur although results of this study do not allow characterizing action of resistance to antifungals employed or observing action of the polyene resistance as a consequence of the previous use of imidazoles agents. We believe that our results can be explained by the small number of strains studied which did not permit the occurrence of this phenomenon. The long period of time in which were maintened "in vitro" previously to the susceptibility test may have favoured the recovery of some important byochemistry pathways. These mentioned ways may be responsible for the sensibility of the yeasts to antifungal agents needing this hypothesis new and accurate researches.

Table 1. Susceptibility variation of *Candida albicans* to polyene and imidazoles antifungal agents in the three studied groups.

Antifungals	Groups	MIC range (ug/ml)	GM	MFC range (ug/ml)	GM
Amphotericin B	I	0,25 – 1,0	0,48	1 – 4	2,74
	II	0,25 – 1,0	0,47	2 – 4	2,90
	III	0,25 – 1,0	0,43	1 – 8	2,79
Nystatin	I	1 – 8	3,87	4 – 32	9,07
	II	2 – 8	4,21	8 – 16	8,90
	III	1 – 8	3,30	4 – 32	8,69
Ketoconazole	I	4 – 64	30,0	32 ≥ 128	>96,39
	II	8 – 64	27,2	4 ≥ 128	>103,41
	III	4 – 64	25,2	32 ≥ 128	>98,38
Miconazole	I	4 – 32	15,50	32 ≥ 128	>68,16
	II	4 – 32	10,44	32 ≥ 128	>51,70
	III	4 – 32	13,69	32 ≥ 128	>62,48

MIC = Minimal inhibitory concentration
MFC = Minimal fungicidal concentration
GM = Geometric mean

Group I = Yeasts from Aids patients using polyene prophylaxis
Group II = Yeasts from Aids patients using imidazole prophylaxis
Group III = Yeasts from Aids patients not using antifungal prophylaxis

RESUMO

Candida albicans isoladas de pacientes com SIDA: sensibilidade "in vitro" aos agentes antifúngicos.

Por meio da determinação da CIM (Concentração Inibitória Mínima) e da CFM (Concentração Fungicoda Mínima), os autores verificaram a sensibilidade de Candida albicans isoladas de pacientes com SIDA, frente a Anfotericina B, Nista-

tina, Cetoconazol e Miconazol. Os autores não verificaram a ocorrência de amostras resistente e discutem o fenômeno da resistência de *C. albicans* aos antifúngicos poliênicos e imidazólicos.

Palavras chave: *Candida albicans*, sensibilidade, antifúngicos, SIDA.

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FIRST ISOLATION AND CHARACTERIZATION OF *YERSINIA ROHDEI* FROM RECREATIONAL WATER IN ARGENTINA

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SHORT COMMUNICATION

SUMMARY

The isolation and characterization of *Yersinia rohdei* from Laguna del Monte, situated in the Provincia of Buenos Aires, which is used for recreational purposes is reported for the first time in Argentina. The strain was classified as belonging to biotype 1, serotype 0:38, phagetype Xo.

Key words: *Yersinia rohdei* - recreational water

Yersinia rohdei was proposed as a single new *Yersinia* species by Aleksic and col. (1) in 1987. This organism was isolated from feces of dogs and humans and from surface water in Germany and the United States. The clinical significance of *Y. rohdei* as a diarrheal agent is not clear yet. Despite the identification of *Y. rohdei* among strains originated from stool specimes from patients with enteritis it is not known whether *Y. rohdei* was the cause of diarrhea in human cases. Aleksic and col (1) hypothesize that the natural habitat of the organism is water, leading to fecal carriage in dogs and humans and possibly an occasional infection.

The organism presents the biochemical reactions typical of the group of *Y. enterocolitica* and *Y. enterocolitica-like*. *Y. rohdei* can be distinguished from other *Yersinia* by its negative reactions in tests for indole, acetoin (VP) and rhamnose and positive reactions in tests for citrate and sucrose.

In Argentina it has been reported the isolation of *Y. enterocolitica* (4, 5, 7, 9, 12, 14), *Y. intermedia* (6, 7) and *Y. frederiksenii* (7) from different sources including feces, foods and waste waters. Despite the increasing number of reports about *Yersinia* species detection, there are no reports of *Y. rohdei* isolation in our country.

In this paper we report the first isolation and characterization of *Y. rohdei* in Argentina from Laguna del Monte, situated in the Provincia of Buenos Aires, which is used for recreational purposes.

The isolation was made in microbiological examination carried out from 51 samples of that surface water collected during March 1988 - May 1990 period (15).

The samples in 500 ml aliquots were concentrated by filtration through a 0.45 nm Millipore membrane filter. The cold enrichment was carried out on PBS – Sorbitol – Bile Salts Medium (11), 21 days at 4°C.

After the incubation a loopful of enrichment broth was streaked directly onto a Mac Conkey Agar plate and Cefsulodin - Irgasan - Novobiocin (CIN) Agar plate (16) for isolation. Agar plates were incubated for 48 hours at 28°C. After the incubation, 167 colonies having *Yersinia* characteristics (either translucent on Mac Conkey Agar

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Table 1. Biochemical reactions of *Y. rohdei* strain PS C 25

Indole production		-		
Methyl red		+		
Voges Proskauer	26°C	-		
	37°C	-		
Citrate	26°C	+		
	37°C	-		
Malonate utilization		-		
H_2S (Kligler Medium)		-		
Urea hydrolysis		+		
Phenylalanine deaminase		-		
L-Lysine decarboxylase		-		
L-Arginine deaminase		-		
L-Ornithine decarboxylase		+		
Motility	26°C	+		
	37°C	-		
Catalase		+		
Lipase		-		
Nitrate reduction to nitrite		+		
Oxidase		-		
o-Nitrophenil-ß-D-galactopiranoside		+		
Pigment production		-		
Esculin hydrolisis		-		
Mucate acid		-		
D-Glucose	acid	+		
	gas	-		
Acid from:				
L-Arabinose		+	Raffinose	+
D-Cellobiose		+	L-Rhamnose	-
D-Galactose		+	Salicin	-
Inositol		-	D-Sorbitol	+
Lactose		-	Sorbose	-
Maltose		+	Sucrose	+
D-Mannitol		+	Trehalose	+
∂-CH_3-D-Glucoside		-	D-Xilose	+

plates or having a deep red center with a rather sharp border and translucent outer zone on CIN Agar plates) were subcultured on Kligler Iron Agar slants and incubated 24 hours at 28° C.

If a typical reaction was obtained (i.e. alkaline/acid without gas or H_2S) the organism was further tested for urease production at 28° C and also for motility at 22° and 37° C. Isolation that gave a urease - positive reaction and that were motile at 22° C but not at 37°C were examined further with biochemical reactions.

Three isolations biochemically identifiable as *Yersinia* spp were sent for biochemical confirmation, serotyping and phage typing to the Laboratorio do Referencia de Yersinia - Araraquara - São Paulo - Brasil.

On of the strains (PS C25) was identified as *Y. rohdei* biogroup 1 (because of melibiose and raffinose fermentation) /0:38/ Xo. The biochemical reactions of *Y. rohdei* (PS C25) are shown in Table 1. Table 2 demonstrate its antibiotic susceptibility pattern as determined by agar diffusion (2).

Although the isolated strain of *Yersinia* belongs to non virulent group (1) further studies

Table 2 - Susceptibility of *Y. rohdei* strain PS C25 to antimicrobial

Antibiotics	disck concn. (ug)	
Amikacin	30	S
Ampicillin	25	S
Carbenicillin	100	R
Cephalothin	30	R
Chloramphenicol	30	S
Clindamicyn	2	R
Colistin	10	S
Erythromicin	10	R
Gentamicin	10	S
Kanamycin	30	S
Lincomicyn	10	R
Nalidixic acid	30	S
Penicillin G	10 UI	R
Rifampin	30	R
Sisomicin	30	S
Streptomycin	15	S
Sulfonamide	300	S
Tetracycline	30	S
Tobramycin	30	S

S: sensitive R: resistant

about its pathogenic potential must be done, since some serotypes no-pathogenic of *Y. enterocolitica* and other species of *Yersinia* are adapting to man and are producing human infections (3, 13).

Testes to assess virulence, such as calcium dependency and Congo Red pigmentation (8) and autoagglutination (10) at 37° C were carried out. All these virulence tests, which indicate the presence of a virulence plasmid in *Y. enterocolitica* were negative in *Y. rohdei* (PS C25). These results suggest that PS C25 strain is an environmental organism rather than a pathogen for humans and warm-blooded animals. Nevertheless further studies about virulence are required.

RESUMO

Primeiro isolamento e caracterização de *Yersinia rohdei* de aguas de recreação na Argentina.

Trata-se do primeiro relato da ocorrência de *Yersinia rohdei* na Argentina, isolada de água de recreação da Laguna del Monte, Província de Buenos Aires. A cepa foi classificada como *Y. rohdei* biotipo 1, sorotipo 0:38 e fagotipo Xo.

Palavras chave: *Yersinia rohdei*, água de recreação.

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ERRATA

Faintuch, B. L.; Sato, S.; & Aquarone, E. – Emprego de algumas fontes nitrogenadas na obtenção de biomassa de *Oscilatória limnetica*

Por um erro na produção da Revista de Microbiologia o artigo acima foi publicado duas vezes.

A referência correta é:

Faintuch, B. L.; Sato, S.; & Aquarone, E. – Emprego de algumas fontes nitrogenadas na obtenção de biomassa de *Oscilatória limnetica.*

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