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SOCIEDADE BRASILEIRA DE MICROBIOLOGIA
AV. Prof. Lineu Prestes, 1374
05508 - São Paulo - S.P.
Brasil
Fone (011) 813-4392
FAX 813-9647

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Endereço

Av. Prof. Lineu Prestes, 1.374
05508 – São Paulo – SP – Brasil
Fone: (011) 813-4392 – Fax: 813-9647

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NEW PATHOGENS OF INTEREST IN FOOD *VIBRIO VULNIFICUS*

Pearl I. Peterkin[1]

SHORT COMMUNICATION

SUMMARY

This paper is a review on *Vibrio vulnificus*. The following aspects are presented: introduction, characteristics of the microrganism, epidemiology of human infections (primary septicaemia and gastroenteritis, wound infections), mechanisms of virulence (effect of iron, role of cytotoxinhaemolisin, role of capsule in virulence), *Vibrio vulnificus* in foods (sporadic cases, incidence and survival in foods), methods of detection in foods (convencional and alternative methods) and control measures. A long and updated list of references is also included.

Key words: *Vibrio vulnificus*, foods.

INTRODUCTION

Vibrio vulnificus is an estuarine bacterial species that has been recently implicated as a cause of serious wound infections, and life-threatening primary septicaemias associated with the consumption of contaminated seafoods (31). It was originally discovered in the early 1970s due to its ability to cause wound infections. It is considered one of the most invasive and rapidly lethal human pathogens, with a 50% mortality rate associated with the septicaemic infection (6). Wound infections by this organism may result in amputation of limbs, or death, with a lower mortality rate of 25% (19). The majority of infections occur in immunocompromised persons, or those with preexisting diseases. Recently, it has also been associated with cases of gastroenteritis, with no fatalities reported (17).

CHARACTERISTICS OF THE ORGANISM

The genus *Vibrio*, which contains several human pathogens including *V. cholerae*, consistis of Gram-negative straight or curved rods, some requiring salt for growth. Though early workers referred to *V. vulnificus* as a "lactose-positive marine vibrio" (6), it was found that wild-type strains of this species were unable to utilize lactose (5). This discrepancy is explained by the ability of many *V. vulnificus* strains to acquire this property by mutation. The use of lactose fermentation as a trait diagnostic for this species should be treated with considerable caution (5).

V. vulnificus is a small Gram-negative curved rod, which is motile, oxidase-positive and ferments glucose and cellobiose. It is easily grown on a variety of media. It is an obligate halophile, requiring at least 0.5% NaCl for growth, with optimal salt concentrations being between 1 and 3% (16). Growth occurs between 20 and 40°C with the optimal growth temperature being 37°C (16). Aeration is also an important factor in stimulating growth. Incubation with shaking at 37°C results in generation times of 22 to 30 min and a final cell density of $\approx 2 \times 10^9$/mL cells/mL. An indole-negative strain has been reported isolated from a wound infection which resulted after cleaning eels (37). *V. vulnificus* is sensitive to cold, and experiences metabolic

1. Bureau of Microbial Hazards, Health Protection Branch Health Canada, Otawa ON, Canada K1A OL2.

damage at low temperatures, explaining the organism's seasonal ocurrence in temperate climates.

EPIDEMIOLOGY OF HUMAN INFECTIONS

V. vulnificus is unusual in this ability to produce disease by two different portals of entry. Table 1 presents a summary of 318 cases of primary septicaemia and 7 cases of gastroenteritis occurring internationally. In both of these ilinesses infectin is usually acquired by the oral route. Table 1 also summarizes 102 cases of wound infection. It should be noted that some of these may be duplicated cases, reported in more than one of studies used in the preparation of Table 1.

cies, renal failure, HIV infection, and patients on immunosuppressant therapy. Following ingestion of contaminated food, usually a seafood, a primary septicaemia develops. This is a systemic illiness with an abrupt onset of fever and chills, usually followed by hypotension, gastrointestinal symptoms, and secondary cutaneous lesions. It is often rapidly progressive, with a 50% mortality rate. In a few cases the septicaemia followed a wound infection (24). The incidence of *V. vulnificus* infections in USA coastal states is 0.5/100,000 population/year, with primary septicaemia accounting for 2/3 of the cases (18). Gastroenteritis caused by *V. vulnificus* has been found in a study of Florida cases reported by Klontz et al. (17). It was characterized by vomiting, diarrhoea or abdominal pain, positive stool cultures

TABLE 1 - Summary of *Vibrio vulnificus* sporadic[a] infections.

	Nº of cases (Nº of deaths)	Year	Country	Source of infection (% of cases)	Nº with chronic diseases	Ref.
Primary septicaemia	57 (32)	Na[b]	USA	Raw seafood (87%)	54	Oliver 1989
	18 (11)	1981-1982	USA	Raw oysters (87%)	16	Tacket et al. 1984
	102 (39)	1978-1987	USA	Raw oysters (100%)	NA	Liston et al. 1991
	38 (21)	1981-1987	Florida	Raw oysters (92%)	36	Klontz et al. 1988
	3 (2)	1979-1985	Europe	NA	NA	Veenstra et al. 1992
	70 (55)	1982-1986	Korea	Raw seafood (63%)	49	Park et al. 1990
	11 (5)	1985-1990	Taiwan	Raw seafood (20%)	11	Chuang et al. 1992
	1 (1)	1991	Australia	Seawater wound	1	Wise & Newton 1992
	18 (13)	1983-1993	California	Steamed oysters Raw oysters (63%)	14	Anon. 1993
Wound infections	54 (12)	NA	USA	Seawater of shellfish (89%)	31	Oliver 1989
	9 (2)	1981-1982	USA	Seawater of shellfish (89%)	5	Tacket et al. 1984
	17 (4)	1981-1987	Florida	Seawater (88%)	6	Klontz et al. 1988
	1 (0)	1985	Canada	Seawater	1	CDWR 1986
	2 (0)[c]	1989	USA	Oyster shell cut Seawater	2	Plotkin et al 1990
	1 (0)[d]	1989	USA	Seawater wound	1	Vartian & Septimus 1990
	1 (0)[e]	1992	Netherlands	Eel	1	Veentra et al. 1992
	17 (6)	1985-1990	Taiwan	Seawater wound (65%)	9	Chuang et al. 1992
GI illness	7 (0)	1981-1987	Florida	Raw oysters (71%)	2	Klontz et al. 1988

a No outbreaks reported to CDC (Liston et al. 1991).
b NA - Data not available
c Multiple infection, with *V. parahaemolyticus*
d Case of osteomyelitis
e Indole-negative *V. vulnificus*

Primary Sipticaemia and Gastroenteritis

There have been reports of *V. vulnificus* primary septicaemias from the USA, 5 countries in Europe and Asia, and Australia (Table 1). The infection usually occurs in people in "at risk" groups, including those with chronic liver disease (especially alcohol-related), haemochromatosis, malignan-

and negative blood cultures for *V. vulnificus,* and no evidence of a wound. No deaths have been reported from this form of the infection.

Wound Infections

There are published reports of serious wound infections caused by *V. vulnificus* from the USA,

Canada, Europe and Asia (Table 1). This syndrome occurs when wounds are exposed to sea water, or when cuts and abrasions occur in sea water. These patients also often have a predisposing condition as with primary septicaemia. These severe wound infections, which may require amputation, have a mortality rate of about 25% (Table 1). Two cases have been reported of dual infections with *V. parahaemolyticus* and *V. vulnificus*; one resulted from a cut finger while shucking oysters and the other from an infected needle used by a drug abuser (28). A case of osteomyelitis resulted from a shin scraped on a rock in brackish water (17).

MECHANISMS OF VIRULENCE

A variety of factors have been implicated as possible virulence determinants for *V. vulnificus* in animal models. These include the ability to acquire iron from transferrin, an extracellular haemolsin and a protease, and the presence of a polysaccharide capsule (39). *V. vulnificus* strain also show a phase variation between virulent and avirulent forms. In the virulent phase, the organisms are encapsulated serum resistant, and acquire iron from transferrin that is > 90% saturated; avirulent variants lack these traits (41).

Effect of Iron

V. vulnificus cells grow better in media supplemented with an iron source such as heme (23), and demonstrate greater virulence in iron-loaded animals. A significant number of human infections with this organism occur in patients with haemochromatosis (24), or alcoholics (7). In both of these conditions, there is close to 100% saturation of serum transferrin. It may be that the greater incidence of *V. vulnificus* infections in males is due to their higher serum iron concentration. It has been suggested that the multiplication of the organism in the blood-stream of patients with haemochromatosis, and suspected of being infected, can be retarded by treatment with human apotrasferrin, or normal human serum or plasma (10). An extracellular protease has been demonstrated as playing a role in the utilization of heme by *V. vulnificus* by eliciting heme liberation from heme-containing proteins (23). Mutants deficient in this protease were unable to utilize this source of iron.

Role of Cytotoxin-Haemolysin

A cytolysin (molecular size of 50.8 kilodaltons) that is lytic for both erythrocytes and Chinese hamster ovary cells has been purified from *V. vulnificus* supernatants. This cytotoxin-haemolysin is separable from both a phospholipase A and a lysophospholipase present in this bacterium (39). This toxin may also be involved in the production of the cutaneous lesions that are characteristic of *V. vulnificus* infections, as it has been shown to degrade mast cells and induce histamine release (43). The determinant (*vvA*) has been sequenced, revealing that two regions show homology to the structural gene for the *V. cholerae* El Tor haemolysin (42). When strains of the organism were made cytolysin-negative by transposon mutagenesis, it was found that inactivation of the cytolysin did not affect virulence (39). Cytolysin-positive and -negative strains had the same 50% lethal dose. It appears that the role of this cytolysin in pathogenesis of *V. vulnificus* infections is of much less importance than other factors.

Role of Capsule in Virulence

V. vulnificus displays two distinct colony morphologies, designated as opaque and translucent. Opaque strains are encapsulated, usually virulent to mice, serum resistant, and can use transferrin-bound iron for growth. In contrast, translucent strains are non-encapsulated, less virulent, serum sensitive, and cannot grow in iron-limited media, even in the presence if transferrin that is 100% saturated (41). Opaque strains shift to a translucent colony morphology at a frequency of 10^{-4}, with accompanying loss of virulence. Two translucent mutants were constructed using only a single transposon insertion each. It was found that loss of capsule was accompanied by decrease in virulence and serum resistance (40). However, the ability to utilize transferrin-bound iron for growth was lost in only one of the unencapsulated mutants. This indicates that utilization of transferrin bound iron is independent of encapsulation (41). The capsular polysaccharide has been purified and its structure determined, showing it was composed of a repeating structure with four sugar residues per subunit (29). Electrophoretic analysis of the lipopolysaccharide (LPS) from opaque and translucent colonies showed that no differences were detected in the patterns of the LPS of these colony variants (4).

VIBRIO VULNIFICUS IN FOODS

Sporadic Cases

Only sporadic cases of *V. vulnificus* infections have been reported; there have been no outbreaks involving two or more individuals reported to CDC in Atlanta (18). In a recent study by Liston et al. (18), 100% of the primary septicaemia cases were associated with the consumption of raw oysters (Table 1). In other studies, the case rate associated with the consumption of raw seafood has varied from 63-92%, with a series of cases in Taiwan reporting a rate of 20%.

Incidence and Survival in Foods

All of the disease-causing vibrios occur naturally in the marine environment, and are thus naturally-occurring contaminants of seafood. This makes it impossible to prevent the contamination of raw products. Between December and March no *V. vulnificus* was recovered from oysters harvested on the USA Gulf Coast; thereafter the level gradually rose to a peak of 10^4 CFU/g oyster meat during the summer months (35). It has been demonstrated that the numbers of the organism increase in the temperature range of 13-22°C (13). Though it survives poorly below 8°C, it can survive in oysters for at least a week at temperatures of 4°C.

METHODS OF DETECTION IN FOODS

Conventional Methods

The method for the isolation of *V. vulnificus* given in BAM (3) involves a 24 h enrichment in saline alkaline peptone water, followed by plating on TCBS and modified CPS agars (see Table 2). Colonies of *V. vulnificus* are green (non-sucrose fermenting) on TCBS agar, and yelow (cellobiose fermenting) on CPC agar. CPC medium, which was developed by Oliver's group (26), uses the antibiotics colistin and polymyxin B, and an incubation temperature of 40°C to select for the organism, and the cellobiose fermentation to give a presumptive identification. Bryant et al. (8) used a medium, SPS agar (Table 2), for direct plating, where polymyxin B provided selectivity, and the colonies were diferentiated based on non-sucrose fermentation and the production of a halo caused by extracellular sulfatase activity. Miceli et al. (21) use VVE as a direct plating medium, where lactose fermentation was used as indicator, followed by the use of a modified SPS as a secondary medium. PMT (Table 2), with polymyxin B as the selective agent, and tellurite as the indicator agent, was originally used in isolating the organism from seawater and shellfish by Kaysner's group, but it does not seem to have been used much since then.

It should be noted that *V. vulnificus*, like other estuarine vibrios and some other species, enters into a "viable but nonculturable state" at water temperatures below 10°C (25). Viability can be demonstrated by the reduction of triphenyltetrazolium chloride, but the cells are no longer culturable in media which normally support their growth. These cells can only by resuscitated by a temperature upshift for 2 to 3 days. The possible presence of cells in this state should be taken into account when culturing *V. vulnificus* cells taken from cold environments.

Alternative methods

Alternative methods for the detection of the organism in food and environmental samples have recently been developed (Table 3). Tamplin et al. (33) developed a monoclonal anti-

TABLE 2 - Selective and indicator Agents in *Vibrio* media.

Medium[a]	Selective Agents					Indicator Agent	Reference
	Thiosulfate	Tellurite	Colistin	Polymyxin	Bile		
TCBS	+	-	-	-	+	sucrose	BAM, 1992
CPC	-	-	+	+	-	cellobiose	Massad & Oliver 1987
SPS	-	-	-	+	-	SDS, sucrose	Bryant et al. 1987
VVE	-	+	-	-	+	cellobiose	Miceli et al. 1989
PMT	-	+	-	+	-	mannose	Kaysner et al. 1987

[a] Abbreviations: TCBS, thiosulfate-citrate-bile-sucrose; CPC, colistin-polymysin-cellobiose; SPS, sodium dodecyl sulfate-polymyxin-sucrose; VVE, *Vibrio vulnificus* enumeration; PMT, polymyxin-mannose-tellurite.

TABLE 3 - Immunological and DNA methods for *V. vulnificus* detection.

Method	Food	Sensitivity (cells/g)	Reference
Immunological			
MAb (sp.-specific epitope)	oysters	8×10^4	Tamplin et al. 1991
DNA probes			
cytotoxin-hemolysin gene (3.2 kb)	oysters	6×10^3	Wright et al. 1985 Morris et al. 1987
cyt.-hemol. gene (recomb. plasmid)	crab, shrimp	10^2	Kaysner et al. 1990
Polymerase chain reaction			
cyt.-hemol. gene, 519 bp	oysters	10^2/g, then O/N incubation	Hill et al. 1991
cyt.-hemol. *(vvh)* gene, 519 bp	-	-	Trost et al. 1993

body to a species-specific epitope, and used it to detect *V. vulnificus* in seawater, sediment and oysters. DNA-based methods started with the cloning of the cytotoxin-haemolysin gene, which was then as a DNA probe in the detection of *V. vulnificus* in seafoods (Table 3). Kaysner's group (15) reported an interesting use of this probe for direct detection of the organism by colony hybridizaiton on hydrophobic grid-membrane filters (HGMF). Primers for a polymerase chain reaction (PCR) detection method were selected from the known sequence of the cytotoxin-haemolysin gene, yielding a 519 bp specific product (Table 3).

CONTROL MEASURES

There appears to be no correlation between the presence of faecal indicator organisms, and the presence of *V. vulnificus* in estuarine waters or shellfish, where it forms part of the normal microflora (24). However, the numbers of the organism in estuarine waters tend to increase as the ambient water temperature rises. This results in the appearance of larger numbers of the organisms in shellfish growing in these waters during the warmer months of the year. Consumption of raw seafoods should thus be avoided during these months. In order to maintain raw seafoods with levels of vibrios as low as possible, they shoud be refrigerated or iced immediately upon harvesting, and then kept at temperatures $\leq 5°C$ until eaten (24).

A study using some "generally recognized as safe" (GRAS) compunds as control agents showed that treatment with diacetyl at 50 ppm produced a six log reduction of *V. vulnificus* within 24 h (30). The organism appears to be quite sensitive to irradiation (1). An accepted tratment to reduce bacterial pollutants in shellfish is exposure to UV-disinfected seawater. However, natural populations of the organism persisted in Gulf Coast oysters following this treatment (32).

All vibrios are rapidly killed by heating foods to an internal temperature of 70°C. Adequate cooking will ensure the safety of seafoods for consumption, and there are no clinical reports associating the consumption of cooked seafoods with human cases of *V. vulnificus* infections. Preventing wound infections by the organism may be more difficult, as these infections can occur in persons in apparently good health.

RESUMO

Novos patógenos de interesse em alimentos: *Vibrio vulnificus*

Essa revisão aborda os seguinte tópicos relativos a *Vibrio vulnificus:* características do microorganismo, epidemiologia das infecções humanas (septicemia primária, gastroenterites e infecções de feridas), mecanismos de virulência (efeito do ferro, papel da citotoxina-hemolisina, papel da capsula na virulência), *V. vulnificus* em alimentos (casos esporádicos, incidência e sobrevivência em alimentos), métodos de detecção em alimentos e medidas de controle. A revisão conta também com uma lista de referências bibliográficas bastante completa e atualizada.

Palavras-chave: *Vibrio vulnificus,* alimentos.

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CHARACTERIZATION OF BRAZILIAN *LISTERIA MONOCYTOGENES* STRAINS USING DNA MACRORESTRICTION PATTERNS

Maria Lúcia Pereira[1]
Carmen Buchrieser[2]
Rolland Brosch[2]
Bénédicte Catimel[2]
Jocelyne Rocourtz[2]
Ernesto Hofer[3]

SUMMARY

Twenty one *Listeria monocytogenes* strains recovered in Brazil from human and foodstuffs in 1989 and 1990 were identified, serogrouped and phage typed according to conventional methods. These strains were also analysed to DNA macrorestriction patterns obtained after its cleavage with ApaI, SmaI and NotI and separation by pulsed field gel electrophoresis. The results of this study presented a greater heterogeneity among these strains: whereas serotyping divided in two serogroups (1/2 and 4), phage typing allowed to define 8 phagovars (including the non-typable strains), and finally DNA macrorestriction patterns allowed to distinguish 12 different groups. These results emphasize the need of characterizing *L. monocytogenes* with at least three different typing systems to ascertain whether isolates are similar or not, especially during epidemiological investigations on the foodborne transmission of human listeriosis.

Key words: *Listeria monocytogenes* - serotyping, phage typing, DNA macrorestriction patterns.

INTRODUCTION

The first case of human listeriosis was described in 1918, but this infection became of real interest only in the 1980's, after the epidemiological investigations conducted during major outbreaks in USA and Europe (6) have demonstrated the foodborne transmission of the disease. Since then, many studies have been undertaken to evaluate the foodborne origin of this infection, including the description of new typing systems to characterize *L. monocytogenes* strains.

Until the middle of the last decade, bacterial strains have been mainly typed using phenotypical markers, such as antigenic structure, susceptibility to bacteriophages production. The introduction and the development of new methods in molecular biology and their subsequent application in clini-

1. Laboratório de *Staphylococci*, Fundação Ezequiel Dias (FUNED). Caixa Postal 26; 30510-010 - Belo Horizonte, MG, Brasil (Author to whom all correspondence should be adressed).
2. Centre Collaborateur OMS pour la listériose d'origine alimentaire et Centre National de Référence pour la lysotipie et le typage moléculaire de Listeria. Département de Bactériologie-Mycologie, Institut Pasteur, 75724 Paris Cedex 15, France.
3. Departamento de Bacteriologia. Instituto Oswaldo Cruz, FIOCRUZ. Caixa Postal 926; 21045-900 Rio de Janeiro, RJ, Brasil.

cal microbiology allowed more refined characterizations of bacteria, mainly based on the characterization of proteins or DNA. This evolution is well illustrated in studies on *L. monocytogenes:* serotyping, described as early as in 1940 (19) and phage typing, later used in routine in the 1980's (16), are now completed with molecular typing methods such as multilocus enzyme analysis (1, 15), ribotyping (13), as well as DNA restriction patterns (2, 3, 14).

Numerous *Listeria* strains have been isolated from various sources in Brazil (7, 8, 9, 10, 11, 12). The goal of this study was to compare *L. monocytogenes* strains isolated from human infections and foodstuffs using conventional typing systems, ie. serotyping and phage typing, as well as DNA restriction patterns obtained after cleavage with rare cutting endonucleases.

MATERIAL AND METHODS

Bacterial strains - Twenty one *L. monocytogenes* strains were selected. They have been recovered in Brazil from human infections (8 strains) and from foodstuffs (13 strains) in 1989 and 1990. They were identified, serogrouped and phage typed according to conventional methods (7, 11, 16, 18).

Preparation of genomic DNA - DNA were extratected with some modifications following a method previously described for bacteria of a phylogenetically related genus (4). Bacteria were grown to midexponential phase in brain-heart infusion broth. Washed cells were ressuspended in a concentration of 109 cells per ml in TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8) and mixed with an equal volume of 1 % low melting agarose (Bethesda Research Laboratories). The mixture was dispensed in a slotformer of 55 µl volume. Agarose plugs were incubated in a lysis solution containing 0.1 M EDTA, 1% sarcosyl (Sigma), deoxycholic acid (sigma) (2 mg/ml) and lysozime (Appligène) (2.5 mg.ml) for 24 h at 37°C. The plugs were washed with TE and deproteinezed by incubating in 0.5 M EDTA containing 0.5 % sarcosyl and proteinase K (Appligène) (2 mg/ml) for 48 h at 50°C. Proteinase K was inactivated by washing the inserts in a phenylmethysulphonylfluoride (PMSF) (Bethesda Research Laboratories) solution (0.04 mg PMSF/ml TE). Plugs were stored in 0.5 M EDTA and were rinced twice in TE before restriction analysis.

DNA cleavage - As *Listeria* is characterized by a low G + DNA content (39%) (16), restriction enzymes with recognition sequences that contain only G and C nucleotides were chosen: A*pa*I (GGGCCC), S*ma*I (CCCGGG) and *Not*I (CGGCCGC). Restriction digests were done using 20-30 units of the enzymes A*pa*I (Boehringer), SmaI and NotI (Amersham) in 150 µl of the buffer recomended by the manufacturer, including 0.1 mg bovine serum albumin (Pharmacia). The plugs were incubated at 37°C for A*pa*I and *Not*I and 25°C for S*ma*I for up to 12 h. The inserts were placed into wells of a 1.1% low melting agarose gel prepared with 0.5 x TBE buffer (1 x TBE is 89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.3) and kept overnight at 4°C before migration. The running buffer (0.5 x TBE) was cooled down to 9°C during migration.

Electrophoresis conditions - A pulse controller (Hoefer PC 750) was used to perform field inversion - field inversion gel electrophoresis as previously described by Carle et al (5). The initial pulse duration was 1.5 s in forward direction and 0.5 s in reverse. The ramp factor and the duration of the migration were chosen according to number and size of fragments generated by the different endonucleases. For A*pa*I and *Not*I digests, a ramp factor value of 0.5 and a migration period of 22 h or 28 h, respectively, were used (i.e., final pulse of 18 s/6s or 22.5 s/7.5 s respectively), whereas for S*ma*I digests, a ramp factor of 0.2 and a migration period of 22 h were chosen (i.e., final pulse of 8.1 s/2.7s). The resulting DNA patterns were visualized by ethidium bromide fluorescence under short wave ultraviolet light and photographed. The estimation of the size of the fragments and the comparison of patterns belonging to different strains were done using labda DNA concatamers (Kindly provided by V. Vincent Levy-Frébault), yeasts chromossomes (Biorad) and by running different combinations of strains together on the same gel.

RESULTS AND DISCUSSION

Among these 21 strains, 10 belonged to serogroup 1/2 and 11 to serovar 4b. Various phagovars characterized 11 strains, and the remaining 10 strains were non phage-typable. Strains were clustered in 12 different groups according to their profile after digestion with A*pa*I (12 profiles), S*ma*I (11 profiles) and *Not*I (11 profiles) - Table 1 and Fig. 1, 2 and 3.

TABLE 1 - Characterization of 21 brazilian *L. monocytogenes* strains.

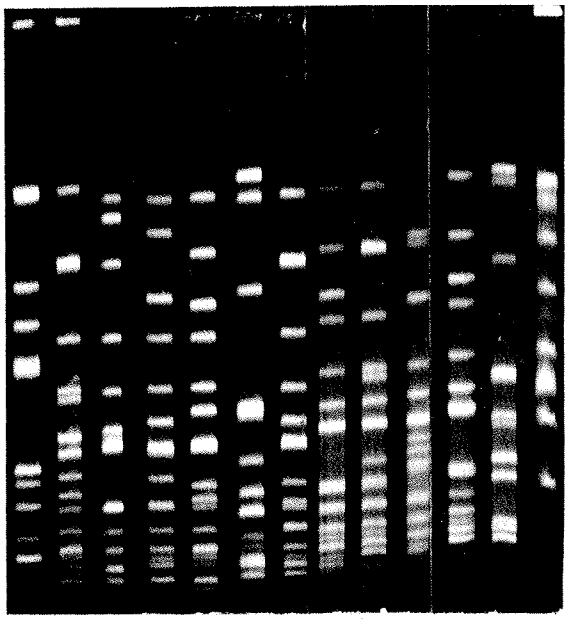
Strain designation	Origin	Serogroup	Phagovar	DNA profile after cleavage with		
				Apa I	I *Sma*	*Not* I
CLIP 16667	food	1/2	575	ml/3	ml/3	ml/3
CLIP 16666	food	1/2	1967/2685	ml/8	ml/23*	ml/3*
CLIP 16722	human	1/2	2685/1806	ml/18*	ml/19*	ml/16*
CLIP 16663	food	1/2	2685	ml/19*	ml/20*	ml/17*
CLIP 16728	food	1/2	2685	ml/19	ml/20	ml/17
CLIP 16729	food	1/2	NT	ml/20*	ml/21*	ml/18*
CLIP 16658	food	1/2	2685	ml/21*	ml/3	ml/19*
CLIP 16660	food	1/2	NT	ml/22*	ml/22*	ml/20*
CLIP 16676	human	1/2	NT	ml/23*	ml/5	ml/21*
CLIP 16677	human	1/2	NT	ml/23	ml/5	ml/21
CLIP 16723	human	4	2389/2425/3274/2671/47/108/340	m4/7	m4/2	m4/3
CLIP 16709	food	4	NT	m4/12	m4/10	m4/7
CLIP 16685	food	4	3552/1444/3274/108/312	m4/18*	m4/18*	m4/8*
CLIP 16688	food	4	3552/1444/3274/312	m4/18	m4/18	m4/8
CLIP 16689	food	4	3274/312	m4/18	m4/18	m4/8
CLIP 16702	food	4	3274/312	m4/18	m4/18	m4/8
CLIP 16659	food	4	NT	m4/19*	m4/19*	m4/9*
CLIP 16673	human	4	NT	m4/19	m4/19	m4/9
CLIP 16674	human	4	NT	m4/19	m4/19	m4/9
CLIP 16678	human	4	NT	m4/19	m4/19	m4/9
CLIP 16724	human	4	NT	m4/19	m4/19	m4/9

* new DNA restriction profile
NT not typable

A greater heterogeneity among these strains could be demonstrated using these three different typing schemes: serotyping divided the strains in two serogroups whereas phage typing allowed to define at least 8 phagovars (including the non-typable strains), and finnaly DNA macrorestriction patterns allowed to distinguish 12 different groups. Taking together all these results, these 21 strains could be separated in 13 groups.

The results of this study confirm that *L. monocytogenes* strains of different serogroups (1/2 vs. 4) do not share the same DNA restriction patterns (2, 3). As previously observed (2, 3), strains with same phagovar may differ in their DNA restriction patterns - strains phagovar 2685 belonged to two different groups of profiles (Table 1). Phage typing of *L. monocytogenes* is presently hampered by the high percentage of non-phage typable strains (around 30-40%); using this molecular typing system, the non-phage typable strains were clustered in 5 different DNA patterns, thus demonstrating a great diversity within these strains, which could be pointed out using phenotipic methods (serogroup 1/2 : 4 non typable strains = 3 DNA macrorestriction groups; and serogroup 4 : 6 non typable strains = 2 DNA macrorestriction groups.

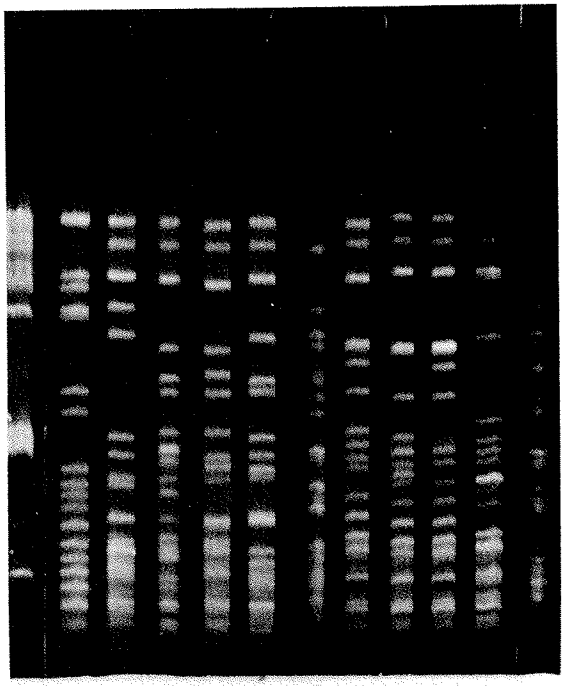
Two indistinguishable strains as far as these markers are concerned, were isolated from a food sample and from a human infection. This confirmed again that contamined foods are potencial hazardous for human health. A few profiles have been already detected in strains isolated in other countries (Spain, Norway and Austria) demonstrating that *L. monocytogenes* are widely distributed in the world. In contrast, several new DNA macrorestriction patterns were observed (table 1). The small



1 2 3 4 5 6 7 8 9 10 11 12 13

lane 1: profile m4/18 (CLIP 16702); lane 2: profile m4/12 (CLIP 16709); lane 3: profile m1/18 (CLIP 16722); lane 4: profile m4/7 (CLIP 16723); lane 5: profile m4/19 (CLIP 16724); lane 6: profile m1/19 (CLIP 16728); lane 7: profile m1/20 (CLIP 16729); lane 8: profile m1/21 (CLIP 16658); Lane 9: profile m1/22 (CLIP 16660); lane 10: profile ml/8 (CLIP 16666); lane 11: profile ml/3 (CLIP 16667); lane 12: profile ml/23 (CLIP 16677); lane 13: lambda concatamers.

FIGURE 1 - DNA restriction patterns of *L. monocytogenes* strains (serogroup 1/2 and 4) after cleavage with *Apa* I.



1 2 3 4 5 6 7 8 9 10 11 12

lane 1: lambda concatamers; lane 2: profile m4/18 (CLIP 16702); lane 3: profile m4/10 (CLIP 16709): lane 4: profile ml/19 (CLIP 16722); lane 5: profile m4/3 (CLIP 16723); Lane 6: profile m4/19 (CLIP 16724); lane 7: profile ml/20 (CLIP 16728): lane 8: profile ml/21 (CLIP 16729: lane 9: profile ml/3 (CLIP 16658); lane 10: profile ml/22 (CLIP 16660); lane 11: profile ml/23 (CLIP 16666); lane 12: profile ml/5 (CLIP 16677).

FIGURE 2 - DNA restriction patterns of *L. monocytogenes* strains (serogroup 1/2 and 4) after cleavage with *Sma* I.

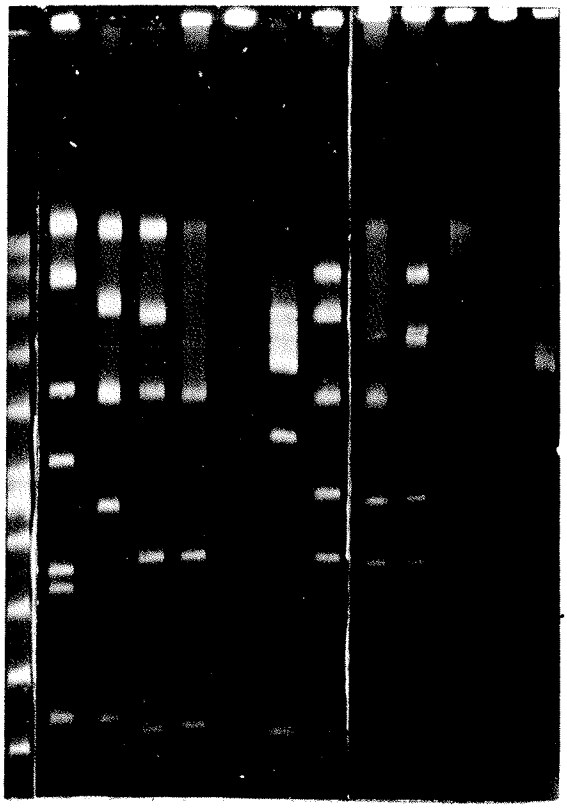
number of strains presently investigated did not allow to suggest whether these strains are specific for Brazil or only new because of the limited number of strains studied with this method - presently 77 strains (2, 3) and 20 not yet published.

Finally, these results emphasize the need of characterizing *L. monocytogenes* strains with at least three different typing systems to ascertain whether isolates are similar or not, specially during epidemiological investigations on the foodborne transmission of human listeriosis.

RESUMO

Caracterização de linhagens brasileiras de *Listeria monocytogenes*, através de perfil de macrorestrição de DNA

Vinte e uma linhagens de *Listeria monocytogenes* amostradas no Brasil, no período de 1989-90, procedentes de material clínico e alimentos foram identificadas, sorotipadas e fagotipadas de acordo com métodos tradicionais. Estas linhagens foram, também, tipadas quanto ao perfil de macrorestrição de DNA, após clivagem com A*pa*I, *Sma*I e *Not*I e separadas por eletroforese em campo pulsado. Os resultados obtidos evidenciaram uma grande heterogeneidade entre as linhagens: enquanto a sorotipagem dividiu dois sorogrupos (1/2 e 4), fagotipagem definiu oito fagovares e, finalmente, perfil de macrorestrição de DNA permitiu distinguir doze diferentes grupos. Estes resultados enfatizam a necessidade de caracterização de *L. monocytogenes* com, pelo menos, três diferentes métodos para definir se isolados são similares ou não, especialmente por ocasião de investigações epidemiológicas da transmissão alimentar da listeriose humana.



1 2 3 4 5 6 7 8 9 10 11 12 13

lane 1: lambda concatamers; lane 2: profile m4/8 (CLIP 16702); lane 3: profile m4/7 (CLIP 16709); lane 4: profile ml/16 (CLIP 16722); lane 5: profile m4/3 (CLIP 16723); lane 6: profile m4/9 (CLIP 16724); lane 7: profile m1/17 (CLIP 16728); lane 8: profile ml/18 (CLIP 16729); lane 9: profile ml/19 (CLIP 16658); lane 10: profile ml/20 (CLIP 16660); lane 11: profile ml/3 (CLIP 16666); lane 12: profile ml/3 (CLIP 16667); lane 13: profile ml/21 (CLIP 16677).

FIGURE 3 - DNA restriction patterns of *L. monocytogenes* strains (serogroup 1/2 and 4) after cleavage with *Not* I.

Palavras-chave: *L. monocytogenes* - sorotipagem, fagotipagem, perfil de macrorestrição de DNA.

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SEROVARS AND MULTIPLE DRUG RESISTANT SALMONELLA SP. ISOLATED FROM CHILDREN IN RIO DE JANEIRO-BRAZIL

Marise Dutra Asensi[1]
Ernesto Hofer[2]

SUMMARY

The Serological characterization and antimicrobial susceptibility of 116 *Salmonella* strains were studied. These isolates were obtained from stool and blood specimens of children who received treatment at the Instituto Fernandes Figueira, FIOCRUZ (Rio de Janeiro, Brazil) between May 1987 and July 1992 .Six serogroups (04; 07; 08; 09; 03,10 and 035) and 18 serovars were detected, the highest frequency being observed for *S. typhimurium* (62.93%) and *S.agona* (7.75%). Antimicrobial susceptibility was tested for ampicillin (Ap), cephalotin (Cf), cefoxitin (Cx), ceftriaxone (Cro), pefloxacin (Pf), gentamicin (Ge),amikacin (Am), sulfamethoxazole-trimethoprim (SxT),chloramphenicol (Cl) and tetracyclin (Te). 92.52% of the *S.typhimurium* isolates had resistance determinants. Twenty-nine patterns of resistance were encountered; the most frequent were Ap, Cf, Pf, Ge, Am, SxT, Cl, Te for *S.typhimurium* (40.57%) and Te for the other serovars (20.83%). The minimum inhibitory concentration analysis of five antimicrobials showed high levels of resistance to Ap and Cl and low levels of resistance to Ge, SxT and Cro.

Key Words - Salmonella sp, Serovars, Frequency, Antimicrobial Susceptibility.

INTRODUCTION

Diarrhea is still a cause of high morbidity and mortality during early childhood in the developing countries (9,11,15). Although progress has been made in the identification of new enteropathogenic bacterial pathogens, *Salmonella* infection continues to be one of the leading causes of bacterial diarrhea among children (12,20).

Antigenic structure determination and antimicrobial susceptibility are important epidemiological tools for the characterization of these agents. Such information identifies the different *Salmonella* serovars in a certain population and provides antimicrobial susceptibility data.

In the present investigation, *Salmonella* serovars were determined from children seen at a public maternal child hospital in Rio de Janeiro over a five year period (May 1987 to July 1992).The antimicrobial susceptibility of these isolates were determined against the antimicrobials normally used for treatment in hospitals.

MATERIAL AND METHODS

Hospital and population description - The Instituto Fernandes Figueira (IFF) is a public maternal child health-care hospital and acts as a technical unit of the Fundação Oswaldo Cruz-FIOCRUZ

1. Bacteriology Laboratory, Fernandes Figueira Institute FIOCRUZ
2. Department of Bacteriology, Oswaldo Cruz Institute FIOCRUZ
Corresponding author:Marise Dutra Asensi, Fundação Oswaldo Cruz Depto. de Bacteriologia / Av. Brasil, 4365 - 21045-900 - Rio de Janeiro / RJ

(Brazilian Ministry of Health).This hospital provides secondary and tertiary care for patients from different localities within the City and State of Rio de Janeiro. Patients came directly or were transferred from other hospitals.

Bacterial strains - A total of 116 isolates were obtained from stool (94) and blood (22) cultures from children who received medical care.

All isolates were characterized as salmonellas by the biochemical methods of Edwards & Ewing (3) and Costa & Hofer (2); the serological identification was done according to Kauffmann-White (16) and the nomenclature of serovars followed that of Le Minor (10).

Susceptibility testing - The antimicrobial susceptibility tests were performed by a disk diffusion method according to NCCLS, 1984 (13). The following concentrations of the antimicrobial drugs (CECON) were used: ampicillin (Ap) - 10 mcg; cephalotin (Cf) - 30 mcg; cefoxitin (Cx) - 30 mcg; ceftriaxone (Cro) - 30 mcg; pefloxacin (Pf) - 5 mcg; gentamicin (Ge) - 10 mcg; amikacin (Am) - 30 mcg; sulfamethoxazole-trimethoprim (SxT) - 23.765/1.25 mcg; chloramphenicol (Cl) - 30 mcg; tetracycline (Te)- 30 mcg.

The Minimum Inhibitory Concentrations (MIC) were made out by dilution in solid media-Mueller-Hinton Agar (DIFCO), according to NCCLS, 1985 (14); the following six antimicrobials were used at concentrations ranging from 256 mcg/ml to 0,0625 mcg/ml: ampicillin (WYETH), gentamicin (SCHERING), chloramphenicol (CARLO ERBA), ceftriaxone (ROCHE), sulfamethoxazole (ROCHE), trimethoprim (ROCHE).

Quality control of the two susceptibility tests was carried out using standard strains of *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27953).

TABLE 1 - Frequency of *Salmonella* serovar isolates.

Serovars	Stools Nº (%)	Blood Nº (%)	Total Nº (%)
Group 04 (B)			
S. typhimurium	53 (56.38)	20 (90.90)	73 (62.93)
S. agona	6	2	8
S. heidelberg	2	-	2
S. bredeney	2	-	2
S. saintpaul	1	-	1
Subtotal	64 (68.08)	22 (100.0)	86 (74.13)
Group 07 (C1)			
S. infantis	6	-	6
S. mabandaka	1	-	1
S. ohio	1	-	1
S. oranienburg	4	-	4
S. oslo	1	-	1
Subtotal	13 (13.82)	-	13 (11.20)
Group 08 (C2 - C3)			
S. hadar	5	-	5
S. newport	1	-	1
S. emek	3	-	3
S. istambul	1	-	1
Subtotal	10 (10.63)	-	10 (8.62)
Group 09 (D1)			
S. berta	2	-	2
S. panama	1	-	1
Subtotal	3 (3.19)	-	3 (2.58)
Group 03, 10 (E1)			
S. give	2 (2.12)	-	2 (1.72)
Group 035 (O)			
S. adelaide	2 (2.12)	-	2 (1.72)
Total	94	22	116

RESULTS

The 18 serovars were distributed in six serogroups. The most frequent was 04, with 86 (74.13%) of the isolates. Of these, 73 (62.93%) were characterized as *S.typhimurium* (Table 1).

The antimicrobial resistance of *S.Typhimurium* (Table 2), was greater than 68% for almost all antibiotics, the exceptions being cefoxitin (1.35%), ceftriaxone (13.69%) and pefloxacin (56%).

Multiple resistance data, presented in Table 3, show that 64 (92.75%) of the *S.typhimurium* strains had the 5 to 9 markers of resistance, while the other serovars presented 1 to 5 markers in 16 (66.6%) of the strains.

A total of 29 multiple antimicrobial resistance patterns (Table 4) were determined, and 89.65% had the ampicillin marker.

Minimum inhibitory concentrations for *S.typhimurium* (Table 5) showed a high resistance level (>=16 Mcg/ml) to ampicillin (90.41%) and chloramphenicol (87.66%). The opposite occurred with ceftriaxone, where only 10 (13.69%) were resistant, the highest MIC being 64 mcg/ml (4.10%)

TABLE 2 - Antimicrobial resistance (%) of *Salmonella* isolates.

Antiicrobial Agents	*S. typhimurium* N = 73		*Salmonella sp.* N = 43		Total N = 116	
	Nº	%	Nº	%	Nº	%
Ampicillin	66	90.41	19	45.23	85	73.27
Cephalotin	64	86.48	15	35.71	79	68.10
Cefoxitin	1	1.35	0	-	1	0.96
Ceftriaxone	10	13.69	2	4.65	12	10.34
Pefloxacin	42	56.75	1	2.38	43	37.06
Gentamicin	64	86.48	11	26.19	75	64.45
Amikacin	61	82.43	7	16.66	68	58.62
Sulfametoxazole+ Trimethoprim	51	68.91	8	19.04	58	50.00
Chloramphenicol	64	86.48	9	2.38	73	62.93
Tetracycline	54	72.97	16	38.09	70	60.34
Total	73		43		116	

TABLE 3 - Antibiotic resistance of *Salmonella* serovars.

Serovar	Nº of markers									
	1	2	3	4	5	6	7	8	9	Total
S. typhimurium		4		1	6	11	11	30	6	69
S. agona	1				1	1	2	1		6
S. oranienburg		1	1			1	1			4
S. adelaide	1									1
S. hadar	4									4
S. bredeney		1								1
S. infantis		1	1		1	1	1			5
S. istambul	1									1
S. saintpaul				1						1
S. heidelberg	1									1
Total	8	7	2	2	2	8	15	31	6	93

TABLE 4 - Resistance patterns among *Salmonella* isolates.

Resistance Pattern	*Salmonella typhimurium* Nº	*Salmonella sp.* Nº	Total
Ap, Cf, Cro, Pf, Ge, Am, SxT, Cl, Te	6	-	6
Ap, Cf, Cro, Pf, Ge, Am, Cl, Te	2	-	2
Ap, Cf, Pf, Ge, Am, SxT, Cl, Te	28	-	28
Ap, Cf, Cro, Ge, Am, SxT, Cl, Te	-	1	1
Ap, Cf, Cx, Ge, Am, Cl, Te	1	-	1
SAp, Cf, Ge, Am, SxT, Cl, Te	7	2	9
Ap, Cf, Pf, Ge, SxT, Cl, Te	2	1	2
Ap, Cf, Cro, Ge, Am, SxT, Cl	1	1	2
Ap, Cf, Cro, Ge, Am, Cl	1	-	1
Ap, Cf, Ge, Am, SxT, Cl	5	-	5
Ap, Cf, Ge, Am, Cl, Te	3	1	4
Ap, Pf, Ge, Am, Cl, Te	1	1	2
Ap, Cf, Pf, Ge, SxT, Te	1	-	1
Ap, Cf, Ge, SxT, Cl, Te	-	1	1
Ap, Cf, Ge, Am, Cl	4	-	4
Ap, Cf, Pf, Ge, Am	1	-	1
Ap, Pf, Ge, Cl, Te	1	-	1
Ap, Cf, SxT, Cl, Te	-	1	1
Ap, Cf, Ge, Am, SxT	-	1	1
Ap, Cf, Ge, Am	1	-	1
Ap, Cf, Ge, Te	-	1	1
Ap, Cf, Te	-	1	1
Ap, Cf, Ge	-	1	1
Ap, Cl	1	-	1
SxT, Te	1	-	1
Cl, Te	2	-	2
Ap, Cf	-	3	3
Ap	-	3	3
Te	-	5	5
Total Nº Resistants	69 (74.19)*	24 (25.81)*	93
Total Nº Sensibles	4	19	23
Total Nº Strains	73	43	116

* Percentage from the total number of resistants strains (93)

TABLE 5 - Minimal inhibitory concentrations for *S. typhimurium* isolates.

Antimicrobial Agent	Minimal inhibitory concentration Mcg/ml								
	>=256	128	64	16	8	4	2	1	<1
Ampicillin	65 (89.04)**	-	-	1	2	2	1	2	-
Chloramphenicol	55 (75.34)	9	-	-	7	-	-	2	-
Gentamicin	28 (38.35)	4	18	8	-	2	-	-	5
Sulfamet. + Trimet.*	27 (36.98)	4	9	9	8	4	5	1	5
Ceftriaxone	-	-	3	6	14	-	6	3	40 (54.79)

* Proportional concentration of S: [19:1]
** Percentage calculated from the total of 73 strains

for three strains. Intermediate levels of resistance to Gentamicin and sulfamethoxazole -trimethoprim were observed, with aproximately 35% of the specimens presenting MIC >=256 mcg/ml.

DISCUSSION

The children which provided the specimens for this study were from the lower socio-economic population which seeks medical care in public hospitals; most were undernourished, suffered from recurring infections and had a history of re-hospitalization. Most of the hospitalized patients presented moderate to severe undernourishment combined with various types of infections, including gastroenteritis and complications arising from septicemia and meningoencephalitis. Many of the latter died as a consequence of infection.

The serovar most frequently encountered in the hospital was *S.typhimurium* (62.93%) and the result is in accordance with that of other investigations (6, 15, 19, 8 and 9).The high frequency of *S.typhimurium* demonstrates its elevated infectious potential as well as its capacity to adapt to the environment; both features are influenced by genetic factors for multiple resistance to antibiotics.

The others serovars mentioned in this study have been described by Fernandes & Gales (5) as important pathogens in Brazil. In their study, these authors showed that 25 (96%) of the salmonellosis cases were due to *S.agona* and 4% to *S.oranienburg*. Other investigations (11; 20; 17) have shown that the importance of these different serovars varies among different regions.

The antimicrobial susceptibility was higher for the non-*S.typhimurium* serovars and most of these strains probably represent community-acquired infections. The predominant serovars had a high rate of resistance and most were from patients with nosocomial infections. This fact is indicated by the resistance to amikacin, used almost exclusively in hospitals. This type of situation may lead to the development of resistant strains capable of transferring R plasmids (18).

The majority of the isolates had multiple markers of resistance. The predominant multi-resistance pattern for *S.typhimurium* was Ap,Cf,Pf,Ge,Am,SxT,Cl,Te, found in 28 (38.35%) of the 73 isolates. Our results are similar to those form a study carried out in Iran (4). Also , 58 isolates of *S.typhimurium* (79.45%) and 6 isolates (13.95%) of the remaining serovars showed resistance to the beta lactam antibiotics (ampicillin, cephalotin) and the aminoglycosides (gentamicin, amikacin), indicating that these were nosocomial in origin (Table 4).

Half of the strains were isolated between 1990 and 1992; most of the pefloxacin resistant strains (66%) were isolated during this period.

The low resistance pattern to the second and third generation cephalosporines probably occurred because recently (last two years) these antibiotics have been used in hospitals as the drugs of choice for the treatment of septicemia, meningoencephalitis and others invasive infections caused by *Salmonella sp* in children..

Antimicrobial resistance to *Salmonella sp* has been shown in various countries and the data were compiled by Murray (12), who reported similar resistance rates for Brazil, Thailand, Chile and Egypt.

The minimum inhibitory concentration test demonstrated a high level of resistance to ampicillin and chloramphenicol suggesting that these drugs have been used over a long period in our hospital. Anderson et alli (1) have emphasized that high rates of MIC could be explained by multiple copies of specific R plasmids.

Two important aspects should be stressed concerning the emergence of antimicrobial resistance. First, the high resistance to "first choice" drugs has made it difficult to treat seriously ill *Salmonella*-infected patients with these drugs and has led to the utilization of third generation cephalosporines. This may lead cause the emergence of new multiple-resistant strains of this pathogen. Secondly, a study by Holmberg et al (7) has shown that both disease severity and the duration of hospitalization are increased with multiple resistant pathogens, a fact that leads to increased costs.

RESUMO

Foram caracterizadas sorologicamente e testadas para a susceptibilidade a antimicrobianos, 116 amostras de *Salmonella* isoladas de coproculturas e hemoculturas efetuadas de crianças durante o período de maio de 1987-julho 1992 no Instituto Fernandes Figueira, Brasil. Detectou-se seis sorogrupos (04; 07; 08; 09; 03; 10 e 035) representados por 18 serovares, sendo a *S. typhimurium* (62.93%) a mais freqüente, seguida da S. agona (7.75%). Os antimicrobianos utilizados foram a ampicilina (Ap), cefalotina (Cf), cefoxitina (Cx), ceftriaxona (Cro), pefloxacin (Pf), gentamicina (Ge), amicacina (Am), sulfametoxazoletrimetropim (SxT), cloranfenicol (Cl e tetraciclina (Te). 94.52% das cepas de *S. typhimurium* apresentaram determinantes de resistência. Foram encontrados 29 padrões de resistência diferentes, sendo o mais freqüente para *S. typhimurium* o Ap, Cf, Ge, Am, Sxt, Cl, Te, Pf (40.47%) e para os outros serovares o Te (20.83%). A determinação da concentração mínima inibitória realizada para cinco dos antimicrobianos citados evidenciou altos níveis de resistência para Ap e Cl, contrapondo-se a níveis mais baixos obtidos para Ge, Sxt e Cro.

Palavras chaves: *Salmonella*, serovares, frequência, susceptibilidade a antimicrobianos.

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NON PRODUCTION OF SHIGA-LIKE TOXINS BY *ESCHERICHIA COLI* SEROGROUP O_{26}

Halha O. Saridakis[1]

SUMMARY

We have investigated the production of Shiga-like toxins (SLTs) by 41 strains of *Escherichia coli* O_{26} belonging to serotypes O_{26}:H$^-$, O_{26}:H_{10}, O_{26}:H_{11}, O_{26}:H_{32} and O_{26}:H_{NT} which were isolated from human and calf specimens and also from meat products. The production of SLTs was tested on HeLa and Vero cell cultures and by DNA hybridization with probes specific for SLT_I and SLT_{II}. None of the Brazilian strains were SLTs producers, whereas 8 reference strains of *E. coli* O_{26}:H_{11} were confirmed as SLT_I producers. These results suggest that SLT may not be an important virulence factor among Brazilian *E. coli* O_{26} isolates.

Key Words: VTEC, SLT, Shiga-like toxins, EPEC, EHEC.

Diarrheagenic *Escherichia coli* strains can be classified into 5 categories according to their pathogenic mechanisms: enterotoxigenic (ETEC); enteroinvasive (EIEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC) and enteropathogenic (EPEC) (1).

The EPEC group, though widely studied, has not been well defined yet, due to variations in virulence factors among its different serogroups and/or serotypes. In the specific case of serogroup O26, the main variation reported is the production of SLTs (Shiga-like toxins or Verotoxins) by some strains belonging to serotypes O_{26}:H_{11} and O_{26}:H$^-$.

Since the first description of O_{26}:H_{11} as an SLT producer(2), this serotype as well as O_{26}:H$^-$ have been frequently associated with hemorrhagic colitis and the hemolytic uremic syndrome (HUS) in Europe and North America (3, 4, 5, 6, 7, 8, 9).

In the present work, we investigated the production of SLTs by strains of serotypes O_{26}:H_{11}, O_{26}:H$^-$, O_{26}:H_{32}, O_{26}:H_{10} and O_{26}:H_{NT} (Table I). These strains were isolated in the cities of São Paulo, Marília and Londrina and mantained in TSB-glycerol (20%) at -70°C. Eight *E.coli* reference strains from Europe and North America known to be SLT_I producers were used (H_{30}, H_{19}, plus six strains kindly supplied by Dr. L. R. M. Marques from the Instituto Adolfo Lutz, SP). The ability to produce SLTs was tested on HeLa and Vero cells cultures (10) and by colony hybridization with specific probes for SLT_I (pNN37-19) and SLT_{II} (pNN110-18) (11).

TABLE 1 - Serotypes and strains sources.

Serotype	Source		
	Human	Calf	Meat Product
O_{26}:H$^-$	16	-	-
O_{26}:H_{10}	1	-	-
O_{26}:H_{11}	9	2	-
O_{26}:H_{32}	1	-	10
O_{26}:H_{NT}	2	-	-

Production of SLTs by the cell-culture method was not detected for any of the Brazilian strains. Likewise, none of Brazilian strains hybridized with SLT_I or SLT_{II} probes. The eight reference strains, however, were confirmed as SLT_I producers.

1. Dept. Pat. Geral - CCB - Universidade Estadual de Londrina - Campus Universitário - Cx. Postal 6001 - Londrina - PR.

Silva et al. (12), studying the production of cytotoxins by enteropathogenic *E. coli* in São Paulo, obtained negative results with O_{26}:H_{11} and O_{26}:H$^-$ strains tested on Vero cell cultures. More recently, Giraldi et al. (13), also in São Paulo, did not detect any SLT producers among O_{26}:H_{11}, O_{26}:H_{10} and O_{26}:H$^-$ serotypes using HeLa and Vero cell-culture assays.

Our results are thus in agreement with those previously described in our country and suggest that the production of SLTs is not a common feature among Brazilian *E. coli* O_{26} isolates. From this stand point, the 41 strains studied in the present investigation may not be considered as EHEC.

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RESUMO

Produção de Toxinas Shiga-Like por *Escherichia coli* do Sorogrupo O_{26}

Foi investigada a produção de SLTs por 41 amostras de *Escherichia coli* O_{26}, pertencente a diferentes sorotipos, isolados de humanos, bezerros e produtos cárneos. Foram realizados ensaios em culturas de células HeLa e Vero e hibridização de colônias com sondas para SLTI e SLTII. Nenhuma das amostras brasileiras foram produtoras de SLTs, enquanto oito amostras padrão O_{26}:H_{11} confirmaram a produção de SLTI. Este resultado sugere que produção de SLTs não é característica freqüente dentre amostras brasileiras deste sorogrupo.

Palavras-chave: VTEC, SLT. Toxinas Shiga-like, EPEC, EHEC.

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ISOLATION OF AUXOTROPHIC AND NIF- MUTANTS OF *BACILLUS AZOTOFIXANS*

Beatriz Dolabela de Lima[1]
Maria do Carmo de Freire Bastos*

SUMMARY

Auxotrophic and Nif- mutants of *Bacillus azotofixans* were obtained from EMS mutagenized cultures using a penicillin enrichment method developed for this microorganism. The frequency of isolation of auxotrophic mutants (0.19%) was three times higher than that obtained for the Nif-mutants (0.063%), although both frequencies were extremely low. Most auxotrophic mutants (8) required amino acids for growth; 3 required nitrogeneous bases and 2 required vitamins. These mutants will be used in further experiments of gene transfer with the aim of locating the *nif* genes on the bacterial chromosome of this species.

Key words: *Bacillus azotofixans,* auxotrophic mutants, Nif- mutants.

INTRODUCTION

Biological nitrogen fixation, i.e., conversion of atmospheric nitrogen to ammonia by the complex nitrogenase, is carried out exclusively by prokaryotic organisms. A complete understanding of N_2 fixation requires a knowledge of the number of genes involved, the organization and control of these genes and the identification of the function of each gene. The organization and regulation of N_2 fixation (*nif*) genes has been studied in most detail in *Klebsiella pneumoniae*, where 17 *nif* genes have been identified in addition to the structural genes for nitrogenase, *nif*H, D and K. These genes are clustered in a 24 kb region of the chromosome and are organized in seven major transcriptional units or operons (12, 26). These genes have been shown to map between the *shi*A (shikimate permease) gene and the *his*D (L-histidinol:NAD+ oxireductase) gene(1, 24). Fine-structure mapping of *nif* genes of *K. pneumoniae* has been achieved by analyses of point mutations (2, 7, 24), Mu-induced mutations (3, 15), complementation analyses (2, 9), transductional analyses (7, 24, 25) and gene cloning experiments (12, 14, 23).

Among *Bacillus* sp strains, N_2 fixation is restricted to the species *B. polymyxa* (5), *B. macerans* (28), *B. circulans* (8) and *B. azotofixans* (17). In a previous study, homology to *nif*H and *nif*D genes of *K. pneumoniae* was found in the chromosome of 22 Nif+ strains of *B. azotofixans* and the possibility of reiteration of *nif*H was suggested (19). *nif*H and *nif*D code for a subunit of nitrogenase reductase and dinitrogenase, respectively (11). No homology was found between the chromosomal DNA of *B. azotofixans* and other *K. pneumoniae nif* genes. Although in most N_2-fixing organisms the homology to *K. pneumoniae nif* genes is limited to the structural genes (4, 14, 16,

1. Present address: Departamento de Biologia Celular, Universidade de Brasilia, Brasilia, DF.
* Corresponding author
Departamento de Microbiologia Geral, Instituto de Microbiologia, UFRJ, CCS, Bloco I, Cidade Universitaria, CEP 21944-970, Rio de Janeiro, RJ.

21, 23), a similar number of genes (20 genes) is probably involved in N_2 fixation in these organisms, including *B. azotofixans*.

As a first step for determining the total number of genes involved in N_2 fixation in *B. azotofixans*, it is necessary to map these genes on the bacterial chromosome. The mapping is generally performed by genetic transfer of the genes either by transduction (24, 25) or by conjugation (1, 13, 27) and by analysis of linkage groups. In this report, we describe the isolation of auxotrophic and Nif$^-$ mutants of *B. azotofixans* type-strain P3L-5 which can be used in further studies for the genetic transfer of nitrogen fixation markers between strains of this species.

MATERIAL AND METHODS

Bacterial strain and culture conditions

Bacillus azotofixans type-strain P3L-5 (17) was used in all experiments. For the growth of *B. azotofixans* cells the following media were used: a, TBN (18); b, TBNC (TBN medium supplemented with casaminoacids at 0.5% mg/ml); c, BL ($MgSO_4.7H_20$, 0.2 g; $FeCl_3.6H_2O$, 0.02 g; $NaMoO_4$, 0.005 g; $CaCl_2.2H_2O$, 0.08 g; glucose, 10 g; K_2HPO_4, 0.8 g; micronutrient solution (6), 1 ml; distilled water, 1,000 ml; final pH 7.4); d, MM (BL medium supplemented with $(NH_4)_2SO_4$ at 5 mg/ml). All incubations were performed at 32°C.

Ethyl methanesulfonate (EMS) survival curve

A cell suspension containing approximately 10^9 cells/ml was prepared in TBN medium supplemented with Tris/HCl 0.2M pH 7.8. A 2.0 ml aliquot of this bacterial suspension was removed and used as control. Three 2.0 ml aliquots of the same suspension were treated with EMS (Kodak) 140 uM for 1 h, 2 h and 3 h, respectively. These aliquots, as well as the control, were diluted in saline solution (0.85% NaCl, w/v) and 0.1 ml of each dilution was plated onto TBN. After 48 h of incubation, 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.

Isolation of Nif$^-$ and auxotrophic mutants

Strains containing point mutations were isolated after penicillin enrichment of mutagenized cultures. After mutagenesis, cells were washed twice in saline solution and grown to stationary phase in 400 ml TBNC medium. The mutated culture was then washed, resuspended in BL medium and grown to 3 to 5 x 10^8 cells/ml. Penicillin G was added at a final concentration of 10 mg/ml. The culture was incubated for 4 h and cells were collected by centrifugation, washed twice in saline solution, resuspended and grown overnight in TBNC medium. Cells were washed twice in saline solution and resuspended in either TBNC or BL medium to 10^8 cells/ml.

a) *Nif$^-$ mutants:* A cell suspension in BL medium was diluted, plated on BL medium supplemented with $(NH4)_2SO_4$ at 100 µg/ml and incubated for 72 h in GasPak (BBL) anaerobic jars adapted for gas phase exchange. After two subsequent washings (evacuation followed by refilling with 100% N_2) these jars received the final N_2-CO_2-H_2 mixture (80%-10%-10%). Any remaining oxygen was removed by the GasPak catalyzer and maintenance of anaerobic conditions was monitored by the GasPak methylene blue redox indicator. Tiny colonies were picked as presumptive Nif$^-$ mutants, purified in TBNC medium and retested at least twice on BL medium supplemented with $(NH_4)_2SO_4$ at 100 µg/ml as previously described.
b) *Auxotrophic mutants:* A cell suspension in TBNC medium was diluted and plated on TBNC. After 48 h of growth the colonies obtained were simultaneously transferred to MM and TBNC plates with the aid of sterilized toothpicks. Plates were incubated for 72 h. Colonies unable to grow in MM were identified in TBNC plates, retested al least twice for growth on MM plates and characterized for auxotrophy.

Charaterization of auxotrophy

A cell suspension containing approximately 10^7 cells of each auxotrophic mutant was prepared in saline solution and plated on the surface of MM plates. A drop (10 µl) of three different solutions (vitamins, 1-5 mg/ml; amino acids, 1 mg/ml and nitrogeneous bases, 50 mg/ml) was applied on the bacterial layer. After incubation for 72 h it was possible to verify which solution had allowed the mutants to grow. A test with each independent component of the solution allowed us to determine the growth factor required for each mutant.

Acetylene reduction tests

Acetylene reduction was tested by measuring the ethylene evolved from cultures in 18-ml vials as previously described (17). The vials were incubated for 24 h at 32°C. *B. azotofixans* P3L-5 was included in all assays as the positive control. Results were corrected for residual ethylene in the acetylene gas.

Biochemical tests

The mutant strains were confirmed as *B. azotofixans* according to the taxonomic tests recommended by Seldin & Penido (18). The tests used were D-xylose, L-arabinose and mannitol fermentations and nitrate reduction.

RESULTS AND DISCUSSION

The EMS survival curve for *B. azotofixans* strain P3L-5 is shown in Figure 1. The time of EMS treatment allowing approximately 5% survival corresponded to 2 h and was used in all experiments to obtain the mutants. All treatments of strain P3L-5 with EMS (140 μM) for 2 h consistently yielded from 4% to 6% survivors.

Fourteen independent mutagenesis experiments with EMS undertaken in this study yielded 7,927 colonies which were tested for the ability to fix atmospheric nitrogen. Five Nif⁻ mutants were isolated: two (N8 and N30) displayed less than 10% and three (N17, N19 and N23) less than 21% nitrogenase activity when compared to the wild type strain (Table 1), although they exhibited a normal growth in the medium used to test N_2 fixation. Mutagenesis of strain P3L-5 with EMS yielded 0.063% Nif⁻ mutants. Four mutants (N3, N14, N16 and N_20) displayed nitrogenase activity ranging from 23% to 29% of the value observed for strain P3L-5. However these mutants were not considered as Nif⁻, but as mutants with low nitrogenase activity. Based on biochemical tests all mutants were confirmed as *B. azotofixans*. All 9 mutants were also shown to be able to grow in MM medium, exhibiting a normal growth rate in this medium (data not shown). When the Nif⁻ mutants were evaluated for reversion to the wild type phenotype, no reversion could be detected under the conditions of our experiments.

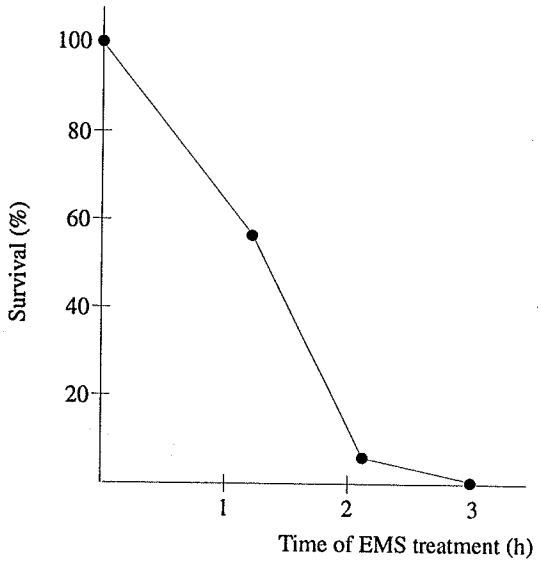

FIGURE 1 - EMS survival curve for *B. azotofixans* P3L-5.

TABLE 1 - Acetylene reduction of mutants with deficient nitrogen-fixation activity. Averages determinations.

Mutant	Acetylene reduction (nmoles ethylene/ml/h)	Percentage of nitrogenase activity in comparison to the wild type strain
N8	32.2±5.1	6.6
N30	18.1±1.3	3.7
N17	58.6±8.7	12.0
N19	68.3±9.0	14.0
N23	97.6±8.5	20.0
N3	112.2±15.4	23.0
N14	141.5±13.6	29.0
N16	134.2±10.2	27.5
N20	122.0±10.1	25.0
P3L-5	488.0±50.4	100.0

Twelve independent experiments of mutagenesis with EMS were carried out for the induction of auxotrophic mutants. A total of 8,855 colonies were tested for the ability to grow in MM and rich media. By this procedure 17 auxotrophic mutants were isolated, at a frequency of 0.19%. The nutritional requirement of each mutant is indicated in Table 2. Most auxotrophic mutants (47.05%) required amino acids for growth, 17.64% required nitrogeneous bases and 11.76% required vitamins. The growth factors required for 4 mutants (23.52%) could not be determined. This result suggests that these mutants must have complex nutritional requirements.

All 17 auxotrophic mutants were tested for their acetylene reducing ability and compared to the wild type strain. Based on biochemical tests, all auxotrophic mutants were confirmed as *B. azotofi-*

TABLE 2 - Nutritional requirement and nitrogenase activity of the auxotrophic mutants compared to the wild type strain P3L-5. Average of three determinations.

Nutritional Requirement	Mutant	Nitrogenase Activity (% of the wild type)
Adeline (Ade)	MA34	201
	MA36	137
Purine (Pur)	MA01	115
Thiamine (Thi)	MA15	82
Arginine (Arg)	MA10	57
	MA19	42
	MA26	38
Tryptophan (Trp)	MA08	44
Methionine (Met)	MA04	27
	MA05	23
Riboflavin (Rib)	MA09	26
Glutamine (Gln)	MA17	26
Histidine (His)	MA18	8
Not determined	MA14	12
	MA20	4
	MA31	12
	MA33	2
P3L-5	Wild type	100

xans. Of the 17 mutants, 5 (MA18, MA14, MA20, MA31 and MA33) behaved as Nif$^-$. With exception of the Pur$^-$, Ade$^-$ and Thi$^-$ mutants, all other mutants showed reduced nitrogenase activity, which varied from 23 to 57% of the value obtained with strain P3L-5. However, this apparent Nif- phenotype or reduced nitrogenase activity shown by most auxotrophic mutants can be explained by the poor growth of these mutants in the medium used to test N_2 fixation, which resulted in lower values of acetylene reduction. On the other hand, auxotrophic mutants with normal nitrogenase activity exhibited normal growth in this medium, which resulted in greater values of acetylene reduction (data not shown) comparable to those for the wild type. Based on biochemical tests, all auxotrophic mutants were confirmed as *B. azotofixans*.

Auxotrophic mutants of strain P3L-5 were isolated at a frequency three times higher than that of Nif$^-$ mutants. The difficulty in isolating Nif$^-$ mutants relates to the nondistinctive character of Nif$^-$ colonies on N-free agar medium, except for their smaller size. In addition, mutants phenotypically deficient in nitrogen-fixation may occur due to mutations affecting respiration, motility, carbon metabolism, NH4+ assimilation, N source utilization, etc, thus decreasing the frequency of nif-specific mutants in the mutagenized population (11).

The frequencies of mutants isolated in the present study were low when compared to the frequencies reported by other authors (from 0.3% to 0.8%). However in most studies on N_2 fixation described in the literature, mutants were isolated by insertional mutagenesis through transposable elements which are known to produce mutations at higher frequencies. Different transposable elements have been shown to have different site-specificities of insertion both within particular genes and in different genes within an operon. Therefore, prophage Mu (3, 15), Tn5, Tn7 and Tn10 (10, 22) have been used to produce insertion mutations. Transposable elements are usually introduced into new hosts carried by plasmids unable to replicate in the new genetic background. However, transposable elements could not be used to generate mutants of *B. azotofixans* since no mechanism of genetic transfer has been characterized so far in this species. Therefore, chemical mutagenesis was used instead. Few studies describe the isolation of Nif$^-$ mutants by chemical mutagenesis. In these cases, the frequencies reported were either comparable to or smaller than the frequencies found in our study.

The mutants described in this report will be used in further experiments that will be performed to generate transductional mapping data, using a phage recently isolated from the strain P3L-5 (20). These studies will certainly contribute to our understanding of the genetic basis of N_2 fixation in *B. azotofixans*. Furthermore the Nif$^-$ mutants should help to answer the question of whether N_2-fixing *Bacillus* species stimulate plant growth yields through nitrogen fixation.

RESUMO

Isolamento de mutantes auxotroficos e Nif$^-$ de *Bacillus azotofixans*

Mutantes auxotróficos e Nif$^-$ de *Bacillus azotofixans* foram isolados a partir de culturas tratadas com EMS, empregando-se um metodo de enriquecimento com penicilina, desenvolvido para este microrganismo. A frequência de obtenção de mutantes auxotróficos (0,19%) foi cerca de três vezes maior do que a de obtenção de mutantes Nif$^-$

(0,063%), embora ambas as frequências fossem extremamente baixas. Os fatores de crescimento exigidos pelos mutantes auxotróficos incluiam aminoácidos (47,05%), bases nitrogenadas (17,64%) ou vitaminas (11,76%). Estes mutantes serão posteriormente usados em experimentos de transferência gênica, realizados com o objetivo de se localizar os genes *nif* no cromossomo desse microrganismo.

Palavras-chave: *Bacillus azotofixans*, Mutantes auxotroficos, Nif- Mutantes.

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ENTEROTOXIGENIC STAPHYLOCOCCI FROM FOOD HANDLERS WORKING IN AN INDUSTRIAL KITCHEN IN BELO HORIZONTE, MG (BRAZIL)

Maria Lúcia Pereira[1]
Luiz Simeão do Carmo[1a]
Maria Auxiliadora de Lara[1]
Ricardo Souza Dias[1]
Merlin S. Bergdoll[2]

SUMMARY

Fifty-five healthy food handlers working in an industrial kitchen in Belo Horizonte, Minas Gerais, were examined for the carriage of enterotoxigenic staphylococci. Thirty-two (58.2%) of the 55 food handlers were *Staphylococcus aureus* carriers, with 17 (30.9%) carriers of enterotoxigenic staphylococci, 8 in their nares, 9 in their throats, and 7 under their fingernails. Eleven individuals carried enterotoxigenic strains in only one of the three sites cultured, five carried them in two of the three sites, and one carried an enterotoxigenic strain (enterotoxin B) in all three sites. Four strains produced enterotoxin A, 10 strains produced enterotoxin B, 4 strains produced enterotoxin C, and 6 strains produced both enterotoxins A and B. The fact that 30.9% of the food handlers in this particular kitchen were colonized with enterotoxigenic staphylococci, in which 80,000 meals were produced per day in 1992, is a very high percentage of carriers.

Key words: *Enterotoxigenic staphylococci*, food handlers, healthy carriers.

INTRODUCTION

Staphylococcal food poisoning, caused by toxins produced by the staphylococci growing in different types of foods, is a leading cause of food poisoning worldwide. (3) Various dairy products, bakery goods, different types of meats, sea foods, and ready to eat meals have been implicated in this type of food poisoning. In recent years many cases of staphylococcal food poisoning have occurred in Brazil. (4,6,15,19) A thorough investigation of these outbreaks has not been done, such as attempting to determine the source of the staphylococci that was responsible for the illnesses. Although these organisms may come from different sources, humans are a major source, as they may be present in the nares, in the throat, and/or on the skin. The percentage of people who are carriers of staphylococci may vary from 30 to 50%, but inadequate information is available about the enterotoxigenicity of human isolates. Although food handlers have been studied, (10,12-14,22,23) the percentage of individuals carrying enterotoxigenic *S. aureus* was given in only one instance, 88 (22%) of 400 food handlers examined. (14) Although staphylo-

1. Fundação Ezequiel Dias, Laboratório de *Staphylococci,* Rua Conde Pereira Carneiro 80, Gameleira, CEP 30510-010, Belo Horizonte, MG, Brasil.
2. Food Research Institut, University of Wisconsin, 1925 Willow Drive, Madison, WI 53706, USA.
a. Author to whom all correspondence should be sent

cocci may be present in raw foods, these are commonly destroyed during the cooking or processing of the food. Usually the foods are contaminated after the cooking by food handlers or in the preparation of foods that do not require heating. In most instances, food handlers are not given proper training in the handling of foods to prevent food poisoning.

This investigation was undertaken because no information is available about the frequency of carriage of enterotoxigenic staphylococci by food handlers in Brazil. A large industrial kitchen in Belo Horizonte was chosen for this study because of the large amount of food needed for their catering service as well as for individual meals.

MATERIALS AND METHODS

Food handlers - Fifty-five food handlers working in a large industrial kitchen in Belo Horizonte, Minas Gerais, were selected for participation in the project. There was no indication of staphylococcal infections in any of the food handlers and none had used any medications for a period of thirty days. Their nares, throats, and fingernails were cultured using sterile swabs.

Isolation of staphylococci - Each swab was placed in a tube of tryptic soy broth containing 10% NaCl and incubated for 24 hours at 37°C. The cultures were streaked on Baird-Parker plates and incubated for 48 hours at 37°C. Five typical colonies (jet black to dark grey, smooth, convex, entire margins, off-white edge; may show an opaque zone and/or a clear halo beyond the opaque zone) and three atypical colonies (gray and mucoid) were selected for further testing. Each colony was transferred to two test tubes containing 1 ml of BHI broth and incubated for 24 hours at 37°C. Tests for coagulase and thermonuclease (TNase) production, anaerobic fermentation of glucose and mannitol, and production of hemolysin using sheep blood were carried out. Any colonies that were positive for these characteristics were considered *S. aureus* and were tested for enterotoxin production.

Enterotoxin production - Inocula were prepared by incubating the staphylococci in brain heart infusion (BHI) broth over night at 37°C. Membrane-over-agar plates were prepared with 25 ml of BHI-agar and covered with a membrane disk made from Spectra/Por membrane dialysis tubing, 6000-8000, 100 mm flat width (Thomas Scientific, Philadelphia, PA, USA)(20). The membrane was inoculated with 0.5 ml of the inoculum and the plates were incubated at 37°C for 24 hours. The cultures were removed from the membranes by washing with 2.5 ml of 0.01 M Na_2HPO_4 in three steps using 1 ml, 1 ml, and 0.5 ml of the phosphate buffer. The cultures were centrifuged and the culture supernatant fluids used for enterotoxin testing.

Enterotoxin testing - The optimum-sensitivity-plate (OSP) method was used(20). In this method, 3 ml of agar (1.2%) is placed in 50 mm plastic petri plates with tight lids: wells are cut according to the original specifications. Specific antisera is placed in the center well, enterotoxin (4 µg/ml) is placed in the two smaller wells, and culture supernatant fluids are placed in the four larger outer wells. Different plates are required for each enterotoxin (SEA, SEB, SEC, SED). The plates are placed in a humidified container and incubated overnight at 37°C. Positive reactions are determined from precipitin lines formed by the culture supernatant fluids that join with the control lines.

RESULTS

Classification as *S. aureus* - All colonies that were coagulase and TNase-positive, fermented glucose and mannitol anaerobically, and were hemolytic on sheep blood agar were considered *S. aureus*. All colonies from the same individual site that were classified as *S. aureus* were combined for enterotoxin production. Although atypical colonies from only one individual were classified as *S. aureus* and used for enterotoxin testing, atypical colonies should not be ignored in the testing.

Frequency of isolation - *S. aureus* was isolated from at least one site in 30 (54.5%) of the 55 food handlers cultured. Isolates from at least one site in 17 (30.9%) of the 55 individuals were enterotoxigenic. Of the 56 *S. aureus* isolated, 24 (42.9%) were enterotoxigenic.

S. aureus was isolated from under the fingernails of 20 individuals,7 (35.0%) of which carried enterotoxigenic staphylococci, from the nares of 17 individuals, 8 (47.1%) of which carried enterotoxigenic staphylococci, and from the throats of 19 individuals, 9 (47.4%) of which carried enterotoxigenic staphylococci. Eleven individuals who carried enterotoxigenic staphylococci carried them in only one site, with five individuals colonized in two sites, and one individual colonized in all three sites (Table 1).

TABLE 1 - Carriage of enterotoxigenic staphylococci by food handlers.

Carrier	Nasal		Throat		Finger Nail	
N°	SE[a]	Non-SE[b]	SE	Non-SE	SE	Non-SE
1	A	-	-	-	-	-
2	B	-	-	+	-	-
3	A + B	-	-	-	-	-
4	A	-	A	-	-	+
5	A	-	A + B	-	-	+
6	C	-	C	-	-	+
7	C	-	-	+	B	-
8	-	-	B	-	-	-
9	-	-	B	-	-	-
10	-	-	A + B	-	-	-
11	-	+	A + B	-	-	-
12	-	-	B	-	A + B	-
13	-	-	-	-	B	-
14	-	-	-	+	B	-
15	-	-	-	-	C	-
16	-	-	-	-	A + B	-
17	B	-	B	-	B	-

a - Type of enterotoxin produced
b - Non-enterotoxigenic staphylococci: -, none; +, carriage.

Enterotoxin production - Twenty-four *S. aureus* strains produced enterotoxin, with 18 producing one enterotoxin and six producing two, SEA and SEB (Table 1).

DISCUSSION

In recent years staphylococcal food poisoning in Brazil has become recognized and investigated. (4,6,15,19) Initially, only isolation of staphylococci from the implicated foods was done which was circumstantial evidence that the illnesses may have been due to staphylococcal food poisoning, providing the symptoms of the illnesses were characteristic of this type of food poisoning. Later, the staphylococcal were examined for the production of an enterotoxin, the cause of the poisoning (4,6,15,19). Finally, the foods were examined for the presence of enterotoxin, proof that the illnesses were actually due to staphylococcal food poisoning. (7,8)

One aspect of the investigations has been neglected, that is, how did the food become contaminated. It is generally recognized that the major source of the contaminating staphylococci is the food handler, (3) but this aspect has not been investigated in Brazil. One food that has been implicated in a number of staphylococcal food poisoning outbreaks in Minas Gerais is white cheese. It is well known that even pasteurized milk in Brazil may be contaminated with staphylococci, either from mastitic cows or from human handlers. The fact that in the examination of foods implicated in staphylococcal food poisoning, only enterotoxin A (SEA) was detectable. (7,8) implicates humans because staphylococci isolated from cows produce either enterotoxin C (SEC) and/or D (SED). (11,18) Without examination of the food handlers to determine the actual source of the contaminating staphylococci it is impossible to know their origin.

A number of studies have been done on the carriage of staphylococci by humans (a number of these done in Brazil, (1,2,5,9,16,17,21,25) the results showing that between 30 and 50% of humans are colonized with *S. aureus* at any given time. However, a relatively smaller percentage are colonized with *S. aureus* strains that may be enterotoxigenic, depending somewhat on the occupation of the individuals being examined. Several studies have been done on food handlers, (10,12-14,22,23) usually with inadequate information presented. Usually, the number of *S. aureus* isolates that were enterotoxigenic is given, but not the percentage of individuals who were actually colonized with them, which is a more important figure. Only one small study had been done in Brazil until this study. (12)

The percentage (58.2%) of food handlers in this study who were found to be colonized with *S. aureus* was above the percentage found by investigators in other countries (27.7-38.5%) (10,12,14,23) for human colonization with *S. aureus,* however, only the nares and/or throats were cultured. This is important, but not as important as the percentage (30.9%) of food handlers in this study who were colonized with enterotoxigenic staphylococci. This was higher than the 22% found by Mori et al., (14) which was the only reliable figure reported. The actual percentage of the *S. aureus* isolates that were enterotoxigenic was high also, and compares to the 57.1% Mori et al. (14) found to be enterotoxin positive. However, this may not be so important because people may be colonized in more than one site, and these could bear the same strain. Two people in this study carried the same strain in two sites and one person carried the same strain in all three sites.

The type of enterotoxin produced by the isolates is of importance because SEA is the major cause of staphylococcal food poisoning. (24) In nime outbreaks in Brazil in which the food was

examined for the presence of enterotoxin, only SEA was detectable. (7,8) The fact that 10 of the *S. aureus* strains isolated in this study produced only SEB was unexpected because the strains isolated from foods implicated in food poisoning seldom produced SEB alone, but usually with SEA. It is possible that some of the strains that produced SEB alone may have produced amounts of SEA below the detectable level of the OSP method used here. Only occasionally has SEB been involved in food poisoning, primarily because it is not readily produced in most foods. One outbreak was reported in Brazil due to SEB, (15) but on closer examination, the *S. aureus* strain was found to produce SEA also. The fact that 10 isolates did produce SEA is important as well as the fact that 4 isolates produced SEC. *S. aureus* isolates from four food poisoning outbreaks that occurred in Curitiba produced only SEC, (4) which implicated this enterotoxin in the food poisoning outbreaks. Although *S. aureus* strains were isolated from two foods implicated in food poisoning in Minas Gerais, the strains also produced SEA, the enterotoxin that was detected in the food. (7)

Although some countries do not allow individuals to handle foods if they are colonized with staphylococci, this is not a practical solution to the problem nor is it easy to control. The best solution is to institute training of food handlers in the proper handling of vulnerable foods to prevent their becoming contaminated. In addition instructions on the proper storage of such foods should be given. If foods are kept either above 45°C or below 4°C during storage any staphylococci present will not be able to grow and produce enterotoxin. (3)

RESUMO

Staphylococci enterotoxigênicos em manipuladores de alimentos de uma cozinha industrial em Belo Horizonte, MG (Brasil)

Cinqüenta e cinco indivíduos sãos, manipuladores de alimentos de uma cozinha industrial em Belo Horizonte, MG, foram examinados quanto a presença de estafilococos enterotoxigênicos.

Trinta e dois (58,2%) apresentaram-se portadores de *S.aureus* e 17 (30,9%) de *S.aureus* enterotoxigênico, estes, assim distribuídos: oito nasais, nove de cavidade orofaríngea e sete de leito subungüeal.

Onze manipuladores carrearam linhagens enterotoxigênicas em apenas um dos três sítios e um carreou linhagem produtora de enterotoxina B, concomitantemente em todos três sítios pesquisados.

Quatro cepas se mostraram produtoras de enterotoxina A, dez de enterotoxina B, quatro de enterotoxina C e seis de enterotoxina A e B.

Considerando-se que, 30,9% dos manipuladores de cozinha industrial que produzia em 1992, em Belo Horizonte, 80.000 refeições/dia, caracterizaram portadores de *S.aureus* enterotoxigênico, o resultado obtido pode ser considerado significativamente elevado.

Palavras-chave: *Staphylococci* enterotoxigênicos, manipuladores de alimentos, portadores sãos.

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PENICILLIN RESISTANT *STREPTOCOCCUS PNEUMONIAE* FROM PATIENTS WITH MENINGITIS

Silvana Tadeu Casagrande*
Ilka Maria Landgraf
Maria de Fatima Paiva Vieira

SHORT COMMUNICATION

SUMMARY

The susceptibility to antibiotics of 146 strains of *Streptococcus pneumoniae* isolate from cerebrospinal fluid (CSF) was studied. Seven strains, 3 of the resistant and 4 of the moderately susceptible phenotype, showed similar results for oxacillin by the disk diffusion method and for penicillin by the minimal inhibitory concentration method. Two strains showed opposite results when comparing the two methods.

All the other strains (137) were inhibited at an oxacillin concentration of 1ug/ml by the diffusion disk test.

Key words: *Streptococcus pneumoniae;* Penicillin susceptibility tests; Penicillin resistance; Oxacillin susceptibility test.

Streptococcus pneumoniae has been the third most frequent pathogen isolated from cerebrospinal fluid in the laboratory of the Institute Adolfo Lutz, São Paulo. Meningitis caused by *Streptococcus pneumoniae* has been widely treated with penicillin yet any degree of resistance to this drug is associated with a poor response and a high mortality, even after high dose penicillin therapy. This outcome is almost certainly due to the relatively poor penetration of penicillin into the cerebrospinal fluid, yielding concentrations lower than or close to the penicillin minimal inhibitory concentration (MIC) of the resistant strains(1,3,6).

Since 1960, there have been many reports penicillin resistant *Streptococcus pneumoniae* in Australia (4), New Guinea(2), South Africa(1,5), and in the United States(6). However, almost nothing is know about the resistance pattern of *Streptococcus pneumoniae* isolated in Brazil.Therefore, a retrospective study of *Streptococcus pneumoniae* susceptibility to penicillin and to a variety of antimicrobial drugs was carried out in the Instituto Adolfo Lutz, São Paulo.

A total of 146 strains of *Streptococcus pneumoniae* isolated from cerebrospinal fluid during the period of 1990 to 1993 was studied. The disk diffusion method was performed according to Bauer-Kirby and the National Committee for Clinical Laboratory Standards(NCCLS)(3,7) methods. The antibiotics and respective concentrations tested using disks were penicillin (10U) and oxacillin (1μg).

Penicillin minimal inhibitory concentration (MICs) were determined as described by NCCLS and Swenson(7,9) for all isolates that showed resistance to oxacillin in the disk diffusion method. Penicillin, obtained as reagent grade powder from Sigma Chemical CO., St. Louis, MO., was tested

* Corresponding Author: Instituto Adolfo Lutz - Seção de Bacteriologia - Av. Dr. Arnaldo, 351 - São Paulo - CEP 01246-902

at twofold incresing concentrations from 0.0015μg/mL to 8.0μg/mL. Isolates were considered susceptible when the MIC was <0.06μg/mL, moderatly resistant when the MIC was between 0.12 and 1.0ug/mL, and resistant when the MIC was >2.0μg/mL. The Minimal Bactericidal Concentration(MBC) for penicillin was determined according to Thrupp(11).

Of the 146 strains studied, 3(2.1%) were resistant to both penicillin (MIC 2-4μg/mL) and oxacillin (disk diffusion test). Four strains (2.7%) showed a moderate resistance to both penicillin and oxacillin. Two strains (1.4%) were moderatly resistant to penicillin by the MIC but resistant when tested for oxacillin. In the disk diffusion test, all but one were susceptible to penicillin. One of these penicillin-resistant strains also showed to be resistant penicillin by the MIC and to oxacillin.

According to Oxley(8), for whom penicillin disks do not provide acceptable predictive accuracy, using a 10U penicillin disk gave a correct identification of penicillin resistance was show only in 14% of the strains. However, using the 1μg oxacillin disk the success rate was 91%. This observation is in aggreement with our results for 9 strains, only 2 of these strains were resistant to penicillin and oxacillin by the disk diffusion test. However, 7 strains showed some degree of resistance to oxacilin but were susceptible to penicillin. Swenson et. al. (10) recommend that the oxacillin screening test should be used to detect penicillin resistance in *Streptococcus pneumoniae*, but not to differentiate between resistant and moderately resistant isolates. Taking into account the possible emergence of *Streptococcus pneumoniae* penicillin resistant strains, alternative therapics should be sought.

RESUMO

Resistência à penicilina em *Streptococcus pneumoniae* isolado de pacientes com meningites

Avaliou-se a susceptibilidade a antibióticos em 146 cepas de *Streptococcus pneumoniae* isoladas de líquido cerebroespinhal. Sete cepas (3 com o fenótipo de resistência e 4 com o fenótipo de susceptibilidade moderada) apresentaram resultados semelhantes para a oxacilina segundo o método da difusão em disco, e para a penicilina segundo o método da concentração mínima inibitória. Duas cepas tiveram comportamentos discrepantes ao comparar-se os resultados dos dois métodos.

Todas as outras cepas (137) apresentaram inibição de crescimento para uma concentração de oxacilina de 1 mg/ml, segundo o método da difusão em disco.

Palavras chaves: *Streptococcus pneumoniae*, testes de susceptibilidade à penicilina, resistência a penicilina, testes de susceptibilidade à oxacilina

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DETERMINATION OF THE RESPONSIBLE MOLECULAR ZONE FOR THE CHALCONES BACTERIOSTATIC ACTIVITY

Nora Beatriz Pappano
Olga Nélida Puig de Centorbi*
Ferdinando Héctor Ferretti

SUMMARY

In order to asses the molecular zones, responsible for the chalcones bacteriostatic action, the bioactivity of compounds with "acetophenone and benzaldehyde structure" was studied by the kineticturbidimetric method". The benzaldehydes and acetophenones assayed did not inhibit the *Staphylococcus aureus* ATCC 25 923 development but were efficient for *Escherichia coli* ATCC 25 922. The obtained results were satisfactorily interpreted by means of the stablished rule for bacteriostatic inhibition mechanism previously proposed. On the other hand, to facilitate the comparison of the similar structure substances it was appropriate to define the drug percentual bacteriostatic efficacy as Pbe = 100/MIC, where MIC is the drug minimal inhibitory concentration in μg/ml and 100 is an arbitrary percentual factor. When the Pbe of the appropriate pairs of acetophenones and benzaldehydes are analyzed, then it results Pbe (acetophenone) + Pbe (benzaldehyde) <<< Pbe (chalcone). The high Pbe of chalcones is chiefly due to the presence of the double bond $C_\alpha = C_\beta$ in the chalcone structure, which enables the electronic deslocalization of the molecule-B-ring over carbonyl group, thus increasing its polarization. It was concluded that, in the chalcones, the molecular zone involving the carbonyl group and the neighbour hydroxyl (2') is mainly responsible for the achievement of the substances activity.

Key Words: chalcone - bacteriostatic activity - acetophenone - benzaldehyde.

INTRODUCTION

Generally flavonoids, particularly chalcones and flavanones, are compounds that can be obtained from different organs of numerous plant species (1,5,8) and through various synthesis procedures (4). They proved to have important antiviral (13), cytoprotective (2), antiinflamatory (20), antifungal (9) and anticytotoxic (12) properties. Oganesyan et al. (15), in numerous reports on synthesis of heterocyclic compounds containing oxygen (chromone, chalcone, flavanone, coumarin and so on), demonstrated their antimicrobial properties. Early, many chalcones have been used by diverse investigators (10,19) to treat microbial infections; they related the drug therapeutic properties with the system formed by the carbonyl group and the double bond $C_\alpha = C_\beta$, which could interact with the microbial protoplasm producing the observed optimal effects.

Cátedra de Química-Física II
* Cátedra de Bacteriologia II, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera, 5.700-San Luis. Argentina.

On the other hand, previous results (16,18) about the bacteriostatic efficacy of simple chalcones against *Staphylococcus aureus* ATCC 25 923 and *Escherichia coli* ATCC 25 922 led us to suggest that the free hydroxyl groups could enhance the biological function of the carbonyl group or participate in other active regions of the molecules. It is well established that the knowledge of molecular regions responsible for the biological properties of a substance contribute to development of new drugs with probable high bioactivity.

In order to asses the molecular zones responsible for the chalcone bacteriostatic action, the bioactivity of compounds with "acetophenone and benzaldehyde structures" involved in the production of drugs with chalcone structure are studied.

MATERIALS AND METHODS

Microbial strains: *S. aureus* ATCC 25 923 and *E. coli* ATCC 25 922, were mantained by successive subcultures in tripticase soy agar (BBL).

Compounds employed: high purity were employed (Fluka, purity degree 99%): acetophenone (I),2-hydroxyacetophenone (II), 2,4 dihdroxyacetophenone (III), 2-hydroxy-4-methoxyacetophenone (IV), benzaldehyde (V), 3-hydroxybenzaldehyde (VI), 4-hydroxybenzaldehyde (VII) and 4-methoxybenzaldehyde (VIII).

Culture media: nutritive agar (Oxoid); nutritive broth (Oxoid) prepared in a phosphate buffer pH 7 (18).

Turbidimetric kinetic method: the 24 h developed culture in slant agar was transfered to 30ml of nutritive broth and incubated for 18 h at 35°C with permanent stirring in order to be used as inoculum. Erlenmeyers provided with a stop-current system containing 100 ml of nutritive broth plus progressive concentrations of the drug to be tested were cultured with 2 ml of inoculum and stirred in a culture chamber, leaving one without drug as control. Aliquots were extracted at intervals of 20 min for 5 h and the transmittance (T) was registered in a Spectronic 21 at 720 nm. These values of T were related with the number cfu/ml (N), through the expressions

$$\ln N_t = 27.4 - 10.3.\ T \qquad (1)$$

$$\ln N_t = 27.1 - 8.56.\ T \qquad (2)$$

for *S. aureus* (16) and *E. coli* (17), respectively, valid in the 0.2 0.95 T range. The initial number of cfu/ml (t=0) for each microorganism was: N_0 *(S. aureus)*: 6.18 x 10^7 cfu/ML; N_0 *(E. coli)*: 1.6x10^8 cfu/ml and the temperature was 35° C.

RESULTS AND DISCUSSION

The benzaldehydes and acetophenones assayed did not inhibit the *S. aureus* development but were efficient for *E. coli*. The number of cfu/ml at different times was obtained by the expressions of the turbidimetric kinetic method. Considering the microbial growth law

$$\ln N_t = \ln N_0 + \mu.t \qquad (3)$$

where t: time in min; N_0: cfu/ml for t=0; N_t: cfu/ml for a time= t; μ: specific growth rate in min^{-1}, from the 1n N_t vst plot during the growth exponential phase, the values for the *E. coli* specific growth rates in media plus progressive concentrations of the drugs to be assayed were obtained. In Figure 1, as example, the results of the *E. coli* growth tests in presence of acetophenone are shown.

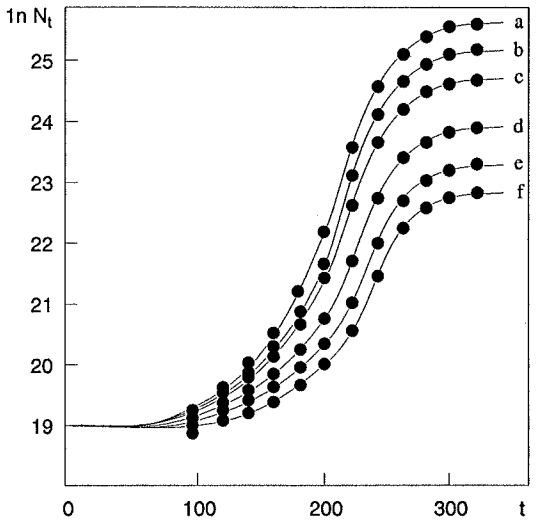

FIGURE 1 - Growth plots *Escherichia coli* ATCC 25 922 in media with *acetophonone* (I) (stirring: 140 rpm, 35°C).
Symbols: t: reading time (min); N_t: number of cfu/ml (Ec.2).
Concentrations (µg/ml): (a) control; (b) 70.0; (c) 113; (d) 203; (e) 258; (f) 316.

In Table 1 are exhibited the values for the microbial specific growth rates and the corresponding drug concentrations added to the culture media.

TABLE 1 - Specific growth rate variation of *Escherichia coli* ATCC 25.922 with acetophenones and benzaldehydes concentrations (nutritive broth, stirring: 140 rpm; 35°C). Symbols: C: drug concentration (μg/ml); μ: specific growth rate (min^{-1}); acetophenone (I); 2- hydroxyacetophenone (II); 2,4-dihydroxyacetophenone (III); 2-hydroxy-4-methoxyacetophenone (IV); benzaldehyde (V); 3-hydroxybenzaldehyde (VI); 4-hydroxybenzaldehyde (VII) and 4-methoxybenzaldehyde (VIII).

I	C	0	70.0	113	203	258	316
	$\mu \times 10^3$	41.5	37.2	34.5	29.8	26.5	23.3
II	C	0	99.4	178	229	279	320
	$\mu \times 10^3$	41.5	37.9	35.3	33.6	31.8	30.5
III	C	0	73.2	105	132	160	205
	$\mu \times 10^3$	41.5	32.7	29.4	27.0	23.2	18.3
IV	C	0	68.8	103	137	162	193
	$\mu \times 10^3$	41.5	31.5	27.8	23.0	20.1	16.2
V	C	0	90.0	121	202	238	282
	$\mu \times 10^3$	41.5	34.9	32.6	27.0	24.6	19.8
VI	C	0	73.8	108	148	206	260
	$\mu \times 10^3$	41.5	36.2	33.8	31.6	26.2	23.0
V!!	C	0	67.8	107	136	191	238
	$\mu \times 10^3$	41.5	35.3	32.3	29.8	25.3	21.0
VIII	C	0	102	170	212	216	306
	$\mu \times 10^3$	41.5	34.4	30.2	27.1	23.9	20.8

The obtained results were satisfactorily interpreted by means of the bacteriostatic inhibition mechanism previously proposed (16). Thus, the variation of the specific growth rate (μ) with the drug concentration follow the relation

$$\mu = \mu_T \frac{k_2 . Y_o . K . C}{1 + K . C} \qquad (4)$$

where, μ: specific growth rate (min^{-1}); μ_T: specific growth rate in a medium without drug (min^{-1}) (control); k_2: specific inhibition rate ($ml.\mu g^{-1} . min^{-1}$); K: formation equilibrium constant of the drug-chemical carrier complex; C: drug concentration (μg/ml); Y_0: total chemical carrier concentration; $k_2.Y_0$: parameter proportional to the specific inhibition rate.

The graphical interpretation of expression (5), rewritten from (4)

$$\frac{1}{\mu_T - \mu} = \frac{B}{A} + \frac{1}{A} . \frac{1}{C} \qquad (5)$$

where $A = k_2 . Y_0 . K$ and $B = K$, it is shown in Figure 2, led to the evaluation of $K_2 . Y_0$ and K constants necessary for the determination substances minimal inhibitory concentrations (MIC), according to

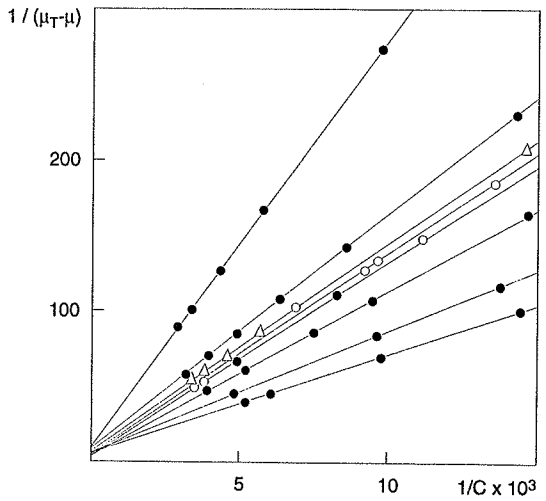

FIGURE 2 -Graphical determination of the stability constant of the drug-chemical carrier complex and the specific inhibition rate *Escherichia coli* ATCC 25 922 by acetophenones and benzaldehydes (Eq. 5).
Symbols: μ_T: control specific growth rate (C=0) (min^{-1}); μ: specific growth rate (in media plus progressive concentrations of the drugs (min^{-1}); C: concentrations in μg/ml; acetophenone (I); 2-hydroxyacetophenone (II); 2,4-dihydroxyacetophenone (III) 2-hydroxy-4-methoxyacetophenone (IV) benzaldehyde (V); 3-hydroxybenzaldehyde (VI); 4-hydroxybenzaldehyde (VII) and 4-methoxybenzaldehyde (VIII)

$$MIC = \frac{\mu_T}{A - \mu_T . B} \qquad (6)$$

In Table 2 the values of $k_2 . Y_0$, K and MIC for all the acetophenones and benzaldehydes assayed againts *E. coli* are detailled.

TABLE 2 - Kinetical, equilibrium and concentration parameters that achieve the inhibition of *Escherichia coli* ATCC 25.922 by acetophenones and benzaldehydes.
Symbols: K: formation equilibrium constant of the drug-chemical carrier complex C-Y (Ec.4); $K_2.Y_0$: parameter proportional to the specific inhibition rate (EC.4). Acetophenones and (I); 2-hydroxyacetophenone (II); 2,4-dihydroxyacetophenone (III); 2-hydroxy-4-methoxyacetophenone (IV); benzaldehyde (V); 3-hydroxybenzaldehyde (VI); 4-hydroxybenzaldehyde (VII); 4-methoxybenzaldehyde (VIII).

Compound	$K \times 10^4$	$K_2 . Y_0$	MIC
I	3.40	0.186	845
II	2.96	0.126	1650
III	7.33	0.171	437
IV	10.70	0.144	378
V	0.113	6.48	570
VI	0.949	0.757	611
VII	5.90	0.159	599
VIII	2.13	0.332	671

Acetophenones structure-activity relationship

It is simple to infer, from the proposed inhibition mechanism, that the stability constant of the drug-chemical carrier complex (K) is directly related with the drug bacteriostatic efficacy. It is observed that the stability of the C-Y complex for the acetophenones, acetophenone (I), 2-hydroxyacetophenone (II), 2,4-dihydroxyacetophenone (III) and 2-hidroxy-4-methoxyacetophenone (IV) raises in the order K(II) < K(I) < K(III) < K(IV), while that for respective MIC do it MIC(IV) < MIC(III) < MIC(I) < MIC(II).

This reciprocal relation between K and MIC values shows the relevance of the C-Y complex stability in the determination of the acetophenones bacteriostatic efficacy. On the other hand, if it is considered the great reactivity of the carbonyl group as its main characteristic (11), its is rational to suppose that the carbonyl oxygen atom of the substances directly participate in the C-Y complex formation.

In this way, the introduction of electron donor substituents in the acetophenone benzene ring (I) would increase the nucleophile of the carbonyl oxygen and thus the stability of the drug-chemical carrier complex. This explains the high values of K in 2,4-dihydroxyacetophenone (III) and 2-hydroxy-4-methoacetophenone (IV), in comparison with I. In the 2-hydroxyacetophenone (II), as a consequence of the tight intramolecular bond involving the hydroxylic and carbonylic oxygen atoms, the eletronic density of the carbonylic oxygen of II in relation to that of I decreases due to the interactions produced. Then, it results that K (2-hydroxyacetophenone) < K (acetophenone) and MIC (II) > MIC (I).

Benzaldehydes structure-activity relationship

In view of the values of the stability constants of the drugchemical carrier complexes (K), specific inhibition rates (k_2 . Y_0) and MIC benzaldehydes (Table 2), to relate the structural characteristics of these compounds with their activity against *E. coli* is not possible as in the above system.

But, it is clear that the phenolic hydroxyls do not constitute themselves the molecular region accounting for the substances bioactivity, considering MIC 3-hydroxybenzaldehyde (IV)> MIC 4-hydrobenzaldehyde (VII)> MIC benzaldehyde (V).

Chalcones structure-activity relationship

It is know that the chalcones are frequently prepared by condensation of acetophenones and benzaldehydes in alkaline media (7,14) and they generally preserve their structural particularities. A drug with a "chalcone structure" comes from, schematically, the "sum" or union of a compound with an "acetophenone structure" and other with "benzaldehyde structure" as it is shown in Figure 3. In medicinal chemistry, this method of obtaining new drugs is called "molecular hybridization".

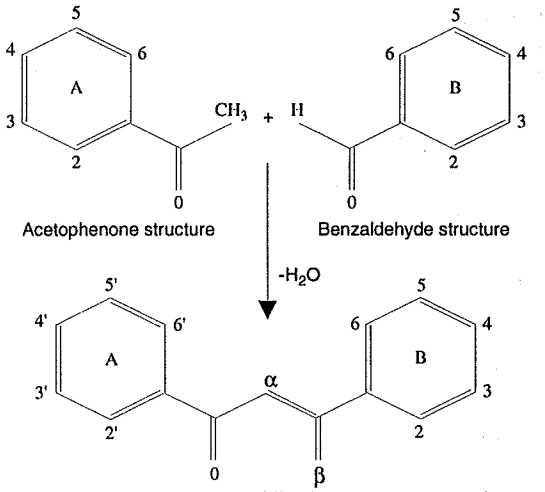

FIGURE 3 - Molecular hybridization between acetophenone and benzaldehyde yields chalcone

In account of the structural similarity of these substances is valid to extend the argument previously described for acetophenones and benzaldehydes. The chalcones bacteriostatic activity against *S. aureus* ATCC 25 923 and *E. coli* ATCC 25 922 was before assayed aplying identical procedure and with optimal results (16,17,18).

In order to facilitate the comparison of the inhibitory action of the similar structure substances it is appropriate to define the drug percentual bacteriostatic efficacy as

$$Pbe = \frac{100}{MIC} \qquad (7)$$

where MIC: drug minimal inhibitory concentration in μg/ml and 100: arbitrary percentual factor.

In table 3 are consigned the values for MIC and Pbe of 2', 4'-dihydroxychalcone (IX), 2',4-dihydroxychalcone (X), 2',3-dihydroxychalcone (XI), 2'-hydroxychalcone (XII), 2'-hydroxy-4-methoxychalcone (XIII) and those of the acetophenones and benzaldehydes studied.

When the Pbe of the appropriate pairs of acetophenones and benzaldehydes are analyzed, then it results

TABLE 3 - Percentual bacteriostatic efficacy of acethophonones, benzadelhydes and chalcones against *E. coli* ATCC 25.922.
Symbols: Pbe: percentual bacteriostatic efficacy; MIC: minimal inhibitory concentration; acetophenone (I); 2-hydroxyacetophenone (II); 2,4-dihydroxyacetophenone (III); 2-hydroxy-4-methoxyacetophenone (IV); benzaldehyde (V); 3-hydroxybenzaldehyde (VI); 4-hydroxybenzaldehyde (VII); 4-methoxybenzaldehyde (VIII); 2',4'-dihydroxychalcone (IX); 2',4-dihydroxychalcone (X); 2',3-dihydroxychalcone (XI); 2;-hydroxychalcone (XII); 2'-hydroxy-4-methoxychalcone (XIII).

Compound	MIC	Pbe
I	845	0.118
II	1650	0.060
III	437	0.229
IV	378	0.265
V	570	0.175
VI	611	0.164
VII	599	0.167
VIII	671	0.149
IX	46.8	2.14
X	66.5	1.50
XI	72.2	1.39
XII	102.0	0.980
XIII	93.8	1.07

Pbe (III) (0.229) + Pbe (V) (0.175) = 0.404 <<< Pbe (IX) (2.14)
Pbe (II) (0.60) + Pbe (VII) (0.167) = 0.227 <<< Pbe (X) (1.50)
Pbe (II) (0.60) + Pbe (VI) (0.164) = 0.224 <<< Pbe (XI) (1.39)
Pbe (II) (0.60) + Pbe (V) (0.175) = 0.235 <<< Pbe (XII) (0.980)
Pbe (II) (0.60) + Pbe (VIII) (0.149) = 0.209 <<< Pbe (XIII) (1.07)

The high Pbe of chalcones is chiefly due to the presence of the doble bond C_α=C_β in the chalcone structure, wich enables the electronic delocalization of the molecule B-ring over the carbonyl group, thus increasing its polarization. This increased polarity of C=0 bond raises the oxygen nucleophile and consequently the drugchemical carrier complex stability, increasing the biological action of the drug. On the order hand, it is observed that the percentual bacteriostatic efficacy of chalcones against *E. coli* decreases in the folowing order: Pbe 2',4'-dihydroxychalcone > Pbe 2',4-dihydroxychalcone > Pbe 2',3-dihydroxychalcone > Pbe 2'-hydroxy-4-methoxychalcone. This sequence clearly shows that the introduction of electron donating groups in the chalcone benzenic rings (inactive substance against *E. coli*) promotes in the derived chalcones the appearance of activity whose intensity depends on the position and nature of substituent groups.

It is concluded that in the chalcone the molecular zone involving the carbonyl group and the neighbour hydroxyl (2') is mainly responsible for the achievement of the substance activity. Besides, considering the oxygen atoms involved, the carbonyl one accounts for the essential functions. In view of the obtained results with the acetophenones and benzaldehydes, the possibility that the phenolic hydroxyls in position 4'(A-ring), 3 and 4 (B-ring) of chalcones can integrate other active molecular region is discarded. So the less microbial efficacy of 2'-hydroxy-4-methoxychalcone (XIII, Pbe=1.07) respect to 2',4- dihydroxychalcone (X, Pbe = 1.50), can't be explained in function of a new active center that includes the hydroxyl joined to C 4, but considering the factors:

a) The greater mesomeric capacity of hydroxyl group in relation to the methoxyl group. It is known that the quantitative effects of substituent groups over diverse chemical and biological reactions is usually determined by means of Hammett constants (6). If if considered that $\sigma^{p\text{-}OCH3} = 0.27$ and $\sigma^{p\text{-}OH} = -0.46$, it is deduced that the electron doning capacity for the hydroxyl group in "para" position almost exceeds a 70% of that shown by methoxyl group.
b) The large size of methoxyl group in comparison with the hydroxyl one. As a consequence of its volume the OCH3 can achieve a steric effect that modifies part of the chalcone molecule that hampers the electronic resonance because of the resulting planar lack.

On account of the factors before cited the greater electronic density of the carbonylic oxygen of 2',4-dihydroxychalcone (X) in relation to that one of 2'-hydroxy-4-methoxychalcone (XIII), the major stability of X-Y comples vs. XIII-Y complex and also the bacteriostatic efficacy of 2',4-dihydroxychalcone and 2'-hydroxy- 4- methoxychalcone against *E. coli* can be satisfactorily explained. More over diverse determinations of spectroscopic and dielectric properties of the previously studied chalcones (3) enhance the relevance of the oxygen functions in the carbonyl group. Thus, if the dipolar moments (DM) or the infrared absorption frequency of 2',4'-dihydroxychalcone (IX), 2',4-dihydroxychalcone (X), 2',3-dihydroxychalcone (XI) and chalcone are analysed, then it is inferred

DM(IX) (4.04 D) > DM(XI) (3.63 D) > DM (chalcone) (2.88 D) and

$V_{c=0}$ (IX) (1640 cm^{-1}) < $V_{c=0}$ (X) 1641 cm^{-1}) < $V_{c=0}$ (XI) (1645 cm^{-1}) < $V_{c=0}$ (chalcone) 1660 cm^{-1})

On the other hand, the MIC of precited compounds against *E. coli* increase in the order MIC 2',4'-dihydroxychalcone (IX) (46.8 µg/ml) < MIC 2',4-dihydroxychalcone (X) (66.5 µg/ml) < 2',3-dihydroxychalcone (XI) (72.2 µg/ml) < MIC chalcone (inactive).

These reciprocal relations between the DM sequences, $\upsilon_{c=o}$ and MIC, ratify the importance of the carbonyl oxygen atom in comparison with the hydroxyl oxygen (C 2') in the chalcone bacteriostatic activity. These results enable to establish that the chalcone synthesis must be directed toward the obtention of compounds with greater bioactivity through the introduction of electron donor groups in the A and/or B rings of molecule that would increase the nucleophilicity of the carbonylic oxygen.

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RESUMO

Determinação da zona molecular responsável pela atividade bacteriostatica de chalconas

Foi estudada a bioatividade de compostos com estrutura de acetofenona e benzaldeído pela metodologia cinético-turbidimétrica. Os agentes testados não inibiram o desenvolvimento de S. *aureus* ATCC 25.923, mas foram eficientes para *E. coli* ATCC 25.992. Os resultados foram satisfatoriamente interpretados, a partir da fórmulas anteriormente proposta, para estudo do mecanismo de ação bacteriostática. Na comparação de substâncias de estruturas similares, definiu-se a Eficácia Bacteriostática Percentual (Ebp) de um fármaco como Ebp = 100/CIM, em que, 100 é o fator percentual arbitrário e CIM é a concentração inibitória mínima do fármaco, em µg/ml. Na comparação das Ebp dos pares de acetofenonas e benzaldeídos apropriados, encontra-se que:Ebp (acetofenona) + Ebp (benzaldeído) <<<< Ebp (chalcona)A maior Ebp, observada para as chalconas deve-se, fundamentalmente, à presença na "estrutura de chalcona" da ligação dupla C_{α}=C_{β}, que possibilita a intensificação do deslocamento eletrônico no anel B da molécula, aumentando a polarização do grupo carbonila e a oxidrila vizinha (2') é a responsável pela presença da atividade bacteriostática destas substâncias.

Palavras chaves: chalconas - atividade bacteriostática - acetofenona - benzaldeído.

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PARAINFLUENZAVIRUS TYPE 1 VARIANTS: ANALYSIS OF HEMAGGLUTINATING, NEURAMINIDASE AND FUSION ACTIVITIES

Ana Maria Viana Pinto[1]
Maulori Curié Cabral[1]
José Nelson dos Santos Silva Couceiro[1*]

SUMMARY

The existence and the character of two variants or subpopulations in a Sendai standard virus sample were studied, and virus variants were selected by a technique based on the receptor-binding activity. The sialidase biological activity of the Sendai variant samples of parainfluenza type 1, their pH-dependence, calcium-dependence and association with hemagglutinating and hemolytic capabilities were studied. Both the standard-like sample and one obtained by an adsorption process exhibited the highest hemagglutinating or sialidase activities at pH values between 5.0 and 7.0. However, the standard-like sample did not exhibit hemagglutinating activity to human O group erythrocytes at all pH values used, but it did show significative sialidase activity. The sialidase activity was calcium-dependent and was shown related with the hemolytic activity. The low hemolytic activity could be explained by efficient enzymatic activity developed for HN structures on cell receptors that could be essential for the fusion process. The samples exhibited diverse hemagglutinating and neuraminidase characters that suggested selection of different viral variants or subpopulations.

Key words: parainfluenzavirus, variants, selection, activities, hemagglutinating, neuraminidase, sialidase, fusion.

INTRODUCTION

Type 1 parainfluenzavirus are members of the genus *Paramyxovirus* classified in the family *Paramyxoviridae* (17), which presents RNA and a lipoproteic envelope with two types of glycoproteic structures inserted in it. These two types of glycoproteic structures, HN (hemagglutinin-neuraminidase) and F protein (fusion protein), exhibit different biological activities that are important in the virus replication cycle (6,7). The HN structure is responsible for virus binding to cell receptors and also for sialidase activity (22). F protein is involved in a process that results in fusion among viruses and membranes of host cells which causes hemolysis when erythrocytes are utilized as a cellular model (16).

Many authors have indicated the possible existense of variants or subpopulations of RNA-virus in virus samples, by genetic, immunologic and receptor studies (4,10,18). Our objective was to analyse the hemagglutinating, sialidase and fusogenic activities of variant virus samples selected by virus-cell receptor adsorption methodology.

1. Departamento de Virologia - Instituto de Microbiologia, Centro de Ciências da Saúde - Bloco I - Cidade Universitária, Ilha do Fundão, CEP 21941-590 - Rio de Janeiro, RJ, Brasil.
* Corresponding author.

MATERIAL AND METHODS

Viral samples studied: two variants were obtained from a standard sample os Sendai virus (miurne strain of parainfluenzavirus type 1 Kindly sent by Dr. J.J. Skehel from National Institute for Medical Research, Mill Hill, London were utilized. The standard virus sample of parainfluenzavirus type 1 was submitted to two different procedures. In the first of them, the sample named P_1 was constituted by allantoic fluid which was obtained after inoculation of the standard sample (P_0) into allantoic cavity of 10-day old embryonated chicken eggs, and incubation at 36°C for 48 hr. The second sample (P_2) was constituted by that allantoic fluid obtained by inoculation into allantoic cavity of 10-day old embryonated chicken eggs and incubation at 36°C for 48 hr of that pellet obtained after virus-cell receptor adsorption process, using erythrocytes as cellular support. In this process, the standard virus sample was diluted to 1.5% in a 10% human O group erythrocytes suspension. The virus-erythrocytes mixture was incubated for 1 hour at 4°C, and then was washed twice at 600 X g at 4°C for 10 minutes in cold 0.15 M phosphate buffer saline (PBS) pH 7.0 (20).

The allantoic fluids obtained in both situations were clarified by centrifugation at 7,500 x g for 30 minutes at 4°C, 50 times concentrated by ultracentrifugation at 100,000 x g for 60 minutes and purified in a potassium tartrate continuous gradient at 180,000 x g for 120 minutes at potassium 4°C (8). Virus bands were collected and diluted in 5 volumes of TESC pH 8.4 (0.01 mM TRIS, 0.01 mM EDTA, 0.10 M sodium chloride, 0.006 mM cystein). The virus bands were centrifuged for 60 minutes at 100,000 x g at 4°C. The pellets from P_1 and P_2 samples were resuspended finally with TESC to recover those same volumes obtained after concentration and stored at -20°C (20).

Erythrocytes: human O group and chicken erythrocytes were collected in 5 volumes of Alsever solution, and used after three washing steps and suspension at 10% concentration in 0.15 M sodium chloride. The final suspensions were prepared with diverse buffers for the different experiments, as described below.

Lectin: peanuts lectin or PNA *(Arachis hypogaea)* obtained from Sigma was titrated and standardized at 25°C for 1 hour to two hemagglutinating units (13).

Hemagglutination Test: Virus samples (25 µl) were diluted serially with 25 µl volumes of 0.15 M PBS pH 7.0, with posterior addition of 25 µl volumes of 1% human O group or 0.5% chicken erythrocytes suspensions prepared in the buffers at different pH values and 0.2% bovine albumin. The results were read after incubation for 2 hours at 4°C.

The reciprocal of the highest dilution of virus responsible for complete agglutination was considered as the numbers of hemagglutinating units (HAU) or titers present (8).

Neuraminidase-Lectin Test: virus samples (25 µl) were diluted seially in duplicates with 25 µl volumes of 0.15 M PBS pH 7.0 and added to 1% human O group or 0.5 chicken erythrocyte suspensions prepared as above in the appropiate buffers. The reactions were incubated at 4°C (negative controls) or 37°C for two hours or until complete reversion of the initial visible hemagglutination, with posterior addition of PNA and homogenization of the content from each well. The titer of the sialidase activity of the virus sample against erythrocytes was considered as the reciprocal of the highest dilution of virus sample which were responsible for complete PNA agglutination after 1 hour of incubation at 25°C (14).

Hemolysis Test: the hemolytic activity at different pH values was analysed in a 3.0 ml total volume with equal 1.0 ml volumes of 1/10 dilution of virus samples. Different virus samples, 1% suspensions of human o group or 0.5% chicken erythrocytes and adequated buffers were put together. The capped tubes were mixed, incubated at 4°C for 20 minutes, incubated at 37°C for 60 minutes, and finally centrifuged at 600 x g for 10 minutes. Hemolysis was revealed by the amount of hemoglobin that was released in the supernatant, as measured by absorbance measurement at 545 nm. Maximal and residual hemolysis were determined after addition of 1.0 ml buffer solution and 1.0 ml of erythrocyte suspensions with or without 0.1% Nonidet P-40 (NP-40), respectively (1,2,12).

Analysis of different parameters of biological activies: Hemagglutinating and sialidase activities at different pH values - The virus samples were anlysed by hemagglutination at 4°C and neuraminidase assays at 37°C using buffers with pH values ranging from 5.0 to 9.0(15,21).

Evaluation of calcium-dependence of the silaidase activity of virus samples at pH 5.8 - The samples were studied by hemagglutination and neuraminidase assays using EDTA - 0.15 M NaCl pH 7.0) 1% human O group and 0.5% chicken erythrocytes in acetate buffer at pH 5.8. EDTA at concentrations between 0 and 10 mM was used as quelating agent of calcium ions.

Evaluation of sialidase-dependence of the hemolytic activity of the virus samples at pH values from ranging 5.2 to 7.8 - The hemolytic activity of the virus samples were analysed by hemolysis assays using 1% human O group and chicken erythrocytes and buffers (15, 20) with pH ranging from 5.2 to 7.8. In buffers, EDTA was used at 2 mM as final concentration.

RESULTS

The utilization of a selection process of virus variants from an initial standard sample (P_0) by a technique based on virus-cell receptor adsorption showed the existence of two different virus variants, propagated standard (P_1) and adsorpted and propagated standard (P_2) samples, in sample P_0. The P_1 sample exhibited titers of 16 and < 2 HAU/25 µl while the P_2 sample showed titers of 32 and 32 HAU/25 µl, when incubated at 4°C with chicken or human O group erythrocytes receptor cells respectively.

Table 1 exhibits results from the analysis of the hemagglutinating and sialidase activities at different pH values, utilizing both selected virus samples (P_1, P_2) and 1% human O group erythrocytes. Table 1 also indicates the results of analysis of hemagglutinating and siliadase activities of the P_1 and P_2 samples using 0.5% chicken erythrocytes, at different pH values.

The results of the calcium-dependence analysis obtained for both Sendai virus samples by hemagglutinating and neuraminidase-lectin assays at 4°C and 37°C using EDTA and human O group and chicken erythrocytes are shown in Table 2. The absence of influence of calcium ions on the sialidase activity is demonstrated here. Hemolysis activity of both samples with human O group or chicken erythrocytes, using EDTA to analyse the mechanism of calcium-dependence of the silaidase activity, was also studied. The highest hemolytic activities were demonstrated at pH values with the lowest sialidase activities (data not shown).

DISCUSSION

The hemagglutinating and sialidase activities of the selected P_1 and P_2 samples showed a clear influence of the selection process used. Burnett & Bull (4) and Burnet et al. (5) observed similar results for analysis of Influenza virus samples after different number of passages in embryonated chicken eggs exhibiting diverse hemagglutinating standards.

The existence of such variant samples had already been indicated by antigenic analysis (11,18).

The analysis of agglutinating titers at diverse pH values, before and after lectin addition, showed the pH values between 5.0 and 7.0 to have

TABLE 1 - Evaluation of hemagglutinating and sialidase activities in HAU/25 µl of the two parainfluenzavirus type 1 samples (P_1, P_2) at different pH values with human O group and chicken erythrocytes.

	Hemagglutination titer* - P_2 sample				Hemagglutination titer* - P_1 sample			
pH**	4°C Human/ Chichen	4°C + PNA Human/ Chicken	37°C Human/ Chicken	37°C + PNA Human/ Chicken	4°C Human/ Chichen	4°C + PNA Human/ Chicken	37°C Human/ Chicken	37°C + PNA Human/ Chicken
5.0 - 5.4	16/32	64/64	<2/<2	512/256	<2/16	<2/16	<2/<2	128/64
5.6	32/32	64/64	<2/<2	256/256	<2/16	<2/16	<2/<2	128/64
5.8	32/32	64/64	<2/<2	128/256	<2/16	<2/16	<2/<2	8/64
6.0 - 6.2	32/32	32/64	2/<2	128/256	<2/16	<2/16	<2/<2	8/64
6.4 - 7.0	32/64	32/128	2/<2	128/128	<2/16	<2/16	<2/<2	8/64
7.2 - 7.4	32/32	32/32	2/<2	128/128	<2/16	<2/16	<2/<2	8/32
7.6	16/32	16/32	2/<2	128/64	<2/16	<2/16	<2/<2	8/32
7.8	16/64	16/32	2/<2	128/64	<2/16	<2/16	<2/<2	8/32
8.0 - 8.2	16/64	16/64	2/<2	64/16	<2/16	<2/16	<2/<2	8/32
8.4 - 8.6	8/16	8/16	2/<2	32/64	<2/16	<2/16	<2/<2	8/32
8.8	8/8	8/8	2/2	32/16	<2/16	<2/16	<2/<2	8/32
9.0	8/8	8/8	4/2	8/16	<2/16	<2/16	<2/<2	8/32

(*) Titers were expressed as the reciprocal of the highest dilution of virus necessary to cause complete agglutination.
(**) Acetate, Sodium Phosphate and Glycine-NaOH buffers used for the different values of ambient pH as Maeda & Ohnishi (1980).
P_1 Standard-like sample.
P_2 Sample obtained by Adsorption Process.

TABLE 2 - Evaluation of hemagglutinating and sialidase activities at pH 5.8 of two parainfluenzavirus type 1 (P_1, P_2) different EDTA concentrations and human O group/chicken and chicken erythrocytes.

	Hemagglutination titer* - P_2 sample - chicken erythrocytes				Hemagglutination titer* - P_2 sample - Human O group erythrocytes				Hemagglutination titer* - P_1 sample - chicken erythrocytes				Hemagglutination titer* - P_1 sample - Human O group erythrocytes			
EDTA** concentration	4°C	4°C + PNA	37°C	37°C + PNA	4°C	4°C + PNA	37°C	37°C + PNA	4°C	4°C + PNA	37°C	37°C + PNA	4°C	4°C + PNA	37°C	37°C + PNA
0 mM	16	64	<2	128	16	32	<2	128	8	32	<2	128	<2	2	<2	64
1 mM	16	32	<2	64	8	16	<2	32	8	32	<2	64	<2	2	<2	32
2 mM	16	32	<2	128	8	16	<2	32	8	32	<2	64	<2	2	<2	32
3 mM	16	64	<2	128	8	16	<2	32	8	32	<2	64	<2	2	<2	32
4-10 mM	32	64	<2	128	8	16	<2	32	8	32	<2	64	<2	2	<2	32

(*) Titers were expressed as the reciprocal of the highest dilution of virus required to cause complete agglutination. P_1 (original-like) and P_2 (obtained for Adsorption Process) samples showed HA titers of $64^{(1)}$, $16^{(2)}$ $32^{(3)}$ and $<2^{(4)}$ HAU/25µl at pH 7.0 (PBS) with "O" group human$^{(1,2)}$ and Chicken Erythrocytes$^{(2,3)}$.

(**) Acetate buffer-EDTA pH 5.8 - 50 mM sodium acetate, 0,15 M sodium chloride. 0,25 M EDTA is added to obtain concentrations from 1 mM to 10 mM at pH 5.8.

P_1 Standard-like sample.

P_2 Sample obtained by Adsorption Process.

the most intense hemagglutinating activity for the variant sample obtained by the adsorption process propagation (P_2). This variant sample showed the highest sialidase activities at pH values from 5.0 to 7.0, with elution times varying from 2 to 2,5 hr when it was analysed at pH 5.0-8.6 pH, using human O group erythrocytes. This same sample exhibited elution times from 1 to 1,5 hr, showing the highest sialidase activities at pH values from 7.2 to 8.6, when chicken erythrocytes were used. This sialidase activity was revealed by the reaction of PNA with galactose residues, which are exposed by sialidase activity in the same pH range. PNA is a lectin that has affinity for binding to residues like ß-D-gal(1-3)DgalNAc, wich are exposed after virus sialidase activity on surface glycoproteic structures of erythrocytes, causing final hemagglutination (Table 1).

The P_1 sample did not show significative variation in hemagglutinating and sialidase activities with chicken erythrocytes (Table 1) and it showed elution in 1,5 hr at pH values from 5.0 to 8.6 (data not shown). However the P_1 sample did not exhibit hemagglutinating activity at 4°C or 37°C, using human O group erythrocytes within the large pH range used, but it exhibited more significative sialidase activity at pH values between 5.0 and 5.6 (Table 1). In this case, subhemagglutinating or sub-eluting quantities of viruses were revealed by analysis of their sialidase activity after addition of lectin.

These results demonstrate the existence of populations with different characters within virus samples, suggesting the presence of variants with different grades of affinity to different types of erythrocyte receptors (11). Our results also suggested that sample P_2 originated from a small set of variants presents in the P_0 sample (data not shown) and also sample P_1, which exhibited no significant hemagglutinating activity to human O group erythrocytes. However, the P_2 sample exhibited significant hemagglutinating activity with erythrocytes from both human and chicken origins. This could be explained by selection and amplification of a small hemagglutinating set in the originally non hemagglutinating samples (P_0 and P_1) to human o group erythrocytes.

Sialidase activity was independent of different EDTA concentrations in both Sendai samples at the optimum pH of 5.8 and in the presence of calcium ions (Table 2), as previously demonstrated in a standard Sendai virus sample by Brostom et al. (3). The presence of a minimal quantity of calcium ions may be sufficient for expression of the sialidase activity.

The hemolytic activity of the two samples was low, but the P_2 sample exhibited significant differences in optical density, with human O group erythrocytes at pH values above 7.2. This low activity could be explained by significatively high sialidase activity of the two samples, that could be responsible for the fast elution with inhibition of the secondary process of hemolysis. These results indicated that the glycoprotein HN was responsible for receptor-binding, and sialidase activities needs to be initially con-

nected with the cell receptors during the hemolysis process. The highest hemolytic activities were demonstrated at pH values (7.2 - 7.8) with the lowest sialidase activities (data not shown), as could be explained by the mechanism described above.

Two different subpopulations were selected with diverse grades of HN hemagglutinating activity to human O group and chicken erythrocytes but always exhibiting significant HN sialidase activity. The existence of virus variants has been indicated by studies of antigenic, genetic and receptor characters, reinforcing our results (10,18,19,23).

Domingo et al. (9) analysed the high genetic variability of RNA-viruses using Vesicular Stomatitis Virus as model, introducing the term "quasiespecies" to define it. The total comprehension of that mechanism is very important for purposes of laboratory diagnosis of virus diseases and production of vaccines. Obtaining virus samples by this process would make selection of virus samples possible that actually represent virus variants responsible for the clinical symptoms of disease and virus samples able to induce a specific and efficient immunization.

RESUMO

Variantes de Vírus Parainfluenza Tipo 1: Análise de Atividades Hemaglutinante, Sialidásica e de Fusão

A existência e o caráter de variantes ou subpopulações derivadas de uma amostra padrão de vírus Sendai foram estudados, usando duas amostras de vírus Sendai selecionadas por técnica baseada na atividade de ligação e receptores, a qual é apresentada por aqueles vírus. Foram estudados o comportamento bilógico da neuraminidase de duas diferentes amostras Sendai de vírus parainfluenza tipo 1, sua pH-dependência, cálcio-dependência e relação com as capacidades hemaglutinante e hemolítica.

Foi observado que ambas as amostras, aquela considerada como semelhante à padrão e aquela obtida por propagação de subpopulação adsorvida à eritrócitos, mostravam mais significativas atividades hemaglutinante e sialidásica em valores de pH entre 5.0 e 7.0. A amostra considerada como semelhante à padrão, embora não mostrasse qualquer atividade hemaglutinante frente a hemácias humanas do grupo O, era capaz de mostrar uma atividade sialidásica significativa. A atividade sialidásica revelava cálcio-dependência e relacionamento com a atividade hemolítica. A baixa taxa de hemólise exibida poderia ser explicada pela eficiente atividade sialidásica de estruturas HN sobre receptores celulares, os quais seriam essenciais para o processo de fusão. As amostras mostravam caráteres diferentes quanto às atividades hemaglutinante e sialidásica, o que sugeriu que a seleção de diferentes variantes ou subpopulações, uma observação já feita por alguns autores, seria muito importante de ser compreendida, para um eficiente direcionamento dos processos de diagnóstico e de seleção de amostras virais vacinais.

Palavras-chave: parainfluezavírus, variantes, seleção, atividades, hemaglutinante, sialidase, fusão.

ACKNOWLEDGMENTS

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MYCELIAL PRODUCTION OF *METARHIZIUM ANISOPLIAE* IN LIQUID CULTURE USING DIFFERENT SOURCES OF CARBON AND NITROGEN

Bonifácio Peixoto Magalhães[1,2]
José Manuel Cabral de Sousa Dias[1]
Cláudia Martins Ferreira[2]

SUMMARY

The liquid fermentation of *Metarhizium anisopliae* for dry mycelium production was investigated. Several culture media based on sucrose, dextrose, yeast-water and yeast extract were tested in agitation cultures (150 rpm). The best medium composition contained sucrose (4%) and yeast extract (1%). Dry mass and pH varied according to the initial concentration of conidia. The highest dry mass was obtained at 10^8 conidia/ml per flask. The most significant change in pH was detected at high concentrations of conidia (> 10^7 conidia/ml). In the fermenter, a dry mass yield of 9.05 g/l was observed at time 72 h of culture, with a 0.13 $g.l^{-1}.h^{-1}$ productivity rate and a 69% level of sugar consumption. Following rehydration (72 h at 25°C), the fungus produced 1.63 x 10^{10} conidia/g of dry mycelium. The viability of the conidia produced was higher than 90%. The use of yeast-water prepared from baker's yeast according to two different methods proved to be a potential replacement for yeast extract in the culture medium.

Key words: Fermentation, entomopathogenic fungi, mass production, microbial control, carbon source, nitrogen source, conidial concentration.

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* is a pathogen of many insect pests worldwide and is considered an effective biocontrol agent in Brazil. The use of this pathogen has been based on its ability to generate infective conidia on solid substrates (1, 2). Alternatively, this pathogen may be obtained as dry mycelium using a simpler and cheaper process of submerged culture. Mycoinsecticides produced as dry mycelium may be cheaper and more efficient than those produced as conidia (12, 13). A few hours after rehydration, the dry mycelium starts to grow and sporulates profusely. If the dry mycelium is appropriately delivered onto the field, the highly viable conidia produced after rehydration can be at least as effective in infecting insect hosts as the conidia produced in solid substrate (13).

The production and formulation of *M. anisopliae* dry mycelium were recently investigated (10). However, there is no information on the influence of different media and initial concentration of inoculum on mycelium production by this fungus. Therefore, the present investigation was undertaken to analyse the effects of different sources of carbon and nitrogen and of the initial concentra-

1. Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, CENARGEN/EMBRAPA - P. O. Box 0233372, 70849-000 - Brasília, DF, Brasil.
2. Bolsista, CNPq.
Correspondence to be sent to: Bonifácio Magalhães CENARGEN/EMBRAPA C.P.02372 70770-000 - Brasília, DF, Brazil

tion of inoculum on *M. anisopliae* mycelium production in submerged cultures.

MATERIALS AND METHODS

Fungal strain: *Metarhizium anisopliae*, CG 46 (Collection of Entomopathogenic Fungi, CENARGEN/EMBRAPA, Brazil), isolated from the spittlebug *Deois flavopicta* was removed from liquid nitrogen and grown at 25°C on SDAY (Sabouraud dextrose agar and 1% yeast extract, pH 6.5). The fungus was transferred weekly and, after sporulation, kept at 4°C until use (up to two weeks).

Culture media: In order to obtain a culture medium based on cheap components easily available from the Brazilian suppliers, sucrose and dextrose were tested as carbon sources, and yeast extract and three types of yeast-water were used as nitrogen sources. Various combinations of these components resulted in 12 types of media, including SDY (4% dextrose, 1% yeast extract and 1% neopeptone) as the standard liquid medium (Tables 1 and 2). The initial pH of all media tested was adjusted to 6.4 - 7.0 before sterilization. Previous observations showed that pH changes after sterilization were insignificant.

Three types of yeast-water were prepared using commercial baker's yeast (Fleischman®) as feedstock. The procedure for these preparations is illustrated in Figure 1. The main aim of these methods was to obtain liquid substrates with a high content of proteins, vitamins and growth factors (3, 4, 9). In the first method (YW1), 150 g/l (wet weight) of the yeast suspension was sterilized at 121°C for 15 min; after cooling, the suspension was next centrifuged at 9000 rpm for 20 min. The same initial procedure was used for YW2; howev-

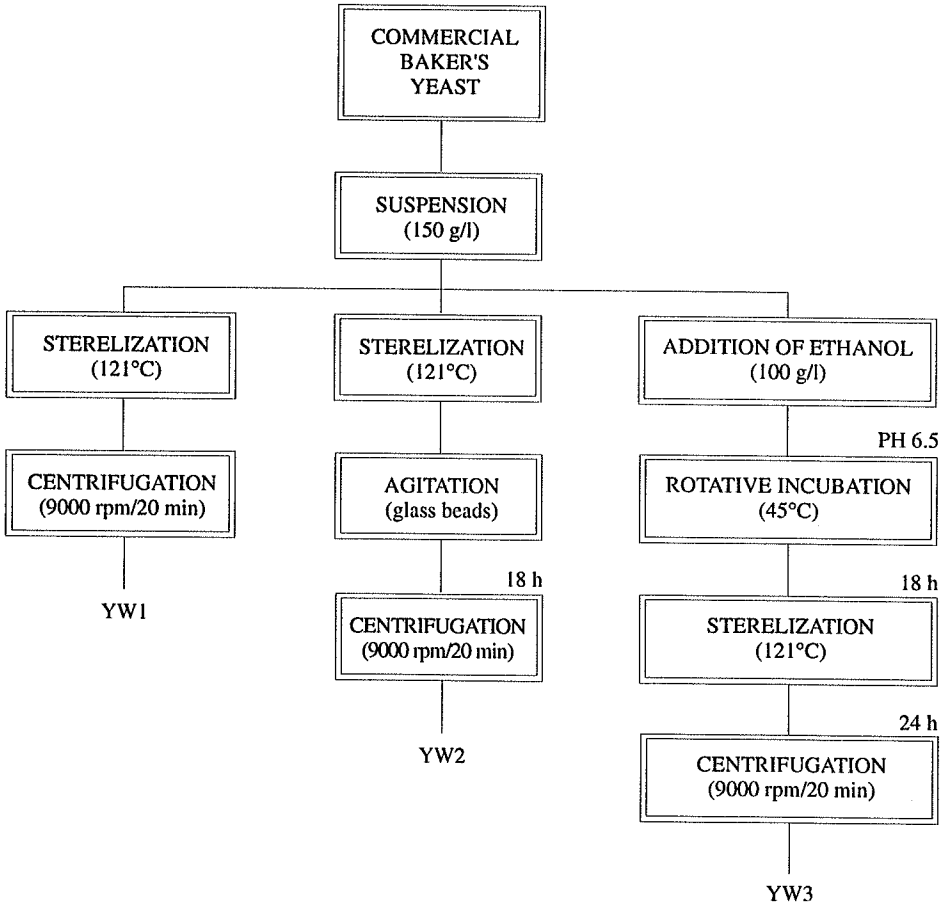

FIGURE 1 - Procedure to obtention of three of yeast-water (YW1, YW2, YW3) from commercial yeast.

er, after sterilization, a suspension of YW2 containing glass beads (0.2 - 0.3 cm diameter) was agitated (rotatory shaker, 150 rpm) during 18 h and subsequently centrifuged as described for YW1. For the preparation of YW3, 100 g/l of ethanol were added to the suspension of baker's yeast (150 g/l), the pH adjusted to 6.5 and the resulting suspension agitated at 45°C for 18 h. After agitation, this suspension was sterilized (121°C; 20 min) and centrifuged after cooling (9000 rpm; 20 min). In all cases, only the supernatants were utilized. It should be noticed that a single thermic method to disrupt yeast cells and release intra-cellular contents was used only with the YW1 preparation. For the YW2 preparation, a mechanical method (agitation) was employed to help break the yeast cells in addition to the thermic process. Concerning the preparation of YW3, ethanol was added to alter cell-wall permeability and the suspension subsequently sterilized to avoid contamination, to hydrolyse proteins, and to remove the ethanol by evaporation.

These three preparations were utilized as the bases for culture media without addition of water or other liquid diluents. Sucrose (2%) was used as carbon source. The media tested were clear and totally filtrable.

Agitation culture: Erlenmeyer flasks (250 ml) containing 50 ml of culture medium were inoculated so as to obtain an initial concentration of 10^6 conidia/ml. The conidia used in all experiments presented 95-100% viability. Conidia were scraped from the solid medium surface and transferred to a solution of Tween 80 (0.1%). The conidial suspension was then filtered through glass wool as described by Leser(5). The experiments were performed in a rotatory shaker (New Brunswick Scientific Co., G-27, Edison, NJ, EUA) at 150 rpm and 26°C, using three replicates unless otherwise stated. Fungal growth was evaluated by measuring final dry mass. Changes in pH were also monitored at the same time intervals.

The effects of initial concentration of conidia on fungal biomass production and pH change were investigated in agitation cultures using sucrose (4%) and yeast extract (1%) as culture medium. Mycelial dry mass was evaluated 84 h after inoculation, and pH was measured every 12 h, starting at 24 h of culture up to 84 h.

Culture in fermenter: The procedure for mycelium production was based on the method developed by McCabe & Soper (7) and is summarized as follows. The inoculum consisted of 500 ml of a mycelial mass grown in agitation cultures (150 rpm, 26°C, 10^6 conidia/ml) for 72 h. The fungus was cultured in 10 flasks (250 ml), each containing 50 ml of SDY liquid medium. The fungal suspension was then transferred to a fermenter (Microferm, New Brunswick Scientific Co.) containing 4.5 l (total capacity = 14 l) of liquid medium based on yeast extract (1%) and sucrose (4%). The fungus was kept at 26°C - 27°C for 72 h with an agitation rate of 200 rpm, which minimizes foam formation and allows satisfactory mycelial growth (unpublished). Sunflower oil (0.1%) was used as an antifoaming agent. Five liters filtered air/minute (1 V.V.M.) were injected into the fermenter. The experiment was performed twice giving similar results.

Harvesting and drying processes: The mycelium was harvested with the aid of a Buchner funnel by filtering 0.5 l of the mycelial suspension at a time through a chromatography filter paper (Whatman®, 3 mm Chr.). In order to completely remove the medium and prevent fungal growth, the mycelial mat was washed with an equal volume of distilled water. After filtering, the mycelium was separated from the filter paper and transferred to aluminum racks for drying at room temperature. The rest of the procedure was as described by McCabe & Soper (7). After drying, the mycelium was ground using a set of sieves (0.2, 0.5 and 1.0 mm) and particles of 250 to 500 µm were selected. The dry mycelium was stored at -18°C in plastic bags containing silica gel particles (0.5 - 1.0 cm).

The reducing sugar content of the filtrate was quantified using the dinitrosalicilic acid method (8). Measurements of fungal sugar consumption can indicate whether it is possible to reduce the amount of sugar added to the medium in the fermenter. This technique is also used to determine if sugar is the limiting nutrient in the medium.

Dry mycelium and conidial viability: Petri dishes lined with saturated filter paper were used to test dry mycelium viability. Ten milligrams of dry mycelium particles were spread on the wet filter paper and incubated at 27°C for 72 h. The viability of conidia produced by the dry mycelium was tested in a yeast extract (0.125%) liquid medium as described by St. Leger et al. (15).

RESULTS AND DISCUSSION

Effects of type and concentration of carbon sources: Results obtained from two replicates of preliminary experiments indicated that it was possible to replace the SDY medium by a simpler me-

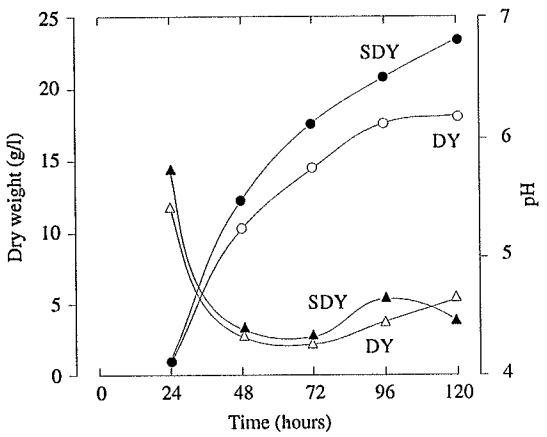

FIGURE 2 - Mycelial mass of *Metarhizium anisopliae* produced in SDY (Sabouraud dextrose and 1% yeast extract) and DY (4% dextrose and 1% yeast extract) at 26°C under agitation (150 rpm); changes in pH are also included.

dium based only on dextrose (4%) and yeast extract (1 %) (DY medium). The dry mass produced by *M. anisopliae* on DY was comparable to that produced on SDY (Figure 2). pH values followed the same trend in both media (Figure 2).

The next step was to test the possibility of replacing dextrose by sucrose. The effects of different initial concentrations of dextrose and sucrose on the growth of *M. anisopliae* are presented in Table 1. No differences were detected between media based on 1% yeast extract combined with 2, 4 or 8% sucrose. This clearly indicates that, for these cultures, the limiting factor was some substance present in the yeast extract. This limitation was confirmed by results obtained from cultures with a lower yeast extract

TABLE 1 - Dry Mycelium production of *Metarhizium anisopliae* using different culture media. The fungus was incubated inside 250 ml flasks under agitation (150 rpm) for 72h at 26°C. The initial inoculum was 10^6 conidia/ml.

Medium	Dry mass (g/l)[1,2]
Sucrose (8%) + yeast extract (1%)	9.5 ± 0.4 ab
Sucrose (4%) + yeast extract (1%)	10.7 ± 0.3 a
Sucrose (2%) + yeast extract (1%)	8.9 ± 0.3 ab
Sucrose (8%) + yeast extract (0.5%)	7.9 ± 0.1 bcd
Sucrose (4%) + yeast extract (0.5%)	8.1 ± 0.1 bc
Sucrose (2%) + yeast extract (0.5%)	8.1 ± 0.6 bc
Destrose (4%) + yeast extract (1%)	10.3 ± 0.5 a
Destrose (4%) + yeast extract (0.5%)	6.0 ± 0.2 de

1 Numbers indicated the mean and 95% confidence interval from 3 replicates of 25 ml of mycelial mass and liquid medium.
2 Means within columns follwed by the same lether are not significantly different according to Tukey's test ($\alpha = 0.05$).

concentration (0.5%). In this case, all final biomass yields from the sucrose containing cultures did not differ among themselves; however, they were significantly different when the concentration of yeast extract was doubled. Similar results were obtained when dextrose was used as the carbon source (Table 1).

Effects of nitrogen source: Results from two types of yeast-water, YW2 and YW3, did not differ from that obtained with 0.5% yeast extract (Table 2). Only one method of yeast-water preparation (YW1) provided significantly less mycelium than the yeast extract.

The possibility of replacing yeast extract by yeast-water prepared from baker's yeast or by-products from the alcohol industry is very interesting. This idea can be even more attractive if one considers that the preparation of YW2 and YW3 can be easily scaled up. However, the choice of one of these methods will depend on the costs involved.

TABLE 2 - Effect of yeast-water on dry mycelium production of *Metarhizium anisopliae*. The fungus was incubated inside 250 ml flasks under agitation (150 rpm) for 72h at 26°C. The initial inoculum was 10^6 conidia/ml).

Medium	Dry mass (g/l)[1,2]
yeast extract (0.5%) + sucrose (2%)	8.1 ± 0.6 ab
yeast-water (YW3) + sucrose (2%)	6.2 ± 1.7 bcf
yeast-water (YW2) + sucrose (2%)	6.4 ± 0.4 bcd
yeast-water (YW1) + sucrose (2%)	5.0 ± 0.5 d

1 Numbers indicated the mean and 95% confidence interval from 3 replicates of 25 ml of mycelial mass and liquid medium.
2 Means within columns follwed by the same lether are not significantly different according to Tukey's test ($\alpha = 0.05$).

Conidial concentration: Dry mass production and pH change were significantly affected by the initial conidial concentration. A regression analysis of the results confirmed that an increase in conidial concentration was linked to a greater production of dry mass ($P < 0.05$) (Figure 3). In addition, an increase in conidial concentration was associated with a major change in pH (Table 3), indicating utilization of nitrogen. A time-course experiment should be performed to establish the best conidial concentration to produce the inoculum for fermenter cultures.

Culture in the fermenter: Growth of *M. anisopliae* in a fermenter was accompanied by a high production of foam, despite the inclusion of sunflower oil (0.1%) as an antifoaming agent and the

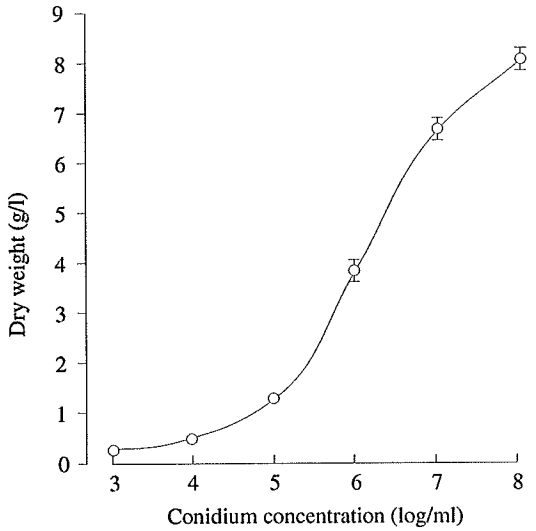

FIGURE 3 - Effect of initial conidial concentration on the production of mycelian mass by *metarhizium anisoplae*. The fungus was incubated for 84h at 26°C under agitation (150 rpm) in a sucrose (4%) and yeast extract (1%) medium.

TABLE 3 - Effect of initial concentration of inoculum of the culture medium of *Metarhizium anisopliae* on pH change. The fungus was grown for 84h inside 250ml flasks under agitation (150 rpm) at 26°C.

Conidia/ml	pH (hours of culture)[1,2]				
	24	36	48	72	84
10^3	6.5	6.3	6.2	5.8	5.8
10^4	6.3	6.2	6.1	5.5	5.7
10^5	6.2	6.1	6.1	4.5	5.1
10^6	6.2	6.0	5.9	4.2	4.5
10^7	6.1	5.9	5.1	4.2	5.8
10^8	5.9	5.1	4.3	5.2	6.2

1 Initial pH = 7.0
2 In culture medium based on sucrose (4%) and yeast extract containing Tween 80 (0.1%).

lower agitation rate (200 rpm). The growth rate inside the fermenter (Figure 4) was inferior to that in the shaker (Figure 2). This might be the result of oxygen limitation caused by low agitation (200 rpm) under fermenter conditions. A regression analysis of the data obtained between 24 and 54 h of culture in the fermenter showed a linear increase of dry mass (X) as a function of time (t) ($X = -0.345 + 1.62\ t$; $R2 = 0.97$; $P < 0.05$). These results support the hypothesis of oxygen limitation, as also observed by Pirt (11) and Slater (14). Final dry mass (72 h) was 9.05 g/l (Figure 4), with a productivity of 0.13 $g.l^{-1}h^{-1}$. As observed in other

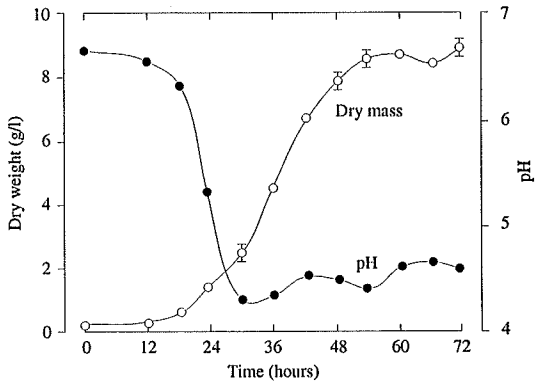

FIGURE 4 - Production of mycelial mass of *Metarhizium anisoplae* in submerged culture (fermenter) at 26 - 27°C under agitation of 200 rpm and air input of 1 V.V.M., and change in pH.

systems (5), the pH value of the mycelial suspension showed a fast reduction from 0 to 18-24 h of culture and a slight raise after 48 h (Figure 4).

Sugar consumption after 72 h of culture was approximately 69% (Figure 5). This result is in accordance with the findings reported by Machado et al. (6) and confirms the possibility of reducing the sucrose concentration of the original medium from 40 to 20 g/l, a change that would permit a cut in the production costs.

Dry mycelium and conidial viability: Co-

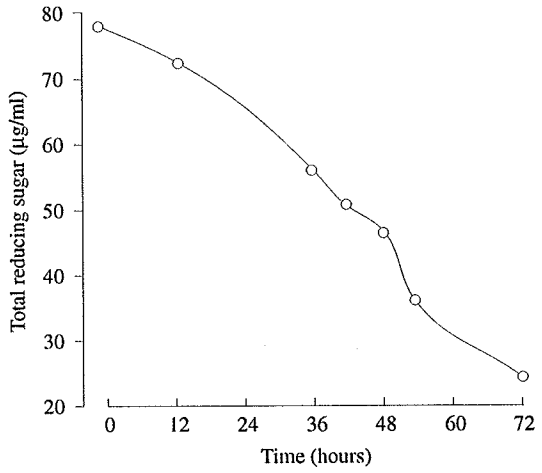

FIGURE 5 - Consumption of sucrose by *Metarhizium anisoplae* during fermentation at 300 rpm and 1 V.V.M.

nidial production was initiated 48 h after rehydration and incubation at 25°C. The fungus produced $1.63 \pm 0.02 \times 10^{10}$ conidia/g of dry mycelium 72 h after being rehydrated. Similar results

were obtained by Pereira & Roberts (10) The conidia obtained before and after drying presented high viability (> 90%).

Conclusions: Compared to dextrose, sucrose is a cheap and effective carbon source for growing *M.anisopliae* mycelium. The production of dry mycelium is proportional to the log of the initial concentration of conidia within the range of 10^5 to 10^8 conidia/ml. In addition, pH changes are less at high concentrations (> 10^7 conidia/ml) than at low concentrations (10^3 - 10^6 conidia/ml) of inoculum. The viability of conidia produced by the dry mycelium (> 90%) is comparable to that of conidia generated from fresh mycelium. Further studies may definitely point out to yeast-water as a substitute for yeast extract in the production of dry mycelium by *M. anisopliae*.

The results presented herein have already been useful for the production of dry mycelium from *Paecilomyces lilacinus* used in the control of nematodes (unpublished). These results may also be of help for the mass production of other fungi potentially interesting as biological control agents of weeds, such as *Alternaria cassia* against sicklepod (*Senna obtusifolia*) and *Cercospora sp.* against the purple nutsedge (*Cyperus rotundus*).

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RESUMO

Produção de micélio de *Metarhizium anisopliae* em meio líquido usando diferentes fontes de carbono e nitrogênio

A produção de micélio seco de *Metarhizium anisopliae* (CG 46, isolado da cigarrinha das pastagens *Deois flavopicta*) foi investigada. Várias fontes de carbono (dextrose e sacarose) e nitrogênio (extrato e água de levedura) foram testadas em cultivos em incubador rotativo (150 rpm). As melhores fontes de carbono e nitrogênio foram sacarose (4%) e extrato de levedura (1%), respectivamente. A concentração celular (massa seca) e pH variaram de acordo com a concentração inicial de conídios. A maior massa seca foi obtida com concentrações altas de conídios (> 10^7 conídios/ml). No fermentador, com meio de cultura à base de sacarose (4%) e extrato de levedura (1%), às 72 h de cultivo, a massa final foi 9.05 g/l com uma produtividade de 0,13 $g.l^{-1}.h^{-1}$. O consumo de açúcar a 72 h de cultivo foi de cerca de 69%. Após rehidratação (72 h, 25°C), o fungo produziu 1,63 x 10^{10} conídios/g de micélio sêco. A viabilidade dos conídios produzidos foi superior a 90%. A utilização de água de levedura, preparada com dois métodos distintos para substituição do extrato de levedura (0.5%) no meio de cultura, mostrou resultados satisfatórios. A vantagem desta substituição é que a água de levedura pode ser fácilmente preparada a partir de sub-produtos de destilarias de álcool.

Palavras-chaves: Fermentação, fungos enteropatogênicos, produção em massa, controle microbiano, fonte de carbono, fonte de nitrogênio, concentração de conídias.

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OCCURRENCE OF MICROFUNGI DURING LEAF LITTER DECOMPOSITION IN A 'CERRADO SENSU STRICTO' AREA OF SÃO PAULO, BRAZIL

Derlene S. Attili*
Sônia M. Tauk-Tornisielo

SUMMARY

Microfungal occurrence during the leaf litter decomposition of samples placed onto the soil surface or buried at a 30cm depth was followed-up throughout one year in a "cerrado" area, using the litter bag technique and washing procedure of leaf discs. The survey was repeated during the following year, completing two periods of investigation. Leaf parameters like moisture, decomposition rate and pH were measured and subjected to statistical analysis. Although the diversity index was significant between buried and surface samples, the following most commonly isolated genera were found associated with both treatments : *Trichoderma* Pers.; *Cylindrocladium* Morgan; *Mucor* Micheli ex Fr.; *Absidia* Tieghem; *Fusarium* Link.; *Verticillium* Nees and also *Mycelia sterilia* representatives. The pH of the leaves inside the surface litter bags was higher but the buried leaves were decomposed faster.

Key words: Microfungi; leaf litter; decomposition; 'cerrado'

INTRODUCTION

The role of microorganisms is relevant to the energy flux and nutrient cycling. Fungi are known to be among the primary agents of plant litter decomposition in microbial communities (4, 23). They have features which make them highly competitive saprobes such as their specific enzymes for litter degradation, their ability to grow under lower pH and dry conditions, their production of antibiotic substances and rapid germination (4, 6).

Very few studies have been carried out on fungal succession during leaf litter decomposition in 'cerrado' areas in Brazil, despite their large agricultural potential and representative extension to the country (9, 10). The influence of vinasse addition on the decomposition of *Ocotea pulchella* leaves (19) or on the properties of the soil (24), as well as the description of hyphomycetes from its vegetation (10), are some examples of the few studies carried out on fungal aspects of the 'cerrado'.

Other tropical and subtropical areas have received more attention resulting in investigations of great relevance (7, 13, 16, 17, 25, 26). All of them have described a high percentage of Deuteromycetes as an important factor for the decomposition of plant material, and have also reported the influence of different climates and conditions on that process.

In the present paper, we verify differences in the occurrence of microfungi and decomposition rate between leaf samples placed on the soil surface and those buried at a 30cm depth.

Corresponding author
Depto. de Ecologia,IB-UNESP, Caixa Postal 199, 13506-900, Rio Claro , SP.

MATERIAL AND METHODS

Study area

The research area is located in Corumbataí city, at approximately 200km from the city of São Paulo (in the southeast of Brazil) between meridians 47°40'- 47°45' of western longitude and 22°10'- 22°15' of southern latitude. It is a 38.8ha 'cerrado' reserve with a maximum altitude of 850m (3). The climate is Tropical, with the rainy season taking place from October to March and the dry season from April to September. The annual rainfall is 1,517.6 mm and the mean temperature ranges from 15.3° to 26.5° C (15). The soil was classified as Red-Yellow Latosol, medium texture (1) and the term 'cerrado sensu stricto' refers to 'orchard-like' vegetation (9). Some of the principal representative plants of the reserve are: *Myrcia lingua* Berg.; *Blepharocalix acuminatum* Berg.; *Daphnopsis fasciculata* (Meissn.) Nevl.; *Ocotea pulchella* (Ness) Mez. and *Vockysia tucanorum* (Spr.) Mart. (5).

Sampling

The experiment was carried out from August 1985 till July 1986 (first period), and then from August 1986 till July 1987 (second period). During July 1985 and July 1987, one hundred and twenty nylon litter bags (25 x 25cm and 2mm mesh), each containing 20g of leaf litter taken from the principal representative plants of the reserve, were placed on the soil surface; the same number of bags was buried at a 30cm depth. All the leaves were dried at 30.0° C for 2 weeks before being placed inside the bags. The recently fallen litter was removed from beneath each surface bag so that its bottom stood in direct contact with the soil. Ten randomly selected litter bags were removed from the soil surface at 8 weeks intervals; wet and dry weights were determined to calculate leaf moisture content (14) and decomposition rate (18). Another set of ten litter bags was collected monthly from the soil surface for fungal analysis. Five g of leaves were crushed in a ball mill, mixed with 30ml of sterile saline (NaCl 0.85%) and shaken for 5 minutes before measuring the pH. The same procedure was used for all buried samples.

Fungal analysis

At the beginning of the experiment, some leaves were retained to identify the fungi already present. The leaf-disc washing technique (20) was employed for microfungal population analysis. Five-mm-diameter discs were cut from leaves using a sterile cork borer and washed to remove all easily detachable propagules from the leaf surface. After 30 1min washes in sterile water, they were dried on sterile filter paper; groups of five discs were then plated on potato dextrose agar (PDA), cellulose agar (CA) or acid rose-bengal agar, (RB), and incubated inside sterile moist chambers. Forty Petri dishes per treatment (buried and surface litter bags) were prepared and incubated at 28.0° C for at least 72h.

Developing fungal colonies were used to prepare slide-mounts. When sterile, mould cultures were submitted to a near uv light emission maximum of about 360 nm to induce sporulation. Fungal identification was done by conventional methods based on morphological characteristics, according to the pertinent literature (2, 6).

A two-way analysis of variance (ANOVA) with replication was applied to the data on pH, moisture and decomposition rate of the leaves (22). The diversity index was also calculated to verify the effect of treatment on the fungal population (21).

RESULTS

Leaf parameters

The moisture content of leaves inside the surface bags averaged 33.2% (maximum = 51% in September 1986 and minimum = 13% in July

TABLE 1 - Moisture content (%) w/w (n = 10) and pH (n = 3) of the leaf litter in the 'cerrado' area. SL = surface leaves; BL = buried leaves.

	Moisture %		pH	
Month/year	SL	FL	SL	FL
Sep / 85	40.4 ± 1.0	35.2 ± 1.3	3.93 ± 0.14	3.89 ± 0.12
Nov / 85	31.3 ± 4.1	37.5 ± 1.7	4.04 ± 0.23	3.67 ± 0.04
Jan / 86	24.1 ± 5.3	35.3 ± 3.6	4.09 ± 0.03	3.47 ± 0.00
Mar / 86	40.2 ± 4.6	38.4 ± 3.3	3.68 ± 0.02	3.23 ± 0.03
May / 86	23.2 ± 1.7	29.8 ± 4.7	3.56 ± 0.03	3.03 ± 0.20
Jul / 86	41.3 ± 3.2	37.2 ± 1.9	3.70 ± 0.00	2.88 ± 0.02
Sep / 86	45.7 ± 3.2	42.2 ± 2.6	3.66 ± 0.05	3.63 ± 0.02
Nov / 86	34.5 ± 5.8	35.8 ± 3.3	3.96 ± 0.05	3.61 ± 0.08
Jan / 87	44.0 ± 2.2	36.5 ± 4.3	4.03 ± 0.00	3.28 ± 0.04
Mar / 87	23.9 ± 3.0	30.5 ± 5.3	3.60 ± 0.02	2.87 ± 0.00
May / 87	44.6 ± 2.4	41.9 ± 2.1	3.79 ± 0.12	3.35 ± 0.16
Jul / 87	21.4 ± 1.0	29.7 ± 3.6	3.46 ± 0.50	3.01 ± 0.27

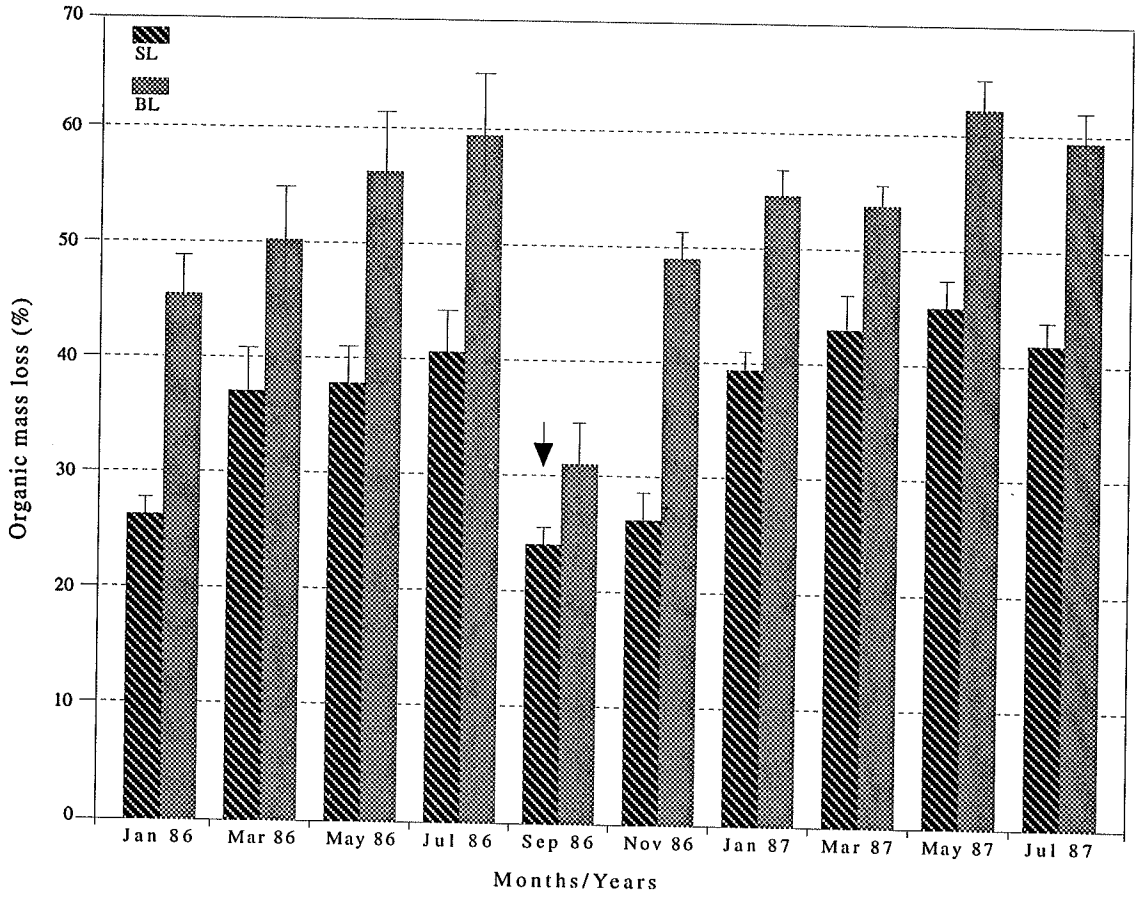

FIGURE 1 - Organic mass loss (%) w/w (n = 10) of the leaf litter in the 'cerrado' area. SL = surface leaves; BL = buried leaves. (The arrow indicates the beginning of the second period of the experiment.

1987) and that of the buried bags averaged 34.6% (maximum of 46.0% in September 1986 and minimum of 24.7% in July 1987) (Table 1). A seasonal variation was found in both cases, but a statistical difference between their moisture values was observed only during the first period of the experiment. The decomposition rate of the buried leaves was higher (Fig.1) and averaged 72.5% per year versus 42.3% for surface samples. The seasonal variation for this parameter in both situations was statistically demonstrated. Mean pH values were 3.8 (4.1-3.5) for the surface leaves and 3.3 (3.9-2.9) for the buried ones (Table 1); these differences were statistically significant.

Fungal analysis

The plant material contained 27 different genera of the Ascomycotina (1), Zygomycotina (4) and Deuteromycotina (22) (Table 2). Eight genera were present in the leaf litter at the beginning of the experiment: *Beltrania* Penzig.; *Cylindrocladium* Morgan; *Fusarium* Link; *Fusicladium* Bonorden; *Mucor* Micheli ex Fr.; *Penicillium* Link; *Trichoderma* Pers. and *Verticillium* Nees. *Mycelia sterilia* representatives, bacteria and yeasts were also isolated. When the experiment was repeated during the following year, it was found that the genera *Beltrania* sp and *Fusicladium* sp had been replaced by *Arthrobotrys* Corda and *Menispora* Pers.

The occurrence of *Cladosporium* Link, *Gyrothrix* (Corda) Corda and *Helminthosporium* Link was very sparse and restricted to leaves maintained on the soil surface. *Acremonium* Link occurred once in the buried leaves. The following genera were isolated only during the first period of the experiment: *Alternaria* Nees; *Cladospori-*

TABLE 2 - Fungal occurrence in the leaf litter of the 'cerrado' area. • = leaf litter; 0 = surface leaves; x = buried leaves.

Microorganisms	First Period													Second Period											
Absidia			x	x			x	0 x	x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	x	0 x	0 x	0 x	0 x	0 x	0 x	0 x
Acremonium																	x								
Alternaria	0 x	0	0		0						0														
Arthrobotrys						0								•	0 x				0						
Aspergillus	0 x	0 x		x		0				x				0 x	0		0 x				x				
Basidiomycetes				0 x	0 x					0	0				0										
Beltrania	•		x	x	x			0		0						0									
Cercospora		x						0 x							0 x										
Cladosporium	0																								
Corimiella									x	x	0 x	0 x			0 x	0 x		x	x	x	0 x	x	x		0
Curvularia	0							x	0				0	x											
Cylindrocladium	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	• 0	0 x	0 x	0 x	0 x	0 x	0 x	0	0 x	0 x	0 x	0 x
Fusarium	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0	0 x	•	0 x	0 x	0			0		0 x	0 x	0 x
Fusicladium	•																								
Gelasinospora			0 x	0 x	0 x	0 x	0 x	0 x	0	x			0			0	0	0		0 x	0			x	x
Gyrothrix															0	0									
Helminthosporium															0										0
Lemoniera			0	x									0												
Menispora					0									•	0		0			0			0 x		
Myxomycetes								0 x								x					0				0 x
Mucor	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x		0	0 x	0	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0	0 x	0 x	0 x	0 x
Mycelia sterilia	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0			x
Paecilomyces			x	x		x					0	0	0												
Penicillium	• 0 x	0 x	0 x	0 x	0		0	0 x	0 x	0	0	0	0 x	• 0 x		0	0 x			0 x			0	0	0 x
Pestalotia	0 x	0 x	0										0 x		0		0								0 x
Rhizopus				0								0	0			x									
Sepedonium							x	0	x													0			
Stylopage		0	0		x																				
Trichoderma	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	• 0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x	0 x
Verticillium	• 0 x	0	x	0 x	0 x	0 x	0 x	0 x	x	x	0		x	• 0 x	x	x					0 x	0 x	0 x	0 x	0 x
Yeasts	• 0 x	0 x	0				0	0 x	0	0 x	x			0							0				
Months	A	S	O	N	D	J	F	M	A	M	J	J		A	S	O	N	D	J	F	M	A	M	J	J
	1985					1986								1987											

um sp ; *Lemonniera* Wild.; *Paecilomyces* Bainier and *Stylopage* Drechsler; in contrast, *Gyrothrix* sp and *Helminthosporium* sp were restricted to the second period.

During the process of leaf decompositon, several microfungi were dominant in both situations: *Trichoderma* sp; *Cylindrocladium* sp ; *Mucor* sp; *Absidia* Tieghem; *Fusarium* sp; *Verticillium* sp and Mycelia sterilia representatives. Their frequencies were between 100.0-60.0%. A small group composed of *Penicillium* sp , *Coremiella* Bubák & Krieger, *Gelasinospora* Dowding and *Aspergillus* sp was regularly observed (frequency of 50.0-25.0%), but most of the fungi were sparse, presenting a less than 20% rate of occurrence during the experiment.

The pattern of fungal occurrence showed many fluctuations of total genera isolated per month and per situation (Table 3). In the second period, there was less diversity of genera in the buried leaves. Other organisms like bacteria, nematodes, Myxomycetes, Basidiomycetes and members of the order Sphaeropsidales were also detected.

TABLE 3 - Total of genera isolated per month from the leaf litter of the 'cerrado' area. SL = surface leaves; BL = buried leaves.

	1 st period			2 nd period	
Date	SL	BL	Date	SL	BL
Ago / 85	11	10	Aug / 86	8	7
Sep / 85	12	12	Sep / 86	6	6
Oct / 85	10	11	Oct / 86	12	10
Nov / 85	8	9	Nov / 86	12	6
Dec / 85	7	7	Dec / 86	4	5
Jan / 86	8	7	Jan / 87	6	6
Feb / 86	11	10	Feb / 87	6	6
Mar / 86	9	9	Mar / 87	8	5
Apr / 86	8	8	Apr / 87	7	6
May / 86	10	6	May / 87	9	9
Jun / 86	8	4	Jun / 87	7	8
Jul / 86	10	7	Jul / 87	9	7

No notable difference was observed in the occurrence of fungi among seasons (Table 2), except for the fact that many of them were detected more often during the first period, when the total rainfall was lower. The genera *Penicillium* , *Verticillium* and *Fusarium* , for example, were absent during the months of heavy rainfall.

DISCUSSION

A few organisms were found to be dominant during leaf litter decomposition. They seemed to be well adapted to the low pH of the leaves and were not affected by climatic changes nor for being buried or non-buried. All of them have been described as soil-borne fungi or fungi very common in soil. This may show that the leaf litter investigated had already been colonized by soil fungi at the beginning of the experiment, indicating a further stage of decomposition.

The total number of genera encountered was considered low but was similar to the results obtained on leaves of *Ocotea pulchella* (Ness.) Mez. from the same study area (19), and it is also in accordance with the data presented by GRANDI (10) who examined 'cerrado' soil and leaf samples.

Filamentous fungi occur in soil as mycelium or spores, but the former stage is the most active and more important in soil metabolism (8). The disc-washing procedure was considered very convenient to select active microorganisms during the leaf litter decomposition studied. However, a high frequency of *Mycelia sterilia* may have resulted from removal of the spores present in the material, although the method was successfully used by other authors (10, 13, 16, 19). The litter bag technique was as useful here as in other studies (7, 12, 19).

Soil fungal communities are frequently dominated by Hyphomycetes (10, 12). They play an important role in terrestrial ecossystems decomposing plant material actively (6, 8, 10, 23), and contributing to soil fertility. Ascomycetes and Basidiomycetes made up a very low percentage of all isolations from the 'cerrado' leaf litter and even less from green and senescent sugar maple leaves (12); however, such populations might have been underestimated due to the methods employed. The fungal succession of beech forest litter had a representative frequency of Mucorales (17, 25).

Most of the taxa found during the first year of the study were also recovered during the second, suggesting a homogeneous composition of the leaf litter in both periods of the investigation. We have assumed that the dominant group of Hyphomycetes reported in this study were important in the decomposition process.

Although the dominant fungi were common in both situations, the decomposition rate of the buried samples was higher. The complex microbial community of the soil probably offered bet-

ter conditions for microbial attack of the buried material. Studying cacao cultivated areas, GRISI (11) observed a higher and faster decomposition of cellulose at a depth of 2.5 cm in shaded soils. In the present investigation, buried leaves were shown to have a higher organic loss in the soil at a depth of 30 cm.

The *Aspergillus* frequency in buried and surface leaves was not very expressive considering the high saprophytic potential of the genus but it is in agreement with data presented for this genus on leaf litter of Japanese forests, probably due to nutritive preferences (2, 26).

The genus *Penicillium* was regularly isolated, except during periods of heavy rain fall, as observed in a previous study (19). This was the dominant genus in the summer months during maple leaf decomposition (12). This ubiquitous genus of saprophytes was easily detected by the dilution plate technique, but frequencies were reduced when the soil washing technique was employed (6).

The genus *Alternaria* was considered to be the first colonizer of green leaves of *Ocotea pulchella* (19). However, its occurrence in our study was limited to the first period, showing unsatisfactory conditions for its development in the second period. *Arthrobotrys* is a predacious fungus (2) and its rare occurrence, may have been encouraged by the nematodes present inside the leaf discs.

Cercospora is rare in soil and is not saprophytic (2), making its occurrence noteworthy. Further information is needed on the substrate exploitation and nutritional pattern of this genus.

Coremiella was mostly isolated in PDA medium. There are no previous reports of this fungus in 'cerrado' areas and thus this is the first time it has been associated to its vegetation.

Cylindrocladium is a polyphagous plant parasite and had not been detected on soil samples from the study area (2). Other known habitats include forest, cultivated soil, and leaf litter. A high humidity is required for the production and germination of the spores of some species (6), but in the 'cerrado' area the fungus was dominant even during the months of minimum leaf moisture.

Fusarium, Trichoderma and *Verticillium* were some of the fungi found to be dominant in the present investigation. Species of the former were prevalent in the litter of black rush (*Juncus roemerianus*) in South Florida (7) but were very rare in the litter of forests in Japan (26). Most species of *Fusarium* and *Verticillium* are soil fungi with cosmopolitan distributions but other representatives are typically pathogenic (26). *Fusarium* is also active in decomposing cellulosic plant substrates or in causing serious storage rots (6).

Trichoderma is one of the most common genera among the hyphomycetes of soil and decaying wood. The species are usually fast growing (8) and often present antifungal activity in addition to their high cellulolytic ability (6), which may explain its predominance in this study. In the fungal succession of mangrove leaves this genus was present at a late stage of degradation, becoming predominant in the leaf fragments (16).

The genus *Pestalotia* is associated with living leaves (26), which may explain its sparse occurrence in our study, since it focused on the litter fraction.

The community composition was generally unstable and may have been influenced by the seasons. *Beltrania* sp, *Curvularia* sp, *Lemonniera* sp, *Menispora* sp, *Paecilomyces* sp, *Sepedonium* sp, *Rhizopus* sp and *Stylopage* sp were transient fungi. However, a small but stable fungal group was predominant during the decomposition process. This may have been due to the substrate characteristics and the heterogenity of the plant material studied fulfilling their requirements for exploitation and colonization.

KUTER (12) reported that microfungal populations which initially colonized the maple leaf litter were partly dependent on seasonal conditions such as temperature and moisture availability. In the 'cerrado', the low values of the leaf litter pH, for instance, may have influenced the development of the community, affecting the competitive abilities of the fungi. The dominant genera were similar in both buried and surface leaves, but their activities were distinct considering the difference found between the decomposition rates.

RESUMO

Ocorrência de microfungos durante a decomposição da fração foliar da serrapilheira na área em São Paulo

Observou-se a ocorrência de microfungos durante a decomposição da fração foliar da serapilheira ao longo de um ano em uma área de cerrado, utilizando-se os métodos de bolsas de náilon e o de lavagem de discos. Algumas amostras foram colocadas na superfície do solo, enquanto outras foram enterradas a 30 cm de profundidade. Repe-

tiu-se o estudo no ano seguinte, perfazendo-se 2 períodos de investigação. Parâmetros como umidade, taxa de decomposição e pH das folhas foram analisados e submetidos a tratamento estatístico. O índice de diversidade entre as amostras enterradas e não enterradas mostrou-se significativamente diferente; no entanto os gêneros mais frequentes foram comuns em ambos tratamentos: *Trichoderma* Pers.; *Cylindrocladium* Morgan; *Mucor* Micheli ex Fr.; *Absidia* Tieghem; *Fusarium* Link.; *Verticillium* Nees, além de representantes do grupo *Micelia sterilia.* O pH das folhas não enterradas foi mais alto, porém a taxa de decomposição deste material foi menor em relação ao enterrado.

Palavras-chave: microfungos; serapilheira; decomposição; cerrado.

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EFFECT OF DITHIOTHREITOL AND SODIUM THIOGLYCOLLATE ON PROTOPLAST PRODUCTION OF *SACCHAROMYCES CEREVISIAE*

Cintia de Moraes Borba
Antonio Manoel Mendes da Silva
Pedrina Cunha de Oliveira

SHORT COMMUNICATION

SUMMARY

The effect of dithiothreitol and sodium thioglycollate on protoplast production of *S. cerevisiae* was investigated. When dithiothreitol was used as pretreatment and added to an enzymatic solution favorable results were obtained starting at 30 min. of digestion. When sodium thioglycollate was used as pretreatment and added to the same solution, protoplast production was inhibited.

Key Words: *Saccharomyces cerevisiae*, dithiothreitol, sodium thioglycollate, protoplasts.

Saccharomyces cerevisiae is a yeast whose cell wall is essentially divided into two layers. The outer layer consists of mannanprotein and the inner layer of ß-1,3 and ß-1,6-glucans (1,6). Chitin is located on the primary septum forming a complex with b-glucan and also on the lateral walls together with ß-1,6-glucan (5,6,12). Disulfide bonds are present in mannan-protein complexes, forming a barrier againts the exit of enzymes located on the inner layer of the wall and against the entry of extracellular glucanases (6). For this reason, thiol treatment is necessary for protoplast derivation (4, 8, 11). It has been suggested that thiols can cleave the disulfide bonds, mading the cell wall more susceptible to digestive enzymes, with consequent optimization of protoplast production (2,9).

The objective of the present study was to investigate the effect of dithiothreitol and of sodium thioglycollate on protoplast production of *S. cerevisiae*.

The organism used was strain 1473 of *S. cerevisiae* Meyen ex Hansen preserved under sterile mineral oil in the Culture Collection of the Department of Mycology, Oswaldo Cruz Institute. The strain was removed from the oil and subcultured several times in Sabouraud medium at room temperature. The cells were grown in Sabouraud medium in 100 ml Erlenmeyer flasks. The initial inoculum prepared in PBS, pH 7.4, was 8.5 x 10^5 cells/ml. Cells were incubated at 30°C in a shaker at 104 oscillations/min for 24h. The entire experiment was carried out in duplicate. Cells were washed twice PBS, pH 7.4, and submitted to two pretreatments: the first with 0.5M sodium thioglycollate (Sigma) in 0.1M Tris (Merck), pH 9.0, and the second with 48mM dithiothreitol (Sigma), both for 15 min and with gentle shaking at 30°C. The cells were centrifuged at 1250g for 5 min and washed twice with 0.9M sorbitol (Sigma) - 0.1M EDTA (Sigma), pH 7.0. The cells pretreated with sodium thiogly-

Departamento de Micologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, FIOCRUZ, Caixa Postal 926, 21040-000 Rio de Janeiro, RJ, Brasil.

collate were incubated with an enzyme solution containing 3.3mg/ml Novozym 234 (Novo Biolabs), 0.42mg/ml chitinase (Sigma), 0.5M sodium thioglycollate, and 0.9M sorbitol - 0.1M EDTA. The cells pretreated with dithiothreitol were incubated with 3.3mg/ml Novozym 234, 0.42mg/ml chitinase, 48mM dithiothreitol, and 0.9M sorbitol - 0.1M EDTA. Both treatments were carried out at 30°C under constant shaking at 104 oscillations/min. The digestion was monitored for 2h at 30 min intervals by phase-contrast microscopy. The amount of protoplasts was calculated in terms of sensitivity to distilled water. Percent lysis was calculated as follows: % lysis= [1-(number of cells in distilled water/number of cells in sorbitol - EDTA) x 100] (3).

Protoplast production occurred in cells submitted to pretreatment with dithiothreitol. Protoplasts began to be observed after 30 min of incubation. Percent lysis during this period was 12%. At the end of 2h of incubation, 70% protoplasts were obtained. Phasecontrast microsocopy revealed vacuolated cells with no cell wall which were sensitive to distilled water. In the cells pretreatment with sodium thioglycollate and incubated with the enzyme solution containing the same concentration of sodium thioglycollate as used for pretreatment, no protoplasts were detected either by phase-contrast microscopy or the basis of sensitivity to distilled water. Sodium thioglycollate at the concentration used, together with Novozym 234 and chitinase, inhibited protoplast formation.

Some thiols have been previously reported to inhibit protoplast formation in *Microsporum gypseum* when used with Novozym 234 (2). Pretreatment of *Acremonium chrysogenum* mycelium with dithiothreitol increases protoplast production, a fact not observed with *Aspergillus* species (7). In *Trichophyton mentagrophytes* and *M. gypseum*, mycelial pretreatment with mercaptoethanol-sodium-lauryl-sulfate greatly reduced protoplast production (2,10). The inhibitory effect was attributed to the high concentration of the compound which killed the hyphae or changed the proteolytic activity of Novozym 234, a fact that may have occurred with the enzyme solution containing sodium thioglycollate in our experiment.

The osmotic stabilizer employed, 0,9M sorbitol, was effective in maintaining the protoplasts intact, confirming the efficiency of sugars-alcohols in stabilization of yeast-like cells (4,8).

RESUMO

Influência do ditiotrutal e do tioglicolato de sódio na produção de protoplastos de *S. cerevisiae*

Investigou-se a influência do ditiotreitol e do tioglicolato de sódio na produção de protoplastos de *S. cerevisiae*. Resultados favoráveis foram obtidos a partir de 30 min. de digestão, usando-se o ditiotreitol como pré-tratamento e adicionado à solução enzimática composta por Novozym 234 e quitinase. O tioglicolato de sódio como pré-tratamento e adicionado à mesma solução enzimática, inibiu a produção de protoplastos.

Palavras-chave: *Saccharomyces cerevisae,* ditiotreital, tioglicolato de sódio, protoplastos.

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BEHAVIOR OF SOME STRAINS OF THE GENUS PLEUROTUS AFTER DIFFERENT PROCEDURES FOR FREEZING IN LIQUID NITROGEN[1]

Gerardo Mata
Dulce Salmones
Rosalía Pérez
Gastón Guzmán

ABSTRACT

Procedures for freezing of *Pleurotus* strains in liquid nitrogen inside polypropylene flasks using glycerol as a cryoprotector are reported. Mycelial recovery after 30, 60, 90 and 120 minutes of contact with the cryoprotector prior to freezing and two procedures for freezing (immediate immersion in liquid nitrogen and 30 minutes pre-freezing at -40°C) were evaluated. The mycelia were defrosted after 7 days. It was observed that the time of appropriate contact with the cryoprotector varied between 60 and 120 minutes. Fifty-eight percent of the samples which were immediately frozen and forty-five percent of those which were pre-frozen were recovered. The recovered mycelia were cultured in a mushroom farm and normal fructifications were obtained.

Key Words: *Pleurotus*, liquid nitrogen, cryogenic storage.

INTRODUCTION

Preservation of the characteristics of fungal strains during long periods of time is one of the features that contribute to the maintenance of their stability. The preservation of strains by traditional refrigeration methods and periodic cultivation increases the risk of aging, contamination and mutation. Consequently, research has been focused on producing methods that ensure the viability of the genetic material of species over long periods of time. One of the most modern methods is the immersion of the mycelium in liquid nitrogen at temperatures near -200°C, also known as the cryogenic system, which facilitates the preservation of the fungus and avoids most of the risks mentioned above (2, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17).

In order to perform immersion in liquid nitrogen, it is necessary to take precautions with respect to the freezing rate and the addition of a substance which acts as a cryoprotector against the formation of water crystals. Two of the cryoprotectors most commonly used in the preservation of mycelia are dimethyl sulfoxide and glycerol, both of which slowly penetrate the hyphae, thus helping to protect them (1, 16, 17).

Cryogenic methods have been developed in the U.S.A., Europe and Japan but has been little work on this subject in Mexico (3, 4). The present study focuses on the investigation of two basic factors for the preservation of mycelia: the time that the strain is in contact with the cryoprotector and the freezing time.

MATERIALS AND METHODS

Five strains of three species of *Pleurotus* were studied: *Pleurotus columbinus* Quél., IE-

1. Part VI of the series: Studies on the genus *Pleurotus*. Research on Project 0088-N9106 financed by CONACyT at Mexico. Instituto de Ecología, Apartado Postal 63, Xalapa 91000, Veracruz, Mexico.

136 from Czechoslovakia; *P. djamor* (Fr.)Boedijn, IE-11 from Guatemala, IE-134 and IE-153 from Mexico; and *P. ostreatus* (Jacq.:Fr.)Kumm., IE-8 from Europe, deposited in the Fungus Strain Collection of the Instituto de Ecología. The mycelia of each strain were grown in Petri dishes on agar with malt extract (BIOXON brand: malt extract, 12.75 g; dextrine, 2.75 g; glicerine, 2.35 g; peptone, 0.78 g; agar 15 g), with the exception of strain IE-153, for which the medium was enriched by boiling 400 g of wheat seeds and 100 g of barley straw in 1 lt of water and the infusion subsequently used to dilute the malt agar medium.

Ten fragments of mycelium were placed in polypropylene flasks with 2 ml of 10% sterilized glycerol (V/V) as a cryoprotector, following the methodology of Butterfield *et al.* (2). Sample recovery was studied in relation to the variables: contact time with the cryoprotector (30, 60, 90 and 120 minutes) and freezing procedure (immediate immersion in liquid nitrogen or 30 minutes pre-freezing at -40°C). Samples were defrosted after seven days. The flasks were then submerged in water (45°C) for 15 minutes and the mycelia fragments subsequently placed in Petri dishes containing the media mentioned above; incubation was carried out at 27°C in the dark. The recovery time and percent of samples recovered after each freezing procedure as well as their basidiomata at the mushroom farm were evaluated.

RESULTS AND DISCUSSION

Table 1 and figures 1 and 2 show the data on sample recovery. There was a greater tendency for higher and faster recovery when strains were exposed to glycerol for 60 to 120 minutes, thus suggesting that those may be the optimal contact-time periods. Some strains (IE-136 and IE-153) showed good recovery for almost all contact times (figure 1). Strain IE-8 recovered similarly, but in a smaller number of samples.

The greatest recovery was obtained for strains IE-136 and IE-153 of *P. columbinus* and *P. djamor*, respectively. Strain IE-136 had the fastest recovery time with an average of 1 to 4.2 days, whereas strain IE-134 of *P. djamor*, took 5.2 to 11 days and was the slowest to recover. Strain IE-8 of *P. ostreatus* had a relatively rapid recovery (2.5 to 5 days) but a low recovery rate (table 1). It seems that the appropriate time for contact of the cryoprotector with the strain depended on the strain, thus it is possible that strains differ in their ability to take up the cryoprotector.

TABLE 1 - Number of samples recovered for the different strains studied after 7 days of freezing.

Species and strain	Contact Time							
	30 minutes		60 minutes		90 minutes		120 minutes	
	A	B	A	B	A	B	A	B
P. columbius								
IE-136	9	9	10	7	10	7	10	10
Czechoslovakia	(2.6)	(2.2)	(1.0)	(4.2)	(2.7)	(3.5)	(1.9)	(2.8)
P. djamor								
IE-11	1	3	4	5	7	0	4	2
Guatemala	(8.0)	(3.6)	(4.5)	(5.4)	(5.0)		(3.0)	(3.5)
IE-134	0	0	1	1	5	4	5	0
México			(6.0)	(11.0)	(5.2)	(10.2)	(7.6)	
IE-135	8	10	10	8	10	8	10	6
México	(4.7)	(5.0)	(4.9)	(5.5)	(4.7)	(5.8)	(4.8)	(4.8)
P. ostreatus								
IE-8	5	2	2	1	5	1	0	6
Europe	(4.4)	(2.5)	(3.0)	(5.0)	(4.6)	(5.0)		(3.5)

A = Immediate freezing B = Pre-frozen
The numbers in parentheses indicate the average number of days which the samples took to recover.

With respect to the freezing procedures, all strains behaved in a similar way. If the samples recovered under each procedure are added, it is possible to see that there was greater recovery from samples which were immediately frozen (58%) than from those that underwent pre-freezing at -40°C (45%) (figure 2). Notwithstanding the difference found between the two freezing methods, the recovery presently reported was greater than that found by García Hinojosa & Martínez Cruz (3) (they obtained 0 % of recovery with *P. ostreatus*) but it was lower than that obtained by Ito & Yokoyama (9) (they found 88.3 % of recovery in Agaricales, not only in *Pleurotus*). The difference found in the freezing procedure is very important as it greatly facilitates the management of mycelia in the laboratory. Of the recovered samples, strains IE-8 and IE-11, considered commercially important for their high rate of production (15), were tested at the mushroom farm by growing them on wheat straw; in both cases, normal fruit bodies were produced in the normal time. The biological efficiency of these two strains was similar to that previously reported by Guzmán *et al.* (5) and Salmones *et al.* (15).

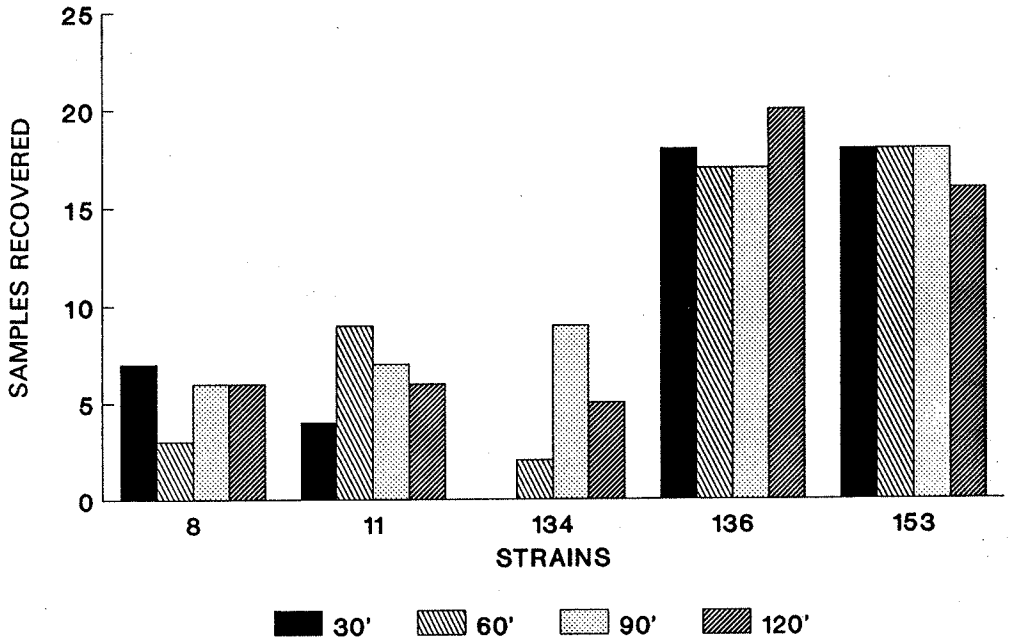

FIGURE 1 - Number of samples recovered after different times of exposure to glycerol.

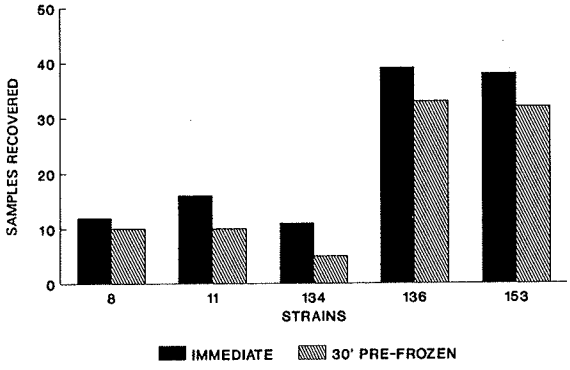

FIGURE 2 - Nuber of samples recovered after application of different freezing techniques.

RESUMO

Comportamento de algumas cepas do gênero Pleurotas após diferentes métodos de congelamentos em nitrogênio líquido

Descrevemos 2 métodos de congelamento de cepas de *Pleurotos* em nitrogênio líquido dentro de tubos de polipropileno, utilizando glicerol como criopreservante.

Avaliaram-se a recuperação de micélios após 30, 60, 90 e 120 minutos de contato com o criopreservante previamente congelado e também 2 procedimentos para congelamento (a saber: imersão imediatada em nitrogênio liquido e pré-resfriamento por 30 minutos a -40°C).

Os micélios foram descongelados após 7 dias, observou-se que o tempo de contato com glicerol mais adequado variou de 60 a 120 minutos.

Recuperava-se 58% das amostras imediatamente congeladas por nitrogênio e 45% das amostras submetidas ao pré-resfriamento a -40°C.

Os micélios descongelados foram transferidos para estufas de cultivo de cogumelos onde se obteve uma frutificacão normal.

Comportamento de algumas aps do gênero Pleurotos após diferentes métodos de congelamentos em nitrogênio líquido.

Palavras-chaves: *Pleurotos*, nitrogênio líquido, estocagem criogênica

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TRYPANOCIDAL ACTIVITY OF 1, 2, 3, 4-TETRAHYDROCARBAZOLES

Gabriel I. Giancaspro[1]
Victor D. Colombari[1]
Sem M. Albonico[1]
Rita Cardoni[2]
Cristina Rivas[1]
Maria T. Pizzorno[1,2]

ABSTRACT

A new series of disubstituted tetrahydrocarbazoles was synthesized and their trypanocidal activity evaluated by the ability to lyse tritium-labeled *T. cruzi*'s epimastigote forms. Twelve compounds of this series proved to be active.

Key words: disubstituted tetrahydrocarbazoles - Trypanomicides - *Trypanosoma cruzi* - Lysis.

INTRODUCTION

About 65 millon people are directly exposed to the risk of *Trypanosoma cruzi* infection and 15 to 20 millon are actually infected (12). Aproximately 10% of infected individuals develop chronic Chagas' cardiopathy. Acording to recent evidence, chronic Chagas'disease may be responsible for up to 10 % of deaths among adult humans in areas which have a high vector population (11). Eradication of Chagas'disease is very difficult and the only effective control has been to attack the vector with insecticides (10).

At present, there are no suitable drugs either for treatment or for the prevention of Chagas' disease, except for some drugs such as nifurtimox and benznidazol that present severe side effects (7,13,14) and others such as allopurinol (9) which are still under trial.

A series of studies on structured-activity relationships of substituted tetrahydrocarbazoles (THC) and dihydrobenzocarbazoles (DHBC) with both *in vitro* (1) and *in vivo* trypanocidal activity, (2,9) which were previously synthesized by our group led us to synthesize seventeen new substituted THC, in order to find new active drugs against *Trypanosoma cruzi in vitro* and to choose between them those that deserve further studies *in vitro* and *in vivo*.

MATERIALS AND METHODS

Synthesis of 1, 2, 3, 4-tetrahydrocarbazoles:

The chemical synthesis of tetrahydrocarbazoles was performed by two different procedures, as already described (5):

Route A: The 1,2,3,4 - tetrahydrocarbazoles 1 and epichlorhydrin in DMSO with KOH react together to give N-oxiranyl-methyl derivatives 2

1. Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires, IQUIMEFA, Consejo Nacional de Investigaciones Cientificas y Tecnicas, Junin 956, 1113 Buenos Aires, Argentina (054-01-961- 9668)
2. Instituto Nacional de diagnostico e Investigacion de la Enfermedad de Chagas "Dr. M. Fatala Chaben", Paseo Colon 568, 1063 Buenos Aires, Argentina.

(figure 1) with yields around 90 %. These compounds react with the suitable amines to give the desired compounds 4-20, with yields over 80 %.

Route B: The 1,2,3,4-tetrahydrocarbazoles 1 react in DMSO containing KOH with N-oxiranylmethyl-N,N-dialkylamines 3 prepared by Gilman's method (6) , to give the desired compounds (Figure 1).

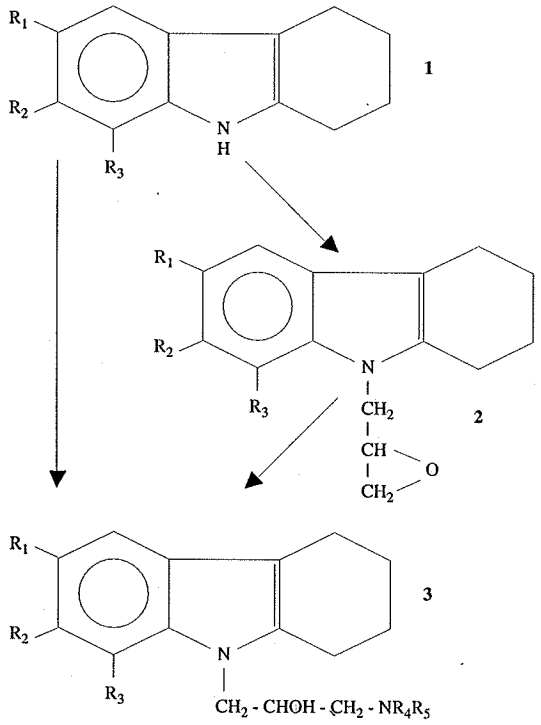


FIGURE 1 - Synthetic routes.

Drugs were identified by 1H NMR, 13C NMR and IR spectroscopy. The purity of the drugs was evaluated by TLC, HPLC and elemental analysis.

Compounds 4-20 were synthesized by one or both methods and are physicochemically characterized in Table 1.

Hydrochlorides and fumarates were dissolved in Ringer-Krebs solution. The drugs obtained as free bases were dissolved in Ringer-Krebs solution with an equimolecular quantity of 0.1 N HCl and the pH of the solution ajusted to 6 ± 1.

Parasites

T. cruzi's epimastigote culture forms of the Tulahuen strain were used.

Epimastigote forms were maintained by weekly passages in LIT medium containg 5 % of heat inactivated fetal bovine serum (FBS). For *in vitro* assays, parasites were cultivated for 5 days in the same medium containing 10 uCi/ml of 3H - uridine (NET 174, NEN, Boston MA.; specific activity 28.5 Ci/mmol). Tritium-labelled epimastigotes were washed three times in RPMI-1640 containing 5 % FBS and resuspended at 2×10^6 parasites/ml.

T. cruzi epimastigote lysis was measured in microcentrifuge tubes containing 100 µl of drug solutions at concentrations ranging from 4 to 200 µl/ml in RPMI-1640 supplemented with 5 % FBS.

Controls containing 100 µl of parasites and 100 µl of one of the following reactants were included : a) culture medium, to determine the spontaneous tritium release; b) Triton X-100 2 %, to determine the total activity incorporated; and c) medium in which the drugs were dissolved. Tubes were incubated for 20 h at 28°C and 37°C for the epimastigote form.

After centrifugation at 5.400 g for 5 min, 50 µl aliquots of the supernatant were used to determine the radioactivity released. Determinations were performed in a LKB Rack Beta Scintillation counter (LKB Inst., Bromma) and lysis of the parasites was evaluated as:

$$\text{Lysis (\%)} = \frac{\text{dpm in presence of drug - dpm spontaneous release}}{\text{dpm in presence of Triton - dpm spontaneous release}} \times 100$$

The total radioactivity incorporated by 105 parasites was 3.300 dpm for the epimastigote forms.

The spontaneous radioactivity released from the epimastigote form was 11 %.

RESULTS AND DISCUSSION

A new series of disubstituted tetrahydrocarbazoles was synthesized on the basis of our previous studies on structure-activity relationship of compounds also produced by our group (1,2,9).

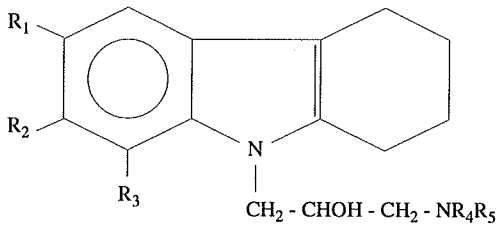
The substituents on the tetrahydrocarbazoles, chlorine and methoxyl on the aromatic ring and an amine chain on the nitrogen of the heterocyclic moiety, were selected among the groups that enhanced the activity in previous series. Furthermore, we introduced a carbinol group in the amine chain in order to improve the hydro-lipophylic balance of the molecule.

Compounds 4-20 were synthesized , and are described in Table 1. The H1 NMR, IR spectra and elemental analysis were in agreement with the proposed structures.

The most active compound synthesized in the past, 6-Cl- N-(1-ethyl-N-dimethylamino)-1,2,3,4-tetrahydrocarbazole, fumarate R(1) was compared with this series to evaluate the modifications introduced (8).

The radiometric assay used here to test the drugs' trypanocidal activity was developed to allow an objetive evaluation of the lysis of *T. cruzi* forms (3).

TABLE 1 - Physicochemical characterization of compounds 4-20.



N	Route	R_1	R_2	R_3	NR_4R_5	Fórmula	M.P.°C	Yield	L50 + SE
4	A, B	H	H	H	$N(C_2H_5)_2$	$C_{19}H_{28}N_{20}$	82-83	70%	70 ± 3
5	A	H	H	H	NC_5H_{10}	$C_{20}H_{28}N_{20}$	77-79	83%	38 ± 6
6	A	H	H	H	NC_4H_8O	$C_{19}H_{26}N_2O_2.HCl$	208-210	92%	> 350b
7	A, B	OCH_3	H	H	$N(C_2H_5)_2$	$C_{20}H_{30}N_2O_2C_4H_4O_4$	124-125	75%	69 ± 6.7
8	A	OCH_3	H	H	NC_5H_{10}	$C_{21}H_{30}N_2O_2.C_4H_4O_4$	192-193	77%	65 ± 8.7
9	A, B	OCH_3	H	H	NC_4H_8O	$C_{20}H_{28}N_2O_3.C_4H_4O_4$	144-147	81%	> 460b
10	A	Cl	H	H	$N(C_2H_5)_2$	$C_{19}H_{27}ClN_2O_4C_4H_4O_4$	189-181	68%	31 ± 2.2
11	A	Cl	H	H	NC_5H_{10}	$C_{20}H_{27}ClN_2OHCl$	208-210	79%	42 ± 2.6
12	A	Cl	H	H	NC_4H_8O	$C_{19}H_{25}ClN_2O_2HCl$	222-225	83%	> 385b
13	A	H	H	OCH_3	$N(C_2H_5)_2$	$C_{20}H_{30}N_2O_2$	109-110	82%	54 ± 6
14	A	H	H	OCH_3	NC_5H_{10}	$C_{21}H_{30}N_2O_2$	105-126	86%	52 ± 5.8
15	A	H	H	OCH_3	NC_4H_8O	$C_{20}H_{28}N_2O_3$	94-95	96%	171 ± 3
16	A	H	H	Cl	$N(C_2H_5)_2$	$C_{19}H_{27}ClN_2O$	124-125	88%	192 ± 2.9
17	A	H	H	Cl	NC_5H_{10}	$C_{20}H_{27}ClN_2O$	126-127	85%	87 ± 3
18	A	H	H	Cl	NC_4H_8O	$C_{19}H_{25}ClN_2O_2$	91-92	92%	> 349b
19	A	H	OCH_3	H	NC_5H_{10}	$C_{21}H_{30}N_2O_2$	88-89	84%	32 ± 3
20	A	H	OCH_3	H	NC_4H_8O	$C_{20}H_{28}N_2O_3$	87-89	91%	> 365b
	Reference								74 ± 3
	Nifurtinox								> 574b

b : Max. Conc. Tested
R : 6-chloro-N-(1-ethyl-N'-dimethylamino)-1, 2, 3, 4-Tetrahydrocarbazole, fumarate.
SE : Standard

The drug concentration which lysed 50 % of the parasites (L50) was calculated from a lysis (%) vs log drug concentration graph obtained from a computerized non-linear curve-fitting program (RNL, TeleVideo graphics Program, Copyright 1984 Televideo Systems, Inc.) and followed a sigmoid curve for the reference standard and for compound 14 (Figure 2). Mean values of L50 interpolated in graphics obtained from independent dilution sets are listed in table 1.

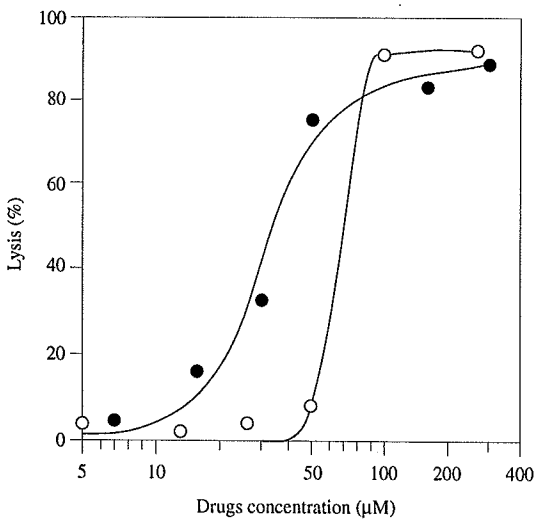


FIGURE 2 - Lytic activity on epimastigote form.
Key: o Compound R (reference)
• Compound N° 5

Our present series exerts patent trypanocidal effect on *T. cruzi* epimastigotes with an activity level several times higher than that of nifurtimox. The compounds with piperidine and diethylamine groups on the hydroxyalkylic chain are the most active, while the morpholine substituted ones are the least effective, no matter how the heterocyclic moiety is substituted.

The change in hydro-lipophilic drug balance in this series, due to the introduction of a carbinol group , makes negligible aromatic ring substitution as compared to more lipophilic series (1,2,10).

Compounds 5, 10, 11, 14, and 19 were selected for further studies *in vitro* and *in vivo*.

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RESUMO

Atividade tripanocida de 1, 2, 3, 4 - Tetrahidrocarbozois

Uma nova série de tetrahidrocarbazois disubstituídos e sua atividade tripanomicida foi avaliada quanto à sua habilidade para lisar formas epimastigotas de *T. cruzi* marcadas com trítio.

Os doze compostos examinados nestas séries mostraram-se ativos.

Palavras-chaves: tetrahydrocarbazoles disustituidos - Tripanomicidas - *Tripanosoma cruzi* - Lise.

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ERRATUM

"The use of fluorochromes in insect and plant mycopathology"

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Volume 25, Nº 2, p. 92, Figure 2. The correct title is "Germling of *Zoophthora radicans* treated with Tinopal (0.1%; w/v) and DAPI (1 µg/ml) to illustrate the nucleaus (arrow) and the cell morphology. Bar = 30 µm."

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